

TET Proteins and 5-Methylcytosine Oxidation in the Immune System

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DNA methylation in the form of 5-methylcytosine (5mC) is essential for normal development in mammals and influences a variety of biological processes, including transcriptional regulation, imprinting, and the maintenance of genomic stability. The recent discovery of TET proteins, which oxidize 5mC to 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine, has changed our understanding of the process of DNA demethylation. Here, we summarize our current knowledge of the roles of DNA methylation and TET proteins in cell differentiation and function. The intensive research on this subject has so far focused primarily on embryonic stem (ES) cells and neurons. In addition, we summarize what is known about DNA methylation in T-cell function.

Methylation of cytosines in metazoan genomes adds epigenetic information onto DNA without changing the genetic information encoded in the DNA sequence. Until recently, the only known modified base in DNA was 5-methylcytosine (5mC), an epigenetic mark established by DNA methyltransferases (DNMTs) (Ooi et al. 2009). In somatic cells, 5mC is almost exclusively found in the CpG sequence context, although non-CpG methylation has been documented in embryonic stem (ES) cells and in neurons (Lister et al. 2009, 2013). The promoters of the most highly expressed genes show the lowest levels of CpG methylation; conversely, dense CpG methylation of promoters is generally associated with decreased gene expression (Suzuki and Bird 2008; Laurent et al. 2010; Deaton and Bird 2011). There is also dense DNA methylation in gene bodies (Lister et al. 2009; Laurent et al. 2010), but the association of gene body CpG methylation with transcriptional regulation is less clear.

Two classes of DNMTs are involved in DNA methylation. The *de novo* DNA methyltransferases DNMT3A and 3B are required to establish DNA methylation patterns, whereas the maintenance DNA methyltransferase, DNMT1, reestablishes DNA methylation patterns following DNA replication (Klose and Bird 2006; Ooi et al. 2009). DNMT1 acts with its cofactor UHRF1, which binds hemimethylated DNA (Avvakumov et al. 2008; Hashimoto et al. 2008), to reestablish symmetrical CpG methylation on the newly synthesized DNA strand, thus maintaining DNA methylation patterns during replication (Bestor et al. 1988; Ooi et al. 2009).

The distribution of 5mC has been mapped at single-nucleotide resolution in human and mouse ES cells, ES cells differentiated to distinct lineages, somatic tissues, cultured cell lines, and various cancer cells (Hansen

et al. 2011; Stadler et al. 2011; Kulis et al. 2012; Gifford et al. 2013; Lister et al. 2013; Xie et al. 2013; Ziller et al. 2013). These studies showed that most of the genome is highly methylated (~80%–90% of CpGs with >50% methylation), with the remainder subdivided into unmethylated regions (UMRs) with <10% methylation and low-methylated regions (LMRs) with 10%–50% methylation (Stadler et al. 2011). UMRs correspond largely to unmethylated CpG islands (CGIs), many of which are located at transcription start sites (TSSs), whereas LMRs often coincide with promoter-distal gene regulatory elements enriched for transcription factor-binding sites (Stadler et al. 2011).

TET PROTEINS ARE 5mC OXIDASES

The recent discovery that TET (ten–eleven translocation) proteins are 5mC oxidases added an additional layer of complexity to our understanding of the biological role of DNA methylation (Iyer et al. 2009; Tahiliani et al. 2009). TET proteins are named after the rare ten–eleven translocation (t(10;11)(q22;q23)) observed in cases of acute myeloid and lymphocytic leukemia, in which the MLL1 (mixed-lineage leukemia 1) gene located on human chromosome 10 is fused with the TET1 gene located on chromosome 11 (Ono et al. 2002; Lorsbach et al. 2003). The three TET proteins in mammals, TET1, TET2, and TET3 (Fig. 1A), were identified by homology with the J-binding proteins (JBPs) of trypanosomes (Iyer et al. 2008) and are members of the larger TET-JBP family of 2-oxoglutarate- and Fe(II)-dependent dioxygenases (Loenarz and Schofield 2008, 2011; Iyer et al. 2009; Tahiliani et al. 2009). JBP1 and JBP2 oxidize the methyl group

