

Regulation of 5-Hydroxymethylcytosine Distribution by the TET Enzymes



John Z. Cao, Anastasia E. Hains, and Lucy A. Godley

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Authors “John Z. Cao” and “Anastasia E. Hains” contributed equally to this chapter.

J. Z. Cao

Committee on Cancer Biology, University of Chicago, Chicago, IL, USA

A. E. Hains

Program on Molecular Pathogenesis and Molecular Medicine, University of Chicago, Chicago, IL, USA

L. A. Godley (✉)

Committee on Cancer Biology, University of Chicago, Chicago, IL, USA

Section of Hematology/Oncology, Department of Medicine, University of Chicago, Chicago, IL, USA

e-mail: lgodley@medicine.bsd.uchicago.edu

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Abstract 5-Hydroxymethylcytosine (5-hmC) has a critical role in the epigenetic regulation of many cellular processes, including differentiation, cytokine responses, and memory formation. This modified base is generated by the TET enzymes through oxidizing 5-methylcytosine and can be further oxidized to 5-formylcytosine and 5-carboxylcytosine. The level and distribution of 5-hmC in the genome is tightly controlled through transcription of *TET* genes, post-translational modifications on the TET enzymes, and various interacting partners of TET enzymes. Deregulation of 5-hmC is often found in pathological conditions and plays crucial roles in disease progression. This chapter will discuss recent findings regarding 5-hmC functions, TET proteins, and their gene regulations, as well as their relationships to cancer.

Keywords TET enzymes · 5-Hydroxymethylcytosine · 5-Methylcytosine · CpG · Demethylation

1 Introduction

In mammalian DNA, cytosine bases found within the context of a 5'-CpG-3' dinucleotide are substrates for covalent modification to 5-methylcytosine (5-mC) by the DNA methyltransferases DNMT1, DNMT3A, and DNMT3B, which then can be oxidized sequentially by Ten-Eleven Translocation methylcytosine dioxygenases TET1, TET2, and TET3 to produce 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC) (Wu and Zhang 2017) (Fig. 1a). With the discovery of TET catalytic activity (Iyer et al. 2009; Tahiliani et al. 2009), there have been great advances in understanding the role of 5-hmC in various biological processes. Emerging evidence indicates that 5-hmC plays important roles in many cellular processes, including differentiation, cytokine responses, and memory formation (Cao 2016; Vogel Ciernia and LaSalle 2016; An et al. 2017). In this chapter, we discuss the regulation of TET enzymes and their roles in modulating the 5-hmC landscape as well as the impact of TET and 5-hmC deregulation in human disease.

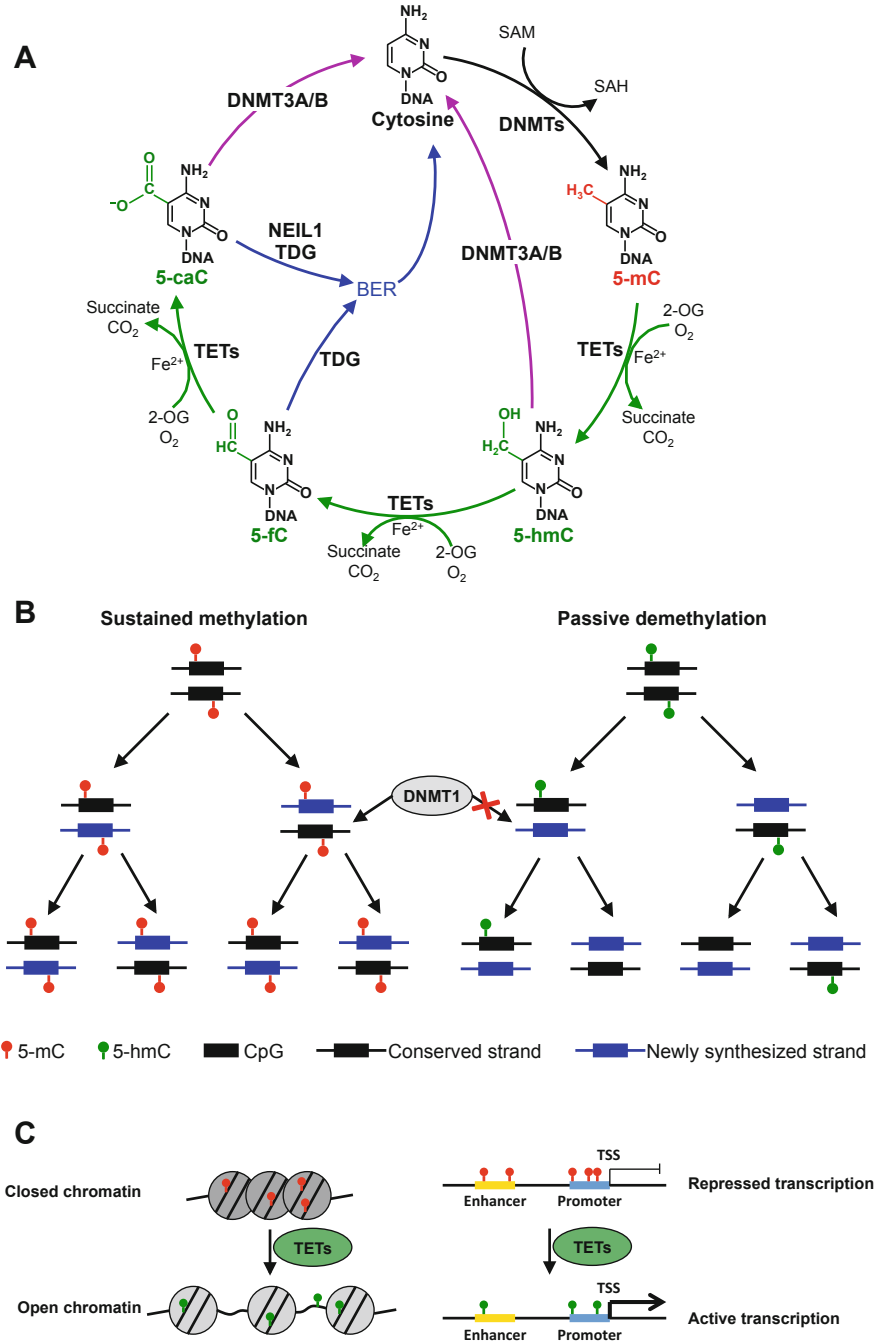


Fig. 1 Regulation and functions of 5-hmC. (a) Biochemical pathways that control cytosine modifications. Unmodified cytosine is methylated to 5-mC by DNMTs using SAM as methyl

2 TET and 5-hmC Functions

2.1 Mammalian Cytosine Modifications

In mammalian genomes, cytosines present within 5'-CpG-3' dinucleotides can be methylated to 5-mC by DNMTs using S-adenosylmethionine (SAM) as the methyl group donor. DNMT1 has optimal activity in methylating hemimethylated cytosines and thus functions mainly to maintain DNA methylation patterns after DNA replication (Lee et al. 2014). In contrast, DNMT3A and DNMT3B function mainly as *de novo* methyltransferases (Lee et al. 2014) (Fig. 1b). Many studies published since the 1980s have established that 5-mC is a base that regulates chromatin function: When found within gene bodies, 5-mC is associated with gene expression (Jjingo et al. 2012; Jones 2012; Varley et al. 2013), and when concentrated in CpG islands that overlap gene promoters, the base represses gene transcription, a phenomenon commonly observed in cancer cells (Feinberg and Vogelstein 1983; Gama-Sosa et al. 1983; Baylin et al. 1986; Feinberg and Tycko 2004).

The first oxidized product of 5-mC, 5-hmC, was originally identified as a specialized base in T2, T4, and T6 bacteriophages (Wyatt and Cohen 1953). Although the presence of 5-hmC was also observed in mammalian DNA, it was believed that this base was the result of oxidative damage (Tardy-Planechaud et al. 1997; Valinluck and Sowers 2007). 5-hmC did not receive major attention as a chromatin regulating base until it was discovered that 5-hmC is the oxidized product of TET dioxygenase activity using 5-mC as a substrate (Iyer et al. 2009; Tahiliani et al. 2009). Since then, multiple 5-hmC specific assays have been developed (Table 1), which have facilitated the elucidation of 5-hmC functions.

Fig. 1 (continued) donor. TETs oxidize 5-mC to 5-hmC, 5-fC, and 5-caC stepwise using 2-OG and O₂ as co-substrates and Fe²⁺ as a co-factor (green arrows). 5-hmC and 5-caC can be converted to unmodified cytosine *in vitro* by direct removal of 5-modifications through DNMT3A/B (purple arrows). 5-fC and 5-caC can be excised by TDG and/or NEIL1 and replaced with unmodified cytosine through base-excision repair (blue arrows). **(b)** 5-hmC promotes passive demethylation through replication. Newly synthesized DNA contains no modified cytosines. When the CpG dinucleotide on the old strand is methylated, DNMT1 can bind and methylate the complementary CpG dinucleotide on the newly synthesized strand, maintaining methylation over DNA replication and cell division. When the CpG dinucleotide in the old strand is hydroxymethylated, however, if DNMT1 fails to bind, the newly synthesized strand will remain unmodified, leading to loss of modification through multiple rounds of DNA replication. **(c)** Stable 5-hmC promotes open chromatin and gene transcription. Abbreviations: 2-OG 2-oxoglutarate, 5-mC 5-methylcytosine, 5-hmC 5-hydroxymethylcytosine, 5-fC 5-formylcytosine, 5-caC 5-carboxylcytosine, 5-mod 5-modified cytosine, BER base-excision repair, SAM S-adenosyl methionine, SAH S-adenosyl homocysteine, TSS transcription start site

