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PRIMER

The roles of TET family proteins in development and stem cells

Jihong Yang[1], Nazym Bashkenova[1], Ruge Zang[1,2], Xin Huang[1] and Jianlong Wang[1,*]

ABSTRACT

Ten-eleven translocation (TET) methylcytosine dioxygenases are enzymes that catalyze the demethylation of 5-methylcytosine on DNA. Through global and site-specific demethylation, they regulate cell fate decisions during development and in embryonic stem cells by maintaining pluripotency or by regulating differentiation. In this Primer, we provide an updated overview of TET functions in development and stem cells. We discuss the catalytic and non-catalytic activities of TETs, and their roles as epigenetic regulators of both DNA and RNA hydroxymethylation, highlighting how TET proteins function in regulating gene expression at both the transcriptional and post-transcriptional levels.

KEY WORDS: TET, DNA demethylation, Pluripotency, Differentiation

Introduction

Pluripotency refers to the ability of specific kinds of cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), to give rise to all cell types of the adult body. The process of DNA methylation – the addition of a methyl group at cytosine on DNA (5-methylcytosine, 5mC) – is closely associated with pluripotency (Wang and Li, 2017) as it plays a key role in regulating gene expression. The effects of DNA methylation on transcription vary by methylation regions: hypermethylation on promoters, cryptic enhancers, and super-enhancers can be repressive (Baribault et al., 2018), whereas high gene-body methylation is reported to be associated with highly expressed genes (Ball et al., 2009; Hon et al., 2012).

The gain, loss and maintenance of methyl marks on cytosine is the result of a balance between three interconnected pathways: the acquisition of *de novo* methylation marks via the action of DNA methyltransferase 3 (DNMT3); the maintenance of existing methylation by DNA methyltransferase 1 (DNMT1); and the active replication-independent erasure of DNA methylation by ten-eleven translocation (TET) proteins. The TET proteins, which include TET1, TET2 and TET3, belong to an evolutionarily conserved family of dioxygenases that can convert 5mC to 5-hydroxymethylcytosine (5hmC) (Hu et al., 2015b). TET1 was the first to be reported for its catalytic ability to convert 5mC to 5hmC (Tahiliani et al., 2009), followed by TET2 and TET3, which carry out similar reactions (Ito et al., 2010). Moreover, TET proteins can further generate 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by oxidizing 5hmC (Ito et al., 2011).

[1]Department of Medicine, Columbia Center for Human Development, Columbia University Irving Medical Center, New York, NY 10032, USA. [2]Clinical and Translational Research Center of Shanghai First Maternity and Infant Hospital, School of Life Sciences and Technology, Tongji University, Shanghai 200092, China.

*Author for correspondence (jw3925@cumc.columbia.edu)

J.W., 0000-0002-2879-9489

5hmC levels are closely associated with transcription. In both ESCs and neural progenitor cells (NPCs), genes with low expression generally exhibit abundant 5hmC at their promoters, whereas genes with high expression show depleted 5hmC at their transcription start site (TSS) regions. However, high 5hmC levels in gene bodies are positively correlated with high gene expression levels in ESCs, but with low gene expression levels in NPCs (Shi et al., 2017; Tan et al., 2013), highlighting that the function of 5hmC can be context dependent. Here, we review recent studies of TET proteins, providing an overview of their structure, functions and roles in pluripotent stem cells and early development.

Distinct features of TETs

Structure-function relationships of TETs

TET protein family members (TET1, TET2, TET3) share a common core catalytic domain at their C termini (Fig. 1), which comprises a double-stranded beta-helix (DSBH) domain (Iyer et al., 2009; Tahiliani et al., 2009) for substrate oxidation (Shen et al., 2014b), a conserved cysteine-rich domain for modulating chromatin targeting (An et al., 2017; Yamagata and Kobayashi, 2017), and a large low complexity insert that plays potential regulatory roles via post-translational modifications, such as phosphorylation and glycosylation (Bauer et al., 2015; Brill et al., 2009). TET1 and TET3 also contain a CXXC domain that aids binding to chromatin at CpG-rich sequences, whereas TET2 pairs up with IDAX protein (or CXXC4), an independent CXXC domain-containing protein that was originally encoded by the *TET2* gene and separated from the catalytic domain of TET2 through chromosomal inversion during evolution (Ko et al., 2013). The CXXC domain is highly conserved and responsible for associating with unmethylated CpG-containing sequences (Ko et al., 2013; Zhang et al., 2010). Of note, the CXXC domain may affect the genomic distribution of TET proteins, as indicated by enrichment of TET1 at promoter CpG islands in mouse ESCs (mESCs) versus enrichment of TET2 in gene bodies or enhancer regions (Hon et al., 2014; Huang et al., 2014; Wu et al., 2011).

