The expanding scope and impact of epigenetic cytosine modifications

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Abstract

Chemical modifications to genomic DNA can expand and shape its coding potential. Cytosine methylation in particular has well-established roles in regulating gene expression and defining cellular identity. The discovery of TET family enzymes opened a major frontier beyond DNA methylation, revealing three oxidized forms of cytosine that could mediate DNA demethylation or encode independent epigenetic functions. Chemical biology has been instrumental in uncovering TET's intricate reaction mechanisms and scope of reactivity on a surprising variety of substrates. Moreover, innovative chemoenzymatic strategies have enabled sensitive detection of oxidized cytosine products *in vitro* and *in vivo*. We highlight key recent developments that demonstrate how chemical biology is advancing our understanding of the extended, dynamic epigenome.

1 **Introduction**

2 Covalent modifications of DNA are a critical component of epigenetic regulation in eukaryotes.
3 The prototypical modification, 5-methylcytosine (mC), is broadly conserved in species ranging from
4 vertebrates to fungi and protists [1]. In mammals, mC has well-studied roles in regulating gene
5 expression, and altered methylation patterns are hallmarks of normal embryonic development as well as
6 tumorigenesis. Our current understanding of cytosine methylation has benefited from chemical biology
7 approaches that illuminated the mechanisms governing proteins that install or recognize methyl marks,
8 most notably DNA methyltransferases (DNMT1 and 3a/b) [2]. In addition, chemoenzymatic techniques
9 such as bisulfite sequencing and methylation-sensitive restriction enzymes made possible both *in vitro*
10 assays and genomic localization of mC bases, which gave insight into epigenetic functions.

11 Amidst these advances, a persistent question has been the mechanism of DNA demethylation,
12 which helps to reset epigenetic marks, particularly in germ cells and early embryos [3]. While DNA
13 replication can lead to a passive reduction in methylation, active enzymatic mechanisms are necessary for
14 rapid, regulated demethylation. Currently, the most compelling model for active demethylation in
15 mammals involves sequential oxidation of mC by ten-eleven translocation (TET) family enzymes,
16 followed by base excision repair (BER) to regenerate unmodified cytosine. Importantly, the bases
17 resulting from TET oxidation are not merely transient intermediates in demethylation but can be stably
18 mapped in various genomes, with mounting evidence to suggest that they encode independent epigenetic
19 functions. With the recognition that the epigenome extends well beyond mC, chemical biology is again
20 providing key mechanistic insights and tools to address prominent questions in the field. We review the
21 most recent advances in our understanding of TET enzymes and the bases that constitute the extended
22 epigenome.

24 **The scope of TET reactivity**

25 One major area of progress involves the mechanisms and reactivity of TET enzymes, the primary
26 writers of the extended epigenome. TET enzymes belong to the superfamily of Fe(II)/ α -ketoglutarate
27 (α KG)-dependent dioxygenases [4]. The three mammalian TET isoforms (TET1, 2, 3) were initially
28 found to oxidize mC to 5-hydroxymethylcytosine (hmC) [5,6]. Later discoveries revealed that they could
29 sequentially oxidize hmC to 5-formylcytosine (fC) and 5-carboxylcytosine (caC) [7-9].

30 All three oxidized mC (ox-mC) bases have been detected in diverse cell types, mostly in the
31 context of cytosine-guanine dinucleotides (CpGs). In general, genomic levels of hmC are 10- to 100-fold
32 lower than mC, while levels of fC and caC are at least 10-fold lower than hmC—approximately 1 in 10^5 -
33 10^6 nucleotides. In line with this trend, studies on several TET homologues have reported decreasing
34 reactivity from mC to hmC to fC [8-11]. Based on recent crystal structures of TET2, it was proposed that

1 conformational restraints on hmC and fC disfavor hydrogen abstraction from the 5-modified group,
2 resulting in lower reactivity compared to the less restrained mC substrate [11]. Decreasing reactivity of
3 the oxidation products, however, raises the question of how oxidation of hmC and fC occurs when the
4 competitor substrate mC is far more prevalent. Biochemical studies showed that Tet2 is capable of
5 iterative oxidation, defined as the ability to generate fC and caC from mC substrate without obligate
6 release of the hmC intermediate [12]. Although hmC is the dominant oxidation product *in vivo*, iterative
7 oxidation provides a feasible mechanism for generating highly oxidized fC and caC bases, which could
8 encode distinct epigenetic functions.