Table 1 Commonly used methods that specifically detect 5-hmC

| Method | Name | Description | Resolution | Limitation | References |
|---------------------|-----------------|---|-------------|--|---------------------------|
| Mass spectrometry | HPLC-MS/MS | Digested nucleoside separated by liquid chromatography and measured by tandem mass-spectrometry | Global | Cannot detect local cytosine modifications | Fernandez et al. (2018) |
| Affinity | hMe-SEAL | Streptavidin pull-down of biotin-labeled 5-hmC in fragmented DNA | 50–100 bp | Requires expensive specialized reagents | Song et al. (2011) |
| Affinity | hMe-DIP | Immunoprecipitation of fragmented DNA with 5-hmC specific antibody | 50–100 bp | Antibody specificity | Nestor and Meehan (2014) |
| Affinity | JBP1-seq | JBP1 affinity pull-down of glycosylated 5-hmC in fragmented DNA | 50–100 bp | Requires specialized beads | Robertson et al. (2012) |
| Redox | TAB-seq | TET-mediated oxidation of 5-mC to 5-caC, while 5-hmC is protected by glycosylation, followed by bisulfite treatment | Single-base | Expensive enzyme | Yu et al. (2012) |
| Redox | oxBS-seq | Selective oxidation of 5-hmC to 5-fC followed by bisulfite treatment | Single-base | Requires parallel bisulfite sequencing | Booth et al. (2013) |
| Restriction enzymes | MspJ1 family | Digest DNA near modified cytosine except glycosylated 5-hmC | Single-base | Sequence specificity of the enzyme | Cohen-Karni et al. (2011) |
| Restriction enzymes | PvuRtsII family | 5-hmC specific restriction enzyme | Single-base | Sequence specificity of the enzyme | Wang et al. (2011) |

2.2 Roles of 5-hmC

5-hmC is a relatively rare base in mammalian genomes, and its abundance is highly tissue dependent. For example, 5-hmC consists of 0.01–0.3% of all cytosine species in myeloid cells (Ko et al. 2010; Madzo et al. 2014); constitutes around 1% of all cytosines in cerebral tissue (Kriaucionis and Heintz 2009; Wen et al. 2014; Field et al. 2015); and is present at levels as high as 5% of all cytosines in undifferentiated embryonic stem (ES) cells (Tahiliani et al. 2009). Currently, two major functions of 5-hmC are proposed: the base is a component of the demethylation process, and the base can also function as a stable epigenetic mark with distinct roles in chromatin structure and transcriptional regulation.

5-hmC facilitates demethylation both within passive and active demethylation pathways (Fig. 1). When DNA is replicated, a newly inserted cytosine on the daughter strand is unmodified at first. If the cytosine base within the parent 5'-CpG-3' dinucleotide is methylated, then DNMT1 methylates the cytosine within

the newly synthesized strand since its highest activity is on hemi-methylated CpG dinucleotides, and DNMT1 sits at the replication fork, thereby maintaining the DNA methylation pattern (Fig. 1b). However, if the cytosine base within the parent DNA strand is hydroxymethylated, then maintenance of this base in the face of DNA replication requires two enzymes, first a DNMT to produce 5-mC, and then a TET to convert that base to 5-hmC. Therefore, in the face of DNA replication, if a DNMT fails to recognize a 5-hmC, that base will result in an unmodified cytosine in the daughter strand. This process is referred to as passive demethylation (Valinluck and Sowers 2007; Seiler et al. 2018). A more active and cell-cycle independent demethylation pathway involves further oxidation of 5-hmC by TETs to 5-fC and 5-caC (Ito et al. 2011), both of which can be replaced by unmodified cytosines through base-excision repair (BER) by thymine-DNA glucosylase (TDG) (He et al. 2011; Maiti and Drohat 2011; Coey et al. 2016; Pidugu et al. 2016) (Fig. 1a). 5-caC, but not 5-fC, can also undergo BER by Nei-like 1 (NEIL1) excision (Slyvka et al. 2017) (Fig. 1a). In addition to BER-mediated demethylation, some studies have also found that DNMT3A/3B have the ability to remove the 5-modification from 5-hmC and 5-caC directly, but not from 5-mC or 5-fC, resulting in unmodified cytosine (Chen et al. 2012; Liutkevičiūtė et al. 2014) (Fig. 1a).

5-hmC can also function as a stable and distinct epigenetic mark associated with active gene transcription. Many reports have found that 5-hmC is strongly associated with promoters, enhancers, and transcription factor binding sites (Madzo et al. 2014; Mariani et al. 2014; Vasanthakumar and Godley 2015) (Fig. 1c). Similarly, 5-hmC was shown to be associated with active transcription and histone modifications that mark open chromatin (Ficz et al. 2011; Szulwach et al. 2011; Madzo et al. 2014; Lin et al. 2017) (Fig. 1c). Evidence for the role of 5-hmC as a stable and actively maintained epigenetic mark is most apparent in the context of cell differentiation, where gene expression and chromatin structure undergo major changes. For example, in the course of erythropoiesis, 5-hmC at certain genomic loci remains highly enriched despite a global decrease in total 5-hmC level and multiple rounds of DNA replication (Madzo et al. 2014). To maintain 5-hmC during consecutive rounds of DNA replication, both DNMTs and TETs must be involved, indicating a functional role of 5-hmC at these loci.

In addition to its role in regulating gene expression and chromatin structure, recent studies have revealed potential involvement of 5-hmC in DNA damage repair. During murine embryonic development, *Tet1* loss-of-function oocytes have a much higher rate of defective meiosis and unresolved double strand breaks compared to those with wild-type *Tet1* (Yamaguchi et al. 2012). In addition, DNA damage in mouse Purkinje cells leads to increased 5-hmC levels (Jiang et al. 2015). Further studies in mouse ES cells showed that knock-out (KO) of all three *Tet* genes led to a high level of mitotic defects, as measured by the presence of chromatin fragments in anaphase, which resulted from a higher sensitivity to replication stress and delayed DNA repair (Kafer et al. 2016). In human cancer cell lines, 5-hmC foci co-localize with DNA damage foci marked by γ H2AX and 53BP1 upon induced DNA damage (Kafer et al. 2016). When *TET2* is knocked-down with shRNA, 5-hmC foci no longer form, suggesting that *TET2* is directly involved in the DNA damage response

(Kafer et al. 2016). It is possible that 5-hmC promotes local chromatin remodeling or serves as an epigenetic mark to recruit additional DNA damage repair machinery (Kafer et al. 2016).

2.3 TET Protein Structure and Function

TET genes are present in all metazoan genomes. In mammals and other gnathostomata, the *TET* gene underwent triplication to generate *TET1*, *TET2*, and *TET3*. During this process, *TET2* likely underwent a local chromosome inversion causing the 5' end of the inversion to create a distinct gene, *CXXC4* (or *IDAX*) (Iyer et al. 2009; Ko et al. 2013). All three *TET* genes are transcribed and translated into catalytically functional TET enzymes.

All three TET proteins share a C-terminal catalytic domain, whereas TET1 and TET3 share an N-terminal CXXC domain (Fig. 2a). The catalytic domains consist of a cysteine-rich domain and a double-stranded β -helix (DSBH) domain, together forming a globular structure with the core DNA-binding DSBH in the center (Hu et al. 2013, 2015) (Fig. 2b). TET catalytic domains specifically recognize and bind CpGs and flip the modified cytosine base into the catalytic cavity, with no preference for flanking sequences (Hu et al. 2013) (Fig. 2b). This base flipping mechanism appears to be conserved evolutionally, as the same mechanism is found in the *Naegleria gruberi* Tet enzyme (Hashimoto et al. 2014, 2015).

TET proteins are 2-oxoglutarate (2OG) and oxygen (O₂) dependent dioxygenases that use Fe(II) as a co-factor. TET catalytic domains share high homology to the previously discovered J-binding proteins (JBP1/2) (Iyer et al. 2009). JBP1/2 are dioxygenases in kinetoplastids that catalyze the oxidation of thymine at the methyl group to carboxyuracil, which is the first catalytic step towards base J. Based on this homology, the catalytic function of oxidizing 5-mC to 5-hmC was identified for the TETs (Tahiliani et al. 2009). Additional studies found that TETs can oxidize 5-hmC further to 5-fC and 5-caC in a stepwise fashion (He et al. 2011; Ito et al. 2011). In mammalian cells, the levels of 5-fC and 5-caC are 2–3 orders of magnitude lower than those of 5-hmC (Ito et al. 2011).

Enzyme kinetic studies showed that the K_m values of TETs for oxygen, Fe(II), and 2OG are about 30 μ M, 4 μ M, and 60 μ M, respectively (Laukka et al. 2015). This study also showed that TETs bind modified CpGs (5-mC, 5-hmC, or 5-fC) at around 100 nM. At a minimum, TET enzymes are two- to threefold more efficient at oxidizing 5-mC compared to 5-hmC or 5-fC due to differences in the conformation of the active sites (Hu et al. 2015), which leads to faster 5-hmC creation than its removal through further oxidation. The catalytic differences, together with TDG/NEIL1 mediated removal of 5-fC and 5-caC, may explain the relative abundance of 5-hmC over 5-fC and 5-caC.

The affinity of TETs for oxygen is much higher than that of the HIF prolyl-hydroxylases, which are 2OG/O₂ dependent dioxygenases like TETs that hydroxylate hypoxia-inducible factor α (HIF α) to induce HIF α degradation (Laukka et al.