The dioxygenase activity of TETs has been dissected by a number of crystallography studies (Hashimoto et al., 2014; Hu et al., 2013, 2015a). For instance, Hu et al. characterized a truncated but catalytically active form of human TET2, revealing that the cysteine-rich domain and the DBSH domain form a compact fold, stabilized by three zinc atoms (Hu et al., 2013). They further showed that the DNA is located above the DSBH domain and that methylated cytosine is inserted into the catalytic cavity and oriented towards catalytic Fe(II) and 2-oxoglutarate (2-OG) (Hu et al., 2013). The TET-mediated reaction can be split into two steps that require Fe(II) and α-ketoglutaric acid (αKG)-dependent dioxygenases. The first step, namely dioxygen activation, requires Fe(II) and αKG to convert a dioxygen molecule into a highly active Fe(IV)-oxo intermediate (Krebs et al., 2007), which then oxidizes the insert substrate on the C-H bond at the second step, namely substrate oxidation (Shen et al., 2014b).

Another study (Hashimoto et al., 2014) reported the crystal structure of *Naegleria* TET-like protein (NgTet1), which shares

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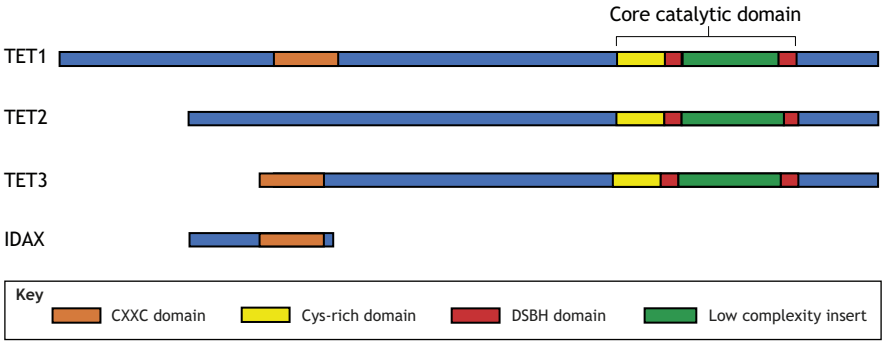

Fig. 1. Domain structure of mouse TET family proteins. The C-terminal core catalytic domain is highly conserved among all three TET family members. It consists of a cysteine (Cys)-rich domain, a DSBH domain, and a low complexity insert. The core catalytic domain supports the oxidizing function of TETs. The N termini of TET1 and TET3 contain a CXXC domain that recognizes CpG-rich motifs on DNA. TET2 lacks the CXXC DNA-binding domain; however, it can interact with a separate CXXC domain-containing protein, termed IDAX.

significant sequence conservation and similarity with mammalian TET1. This study discovered that NgTet1 uses a base-flipping mechanism to access 5mC on DNA, whereby the DNA strand is contacted from the minor groove and bent towards the major groove. In addition, the cysteine-rich region is predicted to insert within the corresponding loop L3, a four-residue short loop among the eight NgTet proteins (Hashimoto et al., 2014). Previous studies reported that loop L3 was involved in the interaction with the minor groove of DNA (Chen et al., 2010, 2013; Cho et al., 1994), and that the cysteine-rich insertion of NgTet1 is in the DNA-binding interface and thus might contribute to DNA binding (Hashimoto et al., 2014).

The expression of TETs

TET3 is highly expressed in oocytes and fertilized zygotes but rapidly disappears during cleavage, whereas TET1 and TET2 expression increases during pre-implantation development (Gu et al., 2011; Iqbal et al., 2011; Wossidlo et al., 2011). Although both TET1 and TET2 act together during the same period of embryonic development, their specific targets do not completely overlap.

TET expression has also been studied in different populations of stem cells. *In vitro*, TET2 protein is highly expressed in naive ESCs but not in primed epiblast-derived stem cells (EpiSCs), whereas TET1 protein expression is activated during the naive-to-primed transition *in vitro* (Fidalgo et al., 2016). *In vivo*, both TET1 and TET2 show high expression in the inner cell mass (ICM) of the mouse pre-implantation embryo (Rasmussen and Helin, 2016). However, TET2 is downregulated after implantation, whereas TET1 maintains its expression up to the post-implantation epiblast at embryonic day (E) 6.5∼7.5 (Khoueiry et al., 2017). After gastrulation (at around E8.5), TET1 and TET3 are weakly expressed in neural tube and head folds, whereas TET2 is not detected. At E9.5-E10.5, all three TET genes are detected in the developing brain (Khoueiry et al., 2017).

Catalytic activity-dependent and -independent functions of TETs

All three members of the TET family proteins possess Fe(II)- and 2-oxoglutarate-dependent enzymatic activity. They remove 5mC by oxidizing it to 5hmC and further oxidation products such as 5caC and 5fC (Fig. 2A). These methylcytosine derivatives can be processed to cytosine through thymine DNA glycosylase (TDG) and a base excision repair (BER) mechanism (Cortellino et al., 2011; He et al., 2011).

TET proteins mainly demethylate DNA at regulatory regions, including enhancers, promoters and other distal regulatory elements (Lu et al., 2014). At these regions, TET proteins may interact with various proteins and non-coding RNAs to exert their regulatory functions (Table 1). For example, the transcription factor NANOG physically interacts with TET1 (and also TET2), and both TET1 and TET2 facilitate NANOG-mediated somatic cell reprogramming in a manner that is dependent on their catalytic activities (Costa et al., 2013). An interesting mechanism involving the interaction of TET1 with the long non-coding RNA (lncRNA) TARID has been described in human cells (Arab et al., 2019). TARID is transcribed in an antisense orientation to the *TCF21* gene and favors the formation of a DNA-RNA structure termed the R-loop at the *TCF21* promoter, which is then bound by GADD45A and TET1 to trigger local DNA demethylation at CpG island sites within the R-loop to activate transcription (Arab et al., 2019). Recently, TET1 catalytic activity was found to be necessary for the expression of PGC7 (DPPA3), which binds and displaces UHRF1 (a key component of mammalian DNA methylation machinery). This, in turn, impairs DNMT1 recruitment and activity, thus driving DNA demethylation (Mulholland et al., 2018).