9 Once established, epigenetic marks often must be maintained by copying parental CpG
10 modifications onto newly synthesized DNA. For mammalian DNA methylation, the basis of this
11 epigenetic “memory” is well understood: *de novo* DNMT3a/b enzymes establish new mC marks
12 irrespective of opposite strand methylation, while maintenance DNMT1 enzymes specifically copy mC
13 onto hemi-methylated DNA after replication [2]. For ox-mCs, epigenetic memory is being actively
14 explored. Isotopic labeling experiments support potentially long-lived modifications [13,14], and
15 sequencing studies suggest that these bases can be stably mapped in many cell lines [15,16]. Recently, the
16 Tet2 isoform was found biochemically to have *de novo* activity [12], in line with the crystal structure
17 showing minimal engagement of the opposite strand CpG [11]. It is unclear if other isoforms could have
18 maintenance activity.

19 Beyond their canonical role in mC oxidation, TET enzymes are capable of broader reactivity that
20 hints at other potential cellular functions (Figure 1). Though initial studies focused on double-stranded
21 DNA, single-stranded 4- to 6-mers are also viable substrates [17]. Likewise, *in vitro* studies showed
22 activity on mC in RNA, and, correspondingly, hmC (and trace fC) were detected in RNA from various
23 species [18,19]. In a particularly interesting example, hmC was discovered in actively transcribing mRNA
24 in *Drosophila melanogaster*, and levels of hmC decreased upon depletion of the organism’s TET
25 homologue [20]. As *Drosophila* lack significant mC in genomic DNA, this opens the door for some TET
26 enzymes to play an added role in RNA biology. At the same time, confirming a physiological role for
27 TET-mediated RNA modification will be important, since the detection of oxidized RNA bases in Tet-
28 null embryonic stem cells [18], as well as in organisms that lack TET homologues [19], indicates that
29 TET-independent mechanisms could contribute.

30 Furthermore, although cytosine analogs are the predominant substrate, TET enzymes were shown
31 to have limited activity on thymine in cells (Figure 1) [21]. The resulting 5-hydroxymethyluracil (hmU)
32 was proposed to trigger DNA repair pathways or serve as another epigenetic base, and novel methods for
33 its localization have been developed [22]. Notably, many dioxygenases in the Fe(II)/ α KG-dependent
34 family, such as the trypanosomal J-binding proteins JBP1 and JBP2, naturally hydroxylate thymine rather

1 than cytosine [23]. By comparing structure-function relationships across these enzymes, future studies
2 could aim to modulate TET activity on various substrates, possibly including unnatural modifications or
3 nucleic acid analogs.

4 Understanding the scope of reactivity goes hand-in-hand with the biological contexts where TET
5 homologues are found (Figure 1). Computational studies have mapped a large family of TET-related
6 proteins spanning the evolutionary tree [24]. Some species harbor a bewildering number of TET
7 homologues—47 in the fungus *C. cinerea*, many catalytically active and associated with transposons
8 [25,26]. As noted, TET enzymes are also present in organisms such as *D. melanogaster* that lack genomic
9 mC, suggesting potential activity on RNA bases or non-cytosine bases such as N6-methyladenosine, or
10 non-catalytic functions [18,20,27]. Thus, while studies on the scope of TET's reactivity highlight its role
11 in DNA demethylation and epigenetic cytosine modification, additional roles in biology will likely
12 continue to come to light.

14 **Structural and chemical properties of ox-mC bases**

15 Another approach to understanding cytosine modification focuses on characterizing the
16 biophysical and chemical properties of ox-mC bases in DNA. By extension, numerous techniques have
17 been developed that exploit the reactivity of ox-mCs to either detect or localize these rare bases, offering
18 insight into their potential functions. Methods of mapping genomic ox-mCs have been expertly reviewed
19 [15,16]; we discuss select topics pertinent to the chemical biology of these bases.