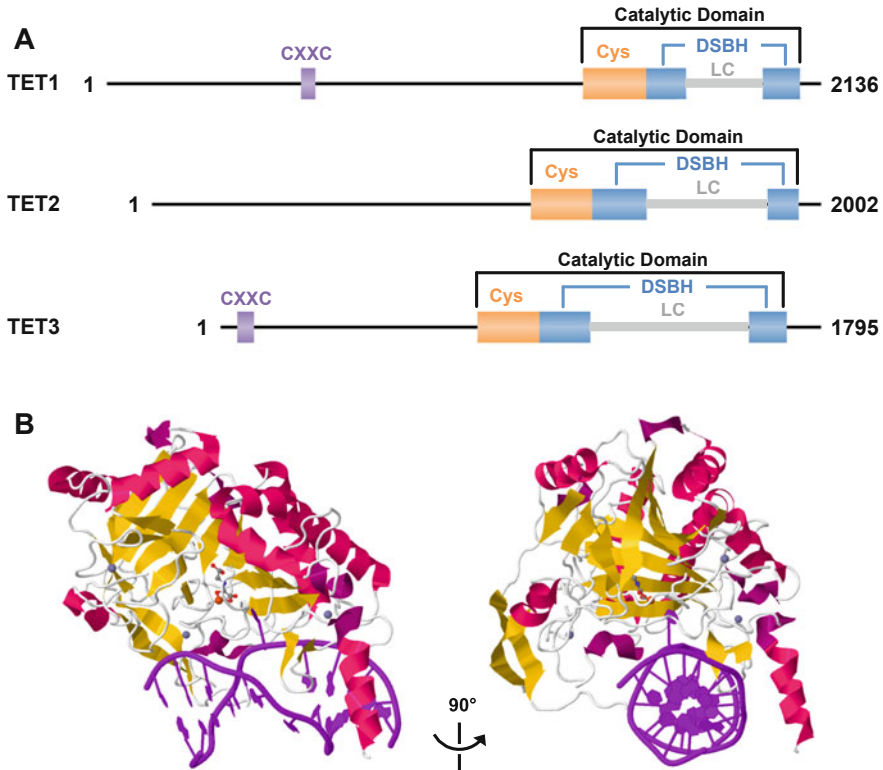


Fig. 2 TET protein domains and binding with DNA. **(a)** Human TET proteins. All three TETs have conserved C-terminal catalytic domain, which consists of a cysteine-rich domain (Cys) and two double-stranded beta-helix (DSBH) DNA binding domains. The two DSBH domains are separated by a low complexity (LC) insert. Both TET1 and TET3 have a CpG binding CXXC domain near the N-terminus. During evolution, the sequences encoding the CXXC domain of TET2 became an independent gene: *CXXC4/IDAX*. **(b)** The crystal structure of TET2:DNA binding (Hu et al. 2013). The TET2 catalytic domain forms a double-stranded beta-helix (DSBH, yellow) structure that binds DNA modified CpG (purple). The DSBH structure is stabilized by several surrounding alpha-helices (red). The modified cytosine base is flipped out from the DNA double-helix and inserted in the active site along with 2-oxoglutarate and oxygen. Protein Data Bank (PDB) ID: 4NM6 (Hu, L., Li, Z., Cheng, J., Rao, Q., Gong, W., Liu, M., Shi, Y.G., Zhu, J., Wang, P., and Xu, Y. (2013). Crystal structure of TET2-DNA complex: insight into TET-mediated 5mC oxidation. *Cell* 155, 1545–1555.), image created with Jmol (Jmol: an open-source Java viewer for chemical structures in 3D. <http://www.jmol.org/>)

2015). Although both of these enzyme families use O_2 as a substrate, the higher affinity for O_2 in TETs suggests that the TETs may remain functional in hypoxic environments. Later studies showed that TETs retain catalytic activity under physiological hypoxia, at O_2 concentrations at which the HIF prolyl-hydroxylases no longer function. The TETs may act with less efficiency or may lose their activities under the pathological hypoxia found in solid tumors (Thienpont et al. 2016).

2OG is produced from isocitrate by the isocitrate dehydrogenases (IDH1/2) in the TCA cycle. It is usually abundant in cells and is not limiting for TET activities. However, 2OG analogs can accumulate in pathological conditions as a result of mutations in genes encoding TCA cycle enzymes (Pollard et al. 2005; Dang et al. 2009). The IDH1 R100/R132 and IDH2 R140/R172 missense mutations commonly found in cancers catalyze the production of R-2-hydroxyglutarate (R-2-HG) (Dang et al. 2009; Losman and Kaelin 2013). R-2-HG competes with 2OG for TET binding, but cannot be used in the oxidation reaction and thus acts as an inhibitor of TETs (Xu et al. 2011a). Consistent with this finding, glioblastomas and leukemias with these *IDH* mutations display global hypermethylation (Figueroa et al. 2010; Ko et al. 2010; Turcan et al. 2012). In addition, *IDH* and *TET* mutations are almost always mutually exclusive in leukemias, strongly suggesting that the two enzymes function in the same cellular processes (Gaidzik et al. 2012; Patel et al. 2012). Two additional TCA cycle metabolites, fumarate and succinate, were found to accumulate in solid tumors with mutated fumarate hydratase (*FH*) or succinate dehydrogenase (*SDH*) (Pollard et al. 2005). Fumarate and succinate are also able to inhibit TET activity by competing with 2OG for TET binding (Laukka et al. 2015). This was demonstrated in SK-N-BE(2) neuroblastoma cells, where 5-hmC levels decreased 10–40% upon fumarate or succinate treatment (Laukka et al. 2015).

TET activity is enhanced by ascorbate (vitamin C) by maintaining the Fe co-factor in a reduced (II) state (Kuiper and Vissers 2014). Ascorbate is essential for the function of collagen prolyl-4-hydroxylase, another 2OG/O₂ dependent dioxygenase similar to the TETs. Ascorbate deficiency causes failure of prolyl hydroxylation and leads to scurvy (Gorres and Raines 2010). The addition of ascorbate to cultured mouse ES cells or mouse embryonic fibroblasts leads to a rapid increase in 5-hmC levels that is Tet dependent (Blaschke et al. 2013; Minor et al. 2013; Yin et al. 2013). Other studies showed demethylation in ascorbate treated human ES cells and mouse embryonic fibroblasts as well as significant changes in the transcriptome (Chung et al. 2010; Blaschke et al. 2013; Chen et al. 2013).

3 Normal Transcription of *TET* Genes and Its Impact on the 5-hmC Landscape

Highly regulated expression of *TET* genes is essential for proper maintenance of the 5-hmC landscape in steady state or epigenetic reprogramming. Despite the functional redundancy, expression of each *TET* gene is distinct depending on the developmental context and tissue type, and disrupted expression patterns can give rise to pathogenic phenotypes. Here, we highlight the regulation of *TET* expression and its impact on 5-hmC by examining ES cells and adult neuronal cells, two cell types where 5-hmC is highly abundant and undergoes dynamic changes. It should be noted that the majority of embryonic development studies were done in murine

systems, but there are supporting data obtained from studies in human cell lines. The species differences are denoted by mouse *Tet* and human *TET*.

3.1 TET Transcription and TET Protein Regulation of 5-hmC in Embryonic Development

Embryonic development is characterized by extensive epigenetic remodeling. Immediately after fertilization, 5-mC in the paternal zygotic genome is oxidized to 5-hmC and is further converted to unmodified cytosine through the demethylation pathways (Fig. 1a) (Mayer et al. 2000; Oswald et al. 2000). Mammalian ES cells have high levels of 5-hmC, which is enriched in enhancers, gene bodies, exons, transcription start sites, and 5'-untranslated regions of genes (Tahiliani et al. 2009; Pastor et al. 2011; Stroud et al. 2011; Wu and Zhang 2011). It is also enriched in the promoters of genes that feature both H3K27me3 and H3K4me3 marks, which are associated with open chromatin (Pastor et al. 2011; Stroud et al. 2011; Wu and Zhang 2011). These enrichments are often associated with elevated gene transcription (Ficz et al. 2011). Furthermore, throughout the development of the embryo, there are dynamic gains and losses of 5-hmC at specific regions of the genome, especially in the primordial germ cells (PGCs). Murine PGCs undergo their own demethylation events, between e9.5 and e10.5, in which there is global erasure of 5-mC (Hajkova et al. 2002; Seki et al. 2005). The TET enzymes are critical for 5-hmC changes in embryonic development and PGCs, as loss of all three *TET* genes is lethal and loss of one or combinations of two has distinct phenotypes (Ficz et al. 2011; Wu and Zhang 2011). During epigenetic remodeling of the developing embryo, expression of *TET1*, *TET2*, and *TET3* is tightly controlled.

3.2 Normal Tet1 Transcription in ES Cells and PGCs

In mammalian ES cells, normal *TET1* expression is very high and plays a role in maintaining the pluripotent state (Koh et al. 2011). *TET1* expression in ES cells is promoted by canonical ES factors, such as OCT3/4, MYC, and NANOG, and is reduced when these factors are absent (Koh et al. 2011; Neri et al. 2015). *TET1* expression decreases as cells differentiate (Neri et al. 2015). The decrease in expression is mediated by the polycomb complex, which epigenetically silences the transcription start sites of *TET1* through deposition of the repressive histone mark, H3K27me (Neri et al. 2015). Although it is known that *Tet1* is expressed as early as e9.25 in PGCs, it is not known what transcription factors control expression of *Tet1* (Vincent et al. 2013).

Truncated *TET1* RNAs have been identified in mouse and human embryonic systems. In mouse ES cells, there is a *Tet1* isoform switch after e14.5. Full length

Tet1 is expressed in early embryos, ES cells, and PGCs, but only a *Tet1* RNA that does not code for part of the N-terminus, including the CXXC domain (*Tet1s*), is expressed in adult tissues (Zhang et al. 2016). Despite having a lower chromatin affinity, *Tet1s* is still capable of binding DNA and its binding pattern is similar to that of full-length *Tet1* (Zhang et al. 2016). Nonetheless, when homozygous *Tet1s* male mice were crossed with WT female mice, the resulting pups featured higher levels of perinatal lethality and lower birth weights compared to pups from WT crosses (Zhang et al. 2016). These experiments indicate that full-length *Tet1* is important for normal embryonic development in mice, but not necessary in adult mice. The regulation of 5-hmC by *Tet1s* has yet to be studied in mice. A similar mechanism has been hypothesized in human systems: full-length TET1 is present in ES cells, and an alternate truncated protein that lack the CXXC domain is present in adult tissues (Good et al. 2017). This smaller isoform of TET1 has been implicated in many cancers, including breast, uterine, and ovarian cancers (Good et al. 2017). This isoform is catalytically active, but does not have as great an impact on gene expression as the full-length TET1 (Good et al. 2017). The mechanism behind this is unclear, although it has been shown that the TET1 short-isoform binds outside of CpG islands, a distinction from full-length TET1 (Good et al. 2017).