TET1 can also indirectly affect the expression of some genes independently of its catalytic activity by binding to certain regulatory factors and protein complexes. For instance, TET1

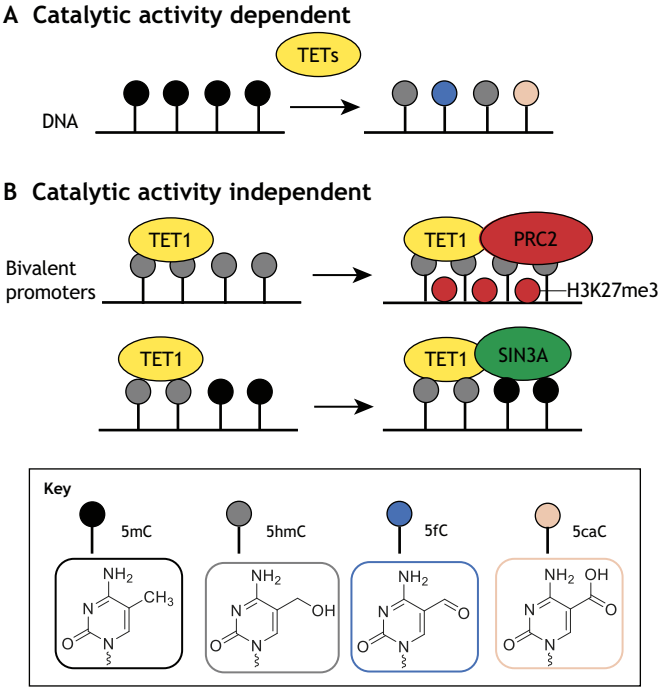

Fig. 2. Regulatory functions of TET proteins. (A) Catalytic activity-dependent functions of TETs. TET proteins carry out the oxidation of 5mC (into 5hmC, 5fC and 5caC) on DNA. (B) Catalytic activity-independent functions of TETs. TET1 can recruit the PRC2 complex to CpG-rich sites at bivalent promoters of PRC2-targeted genes, resulting in H3K27me3 at these regions (top). TET1 can also recruit SIN3A and colocalizes with the SIN3A co-repressor complex (e.g. SIN3A, HDAC1/2 and RBBP7, etc.) to repress target genes (bottom).

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Table 1. TET protein interactions/protein complexes and their related functions

TET complex/interacting protein	Functions	References
TET1↔GADD45A	TET1 is recruited by GADD45A to the R-loop formed by *TARID* RNA at the *TCF21* promoter for demethylation, thus activating the tumor suppressor gene.	Arab et al., 2019; Arab et al., 2014
TET1↔PRC2	TET1 contributes to silencing of PRC2-targeted developmental regulators by facilitating recruitment of PRC2 to CpG-rich promoters.	Wu et al., 2011
TET1↔SIN3A	TET1 and SIN3A form a complex and co-activate the *Lefty1* promoter by recruiting TET1 to demethylate the *Lefty1* promoter. The PAH1 domain of SIN3A is essential for the interaction, which is important for ESC pluripotency.	Williams et al., 2011; Zhu et al., 2018
TET1/2↔OGT↔SIN3A↔HDAC1	OGT binds to CpG-rich gene promoters to affect TET1 hypomethylation activity at these sites.	Shi et al., 2013; Vella et al., 2013
TET1↔NANOG	TET1 binds to the *Nanog* promoter, preventing it from being hypermethylated. NANOG physically interacts with TET1/TET2, and the TETs facilitate NANOG-mediated reprogramming in a catalytic activity-dependent manner.	Costa et al., 2013
TET2↔PSPC1	PSPC1 recruits TET2 to newly synthesized *MERVL* RNA for its demethylation, which destabilizes the *MERVL* RNA to restrict the 2C population in mESCs.	Guallar et al., 2018
TET1⊣ DNMT3B	TET1 inhibits the binding of DNMT3B to bivalent promoters. TET1 maintains the *PAX6* bivalent promoter in a hypomethylated state in human ESCs, thus regulating the cellular differentiation.	Verma et al., 2018
TET2⊣ DNMTs	TET2 balances transcription factors (e.g. OCT4, SOX2) occupancy on enhancers, which are more prone to methylation by DNMTs, thus regulating the timing of transcriptional changes during the differentiation.	Hon et al., 2014
TET1↔ZFP281	ZFP281 interacts with TET1 to activate miR-302/367, thus repressing TET2 expression to establish and maintain primed pluripotency.	Fidalgo et al., 2016