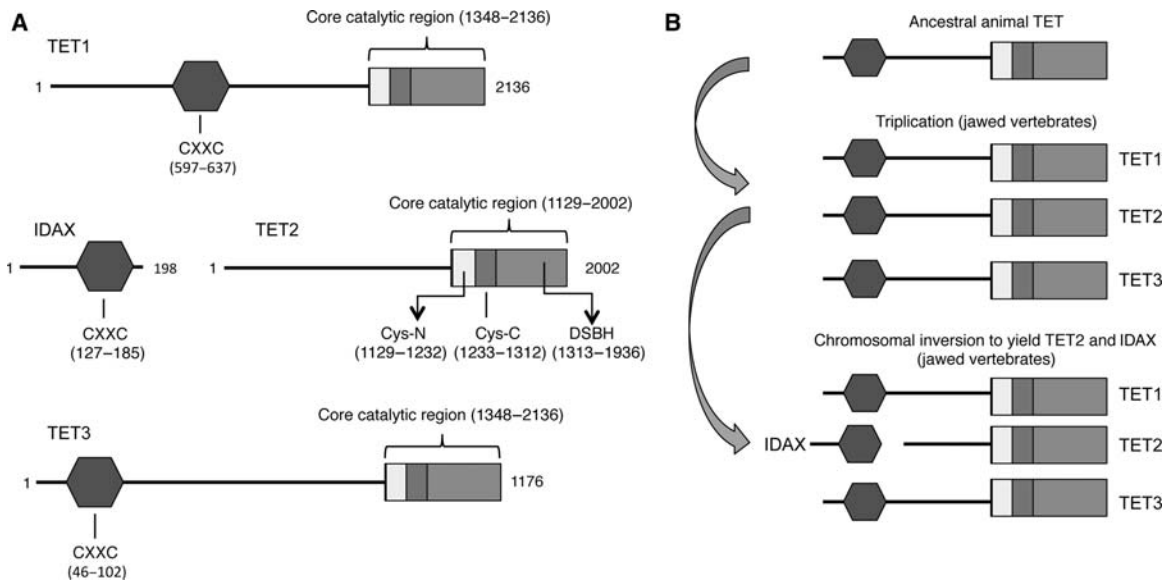


Figure 1. (A) Schematic representation of the predicted functional domains of mammalian TET proteins. Depicted is the CXXC domain that is present in TET1 and TET3, the carboxy-terminal catalytic domain that contains a cysteine-rich (Cys-rich) region, and the double-strand β -helix (DSBH) region, all three of which are common features in all three members of the TET family. The crystal structure of human TET2 reveals that the Cys-rich region is divided into amino- and carboxy-terminal regions that flank the DSBH domain (Hu et al. 2013). Also depicted is IDAX, a CXXC-domain protein that was part of TET2 before chromosomal inversion (B). The numbers indicate the amino acids in human TET proteins. (B) Evolutionary history of TET proteins. The original gene encoding TET underwent triplication in jawed vertebrates, giving rise to the three members of the TET family. A subsequent chromosomal inversion resulted in the separation of the TET2 catalytic domain from the CXXC domain, which became a separate gene, *IDAX/CXXC4*.

of thymine; the resulting 5-hydroxymethyluracil is then glycosylated to generate Base J (Iyer et al. 2009; Pastor et al. 2013). The mammalian TET proteins 2-oxoglutarate- and Fe(II)-dependent dioxygenases oxidize 5mC to generate 5hmC, 5fC, and 5caC (Fig. 1B; for review, see Pastor et al. 2013) (Tahiliani et al. 2009; He et al. 2011; Ito et al. 2011). Representatives of the TET/JBP superfamily are found in every metazoan organism that uses DNA methylation (Iyer et al. 2009), suggesting a major role for TET proteins in regulating DNA methylation status through production of oxi-mC (Kohli and Zhang 2013; Pastor et al. 2013).

The prediction that the core catalytic domain of TET proteins is a double-strand β helix (DSBH) adjacent to a unique cysteine-rich domain involved in DNA recognition (Iyer et al. 2009) was confirmed experimentally by studies on recombinant human TET1 expressed in insect cells, which showed that the DSBH region was catalytically inactive unless the Cys-rich region was present (Tahiliani et al. 2009), and subsequently by determination of the crystal structures of the human TET2 catalytic domain (Hu et al. 2013) and a TET-like dioxygenase from *Naegleria gluberi* (Hashimoto et al. 2013). In the human TET2 structure, the DSBH region forms the active site with the signature His-Xaa-Asp motif (where Xaa is any amino acid) and a conserved Arg residue that binds 2-oxoglutarate, whereas the Cys-rich region wraps around the DSBH core and stabilizes DNA binding (Hu et al. 2013). Notably, the TET2 catalytic domain binds CpG-containing and methyl-CpG-containing DNA with similar

affinity; the fact that the methyl group itself does not contact TET2 presumably allows the catalytic cavity to accommodate the partly oxidized 5mC, derivative 5hmC, and 5fC for further oxidation to 5caC (Hu et al. 2013).

TET1 and TET3 contain amino-terminal CXXC domains, whereas TET2 became separated from its CXXC domain as a result of a chromosomal inversion (Fig. 1B) (Iyer et al. 2009; for review, see Pastor et al. 2013). The separated CXXC domain is now a distinct gene, *IDAX* (inhibition of the Dvl and axin complex; also known as *CXXC4*), whose product, when DNA bound, recruits TET2 and regulates TET2 protein levels through caspase activation (Ko et al. 2013). CXXC domains typically bind unmethylated CpGs and are found in many chromatin-associated proteins, including the maintenance methyltransferase DNMT1 and MLL1, a component of the SET/COMPASS complex.