3.3 *Loss of Tet1 Disrupts 5-hmC Levels and Distribution in ES Cells and PGCs*

Loss of *Tet1* in ES cells has a measurable impact on total levels and distribution of 5-hmC. When *Tet1* was knocked down in mouse ES cells, there is a decrease of about 35–50% of total 5-hmC levels (Tahiliani et al. 2009; Dawlaty et al. 2011; Hon et al. 2014). Correspondingly, there is an increase in total 5-mC levels (Ito et al. 2010; Dawlaty et al. 2011). Loss of 5-hmC in *Tet1* knock-down (KD) ES cells has been localized to promoters, gene bodies, and a few intergenic regions (Wu and Zhang 2011; Huang et al. 2014). Similarly, *Tet1* KD in PGCs results in an increase in global 5-mC levels and a 45% reduction in 5-hmC (Yamaguchi et al. 2012).

In addition to aberrant cytosine modification patterns, loss of *Tet1* in ES cells and PGCs leads to deregulation of gene expression (Dawlaty et al. 2011). Specifically, there is reduced expression of pluripotent factors *Nanog*, *Oct4*, and *Sox2*, as well as other genes associated with pluripotency (Ito et al. 2010; Ficiz et al. 2011). In PGCs, loss of *Tet1* was found to reduce expression of some meiotic genes (Yamaguchi et al. 2012).

Loss of *Tet1* in ES cells and PGCs results in reduced growth and up-regulation of differentiation-associated genes (Ito et al. 2010; Pastor et al. 2011). When mouse ES cells that lack *Tet1* undergo differentiation, there is upregulation of genes encoding primary trophoctoderm factors (*Cdx2*, *Eomes*, and *Elf5*) and downregulation of genes encoding neuroectoderm factors (*Pax6* and *NeuroD2*) (Ito et al. 2010; Dawlaty et al. 2011; Koh et al. 2011; Xu et al. 2011b). Further, as mentioned previously, depletion of

Tet1 in PGCs leads to defective meiotic prophase that includes DSBs and formation of univalent chromosomes (Yamaguchi et al. 2012).

Tet1 KO mouse ES cells are able to differentiate and form living mice (Dawlaty et al. 2011). However, a number of defects have been reported in mice that lack *Tet1* (Dawlaty et al. 2011; Rudenko et al. 2013; Zhang et al. 2013; Kumar et al. 2015). Some *Tet1* deficient mice have a smaller body size compared to WT (Dawlaty et al. 2011). Mice that lack *Tet1* have memory extinction impairment and abnormally enhanced hippocampal long-term depression, indicating that the downregulation of neuroectoderm factors impacts normal brain function (Rudenko et al. 2013). Others have reported impaired spatial memory, object location memory, and threat recognition memory in *Tet1* deficient mice (Zhang et al. 2013; Kumar et al. 2015). The meiosis defects mentioned above in mouse PGCs lead to loss of oocytes and consequent decrease in mouse litter size (Yamaguchi et al. 2012).

3.4 *Loss of Tet2 Transcription and Its Impact in ES Cells and PGCs*

Tet2 is expressed about fivefold less than *Tet1* in ES cells, and its expression has been shown to be under the control of Oct4 (Koh et al. 2011; Sohni et al. 2015). Loss of *Tet2* results in about a 50–90% reduction in 5-hmC (Koh et al. 2011; Hon et al. 2014). This loss of 5-hmC in *Tet2* deficient cells is localized to the gene bodies and exon-intron boundaries of highly expressed genes (Huang et al. 2014). An additional report found *Tet2* deficient cells to have increased 5-hmC around the *Nanog* promoter, which is believed to be the result of compensatory *Tet1* expression (Huang et al. 2014; Langlois et al. 2014). Similar to *Tet1* deficient cells, there is an increase in 5-mC in reaction to the loss of *Tet2*, but specifically at enhancers under this condition (Hon et al. 2014).

Overall, loss of *Tet2* has a less dramatic impact on downstream gene expression than the loss of *Tet1* (Koh et al. 2011; Huang et al. 2014). Broadly, there is delayed gene induction in *Tet2* deficient ES cells during differentiation (Hon et al. 2014). In one study, there was only a modest increase in neuroectoderm markers *Pax6*, *NeuroD1*, *Lefty1*, and *Lefty2*, the opposite effect of *Tet1* KD (Koh et al. 2011). When *Tet2* deficient cells were allowed to differentiate, there was a bias towards neuroectoderm differentiation in one mouse and one human model (Koh et al. 2011; Langlois et al. 2014). However, according to another group, loss of *Tet2* has no impact on gene expression or ES cell morphology (Ito et al. 2010). Differentiation of *Tet2* deficient cells resulted in a decrease in both number and clonogenic capability of hematopoietic progenitor cells (Langlois et al. 2014). In PGCs, *Tet2* is heterogeneously expressed at e9.5 and e10.5 and is generally regarded as redundant with *Tet1* (Hackett et al. 2013; Vincent et al. 2013). When *Tet1* and *Tet2* are silenced together, demethylation of genes in PGCs is inhibited (Hackett et al. 2013).

The evidence for the roles of *Tet2* in ES cells is not always consistent across studies. First, as previously stated, the loss of *Tet2* has highly variable impacts on the level of 5-hmC (50–90% reduction) (Koh et al. 2011; Hon et al. 2014). There are also differing reports on the impact of *Tet2* on gene expression. Loss of *Tet2* promoted the expression of neuroectoderm markers in two studies (Koh et al. 2011; Langlois et al. 2014), but two other groups found that loss of *Tet2* negatively regulated *Lefty1* and had no impact on global gene expression (Ito et al. 2010; Hon et al. 2014). In addition, *Tet2* is frequently studied within the context of *Tet1* loss in embryonic development (Ficz et al. 2011; Koh et al. 2011; Vincent et al. 2013). Few studies have looked at the role of *Tet2* independent of *Tet1* in ES cells and PGCs.

3.5 *Tet3* Transcription and Its Epigenetic Role in the Zygote

Tet3 expression is essential for normal 5-hmC epigenetic remodeling of the zygote, but is nearly undetectable during very early embryonic development before the gene gets re-expressed in specific adult tissues (Gu et al. 2011; Koh et al. 2011; Wossidlo et al. 2011). *Tet3* expression is high in oocytes and zygotes and is significantly decreased in two-cell embryos (Gu et al. 2011; Wossidlo et al. 2011). When *Tet3* is knocked down, the conversion of 5-mC to 5-hmC in the paternal DNA is abrogated, and there is little 5-hmC (Gu et al. 2011; Wossidlo et al. 2011). Consequently, the demethylation of key paternal embryonic genes (*Oct4*, *Lemdl*, and *Nanog*) is impaired (Gu et al. 2011). The pattern of 5-hmC enrichment has yet to be investigated at other regions of the genome. As a result, the *Oct4* gene activation is delayed in the early embryo (Gu et al. 2011). The loss of *Tet3* from the maternal genome causes developmental failure in mouse embryos, due to retention of paternal 5-mC (Gu et al. 2011).

Although *Tet3* expression is low in ES cells, it increases during neuronal differentiation (Li et al. 2015b). *Tet3* KO cells are capable of expressing neuronal differentiation markers but often undergo apoptosis (Li et al. 2015b). *Tet3* is also critical for *Xenopus* eye and neural development (Xu et al. 2012).

3.6 *Loss of Multiple Tets Has Serious Functional Consequences in Embryonic Development*

Double and triple KO (DKO and TKO) of *TET* genes in embryonic systems has dramatic effects on 5-hmC maintenance and cell phenotype. When *Tet1* and *Tet2* are silenced together, there is loss of 75–80% of 5-hmC in ES cells, greater than those of the effects of either single KD (Koh et al. 2011). *Tet3* is upregulated in the double silenced cells, presumably to compensate the loss of *Tet1* and *Tet2* (Koh et al. 2011). In *Tet1/Tet2* DKO cells, the changes in gene expression are similar to *Tet1* KD alone, suggesting that *Tet1* has a dominant role on transcription over *Tet2* in ES cells (Koh

et al. 2011). During differentiation of mouse embryos, *Tet1/Tet2* DKO cells remain depleted of 5-hmC, and there are developmental defects in chimeric embryos consistent with perinatal lethality (Dawlaty et al. 2013). The defects *Tet1/Tet2* DKO embryos exhibit include exencephaly, hemorrhages in the head, or profound growth retardation. However, some *Tet1/Tet2* DKO mice are viable (Dawlaty et al. 2013). Although both sexes of the DKO mice are fertile, females have smaller ovaries and reduced fertility (Dawlaty et al. 2013).

Tet1/Tet3 DKO in embryonic systems also has severe biological consequences, including abnormal mitosis and nuclear blebbing in the two-cell stage (Kang et al. 2015). Although not well studied in ES cells, gestation of *Tet1/Tet3* DKO mouse embryos had been assessed in one study (Kang et al. 2015). In these embryos, 5-hmC was undetectable by antibody staining, and only about 70% of them survived to eight-cell embryos. By e3.5, 60% of the remaining embryos had aberrant *Nanog* expression. During the entire process, the transcriptomes of the embryos featured very high variability. Ultimately, embryos were not viable beyond e10.5.

tet2/tet3 DKO has only been examined in the context of embryonic development in zebrafish (Li et al. 2015a). In this model, *tet2* and *tet3* are the predominant 5-hmC regulators in embryos. *tet2/tet3* DKO resulted in a 30-fold loss of 5-hmC, although the distribution of 5-hmC was not characterized. These embryos featured abnormal differentiation, including altered brain morphology, smaller eyes, and a lack of a definitive hematopoietic stem cell population, and ultimately did not survive beyond the larval stage. Gene expression was not assessed, so it is not known how loss of *tet2* and *tet3* impacted the transcriptome of these animals.