interacts with Polycomb Repressive Complex 2 (PRC2) (Neri et al., 2013), a repressive complex that has histone methyltransferase activity and is required for long-term epigenetic silencing. TET1 is involved in the repression of PRC2-targeted developmental genes through its recruitment of the PRC2 complex to chromatin. TET1 binds to CpG-rich regions at the promoters of both transcriptionally active and Polycomb-repressed genes. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) studies have identified a group of TET1/PRC2 co-bound TET1-dependent targets, among which are many lineage-specific genes including the primitive endoderm markers *Gata6* and *Sox17*, and the trophectoderm markers *Cdx2* and *Eomes* (Wu et al., 2011). ChIP experiments have been used to further validate that TET1 recruits PRC2 to the promoters of these genes to repress transcription in ESCs (Wu et al., 2011) (Fig. 2B, top). Interestingly, this catalytic activity-independent function in repressing lineage-specific genes applies to only TET1 but not TET2 (Koh et al., 2011), perhaps due to the lack of a CXXC DNA-binding domain in TET2.

Another interaction partner of TETs is the SIN3A/HDAC complex (Vella et al., 2013; Williams et al., 2011), a histone deacetylation activity-associated transcriptional repression complex that is involved in early embryo development (Grzenda et al., 2009). TET1 can recruit SIN3A to repress its target genes through catalytic activity-independent functions (Williams et al., 2011) (Fig. 2B, bottom). Moreover, both TET1 and TET2 are involved in forming a multiprotein complex with the OGlc-NAc transferase OGT, SIN3A and HDAC1. The recruitment of OGT to CpG-rich promoters stabilizes TET1 at CpG-rich sites and facilitates its function in promoting hypomethylation (Vella et al., 2013). On the other hand, SIN3A can operate as a transcriptional co-activator of *Lefty1*, which encodes a Nodal antagonist, by interacting with TET1 to demethylate the *Lefty1* promoter (Zhu et al., 2018). Taken together, these studies highlight that both catalytic activity-dependent and -independent roles of TETs contribute to their ability to regulate gene expression in different kinds of cells.

TET protein functions in early mouse embryo development

During mammalian embryo development, cells undergo two dynamic waves of demethylation. First, following fertilization, both maternal and paternal genomes are globally demethylated in zygotes (Saitou et al., 2012; Wu and Zhang, 2014). Global DNA methylation reaches a relative low level in the pre-implantation embryo, which is followed by increased methylation after the onset of implantation (Zhang et al., 2018). The second wave of demethylation then occurs in primordial germ cells (PGCs) of the post-implantation embryo. PGCs are specified at around E6.25 during mouse embryonic development, and then undergo two distinct DNA demethylation phases (Guibert et al., 2012; Seisenberger et al., 2012), namely a genome-wide demethylation phase and a locus-specific demethylation phase. The first phase occurs from E7.25 to E9.5, and is predominantly induced by passive demethylation (e.g. by replication and/or reduced DNMT activity). The second phase occurs from E9.5 to E13.5, and involves both TET1- and TET2-mediated oxidation (Piccolo et al., 2013; Vincent et al., 2013) (Fig. 3).

During the first wave, TET3 mediates the rapid decrease of 5mC signals, resulting in the generation of 5hmC, 5fC and 5CaC on the paternal genome (Inoue et al., 2012, 2011; Iqbal et al., 2011; Wossidlo et al., 2011). The maternal genome also undergoes TET3-mediated oxidation (Guo et al., 2014), but to a lesser extent (Peat et al., 2014; Shen et al., 2014a). However, for both parental genomes, it is DNA replication, not TET3, that makes the dominant contribution to demethylation, which was demonstrated by the finding that DNA replication inhibition blocks demethylation independently of TET3 function (Shen et al., 2014a). Moreover, in terms of the difference in oxidation between the maternal and paternal genomes, one possible explanation might be that PGC7 partially protects 5mC from TET3-mediated conversion to 5hmC by binding to maternal chromatin containing dimethylated histone H3 lysine 9 (H3K9me2) (Nakamura et al., 2012). Another study showed that PGC7 is recruited to the maternal genome and interacts with TET3 to suppress its enzymatic activity, thus protecting the maternal genome from demethylation (Bian and Yu, 2014).

In the second wave of demethylation in PGCs, TET1 and TET2 regulate locus-specific demethylation, but not genome-wide DNA demethylation (Vincent et al., 2013; Yamaguchi et al., 2012, 2013). A crucial set of germline reprogramming-responsive genes, such as *Dazl*, *Mael* and *Rad51c*, can be activated via TET1-dependent and