20 Ox-mCs form high-fidelity, Watson-Crick base pairs with guanine and are generally not prone to
21 spontaneous deamination and oxidation events [28,29]. The 5-modified group occupies the major groove
22 of B-form DNA and appears to have subtle but potentially significant impact on helical thermodynamics
23 and stability [28,30]. For example, the electron-withdrawing character of 5-formyl and 5-carboxyl groups
24 weakens the N-glycosidic bond and decreases the pK_a of the base, resulting in less stable base pairing and
25 possibly promoting base excision by thymine DNA glycosylase (TDG, see next section) [31,32]. Repeats
26 of fC-containing CpGs, which can occur in the genome, were also found to underwind the DNA helix,
27 resulting in a distinct F-form conformation that could influence chromatin packaging or interaction with
28 fC-specific protein readers [33]. Moreover, DNA containing at least one fC displayed greater flexibility in
29 a single-molecule cyclization assay, and the increased DNA flexibility correlated with enhanced
30 nucleosome stability [34]. Overall, however, the evidence suggests that 5-modification of cytosine largely
31 maintains the structural and sequence integrity of DNA while providing an accessible handle for
32 epigenetic readouts.

33 The 5-modified groups also offer opportunity for chemical or enzymatic manipulation, which is
34 particularly important for designing assays to distinguish the identity of ox-mC bases (Figure 2). The

hydroxymethyl group can be glucosylated using UDP-glucose, a reaction typically catalyzed by T4 β -glucosyltransferase. The glucose moiety can itself be modified, such as with an azide group for downstream biotinylation, or oxidized with sodium periodate to create an aldehyde handle for pulldown of hmC-enriched DNA [35,36]. The aldehyde of fC also makes it directly amenable to reaction with probes such as primary amines [37], hydroxylamines [9,38], and hydrazines [39], while caC can react specifically with carbodiimides [8,40]. Although signal-to-noise often complicates readout of rare modifications, such chemical tools have made great strides toward sensitive quantification and localization of ox-mCs in various genomes.

Protein engagement with ox-mC bases

Along with the chemical reactivity of ox-mC bases, there has been great interest in proteins that engage specific ox-mCs (Figure 2). Exogenous proteins such as restriction endonucleases provide a powerful tool to probe and manipulate ox-mC bases *in vitro* and *in vivo* [41]. For example, AbaSI, initially found to restrict T-even phage, selectively cleaves DNA containing hmC or glucosyl-hmC; one sequencing method harnessed this enzyme to localize hmC in mammalian genomes [42]. Another recent technique used transcription activator-like effectors (TALEs) to distinguish C, mC, and hmC bases in DNA [43], and the fusion of TET enzymes to zinc fingers or TALEs can generate ox-mCs at targeted genomic locations [44,45].

Endogenous proteins likewise offer experimental applications but bear increased significance as potential readers, writers, and erasers of ox-mC modifications (Figure 2). The best characterized example is TDG, which specifically excises fC and caC from CpGs, leaving an abasic site that can undergo BER to regenerate unmodified C [7,46]; this demethylation cycle has recently been reconstituted *in vitro* [47]. TDG is therefore considered the leading candidate protein to initiate BER for active demethylation. Also intriguing are proposed roles for DNMTs—classically the writers of methylation—in erasure of ox-mCs via direct decarboxylation of caC [48] or dehydroxymethylation [49,50], though these activities have yet to be observed *in vivo*.

Proteomics analyses revealed many potential reader proteins for each of the cytosine derivatives [51,52]. Transcription factors have been one area of focus; for example, methyl-CpG-binding protein 2 (MeCP2) was identified as a major hmC-binding protein in the brain, with similar affinity for mC and hmC [53], while Wilms tumor protein 1 (WT1) can recognize caC as well as C and mC [54]. The yeast RNA polymerase II elongation complex was also reported to form hydrogen bonds specifically with caC, resulting in transient transcriptional pausing [55]. These studies are beginning to shed light on the molecular mechanisms of reading specific ox-mC bases and how these interactions might translate into epigenetic functions.