TET PROTEINS AND oxi-mC IN THE PATHWAY TO DNA DEMETHYLATION

The oxidative derivatives of 5mC that are generated by TET proteins are likely to serve as intermediates in DNA demethylation (Kohli and Zhang 2013; Pastor et al. 2013). Two main pathways for demethylation have been proposed: Passive dilution of the oxidized base during DNA replication and active DNA demethylation through DNA repair (Fig. 2). We note, however, that 5hmC is abundant in postmitotic neurons—in Purkinje

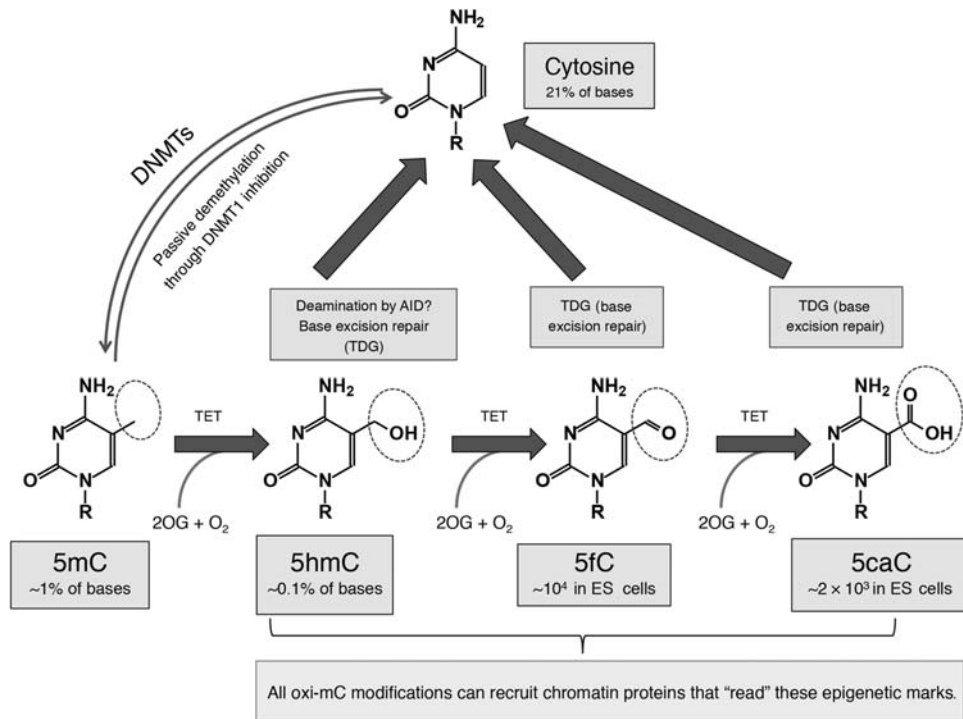


Figure 2. The cycle of DNA demethylation by TET proteins and the generation of oxi-mCs. DNA methyltransferases (DNMTs) add a methyl group to the 5 position of cytosine, and thus are responsible for DNA methylation. DNA demethylation can occur passively by inhibition of the maintenance methyltransferase DNMT1, especially in the presence of oxi-mCs, which inhibit DNMT1, resulting in dilution of the methyl mark during replication. TET proteins in the presence of 2-oxoglutarate (2OG) can oxidize mC to 5hmC, which is further oxidized by the TET proteins to 5fC and 5caC, which are much less abundant than 5hmC in the genome. 5fC and 5caC are recognized and excised by thymine DNA glycosylase (TDG) and subsequently replaced through base excision repair with unmethylated C. 5hmC has also been suggested to be directly demethylated by deamination through AID, followed by base excision repair mediated by TDG. Notably, the oxi-mCs (5hmC, 5fC, and 5caC) can exert functions beyond mediating DNA demethylation, acting as marks that recruit chromatin-bound regulatory proteins.

neurons, where it comprises ~40% of the level of 5mC (Kriaucionis and Heintz 2009), as well as in other neurons in the brain (Szulwach et al. 2011b; Mellen et al. 2012; Hahn et al. 2013)—indicating that like 5mC itself, oxidized methylcytosines are likely to function as epigenetic marks. Indeed, many oxi-mC “readers” have been identified by mass spectrometry (Spruijt et al. 2013); these include diverse transcription factors and chromatin regulatory proteins, but the importance of oxi-mC recognition for gene regulation remains to be defined.

Passive Dilution of the Oxidized Base during Replication

Blocking the action of the maintenance DNMTs would result in loss of DNA methylation during replication, a process known as passive DNA demethylation (Fig. 2). Conversion of 5mC to 5hmC could promote passive demethylation as a consequence of replication by inhibiting the ability of DNMT1 to recognize 5hmCpGs as observed *in vitro* (Valinluck and Sowers 2007). However, the obligate DNMT1 partner UHRF1 is reported to bind 5mC and 5hmC with similar affinities (Frauer et al. 2011), indicating that further investigation is needed. Current *in vivo* evidence from preimplantation embryos suggests

that 5hmC is passively removed through replication (Inoue and Zhang 2011). Passive dilution of 5hmC has also been reported in primordial germ cells (PGCs) during the second wave of DNA demethylation observed in these cells (Hackett et al. 2013). Notably, 5hmC levels are dramatically reduced during *in vitro* expansion of activated mouse CD4⁺ and CD8⁺ T cells (Tsagaratou and Rao, unpubl.) as well as in many cancers (Ko et al. 2010; Pfeifer et al. 2013) and normal proliferating tissues (Pfeifer et al. 2013), suggesting that passive 5hmC dilution occurs in these cases as well.