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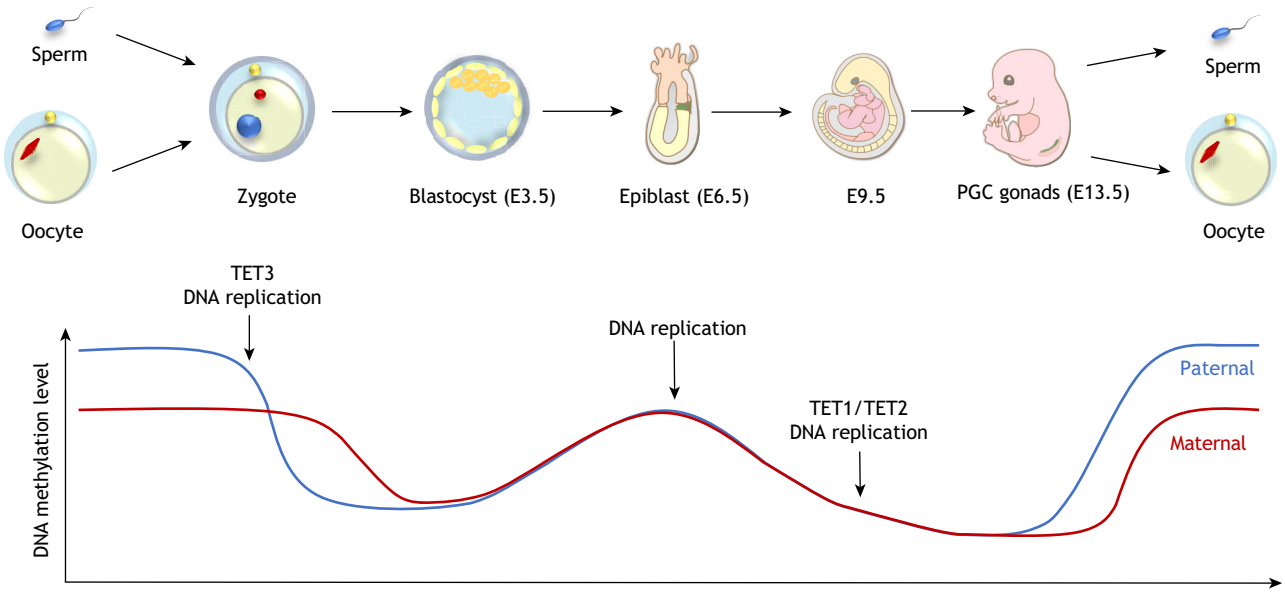

Fig. 3. Overview of TET protein functions during early development. After fertilization, DNA methylation levels are quickly decreased through replication-dependent passive DNA demethylation and TET3-mediated oxidation. Demethylation of the maternal genome occurs to a lesser extent than that of the paternal genome. 5mC levels are relatively low at the blastocyst stage (E3.5). This is followed by DNA methylation re-establishment, with 5mC levels reaching a high at the epiblast stage (E6.5). During the period of E7.25 to E9.5, the genome is demethylated in a replication-dependent but TET-independent manner. TET1 and TET2 then convert the remaining 5mC to 5hmC. This is then followed by a replication-dependent process to complete demethylation during PGC reprogramming. Finally, 5mC levels are regained and return to a high level during oogenesis and spermatogenesis.

-independent functions. Such genes are involved in gamete generation and meiosis (Hill et al., 2018). In addition, PGC7 has been reported to interact with TET2 and TET3 to suppress their enzymatic activity, and PGC7 knockdown induces DNA demethylation at imprinting loci, such as *Peg1* (*Mest*), *Peg2* (*Igf2*) and *H19* (Bian and Yu, 2014). Thus, PGC7 may have potential roles in regulating the second demethylation wave.

The roles of TET1 and TET2 later in development have also been explored. Both TET1 and TET2 are thought to be dispensable for embryonic development, and corresponding single knockout mice are viable and fertile (Dawlaty et al., 2011; Li et al., 2011). However, *Tet1* mutant mice display a slightly smaller body size at birth (Dawlaty et al., 2011), as well as reduced female germ cell numbers and fertility due to defects in meiosis in oocytes (Yamaguchi et al., 2012). In addition, only 30% of the expected number of *Tet1* knockout (KO) pups survive to birth, indicating potential embryonic lethality of the *Tet1* KO phenotype. In order to understand better the roles of TET1 during embryonic development, Khoueiry et al. (2017) generated new *Tet1* KO mice in which both the catalytic domain and the 5′ coding sequence are ablated. These mice exhibit deformities in forebrain development at late gastrulation (E9.5) and high mortality rates, in line with the early embryonic defects caused by loss of *Tet1*. *Tet2* deletion leads to the development of myeloid malignancies in mice (Li et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011). Both sexes of *Tet1* and *Tet2* double knockout (DKO) mice are fertile albeit displaying reduced fertility and smaller ovaries in the case of females (Dawlaty et al., 2013). However, the DKO of *Tet1* and *Tet3* is embryonic lethal, and $Tet1^{-/-}Tet3^{-/-}$ pups do not survive to birth (Kang et al., 2015). Early developmental abnormalities are evident in *Tet1/3* DKO eight-cell embryos, as characterized by delayed or aborted development. *Tet1/2/3* triple knockout (*Tet* TKO) embryos develop past the implantation stage, indicating that TET function is dispensable for the development of the egg cylinder (Dai et al., 2016). Even until the onset of gastrulation (E6.5), *Tet* TKO mutants appear similar to control embryos. However, *Tet* TKO embryos are much smaller compared with control embryos and exhibit defective mesodermal migration at E7.5. Apparent gastrulation defects are also observed at E8.5, with embryos having unrecognizable headfolds, heart, somites and gut tube. These knockout studies highlight the functional redundancy of TETs, which may mask developmental defects in single or double KO embryos. Overall, *Tet1* and *Tet3* deficiencies are likely to cause gastrulation defects, whereas *Tet2* deficiency causes myeloid malignancies.