Tet1/Tet2/Tet3 TKO is invariably lethal and leads to complete depletion of 5-hmC in ES cells (Dawlaty et al. 2014; Hu et al. 2014; Lu et al. 2014). Promoters and enhancers are hypermethylated, and many genes are deregulated (Lu et al. 2014). However, studies disagree about whether the majority of genes are upregulated or downregulated in TKO cells (Dawlaty et al. 2014; Lu et al. 2014). One study found that when the TKO cells underwent differentiation, there was reduced expression of mesodermal and endodermal markers (Dawlaty et al. 2014). In addition, the cells formed fewer differentiated structures. When injected into embryos, there was a lower incidence of chimeric embryos formed from TKO cells compared to controls, indicating that the *Tets* are essential for normal embryonic development.

3.7 Cocaine Stimulation Remodels the 5-hmC Landscape Through Tet1 Expression

The brain is particularly enriched with high levels of 5-hmC (Kriaucionis and Heintz 2009; Globisch et al. 2010), where the base is thought to play a major role in neuronal adaptation to stimuli, which requires epigenetic remodeling. Once considered static epigenetic marks, we now appreciate that covalent cytosine modifications in the brain

are reversible and dynamic, with frequent remodeling of 5-mC and 5-hmC, due in part to *TET1* expression (Reik 2007; Guo et al. 2011a, b). Changes to the 5-hmC landscape occur in response to neuronal activity, indicating specific stimuli have the potential to remodel 5-hmC level and distribution (Guo et al. 2011a). One such stimulus that has been studied within the context of brain tissue is cocaine exposure (Feng et al. 2015). This study found repeated cocaine administration resulted in downregulation of *Tet1* expression and increased 5-hmC levels within the nucleus accumbens of mice. The increases of 5-hmC were enriched around the enhancers and gene bodies of genes known to be involved in drug addiction. The downregulation of *Tet1* and altered 5-hmC appeared to enhance behavioral responses to cocaine. Nonetheless, the mechanism of how *Tets* are regulated in the brain is yet to be identified. Despite being a tissue enriched in 5-hmC, very little is known about the expression and function of the *Tets* in this system.

Epigenetic reprogramming of the 5-hmC landscape is highly dependent on rigorous regulation of expression from the *TET* genes. It is clear that each TET enzyme has a unique role in each process, and the loss of one enzyme can only be partially compensated by the other two, indicating non-overlapping functions.

4 Mutations of *TET* Genes in Cancers

Epigenetic abnormalities such as global hypomethylation and site-specific hypermethylation have been known to be characteristics of cancers since the 1980s (Feinberg and Vogelstein 1983; Gama-Sosa et al. 1983; Baylin et al. 1986; Feinberg and Tycko 2004). Since the discovery of TET function, the role of 5-hmC in pathologic conditions has become a new focus in epigenetics. However, many techniques used to study 5-mC, including sodium bisulfite treatment and several methylation-sensitive restriction enzymes, cannot distinguish 5-mC from 5-hmC. For this reason, many interpretations of ‘methylcytosine’ should be more properly described as ‘5-mC or 5-hmC’. The recent development of 5-hmC specific techniques (Table 1), such as TAB-seq (Yu et al. 2012) and hMe-SEAL (Song et al. 2011), allow specific detection of 5-hmC. Through the use of these new techniques, widespread 5-hmC alterations were found in blood and solid tumor cancers (Vasanthakumar and Godley 2015). Here, we will discuss some of the most common causes of 5-hmC deregulation in cancer, including mutations of *TET*s and changes in *TET* expression.

4.1 *TET1* Mutations and Transcriptional Changes in Cancer

The *TET1* gene was originally discovered as a fusion partner of *KMT2A* (*MLL*) in cases of t(10;11)(q22;23) acute myeloid leukemia (AML) (Ono et al. 2002; Lorsch et al. 2003). *MLL* encodes a histone methyltransferase, and leukemia-specific

chromosomal fusions involving this gene frequently result in production of *MLL*-fusion proteins. The *MLL-TET1* fusion is found in about 0.3% of all *MLL*-fusion leukemias, resulting in the fusion of the N-terminus of *MLL* with the C-terminal catalytic domain of *TET1* (Lee et al. 2013; Meyer et al. 2013). The *MLL-TET1* fusion has also been found in some acute lymphoblastic leukemia (ALL) cases and has the capability to drive a phenotypic switch from ALL to AML (Burmeister et al. 2009; Ittel et al. 2013). *MLL-TET1* fusion causes translocation of the *TET1* catalytic domain, which is normally absent in myeloid systems, therefore promoting AML progression (Huang et al. 2013). In fact, *TET1* is a direct target of all *MLL*-fusion proteins and is found to be significantly upregulated in *MLL*-rearranged leukemia samples compared to normal bone marrow samples (Huang et al. 2013). This alteration results in increased global level of 5-hmC (Huang et al. 2013, 2016). However, genomic enrichment of 5-hmC in *MLL*-rearranged leukemia cells has yet to be studied.

TET1 mutations not involving chromosomal fusion are rare in hematopoietic malignancies, especially compared to *TET2* mutations. A study of 83 patients revealed only 2 cases with *TET1* mutations in myelodysplastic syndrome (MDS) or chronic myelomonocytic leukemia (CMML) patients (Lasho et al. 2018) (Fig. 3), but it remains unclear whether these are driver or passenger mutations.

The role of *TET1* in the pathogenicity of cancers is usually linked to its gene expression. The 5-hmC levels in solid tumors are often low compared to surrounding normal tissue in solid tumors, including liver, brain, kidney, lung, colorectal, and gastric cancers (Kudo et al. 2012; Vasanthakumar and Godley 2015). Knock-down

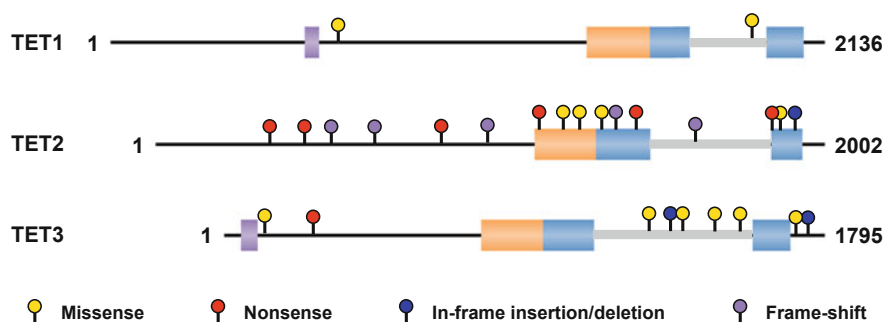


Fig. 3 Mutation effects in TET proteins. *TET1* and *TET3* are rarely mutated in human diseases. Most of the mutations identified in *TET1* and *TET3* are missense mutations or in-frame insertion/deletion (indel), leading to single amino acid substitutions in the protein. All of these mutations occur outside of the core catalytic domain or the CXXC domain, likely maintaining the catalytic function of the protein. A notable exception is a nonsense mutation in *TET3*, which truncates the protein before the catalytic domain, leading to complete loss of the catalytic domain in the protein. Numerous *TET2* mutations have been described over the last decades, most of which lead to loss of function in *TET2* protein. In contrast to *TET1* and *TET3* mutations, *TET2* missense mutations often directly impact the catalytic domain and cause impaired catalytic activities. Nonsense and frame-shift mutations are commonly found throughout the entire coding sequence, also leading to loss of *TET2* catalytic activity

of *Tet1* alone in 3T3 fibroblasts is sufficient to replicate the 5-hmC loss seen in tumors, but not enough to induce malignant transformation (Kudo et al. 2012). Although many of the earlier studies on 5-hmC did not provide mechanistic links between 5-hmC alterations and pathogenic phenotypes, more recent studies began to provide better insight into the roles of 5-hmC regulation in cancers.

For example, we have recently gained insight in how TET1 is involved in hypoxic responses in certain solid tumors. When *MYCN*-amplified neuroblastoma cell lines are exposed to hypoxia, 5-hmC levels increase (Mariani et al. 2014). This result was surprising given, as discussed earlier in this chapter, TETs rely on oxygen for activity. The increased 5-hmC was the result of hypoxia-induced expression of *TET1*. In hypoxia, 5-hmC was enriched along the gene bodies of direct targets of HIF-1, the master regulator of hypoxia. These HIF-1 targets are known as canonical hypoxic response genes. Upon loss of HIF-1 α , *TET1* expression no longer increased, and there was no induction of 5-hmC under hypoxic conditions. Loss of hypoxia induced-*TET1* resulted in decreased expression from canonical hypoxic response genes in comparison to hypoxic controls, demonstrating TET1 facilitates expression from canonical hypoxic response genes in hypoxia (Mariani et al. 2014).

4.2 TET2 Mutations and Transcriptional Changes in Cancer

TET2 is the most commonly mutated gene among the three *TET* genes (Fig. 3). In hematologic malignancies, *TET2* mutations are found in 20–30% of AML, 20–30% of MDS or myeloproliferative neoplasm, and up to 58% of CMML (Delhommeau et al. 2009; Jankowska et al. 2009; Langemeijer et al. 2009; Yamazaki et al. 2012, 2015; Itzykson et al. 2013). *TET2* mutations are also found in other types of cancers at lower frequencies: 12% of T-cell ALL (Quivoron et al. 2011), 7% of clear cell renal cell carcinoma (clear cell RCC) (Sato et al. 2013), 12% of stage IV non-small cell lung cancer (Jin et al. 2016), and in metastatic prostate cancer (Nickerson et al. 2013). Copy number alterations of *TET2* are found in about 5.6% of various hematologic malignancies, 70% of which also have cytogenetic alterations (Bacher et al. 2012). Notably, most in-frame deletions and missense mutations occur in exons that code for the C-terminal catalytic domain, whereas nonsense and frameshift deletions can occur along the entire gene (Fig. 3) (Langemeijer et al. 2009; Euba et al. 2012). Most of the known mutations result in truncated protein or impaired catalytic functions (Langemeijer et al. 2009; Euba et al. 2012). One study found that many of the leukemia associated missense mutations in the catalytic domain of TET2 reduced the affinity of the enzyme for Fe(II) and 2OG and greatly reduce its activity (Laukka et al. 2015).