Active DNA Demethylation through DNA Repair

The DNA repair enzyme TDG (thymine DNA glycosylase) was originally identified as recognizing and repairing T:G mismatches in DNA. However, TDG also efficiently excises 5fC and 5caC that are properly paired to G in double-strand DNA (Fig. 2) (He et al. 2011), and depletion of TDG in ES cells results in increased levels of 5fC and 5caC at specific genomic locations (Shen et al. 2013; Song et al. 2013). Consistent with the hypothesis that TDG-mediated excision of 5fC and 5caC constitutes the last step of a pathway of TET-mediated active DNA demethylation, TDG is required for embryonic develop-

ment and TDG-null embryos as well as embryos that bear a catalytically inactive mutation of TDG and show epigenetic abnormalities, with predominant reduction in the expression of *Hox* genes because of aberrant methylation observed in their regulatory sequences (Cortazar et al. 2011; Cortellino et al. 2011).

BIOLOGICAL FUNCTIONS OF TET PROTEINS AND 5mC

TET enzymes are widely expressed, with at least one member being represented in every cell type examined. Tet1 and Tet2 are the two major TET proteins expressed in mouse ES cells, whereas Tet3 is barely expressed (Fig. 3); Tet1 and Tet2 are also high in PGCs and in the inner cell mass of the mouse embryo from which ES cells are derived. Tet1 mRNA expression drops rapidly in differentiating mouse ES cells (Koh et al. 2011), whereas Tet2 protein levels are regulated by Idax during ES cell differentiation (Ko et al. 2013). Tet2 and Tet3 are the major TET proteins expressed in differentiated tissues and cell types (Fig. 3). Tet1-deficient mice on a pure C57BL/6 genetic background are born below Mendelian ratios and display female sterility (Kang and Rao, unpubl.) and Tet2-deficient mice are viable and fertile (Ko et al. 2011) with a mild hematopoietic phenotype, whereas Tet3-deficient mice are perinatally lethal (Gu et al. 2011; Li and Rao, unpubl.).

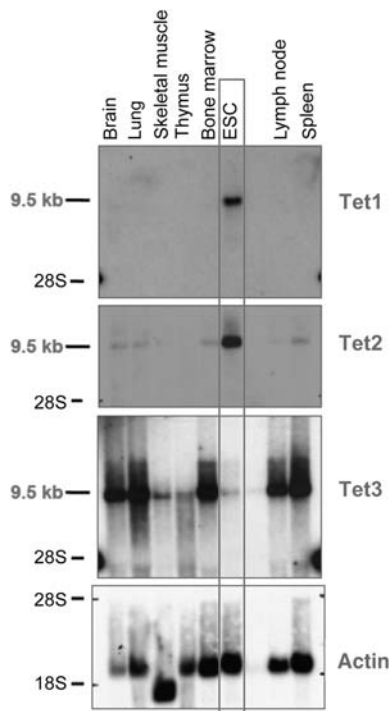


Figure 3. Northern blot analysis of TET mRNA expression in different murine tissues. Probes for actin were used for normalization. Mouse embryonic stem cells (ESCs), which primarily express Tet1 and Tet2, are highlighted. Tet3 is not expressed in ESC but is abundantly expressed in all of the differentiated cell types.

There have been extensive studies of 5hmC distribution in ES cells and brain. 5hmC is found predominantly in euchromatic regions, suggesting that it is associated with gene activity (Ficz et al. 2011). In mouse ES cells, Tet1 and 5hmC are enriched at gene bodies, TSSs, promoters, and genomic regions with moderate CpG density (Ficz et al. 2011; Pastor et al. 2011; Williams et al. 2011; Wu et al. 2011a,b; Xu et al. 2011). CGIs that bind Tet1 in undifferentiated ES cells show an increase in 5mC upon Tet1 deletion (Wu et al. 2011a), implicating Tet1 in maintaining CGI hypomethylation (Williams et al. 2012), either by blocking the DNA methylation machinery or by promoting DNA demethylation. There is also evidence for specific enrichment of 5hmC at “bivalent” gene promoters marked with H3K4me3 and H3K27me3 histone modifications, associated with transcriptionally active and silenced genes, respectively (Gu et al. 2011; Pastor et al. 2011; Williams et al. 2011; Wu et al. 2011a). This dual modification is thought to mark genes that are poised for activation in response to an external signal (Bernstein et al. 2006). Finally, 5hmC is located at many intergenic *cis*-regulatory elements such as active enhancers, pluripotent transcription factor-binding sites, and insulator-binding sites (Ficz et al. 2011; Pastor et al. 2011; Wu et al. 2011a; Yu et al. 2012); indeed LMRs, which correspond generally to gene-distal regulatory elements, show enrichment for TET1 binding and 5hmC (Stadler et al. 2011).