TET functions in the totipotent state

Following fertilization, the resulting zygote starts a developmental program to give rise to a new organism. At the two-cell stage, each blastomere of the mouse embryo is capable of differentiating into a complete organism. This ability of a single cell to generate an entire organism, including embryonic and extra-embryonic structures, is called totipotency. The key features of totipotent embryos are: activation of transposable elements such as LINE-1 and MERVL; expression of *Zscan4*, a two-cell-specific gene that is responsible for genome stability and telomere elongation; changes in DNA and histone modifications; and chromatin reconfiguration (for a review, see Lu and Zhang, 2015). A number of studies have shown that TET proteins play a role in controlling totipotency. For example, Lu et al. (2014) demonstrated that TET proteins regulate the two-cell embryo (2C)-like state of ESCs. Specifically, they showed that *Tet* TKO mouse ESCs exhibit increased expression of Zscan4, which leads to telomere elongation by promoting telomere-sister chromatid exchange. GADD45 (growth arrest and DNA damage 45) proteins, which are regulators of TET-mediated demethylation, can also promote the 2C-like state and two-cell embryos, and *Gadd45a/b/g* TKO mESCs show impaired transition to a 2C-like state. Locus-specific DNA hypermethylation of ∼7000 sites and the consequent misregulation of TET and DNMT targets in *Gadd45a/b/g* TKO mESCs (Schule et al., 2019) are speculated to be responsible for this impaired transition, although the regulatory relationship

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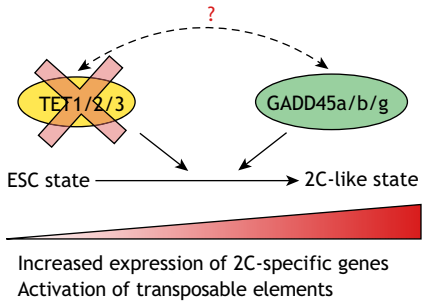

Fig. 4. TET protein functions in regulating the totipotent state. TET1/2/3 triple knockout promotes the transition from an ESC state to a two-cell-like (2C) state that exhibits features of totipotency (Lu et al., 2014). However, the triple knockout of *GADD45a/b/g*, which encode regulators of TET-mediated demethylation, impairs this transition (Schule et al., 2019). The regulatory relationship between TETs and GADD45 remains to be determined (as indicated by the dashed line).

between TETs and GADD45 in the transition remains to be determined (Fig. 4). Nonetheless, these studies suggest that the timely expression of TETs and their regulators may be closely associated with establishment of the 2C state.

The role of TETs in maintaining pluripotency of mESCs

ESCs maintain their pluripotent state through a dynamic network of transcription factors and enzymes (Kim et al., 2008; Loh et al., 2006; Pan and Thomson, 2007; Wang et al., 2006). One of the main pluripotency factors, OCT4 (POU5F1), directly upregulates the expression of *Tet2* by binding to its proximal promoter region (Koh et al., 2011; Wu et al., 2013). Regulation of the *Tet1* gene is also mediated by OCT4 (Koh et al., 2011) together with two other stemness factors, NANOG and MYC (Neri et al., 2015). *Nanog* is also a direct target of TET1: by binding to the *Nanog* promoter in mESCs, TET1 prevents it from hypermethylation. Compared with *Tet1* KO mESCs, *Tet* TKO cells show mostly unaltered expression levels of pluripotency factors, such as OCT4, NANOG and SOX2, as well as similar proliferation and colony formation rates as wild-type control cells (Dawlaty et al., 2014). These data demonstrate that *Tet* TKO has no obvious effect on the maintenance of ESCs. On the other hand, during the naïve to primed pluripotency transition, the transcription factor ZFP281 interacts with TET1 to activate the transcriptional target *miR-302/367*. By activating this primed state-specific miRNA and physically interacting with HDAC co-repressor complexes, ZFP281 represses *Tet2* expression at both the transcriptional and post-transcriptional levels to establish and maintain primed pluripotency. Conversely, ectopic TET2 alone efficiently reprograms primed cells towards naïve pluripotency (Fidalgo et al., 2016).

TET proteins can also regulate pluripotency by controlling the length of telomeres, which help sustain genomic stability and are required for self-renewal and pluripotency of ESCs and iPSCs (Huang et al., 2011; Liu, 2017). Studies conducted in mESCs deficient for TET1, TET2, or both have indicated a reduction in telomere length (Yang et al., 2016). Indeed, *Tet1* or *Tet2* single knockdown cells show decreased expression of telomere recombination genes, such as *Dmc1*, *Rad50* and *Smc1b*, and a minimal change in the expression of telomerase genes (Yang et al., 2016). In addition, the methylation writer and maintenance factors DNMTs and the methylation eraser TETs are closely related and influence telomeres (Gonzalo et al., 2006; Lu et al., 2014; Yang et al., 2016). For example, *Tet1/Tet2* double knockdown or knockout induces the expression of *Dnmt3b*, resulting in an elevated ratio of 5mC/5hmC, which leads to telomere shortening and chromosome instability (Lu et al., 2014; Yang et al., 2016). These studies demonstrate that TET proteins are important for telomere maintenance and thus play important roles in maintaining pluripotency (Fig. 5A).