The effect of *TET2* mutation on 5-hmC and 5-mC has been studied, but not thoroughly. It is clear that *TET2* mutated leukemia cells have globally decreased 5-hmC (Ko et al. 2010; Madzo et al. 2014), but the mechanism behind this loss is not understood. Early studies of *TET2* mutated samples found no global difference in 5-mC, but showed site-specific hyper- and hypomethylation (Ko et al. 2010). In

contrast, other studies have shown global hypermethylation in leukemic cells (Madzo et al. 2014). It should be noted that Ko et al. measured global methylation using the Illumina Infinium 27K array that probes 27,578 selected CpG sites, whereas Madzo et al. used mass spectrometry that measure all nucleotides in the genome, which may explain the discrepancy in these findings. Curiously, in this study, distribution of 5-hmC in *TET2*-mutated leukemia is dramatically different from normal, which is not explained solely by the global decrease of 5-hmC. Although most 5-hmC peaks decrease correspondingly with the global decrease of 5-hmC, certain sites gain high density of 5-hmC not seen in normal control. This redistribution process suggests that *TET1* or *TET3* may compensate for the loss of *TET2*, resulting in skewed 5-hmC distribution. Lastly, in CMML cells, *TET2* mutation-induced cytosine modification changes, especially hyper-modifications, are enriched in enhancer regions and negatively correlate with gene expression changes (Meldi et al. 2015; Yamazaki et al. 2015). Note that these studies used bisulfite sequencing based methods to characterize genome-wide cytosine modification changes, which cannot distinguish 5-mC from 5-hmC. Using modification status from as few as 14 sites, a support vector machine model was able to achieve 79% accuracy in predicting patient response to decitabine, a hypomethylating agent commonly used to treat CMML (Meldi et al. 2015). This result suggests that the changes in cytosine modifications have prognostic value to risk-stratify patients at diagnosis.

In addition to *TET2* mutations, it is becoming increasingly clear that *TET2* expression is a target of microRNAs in blood cancers (Cheng et al. 2013; Song et al. 2013). *TET2* has been found to be downregulated by miR-22, miR-29b, miR-125a, miR-26, miR-101, and miR-520d. 5-hmC levels were found to be decreased when *TET2* was down-regulated by miR-22, miR-29b, or miR-125a. Some of these microRNAs were also shown to regulate *TET1* and *TET3*, but an effect on 5-hmC level or distribution was not demonstrated (Cheng et al. 2013). Expression of miR-22, miR-29b, or miR-125a increases the oncogenic traits of cells, including increased replating ability of hematopoietic stem cells. In addition, mouse models with overexpression of these miRs featured development of MDS, and splenomegaly (Cheng et al. 2013; Song et al. 2013).

Although *TET2* has been investigated mostly in the context of blood cancers, there have been some instances of abnormal *TET2* expression in solid tumors. Low 5-hmC levels correlate with down-regulated *TET2* expression in oral cell carcinoma and esophageal squamous cell carcinoma (ESCC) (Jäwert et al. 2013; Shi et al. 2016). In both tumors, low 5-hmC was associated with low *TET2* expression. ESCC tumors have significantly decreased *TET2* and *TET3* expression compared to a normal tissue control (Shi et al. 2016). It is unknown how downregulation of *TET2* and low 5-hmC impact tumor oncogenicity, but loss of 5-hmC is an unfavorable prognostic factor in ESCC (Shi et al. 2016).

4.3 *TET3 Mutations and Transcriptional Changes in Cancer*

TET3 mutations are rare compared to *TET2* mutations in cancer. A previous study with a cohort of 408 leukemia patients did not find any *TET3* mutations (Abdel-Wahab et al. 2009). More recently, two loss-of-function mutations were identified in *TET3* in the bone marrow of 28 CMML patients, both coexisting with *TET2* mutations (Merlevede et al. 2016) (Fig. 3). In addition, another study identified seven *TET3* mutations in a study of 83 leukemia patients (Lasho et al. 2018) (Fig. 3). None of the newly identified mutations co-occurred with *TET2* mutations. Six of these mutations were found in CMML patients (16% of all CMML patients), two of which were found in the same patient, and one of which co-occurred with a previously mentioned *TET1* mutation (Lasho et al. 2018). These findings suggest that *TET3* mutations preferentially occur in CMML, although the significance and consequences of *TET3* mutations remain to be studied.

There are emerging data that *TET3* expression is associated with an aggressive phenotype in RCC and head and neck cancer (Chen et al. 2017a; Misawa et al. 2018). High expression of *TET3* in RCC is associated with poor survival, but the effect on the 5-hmC landscape is unknown (Chen et al. 2017a). *TET3* is expressed at low levels in head and neck cancer, but perhaps more interestingly, the cytosine modification status of its promoter is negatively associated with disease survival (Misawa et al. 2018). The mechanism behind this finding is not yet known.

4.4 *Deregulated Expression of Multiple TETs in Cancer*

Many other cancers with low 5-hmC levels feature low expression or no change in expression of all three *TET* enzymes, indicating a more complex mechanism behind the aberrant 5-hmC landscape. Low levels of 5-hmC have been identified in brain, breast, liver, lung, pancreatic, prostate, and skin tumors (Jin et al. 2011; Lian et al. 2012; Yang et al. 2013). Breast, liver, and skin cancers all demonstrate low expression of all three *TET* enzymes (Lian et al. 2012; Yang et al. 2013; Chen et al. 2017b). The mechanism of low *TET* expression has only been explored in liver cancer. Low 5-hmC correlated with metastasis and poor prognosis of hepatocellular carcinoma (HCC) (Chen et al. 2017b). Expression from all three *TET* enzymes was downregulated by elevated expression of miR-29a, which in turn, resulted in decreased 5-hmC and increased 5-mC levels. The promoter of tumor suppressor gene *SOCS1* was found to be hypermethylated, and cell motility was increased.

In contrast, there has been no reported change in expression in any of the *TETs* in lung and brain cancer (Jin et al. 2011). Tumors that arise from pancreatic and prostate tissue have featured low levels of 5-hmC, however *TET* expression level has yet to be investigated.

Correcting altered transcription from *TET* genes is an attractive goal because it has the potential to ‘reset’ the 5-hmC landscape to pre-cancerous status. Further work to identify mechanisms of *TET* transcription regulation is required to achieve this goal.

5 Post-Translational Modifications (PTMs) of TET Enzymes

TET proteins are subject to an assortment of PTMs, many of which are poorly understood functionally. However, TET PTMs might reconcile a major discrepancy in studies concerning the DNA binding properties of TETs. There is conflicting research into the binding of TET to DNA: some studies have found that TET does not bind DNA at all, and others have found TETs can bind sequences containing unmodified, methylated, and hydroxymethylated cytosine (Zhang et al. 2010; Frauer et al. 2011b; Xu et al. 2011b). Preliminary research indicates that PTMs play a role in stability, activity, and localization of the TET enzymes. Considering the role of TETs in the pathogenesis of diseases, it is likely the role of TET PTMs and their effects on any aberrant activity will become a target for more studies. Known PTMs of the TET proteins include phosphorylation, O-GlcNAcylation (O-GlcNAc), acetylation, and ubiquitination (Fig. 4) (Bauer et al. 2015; Nakagawa et al. 2015; Zhang et al. 2017).

5.1 Phosphorylation of TET Proteins

A study performed in mouse and human systems indicated phosphorylation of the three TET proteins largely occurs in the N-terminus (Fig. 4a) (Bauer et al. 2015). This modification can occur singly or in tandem with another modification. For example, in TET2, phospho-Ser-23 is found only with phospho-Ser-15. The kinase (s) responsible for the phosphorylation modifications on the TET proteins are largely unknown at this time. Recent studies are starting to reveal the pathways regulating TET2 phosphorylation. For example, a specific site on TET2, Ser-99, was found to be phosphorylated by AMP-activated kinase (AMPK) (Wu et al. 2018). TET2 with this phosphorylation mark was found to have a longer half-life than TET2 without this mark. A Ser-99-Asp phospho-mimic TET2 was found to increase 5-hmC levels when expressed. However, distribution of 5-hmC was not investigated. Further research on TET2 phosphorylation will shed light on other kinases that modulate TET2 activity and stability. However, because modifications are enriched in the non-catalytic domains, it is hypothesized that TET phosphorylation may also play a role in interaction with TET binding partners (Bauer et al. 2015; Liu et al. 2018).

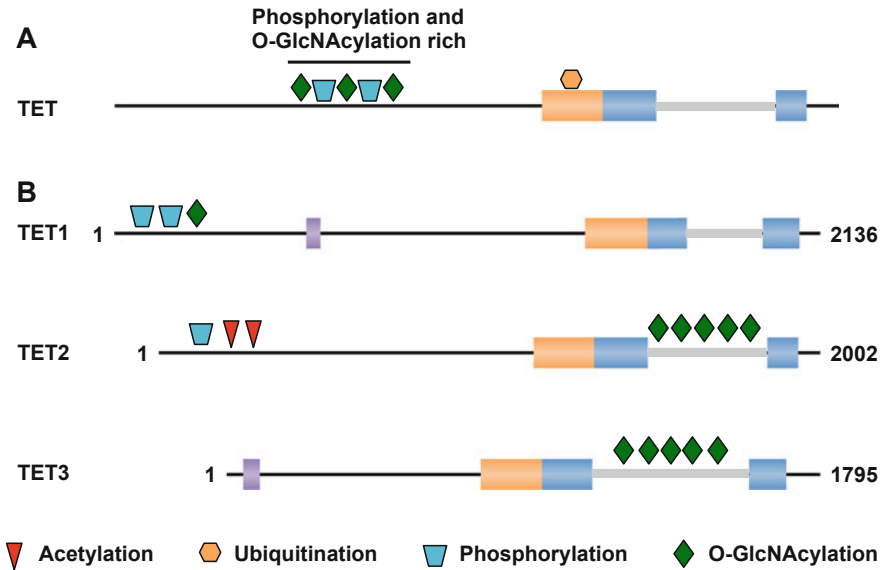


Fig. 4 General distribution of TET PTMs. **(a)** Schematic representation of the common TET structure featuring modifications that are consistent across all three TET enzymes. A phosphorylation and O-GlcNAcylation-rich region is comprised of a large number of phosphate and O-GlcNAc marks that are variably distributed. A monoubiquitination modification is also found in the cysteine-rich domain of all three TET enzymes. **(b)** General distribution of modifications unique to each TET protein. Colors: Red: Acetylation, Orange: Ubiquitination, Blue: Phosphorylation, Green: O-GlcNAcylation