The presence of 5hmC in gene bodies correlates with gene expression in mouse and human ES cells (Ficz et al. 2011; Pastor et al. 2011; Stroud et al. 2011; Szulwach et al. 2011a; Wu et al. 2011a; Xu et al. 2011); in neural cells (Mellen et al. 2012) during neurogenesis, where the gain of 5hmC is accompanied by loss of H3K27me3 at promoters and gene bodies (Hahn et al. 2013); in mouse and human melanomas (Lian et al. 2012); in diverse other cell types and tissues including thymocytes and peripheral T cells (Tsagaratou and Rao, unpubl.). On the basis of our studies in undifferentiated mouse ES cells (Koh et al. 2011; Huang et al. 2014), it seems likely that Tet2 is primarily responsible for depositing gene-body 5hmC. Briefly, the use of ES cells depleted of either Tet1 or Tet2 showed that Tet1 primarily regulated 5hmC levels at promoter/TSS regions, whereas Tet2 primarily regulated 5hmC in the gene bodies of highly expressed genes (Huang et al. 2014). This difference may reflect the fact that Tet1 possesses a CXXC domain that would be expected to bind unmethylated CpGs and therefore would be targeted to CpG islands and high CpG promoters, as is in fact observed (Wu and Zhang 2011; Williams et al. 2012); in contrast, Tet2 would not be expected to be targeted preferentially to promoters because IDAX, the separated CXXC domain of Tet2, is not expressed in undifferentiated ES cells (Fig. 1B) (Ko et al. 2013). The ability of Tet2 to control 5hmC deposition at gene bodies may reflect its reported association with the SET1/COMPASS complex (Deplus et al. 2013) that travels with RNA polymerase II (Shilatifard 2012). Overall, Tet1 and Tet2 have distinct as well as redundant functions in mouse ES cells; similarly, Tet2 and Tet3 may have some overlapping as well as distinct functions in differentiated cell

types, including T cells and hematopoietic precursor cells (Tsagaratou and Rao, unpubl.).

DNA METHYLATION, TET PROTEINS, AND 5hmC IN T CELLS

It is well established that gene expression in T cells, as in other cell types, is influenced by DNA methylation: (1) Genes such as *Cd4* are sequentially activated and repressed during different stages of T-cell development, in a manner thought to be epigenetically controlled (Taniuchi et al. 2002). (2) The up-regulation of *Lck* gene transcription from the DN1 to the DN3 stage of thymic development is correlated with progressive demethylation of CpGs in *Lck* exon 1 through intron 2 (Ji et al. 2010). (3) Naive CD4 T cells show an impressive plasticity and readily differentiate to Th1, Th2, and Th17 cells depending on the cytokine milieu and the environmental cues (inflammatory stimuli, micro-organisms) that they receive (Ansel et al. 2003; Zhou et al. 2009; Mukasa et al. 2010). Demethylation within the transcribed sequences of *Il4* and *Ifng* correlates with high expression levels of these cytokines in Th2 and Th1 cells, respectively (Lee et al. 2002; Ansel et al. 2006). (4) A small region in the promoter–enhancer of the *interleukin-2 (Il2)* gene is demethylated in T cells following activation (Bruniquel and Schwartz 2003). Demethylation occurs rapidly after transcriptional activation, suggesting that it may involve an active process independent of DNA replication (Bruniquel and Schwartz 2003; Murayama et al. 2006). *Il2* promoter demethylation stabilizes Oct1 binding and ensures that secondary activation is more rapid and intense (Murayama et al. 2006). (5) Naive T cells that lack expression of the DNA methyltransferase DNMT1 up-regulate sets of cytokines that are normally silenced and methylated (Lee et al. 2001), whereas *Dnmt1*-null CD8 T cells aberrantly up-regulate CD4 T-cell cytokines (Makar and Wilson 2004). (6) The methylation status of conserved noncoding sequence 2 (CNS2) in the first intron of the *Foxp3* gene influences the stability of *Foxp3* expression in the regulatory T-cell (Treg) lineage. The transcription factor Runx1 and its obligate partner, core-binding factor β (Cbf β), bind to this locus in conjunction with rapid demethylation, providing a window for *Foxp3* binding; this in turn stabilizes lineage progression over ensuing cell divisions in an autoregulatory loop (Zheng et al. 2010). The conclusion is that localized loss of DNA methylation at promoter–enhancer regions is associated not only with transcription factor binding but also with stabilization of DNA–protein interactions to ensure robust expression of target genes after their activation.