The role of TETs in maintaining pluripotency in human ESCs

Human ESCs (hESCs), derived from the pre-implantation stage ICM (Crook et al., 2017), differ significantly from mESCs at both molecular and functional levels. They are considered to represent a later state of epiblast development, resembling primed mEpiSCs with regards to their morphology, developmental potency, gene expression and epigenetic modifications (Hackett and Surani, 2014; Rossant, 2015; Smith, 2017). Based on studies of mESCs (Koh et al., 2011; Olariu et al., 2016), and on recent studies of hESCs, it has been proposed that TET proteins are crucial players in maintaining pluripotency networks in human cells, acting through their physical and functional interactions with core pluripotency factors. For example, TET2 can bind to the *NANOG* promoter in hESCs, whereas high *NANOG* promoter methylation and low *NANOG* expression are detected in TET2-deficient cells (Langlois et al., 2014). On the other hand, a recent study from the Huangfu group (Verma et al., 2018) showed that TET proteins are crucial for preserving bivalent promoters in hESCs in a hypomethylated state. Using an iCRISPR platform, they generated *TET1*, *TET2* and *TET3* individual knockout hESC lines, as well as *TET* TKOs. *TET* TKO hESCs show no difference in morphology, self-renewal capacity or pluripotency gene expression compared with wild-type hESCs. However, *TKO* hESCs are unable to form teratomas and are also impaired in embryoid body differentiation, indicating crucial roles of TETs in regulating cellular differentiation. They further found and confirmed hypermethylation of bivalent promoters of several developmental genes, such as the endoderm marker *FOXA2*, the neuroectoderm marker *PAX6*, and the neural crest marker *SOX10* (Verma et al., 2018). By focusing on the *PAX6* bivalent promoter, the researchers observed that, despite promoter hypermethylation, PAX6 expression shows no change in hESCs, possibly due to a low level of *PAX6* expression at this stage. However, hypermethylation of the *PAX6* promoter prevents PAX6 activation during hESC differentiation, leading to impaired neuroectoderm formation. In addition, they found that, upon *TET* depletion, *de novo* methylation of bivalent promoters is carried out by DNMT3B, global inactivation of which partially reverses the *PAX6* promoter hypermethylation in TKO hESCs, demonstrating the dynamic competition between TET proteins and *de novo* methyltransferases in controlling cell lineage specification.

TET functions during ESC differentiation

TET proteins have also been associated with a broad range of differentiation processes (Fig. 5B). Individual knockdown of *Tet1* and *Tet2* causes alterations in different sets of genes. Deletion of *Tet2* leads to extensive 5hmC loss at enhancers, along with enhancer hypermethylation. For example, the enhancer that physically interacts with the *Lefty1* gene is hypermethylated and hypoacetylated in $Tet2^{-/-}$ mESCs, possibly explaining the decreased expression of this gene observed in these cells (Hon et al., 2014). When $Tet2^{-/-}$ ESCs are induced to differentiate into NPCs, they show delayed induction of some neural marker genes, such as *Slit3*, *Lmo4* and *Irx3* (Hon et al., 2014). On the other hand, knockdown of TET1 results in selective upregulation of differentiation genes, such as the trophectoderm markers *Cdx2*,

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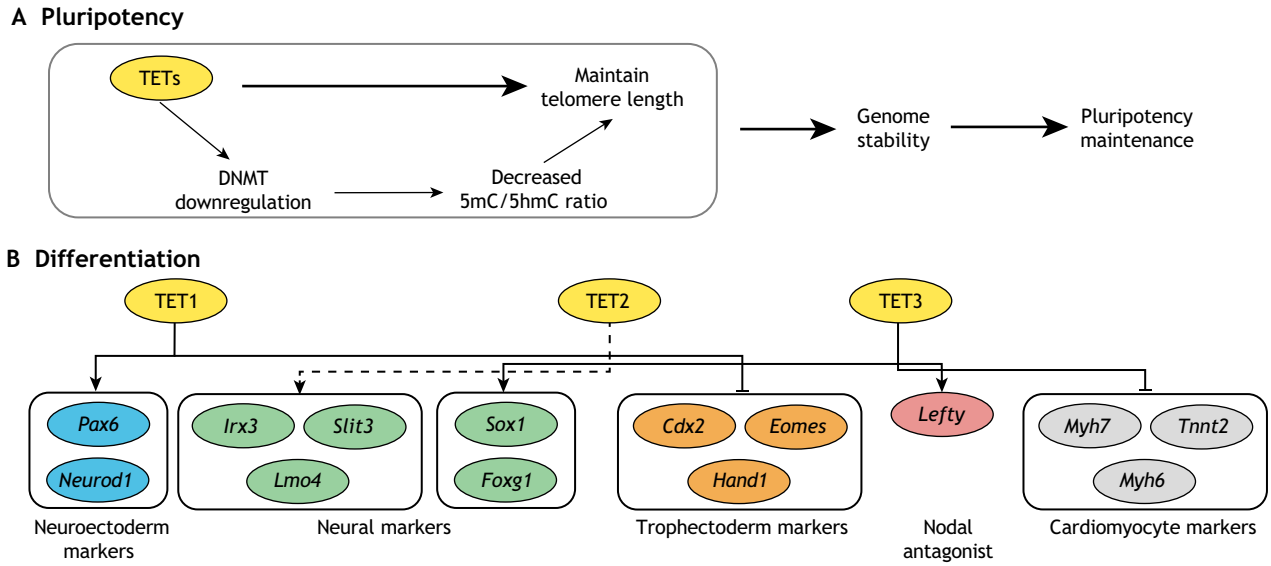