5.2 *O-GlcNAc Modifications Facilitate TET Protein Stability, Activity, and Localization*

O-GlcNAc and phosphorylation modifications are occasionally in competition with each other (Bauer et al. 2015). Specifically, Ser-97 and Ser-374 of TET2 and Ser-362 and Ser-557 of TET3 could be phosphorylated or O-GlcNAcylated. Overall, some TET phosphorylation marks are stable, such as Ser-950 of TET1, but some are lost when *OGT* is highly expressed, such as Ser-2016 of TET1. *OGT* encodes the enzyme *O*-linked *N*-Acetylglucosamine Transferase (*OGT*) which is responsible for the addition of an O-GlcNAc group to serine and threonine. Similar to phosphorylation modifications, O-GlcNAc modifications are generally localized to the N-terminus and the low complexity insert (Fig. 4a, b). However, there is some distinction of the O-GlcNAc modification patterns between the three TET proteins (Fig. 4b). TET1 features fewer O-GlcNAc modifications than TET2 or TET3, with most found in the N-terminus and hardly any in the C-terminal region. In addition, O-GlcNAc modifications of TET1 have been shown to be highly dynamic (Shi et al. 2013; Bauer et al. 2015). TET2 and TET3 display most O-GlcNAc in the low complexity insert region in one comprehensive study (Bauer et al. 2015). In addition, the first 350 amino acids of TET3 are unmodified. In mouse and human systems,

there appears to be crosstalk between the phosphorylation and the O-GlcNAc modifications of the TET proteins. Although increase in *OGT* expression is associated with decreased phosphorylation in TETs, phosphate and O-GlcNAc groups are rarely found on the same residue. Instead of direct competition, it appears that neighboring residues are engaging in crosstalk as several modifications of the same type often occur in close proximity. There have been many proposed functions of TET O-GlcNAc modifications (Vella et al. 2013; Bauer et al. 2015; Hrit et al. 2018). Several studies have demonstrated that this modification increases Tet1 protein stability and activity (Vella et al. 2013; Hrit et al. 2018). Others have implicated O-GlcNAc groups in mediating binding of TET partners and localization (Bauer et al. 2015). Studies in mouse embryonic development have shown that loss of O-GlcNAc modifications on Tet3 resulted in nuclear rather than cytosolic subcellular localization of the protein and substantially increased global 5-hmC levels (Zhang et al. 2014). Interestingly, O-GlcNAcylation of Tet1 and Tet2 had no impact on their respective subcellular localizations (Zhang et al. 2014).

5.3 Acetylation Has Been Implicated in the Stability of TET2

Acetylation has been identified as a modification of only TET2 in human cell lines. One study found TET2 is acetylated by the epigenetic modifier p300 (Zhang et al. 2017). TET2 is acetylated at multiple lysines in the N-terminus, and the presence of two (K110 and K111) are necessary for p300-mediated acetylation (Fig. 4b). Although there is no accumulation of TET2 protein, global 5-hmC level increases in the presence of acetylated TET2, indicating that acetylation of TET2 enhances its enzymatic activity. The acetylation marks were also found to inhibit polyubiquitination of TET2, therefore promoting its stability. Lastly, TET2 acetylation has also been implicated in potential binding of DNMT1.

5.4 TETs Are Monoubiquitinated

Monoubiquitination of all three TET proteins occurs on a lysine, mediated by VprBP, in the highly conserved cysteine-rich domain (Fig. 4a) (Nakagawa et al. 2015). In vivo ubiquitination assays with TET proteins yielded a single band on a gel, indicating addition of a one ubiquitination group. The monoubiquitination occurs on K1589 in TET1, K1299 in TET2, and K859 in TET3. Loss of monoubiquitination prevents binding of TETs to DNA and therefore results in an overall loss of 5-hmC in in vitro and in vivo experiments. Several oncogenic mutations of TET2 disrupt its ubiquitination, indicating this modification is important for normal TET2 activity.

6 Proteins Interact Directly with Modified Cytosine and TET Binding Proteins

Stable epigenetic marks interact with epigenetic “reader” proteins that mediate their downstream effects. Although there is some overlap between binding partners, 5-mC, 5-hmC, and TETs each have their own unique set of interactors.

6.1 Proteins Recognize, Bind, and Read 5-mC and 5-hmC

Identified readers of 5-mC all contain the conserved methyl-binding domain, but not all proteins that feature a methyl-binding domain bind 5-mC (Hendrich and Tweedie 2003). Most proteins that read 5-mC are thought to reinforce the repression of genes with 5-mC rich promoters (Boyes and Bird 1991; Nan et al. 1997; Hendrich and Bird 1998). In fact, it is hypothesized that the ability to bind 5-mC may be cell type specific (Iurlaro et al. 2013). Therefore, this section will only discuss the most universal 5-mC readers. The very first proteins identified that read 5-mC were MeCP1 and MeCP2 (Meehan et al. 1989; Lewis et al. 1992). Although it was originally thought that 5-mC prevents transcription exclusively by directly blocking the binding of transcription factors, it is now known that MeCP1, MeCP2, and MBD1/2/4, are all indirect mediators of the biological function of the 5-mC mark by recruiting chromatin remodeling machinery such as NuRD complex to downregulate gene expression (Boyes and Bird 1991; Nan et al. 1997; Hendrich and Bird 1998; Hendrich and Tweedie 2003; Pan et al. 2017).

Researchers have tried to identify proteins that mediate the biological function of 5-hmC using a variety of screening methods (Iurlaro et al. 2013; Spruijt et al. 2013). Unfortunately, many studies have been unable to replicate results presented by other groups. This may be due to a lack of rigorous testing across multiple model systems, but also may indicate many 5-hmC readers are cell type specific, implying a more complex mechanism at work than simple hydroxymethylcytosine recognition (Iurlaro et al. 2013). Some proposed 5-hmC readers include RBM14, PRP8, RPL26, MSH6, PNKP, Wdr76, and Thy28. However, each of these has only been identified in a single study, and the findings remain to be validated (Hendrich and Bird 1998; Frauer et al. 2011a; Yildirim et al. 2011; Mellén et al. 2012; Iurlaro et al. 2013; Spruijt et al. 2013). Only three proteins, NP95/UHRF1, MeCP2, and MBD3, have been confirmed in more than one study (Sharif et al. 2007; Arita et al. 2008; Frauer et al. 2011a; Yildirim et al. 2011; Mellén et al. 2012; Baubec et al. 2013; Iurlaro et al. 2013; Spruijt et al. 2013). NP95/UHRF1 has been identified as a 5-hmC reader in three studies in two cell-free assays and in mouse ES cells (Frauer et al. 2011a; Iurlaro et al. 2013; Spruijt et al. 2013). Uhrf1 is traditionally recognized as a binder of hemimethylated DNA and functions in DNA methylation maintenance, but has lately been characterized as a reader of both 5-mC and 5-hmC (Sharif et al. 2007; Arita et al. 2008; Frauer et al. 2011a). The functional consequences of NP95/UHRF1

binding 5-hmC have yet to be examined in vivo (Frauer et al. 2011a). MeCP2, already established as a 5-mC binding protein, has also demonstrated 5-hmC binding in two studies in neural tissues and mouse ES cells (Mellén et al. 2012; Spruijt et al. 2013). It is unclear how the MeCP2:5-mC binding profile differs from MeCP2:5-hmC binding profile or whether the function of this protein depends on its ligand. However, a mutated version of *MeCP2* is expressed in Rett Syndrome: R133C. Experiments with electrophoretic mobility shift assays indicated that this MeCP2 protein is incapable of binding 5-hmC (Mellén et al. 2012). This indicates that readers of 5-hmC may have an essential role in normal neuronal phenotype. Finally, MBD3 has been identified as a weak binder of 5-hmC in two studies (Yildirim et al. 2011; Mellén et al. 2012). One study that indicated MBD3 as a 5-hmC reader also indicated Tet1:Mbd3 complexes bind 5-hmC rich regions of the genome in ES cells, and identified Mbd3 as both a recruiter of Tet1 and an effector that mediates gene expression (Yildirim et al. 2011). However, other studies have found that MBD3 binds the same genomic regions independent of the presence of 5-mC and 5-hmC, contradicting its proposed function as a 5-hmC reader (Iurlaro et al. 2013; Spruijt et al. 2013). This also potentially complicates the findings of the relationship between Tet1 and Mbd3 (Baubec et al. 2013; Iurlaro et al. 2013; Spruijt et al. 2013). The significance of MBD3:5-hmC binding is under debate.

Overall, although many potential proteins that bind 5-mC and 5-hmC have been identified, the function of many of these interactions is mostly unknown. Future studies of the biological functions of these interactions are needed to better understand how cytosine modifications regulate the genome.

6.2 *Some Binding Partners Interact with All Three TETs*

Current research on TET binding proteins shows that these binding partners can modify TET activity, localization, and stability (Fig. 5). Many of the identified proteins are not yet fully characterized and may have functions outside of their relationship with TETs.