We previously showed that all three Tet proteins are expressed in CD4⁺ CD8⁺ double-positive (DP) thymocytes as well as CD4 single-positive (SP) and CD8 SP thymocytes (Ko et al. 2010). 5hmC levels in DP cells are lower than in CD4 and CD8 SP cells (0.7 compared with 2.5–3 pmol 5hmC/ μ g DNA, respectively); Th1 and Th2 cells derived from naive CD4 T cells and effector cytolytic T cells derived from naive CD8 T cells have

even lower levels of 5hmC (Tsagaratou ko, and Rao, unpubl.). Global or conditional deletion of individual Tet proteins in T cells (using *Tet2*^{-/-} mice and *Tet3fl/fl CD4Cre* mice) resulted in relatively normal T-cell development and function, but T cells that lacked Tet3 showed compensatory up-regulation of *Tet2* mRNA (Tsagaratou and Rao, unpubl.). Thus, for a full understanding of T function in T cells, it will be necessary to analyze the phenotypes of *Tet2*^{-/-} *Tet3 flx/flx CD4cre* mice, which lack both Tet2 and Tet3 in T cells.

METHODS FOR DETECTION AND MAPPING OF 5mC AND oxi-mC

Several methods have been developed to map modified cytosine species in the genome (summarized in Table 1). They can be roughly divided into affinity-based approaches for enriching genomic regions that contain the modified cytosines and methods for mapping the modified bases at single-nucleotide resolution (Song et al. 2012; Pastor et al. 2013; Wu and Zhang 2014). We describe some of the most useful methods here; commercial kits have been developed for hMe-Seal, TAB-seq, and oxidative bisulfite (oxBS).

Affinity Approaches

Immunoprecipitation of modified cytosines. The development of specific antibodies for each of the known modified cytosines—5mC, 5hmC, 5caC, and 5fC—enabled the immunoprecipitation (IP) and sequencing of genomic regions enriched for these modifications (Ficz et al. 2011; Williams et al. 2011; Wu et al. 2011a; Shen et al. 2013). The methylated DNA-IP (MeDIP) approach allows enrichment of methylated DNA by using an antibody that specifically recognizes methylated cytosines, whether or not they are in a CpG context (Weber et al. 2005). The DNA is denatured to convert it to a single-strand form, thus exposing the 5mCs to permit efficient IP of genomic fragments enriched for 5mC (Weber et al. 2005). In all these cases, the efficiency of IP is markedly density dependent, and the methods described below are preferred for 5hmC mapping (Pastor et al. 2013).

Table 1. Summary of the available methods used to detect 5mC and oxi-mCs

Base	Affinity-based approach	Single-nucleotide resolution
5mC	MeDIP	WGBS, RRBS
5hmC	CMS-IP, GLIB, 5hmC-IP, hMe-Seal, JBP1 IP	TAB-seq, oxBS-seq, oxRRBS
5fC	5fC-IP, 5fC-pull-down, 5fC-Seal	fCAB-seq
5caC	5caC IP	caCAB-seq

MeDIP, methylated DNA IP; WGBS, whole-genome bisulfite sequencing; RRBS, reduced-representation bisulfite sequencing; CMS, cytosine methylenesulfonate; GLIB, glucosylation, periodate oxidation, biotinylation; hMe-Seal, 5-hmC selective chemical labeling; TAB-seq, TET assisted bisulfite sequencing; oxBS-seq, oxidative bisulfite sequencing; 5fC-Seal, 5fC selective chemical labeling; CAB-seq, chemically assisted bisulfite sequencing.

Biotin modification of 5hmC followed by streptavidin pull-down. The enzyme β -glucosyl transferase from phage T4 very efficiently attaches glucose to the hydroxyl group of 5hmC, after which the glucose can be modified by oxidation with sodium periodate, followed by treatment with an aldehyde-reactive probe in a method termed GLIB (glucosylation, periodate oxidation, biotinylation) (Pastor et al. 2011, 2012). A similar method that uses click chemistry to attach the biotin has been developed (5hmC-selective chemical labeling, hMe-Seal [Song et al. 2011, 2012]).

IP of the 5hmC derivative, cytosine 5-methylenesulfonate. This method takes advantage of the fact that treatment of DNA with sodium bisulfite efficiently converts 5hmC in single-strand DNA to a stable and highly antigenic derivative, cytosine 5-methylenesulfonate (CMS) (Ko et al. 2010). Antibodies to CMS can thus be used to immunoprecipitate and sequence 5hmC containing fragments of genomic DNA with high specificity and low background; the method is much less density dependent

than IP with antibodies to 5hmC itself (Pastor et al. 2011; Huang et al. 2012). Numerous studies have used this method to map 5hmC efficiently in different systems (Pastor et al. 2011; Jeong et al. 2013; Lister et al. 2013; Vincent et al. 2013; Huang et al. 2014).

Single-Nucleotide Resolution Approaches

Most single-base-resolution methods used to map oximC species involve a step of treatment of the DNA with sodium bisulfite. Bisulfite treatment has long been used to distinguish unmodified cytosine from 5mC, because it results in the deamination of unmodified cytosine to uracil, which is subsequently read as thymine after polymerase chain reaction (PCR) amplification. In contrast, methylated cytosines (and CMS) are resistant to bisulfite conversion and are read as cytosines, enabling quantitative discrimination between unmodified cytosines and 5mC (Frommer et al. 1992). It is now recognized, however, that bisulfite treatment cannot distinguish 5mC from 5hmC or C from 5fC and 5caC (Fig. 4A; Table 2).