Pathological perturbations of ox-mC bases

The myriad connections between TET enzymes, ox-mC bases, and epigenetic functions suggest complex regulatory mechanisms. Loss of proper regulation gives rise to many pathologies, especially the genesis and progression of various cancers, as recently reviewed in depth [56]. Numerous solid tumors and hematologic malignancies harbor mutations in TET1, 2, or 3, and global reduction in hmC frequently correlates with tumor growth and metastasis. In addition, dependence on α KG places TET enzymes at a potential crossroads of epigenetic and metabolic pathways, which can also be subverted in disease. Metabolites such as fumarate and succinate have been shown to compete with α KG [57]. An even more intriguing example involves the products of isocitrate dehydrogenase (IDH1 and IDH2) enzymes, which normally convert isocitrate to α KG (Figure 3). Cancer-associated, gain-of-function mutations in IDH result in aberrant generation of the oncometabolite 2-hydroxyglutarate (2HG) [58,59], which competitively inhibits α KG-dependent dioxygenases, including TET [60]. A photocaged variant of the mutant IDH, generated using an expanded genetic code, has been used to show rapid metabolic perturbations and changes to ox-mC levels upon activation of the neomorphic enzyme [61]. Another intersection of cytosine modifications and cancer comes from a recent study on nucleotide salvage pathways. Upregulation of the salvage enzyme cytidine deaminase (CDA) in some cancer cell lines resulted in high levels of the deamination products hmU and fU, which could promote cell death when incorporated into DNA [62]. These advances point to novel angles on the biology of ox-mCs and potential therapeutic strategies that merit further exploration.

Conclusions

In defining the scope of TET and ox-mC reactivity, developing specialized chemoenzymatic tools, and probing the molecular mechanisms of protein-DNA interactions, chemical biology approaches have helped to transform our knowledge of the dynamic epigenome beyond 5-methylcytosine. These approaches have a critical role to play in the continued effort to understand TET enzymes and ox-mC bases *in vitro* and *in vivo*, as normal residents of highly diverse cell types and as markers of many human diseases.

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7 Certain cancer cell lines that overexpress CDA are shown to be susceptible to treatment with
8 hmC and fC nucleosides, likely because cytotoxic DNA damage accumulates with genomic incorporation
9 of the deamination products hmU and fU.

10

Figure Legends

Figure 1. The broad scope of TET reactivity. Summary of the generation of various nucleobases (top row) in both DNA and RNA (middle) of diverse species (bottom), with emphasis on the strongest exemplar (underlined). Canonically, TET enzymes sequentially oxidize mC in dsDNA to hmC, fC, and caC, with decreasing reactivity on more highly oxidized substrates. In addition, mC, hmC, and fC have been detected in RNA, with evidence for TET-dependent oxidation, as well as potential alternative mechanisms. Finally, TET enzymes can, to a limited extent, convert T to hmU in DNA, similar to the well-known JBP1/2 enzymes in Trypanosoma.

Figure 2. Ox-mCs as targets for both chemical manipulation and protein interactions. Top: Summary of reaction products after treating hmC (red), fC (blue), or caC (green) with classes of chemical modifiers. These modifiers often contain fluorescent labels or provide handles for purification, detection, or localization of ox-mCs in the genome. Bottom: Exogenous and endogenous proteins—some well-characterized and others only recently proposed—can serve as writers (black), erasers (gray), and readers (white) of epigenetic cytosine modifications.

Figure 3. Intersection of ox-mCs, metabolism, and pathology. Physiologically (green), TET enzymes utilize the cofactor α KG supplied by isocitrate dehydrogenase (IDH) to generate ox-mCs, which may contribute to DNA demethylation and other epigenetic functions. Pathologically (red), in some hematologic cancers, gain-of-function IDH mutants (IDH*) instead produce the oncometabolite 2-hydroxyglutarate (2HG), which acts as a competitive inhibitor of TET. As another example, several cancer cell lines upregulate cytidine deaminase (CDA), which can deaminate hmdC and fdC nucleosides. Incorporation of the resultant hmdU and fdU into DNA can lead to DNA breaks and potential cytotoxicity.

Figureg

Figure1

Figure2

Figure3