The interaction between Tet and Ogt has been studied extensively. Independent of the O-GlcNAcylation activity, stable Tet:Ogt interaction promotes Tet activity and regulates Tet genomic localization. OGT binds TET1 through a conserved C-terminal domain, and is thought to form a larger complex with at least SIN3A (Vella et al. 2013; Hrit et al. 2018). Ogt and Tet1 colocalized at unmethylated CpG-rich promoters (Vella et al. 2013). Additional studies have found that loss of Ogt protein resulted in abnormal Tet1 localization in ES cells, indicating Ogt is important for Tet1 binding of some genomic sites (Shi et al. 2013). Multiple groups have reported that the loss of Tet1:Ogt interaction also results in reduced 5-hmC level and expression from Tet1-activated genes (Shi et al. 2013; Hrit et al. 2018). Ultimately, these experiments indicate that the role of Tet1:Ogt binding is to increase its activity and recruit it to genomic sites (Fig. 5a) (Shi et al. 2013; Hrit et al. 2018). TET2/3:OGT binding does not appear to have a significant impact on the 5-hmC

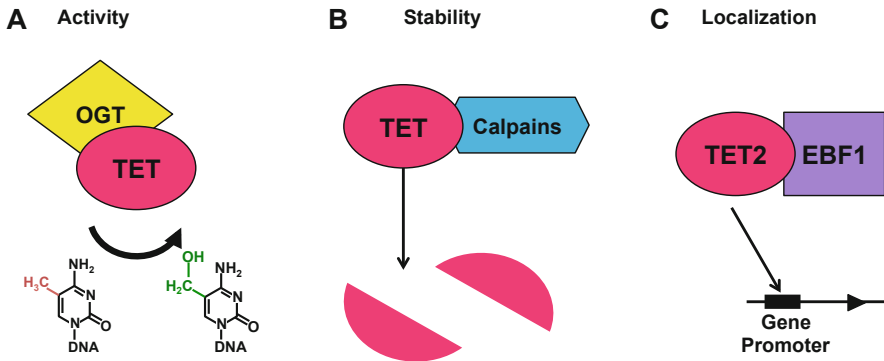


Fig. 5 TET binding partners impact TET activity, localization, and stability. (a) Binding of OGT increases TET activity when bound to DNA. (b) Calpains bind and degrade TETs. (c) EBF1 brings TET2 to promoters of genes, where it hydroxylates 5-mC to 5-hmC

epigenetic landscape, instead playing a scaffolding role for OGT (Deplus et al. 2013; Ito et al. 2014).

Tet stability appears to be regulated, at least in part, by calpains in mouse ES cells (Wang and Zhang 2014). Calpains 1 and 2 regulate TET stability via direct binding and cleavage of TET enzymes (Fig. 5b) (Wang and Zhang 2014). This mechanism of TET protein degradation is predominantly found in certain stages of development when *TET* expression is high (Wang and Zhang 2014). Tet1 and Tet2 are degraded by calpain1 in mouse ES cells, whereas Tet3 is degraded by calpain2 during ES cell differentiation (Wang and Zhang 2014). Loss of calpains resulted in twofold higher 5-hmC levels, downregulation of *Cdx2* and *Eomes*, and upregulation of *Ngn2* and *Pax6* in ES cells (Wang and Zhang 2014). Therefore, TET:calpain complexes are required for normal 5-hmC regulation and gene expression in ES cells.

TDG, MBD4, SMUG1, NEIL1, NEIL2, NEIL3, PARP1, LIG3, and XRCC1 are part of the BER pathway and interact with TET during this process (Müller et al. 2014). Their colocalization with TETs is thought to facilitate removal of 5-caC and repair of the site. However, a role for direct binding between TET and each specific BER protein has yet to be described (Müller et al. 2014).

Beyond the shared interactors, the TET proteins have been shown to have binding partners unique to only one or two of them. These unique partners may explain the non-overlapping functions of TETs and provide further insight to the regulation of 5-hmC.

6.3 *TET1* Binding Proteins

The function of most described interactors is regulating the localization of TET1 within the genome. The binding partners of TET1 are chromatin-binding proteins:

transcription factors and epigenetic modifiers. They are thought to function at different levels of TET1 localization, from promoting TET1 binding to DNA to recruiting TET1 to target regions in the genome.

Proteins that are thought to facilitate TET1 binding to DNA include MeCP2, HDAC1/6/7, EZH2, mSin3A, PCNA, and LSD1 (Cartron et al. 2013). All of these proteins were found to bind TET1 directly and each other, thus stabilizing the TET1-DNA interaction. Further experimentation revealed that, in addition to this function, TET1:EZH2 and TET1:SIN3A are thought to recruit TET1 to specific genomic elements (Chandru et al. 2018). It should be stated that many proteins labeled as ‘recruiters’ of TET1 have not been thoroughly tested on a genomic scale and are usually tested only at selected targets (Williams et al. 2011; Cartron et al. 2013). TET1:EZH2 and TET1:SIN3A have been implicated in the regulation of *HOXD12* and *NES1* respectively. Loss of the complexes resulted in increased methylation at each promoter and, therefore, decreased expression. Another study looked at the impact of Tet1:Sin3a complex on global genomic targets in mouse ES cells and showed that Sin3a recruited Tet1 to demethylate the promoter of *Lefty1* (Zhu et al. 2018). Loss of Sin3a resulted in altered localization of Tet1 and deregulation of 111 genes. However, the level and distribution of 5-hmC was not assessed.

Other identified proteins that act to recruit TET1 to genomic sites include PCNA and the Mbd3/NuRD complex (Williams et al. 2011; Yildirim et al. 2011; Cartron et al. 2013; Costa et al. 2013). Complexes containing TET1:PCNA are thought to play a role in the regulation of *MUC2* and *RRMI*, as experiments identified both proteins bound to these genes through chromatin-immunoprecipitation (ChIP) with α -PCNA antibody and sequential ChIP with α -TET1 antibody.

6.4 TET2 Binding Proteins

Identified binding partners of TET2 promote its localization and stabilization. Two factors that recruit TET2 to genomic sites are EBF1 and WT1 (Fig. 5c). EBF1 and TET2 are part of the same complex, which localize to hypermethylated promoters in the context of *IDH* mutations in chondrosarcoma (Fig. 5c) (Guilhamon et al. 2013). It is hypothesized that TET2:EBF1 complexes act to target tissue specific demethylation sites, but further research is required to identify changes in the 5-hmC landscape and impacts on gene expression. The relationship between WT1 and TET2 was discovered in hematopoietic cells, which commonly feature mutually exclusive mutations in *WT1* and *TET2* in AML (Rampal et al. 2014). The TET2:WT1 complexes are believed to play a role in maintaining normal 5-hmC levels and localization in hematopoietic cells. Another study showed that TET2 occupied the promoters of WT1 target genes, and this occupancy is lost when *WT1* expression was depleted, indicating WT1 recruited TET2 to genomic sites (Wang et al. 2015). Further, mutation of either WT1 or TET2 resulted in lower total 5-hmC levels and aberrant distribution at enhancers, gene bodies, and distal regulatory elements. Conversely, when *WT1* and *TET2* were coexpressed in HEK293T cells, 5-hmC

levels increased at promoters and transcription start sites of WT1 targets. When WT1 was depleted, 5-hmC enrichment at WT1 target genes was lost. When *IDH*, *WT1*, and *TET2* were mutated separately, 5-hmC changes in the gene body and distal regulatory regions had a positive correlation with gene expression changes (Rampal et al. 2014). When *TET2* was ectopically expressed in HEK293T cells, there was increased gene expression from WT1 targets, which was abrogated upon loss of TET2 catalytic activity (Wang et al. 2015). Finally, proliferation of leukemia cells was stimulated by the loss of either TET2 or WT1 but the loss of both was not additive, indicating that TET2 and WT1 function in the same pathway that controls cell proliferation. Taken together, evidence suggests that the TET2:WT1 complex acts to maintain 5-hmC and normal gene expression, and that loss of either protein results in an oncogenic phenotype (Rampal et al. 2014; Wang et al. 2015).

Idax and DNMT1 have been found to be involved in TET2 protein degradation and stability (Ko et al. 2010; Rampal et al. 2014; Wang et al. 2015; Zhang et al. 2017). A study shows that *Idax* and *Tet2* bind to each other, and the coexpression of *Idax* and *Tet2* in HEK293T cells led to a decrease of *Tet2* protein and a decrease in 5-hmC, but only when the DNA binding domain of *Idax* was present (Ko et al. 2013). This evidence suggests that *Idax* binds DNA and *Tet2* then recruits an unknown degradation complex to target *Tet2*, which results in decreased 5-hmC. Binding of TET2 to DNMT1 occurs after the acetylation of TET2, and appears to promote protein stability (Zhang et al. 2017). The impact of TET2:DNMT1 on 5-hmC has yet to be explored, but it appears to prevent abnormal methylation of DNA. The location of 5-hmC loss and any impact on gene expression have yet to be investigated.

6.5 TET3 Binding Proteins

The known binding partners of Tet3, *Rest* and WT1, are believed to be involved in TET3 genomic localization (Rampal et al. 2014). The Tet3:*Rest* complex forms during murine retina maturation, which requires the formation of a complex neural network (Perera et al. 2015). *Rest* binds a Tet3 isoform that lacks the CXXC domain, indicating it may serve as a DNA targeting factor for Tet3. Coexpression of *Tet3* and *Rest* leads to a dramatic increase in 5-hmC levels, more than overexpression of *Tet3* alone. *Rest* target genes were enriched in a subset of genes that gained 5-hmC, suggesting a synergistic interaction. Measurement of global protein levels indicated that the protein levels of many *Rest* gene targets were significantly increased when *Tet3* was overexpressed. However, this increase was lost when *Rest* was not present, meaning both Tet3 and *Rest* are required for the observed phenotype. Finally, TET3:WT1 forms a complex following the depletion of TET2 in hematopoietic models, but this is considered a compensatory mechanism for the loss of TET2 (Rampal et al. 2014).

7 Conclusions

The 5-hmC epigenetic landscape is modulated by the regulation of TET enzymes at the genetic, transcription, and protein levels. As discussed above, the activity of TET enzymes is tightly controlled at each of these levels. However, much of our knowledge is incomplete. More research is needed to understand the mechanisms that control *TET* gene expression and TET PTMs. In addition, although many TET binding partners have been identified, more research is required to determine their functions and mechanisms of action. Loss or change of any of these regulation mechanisms causes TET deregulation, which can alter the 5-hmC landscape and play a pathogenic role in disease. For this reason, study of the TET enzymes, in particular how they are regulated and impact the 5-hmC landscape, will provide answers to questions about the role of the epigenetic state in health and disease.

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