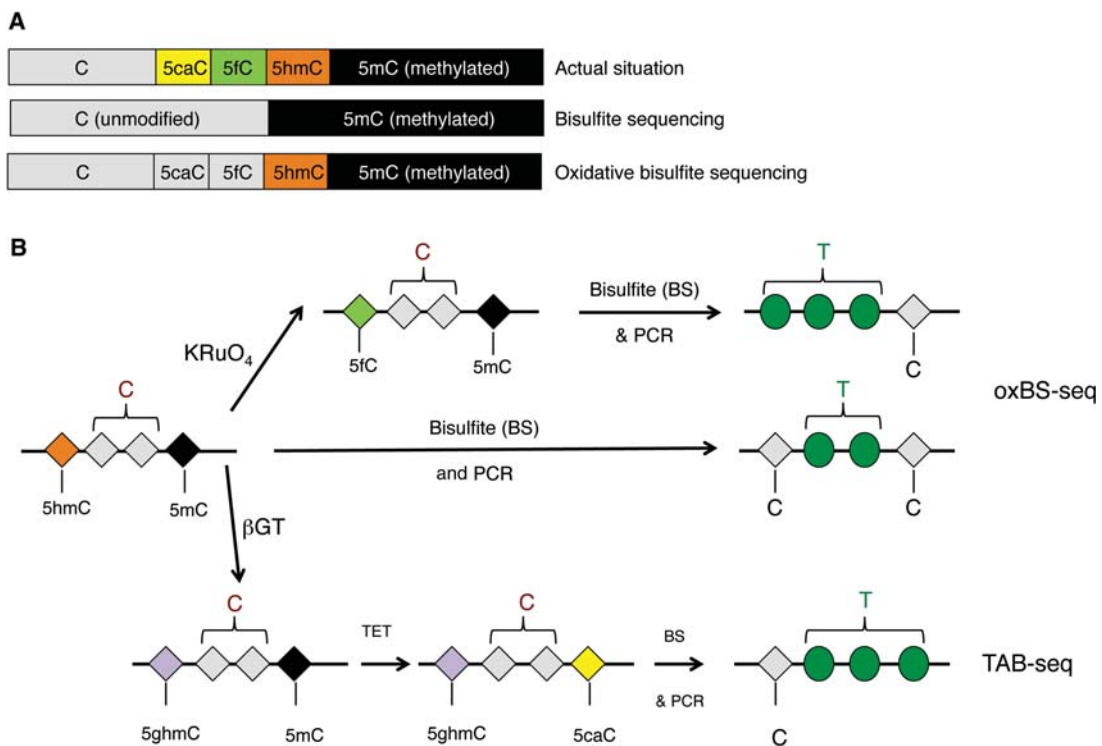


Figure 4. (A, top) Bisulfite sequencing (BS-seq) cannot discriminate methylated C from 5hmC, because both remain unconverted after bisulfite treatment and PCR amplification. (Middle) The actual state of C in the genome can be unmethylated C, 5caC, 5fC, 5hmC, or 5mC. (Bottom) The advent of oxidative bisulfite (oxBS) sequencing, which introduces chemical treatment of DNA before the bisulfite treatment, allows discrimination of 5hmC from 5mC; however, the unmodified C is still not distinguished from 5caC or 5fC. (B) Schematic representation of oxBS-seq and TAB-seq, two methods that enable detection of 5hmC at single-nucleotide resolution when combined with bisulfite sequencing. In the oxBS approach (top), DNA is treated with potassium perruthenate ($KRuO_4$) that oxidizes 5hmC to 5fC. Subsequent bisulfite treatment converts 5fC (as well as 5caC and C) to U that will be read as T after PCR amplification, so only 5mC stays unconverted and is thus read as C after PCR amplification. In contrast, in DNA samples that have been subjected exclusively to bisulfite treatment, both 5hmC and 5mC are read as C after PCR amplification, so 5mC, 5hmC, and “unmodified” C can be discriminated by subtracting the results of BS-seq from those of oxBS. In TAB-seq (TET-assisted bisulfite sequencing), the DNA is treated with T2 phage β -glucosyltransferase (β GT) that specifically recognizes and glycosylates 5hmC, generating glucosylated 5hmC (5ghmC). Subsequent treatment with purified and active TET1 leads to oxidation of 5mC to 5caC, and finally, treatment of the sample with bisulfite results in complete conversion of all the bases to U, read as T after PCR amplification, with the exception of 5ghmC that stays unconverted and thus is read as C.

Table 2. Behavior of 5mC and its oxi-mCs in bisulfite conversion and the following PCR step

Base	Bisulfite conversion	BS sequencing result
5mC	5mC	C
5hmC	CMS	C
5gmC	5gmC	C
5fC	U	T
5caC	U	T
C	U	T

CMS, cytosine, methylenesulfonate; 5gmC, i.e., cytosine 5- β -glucosyl-5-hydroxymethylcytosine.

TAB-seq. TET-assisted BS-seq has been used to map 5hmC quantitatively at single-base resolution (Yu et al. 2012). In this method, treatment with β GT blocks 5hmC, and subsequently, recombinant mouse TET1 is used to convert 5mC to oxi-mC—ideally, all the way to 5caC (Fig. 4B). 5caC (originally 5mC) and unmodified C are both converted by bisulfite treatment to uracil; thus, the only base that is read as cytosine is the glucosylated 5hmC (Fig. 4B). The main caveat of this approach is that highly active TET enzyme is required for efficient conversion of 5mC to 5caC: If the conversion is inefficient such that there is no conversion of the 5mC or the conversion does not proceed beyond 5hmC, there will be false-positive base calling of 5hmC at sites that were actually 5mC. Bisulfite treatment can also be inefficient: Even with a high efficiency of 5mC oxidation by TET1 at sites with 80%–100% 5mC, if only 95% of the unmodified C is converted to uracil, the remaining 5% of unconverted 5mCs will be falsely identified as 5hmC (Yu et al. 2012).

Oxidative bisulfite sequencing. This is a chemical conversion method that also allows the quantitative mapping of 5hmC in genomic DNA at single-nucleotide resolution. Potassium perruthenate selectively oxidizes 5hmC to 5fC, and subsequent bisulfite treatment of the DNA converts 5fC to U (Fig. 4B). By comparing the data from oxidized and bisulfite-treated DNA with those from only bisulfite-treated DNA, it is possible to distinguish 5mC, 5hmC, and unmodified C from one another. However, bases that are read as T can be derived from unmodified C, 5fC, or 5caC (Booth et al. 2012). The method is more cost effective than TAB-seq; however, one needs to be cautious, because the method is very sensitive to contaminants in the isolated genomic DNA (Booth et al. 2013) and also tends to damage DNA, resulting in biases because of overamplification.

IP of the 5hmC derivative CMS. An advantage of the IP of the 5hmC derivative CMS (CMS-IP) method is that the modification status of cytosines in the immunoprecipitated DNA fragments can be estimated by determining whether they are read as T (i.e., were originally C, 5fC, or 5caC) or remain as C (in which case they were originally 5mC or 5hmC converted to CMS). Moreover, the input DNA can be analyzed by BS-seq to yield the methylation status of all cytosines in the genome.

Effect of Sequencing Depth on Mapping Performance

Because of the relatively low levels of 5hmC in the genome, high coverage is needed to sequence this base quantitatively. Furthermore, for the oxBS and TAB-seq single-base resolution methods, a subtraction is needed to obtain the actual levels of 5hmC. As a result, there is an increase in the “noise level,” thus further increasing the need to increase both coverage and the number of biological replicates.

In our experience and at current sequencing costs, a range of ~ 30 million reads per sample for MeDIP-seq, CMS-IP, and hMe-Seal constitutes a viable compromise between breadth and depth of sequencing. In contrast, whole-genome bisulfite sequencing and oxBS provide comprehensive genomic coverage at the cost of having to sequence more than a billion reads per sample, which increases the cost of the experiment tremendously. An alternative approach would be to combine TAB-seq and oxBS-seq with reduced-representation bisulfite sequencing (RRBS), which allows selective, but deep, sequencing of a fraction of the genome that is highly enriched for CpG islands (Meissner et al. 2008); in this case, only ~ 25 million 40-bp short reads are required to achieve an average sequencing depth of $\sim 120\times$ by combining oxBS-Seq with RRBS (oxRRBS) (Booth et al. 2012), making this method cost efficient and suitable for some applications, although it captures only a fraction of 5mC and thus 5hmC sites in the genome ($< 10\%$ of the 28 million CpGs [Bock et al. 2010] and $\sim 11.5\%$ of the dynamically active CpGs that overlap with differentially methylated and hydroxymethylated regions, e.g., during development or during different time points of differentiation/treatment with different substances [Ziller et al. 2013]).

Overall, in our opinion, an approach that is cost efficient without compromising the quality of the data and thus the confidence of the conclusions deduced would be to combine affinity-based approaches such as CMS-IP to assess 5mC and oxi-mC at a genome-wide level and then apply single-nucleotide resolution approaches to specific loci of interest.

CONCLUSIONS

The oxi-mC species, 5hmC, 5fC, and 5caC, are now established as important intermediates in DNA demethylation as well as potential epigenetic marks that regulate chromatin structure by recruiting interacting proteins. Despite extensive research in numerous biological systems, the physiological functions of these modifications remain elusive. For instance, what targets TET proteins to specific regions of DNA? Is the correlation of high gene-body 5hmC with high gene expression merely a by-product of gene transcription or does the presence of 5hmC actually facilitate transcription? The large data sets that have been generated provide stimulating ideas but are far from providing conclusive information. The advent of new sequencing approaches, the increase in depth of sequencing coverage, the study of more biological systems

in combination with detailed experimental strategies for monitoring gene expression, the use of incisive mouse models, and a strong focus on individual genes will allow us to gain a better understanding of the biological functions of TET proteins and the oxi-mC modifications that they generate, in the context of physiological development as well as disease.

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