Fig. 5. TET functions in pluripotency and cell differentiation. (A) TET proteins regulate pluripotency by maintaining telomere length (Lu et al., 2014; Yang et al., 2016). They can also downregulate the expression of Dnmts, such as *Dnmt1*, *-3a* and *-3b*, which decreases the ratio of 5mC/5hmC, thus contributing to the maintenance of telomere length. This process is important for genome stability, which is essential for maintaining pluripotency. (B) TET functions in differentiation. TET1, TET2, and TET3 affect the expression of diverse differentiation marker genes. Solid arrowheads indicate increased expression, inhibitory symbols indicate decreased expression, and the dashed lines indicate delayed induction.

Hand1 and *Eomes*, and downregulation of the neuroectoderm markers *Neurod1* and *Pax6*. *Tet1* knockdown ESCs also display *Lefty1* downregulation and tend to differentiate into endoderm-mesoderm lineages in embryoid bodies (Koh et al., 2011). Consistent with this, increased endoderm and reduced neuroectoderm differentiation is observed in hemorrhagic teratomas formed by *Tet1*-depleted ESCs (Koh et al., 2011). *Tet1/2* double knockdown results in downregulation of pluripotency-related genes such as *Esrrb* and *Prdm14* (Ficz et al., 2011), which are reported to safeguard embryonic cells from adopting an endoderm cell fate (Ivanova et al., 2006; Ma et al., 2011), resulting in increased extra-embryonic lineage differentiation (Ficz et al., 2011).

TET3 knockout causes the promoter hypermethylation of secreted frizzled-related protein 4 (*Sfrp4*) as well as deceased gene expression, which may partially impair neuroectoderm formation in serum-free embryoid body assays (Li et al., 2016). Finally, *Tet1/2/3* TKO mESCs show global impaired differentiation ability, as characterized by poorly differentiated embryoid bodies and teratomas (Dawlaty et al., 2014), which is consistent with the impaired differentiation that occurs in human *TET* TKO ESCs (Verma et al., 2018). Taken together, these studies indicate that single and double TET deficiencies skew differentiation to certain lineage-specific cell fates, and loss of all three TETs compromises proper differentiation.

TETs and RNA modification

The recent discovery of reversible mRNA methylation has provided new insights into post-transcriptional gene regulation in eukaryotes. In addition to 5′ caps and 3′ poly(A) tail modifications, eukaryotic mRNA harbors several chemical modifications with apparent regulatory functions. These modifications affect almost every stage of mRNA metabolism: altering folding and structure, regulating mRNA maturation, enhancing nuclear processing and export to the cytoplasm, promoting mRNA translation, and facilitating its decay (for a review, see Roundtree et al., 2017). Although one of the most abundant mRNA modifications is N6-methyladenosine (m6A) (Dominissini et al., 2012), several other mRNA modifications exist, such as N1-methyladenosine (m1A), pseudouridine (Ψ), 5-methylcytosine (referred to here as m5C to distinguish from the DNA modification 5mC) and 5-hydroxymethylcytosine (referred to here as hm5C to distinguish from DNA modification 5hmC). 5mC has long been known as a DNA epigenetic mark. However, recent bisulfite treatment experiments have revealed several m5C sites on tRNAs (Khoddami and Cairns, 2013), which have been shown to have protective functions against stress-induced tRNA cleavage (Schaefer et al., 2009). Moreover, the oxidative derivative of m5C, hm5C, has also been detected in RNAs from *Drosophila* (Fu et al., 2014) to mammalian cells (Huber et al., 2015) and brain tissues (Miao et al., 2016).

Interestingly, both TET1 and TET2 have been identified as novel RNA-binding proteins (RBPs) (He et al., 2016), and it has also been demonstrated that TET proteins possess catalytic activity that oxidizes m5C to hm5C on RNA *in vitro* (Fu et al., 2014). We have also shown that TET2 is recruited to chromatin through a DNA-/RNA-binding protein, PSPC1, which is a component of paraspeckle complexes (Guallar et al., 2018). By binding to ERVL- and ERVL-associated transcripts, TET2 and PSPC1 regulate the expression of these genes at the post-transcriptional level. Specifically, TET2 oxidizes m5C into hm5C on newly synthesized *MERVL* RNA, dramatic enrichment of which is a hallmark of the 2C population (Macfarlan et al., 2012). The increased hm5C mark on *MERVL* RNA facilitates its destabilization and further degradation (Fig. 6). We therefore propose that TET2-mediated RNA hydroxymethylation may provide an additional regulatory layer to properly control ERV expression and hence restrict the 2C population in maintaining pluripotency of mESCs. However, studies on mRNA m5C/hm5C modifications are just beginning, with many unresolved issues. In one study, it was shown that TET2 is able to oxidize m5C to hm5C on *Socs3* mRNA in the context of myelopoiesis (Shen et al., 2018). In contrast, mass spectrometry analysis showed very rare or absent hm5C modifications on mRNAs

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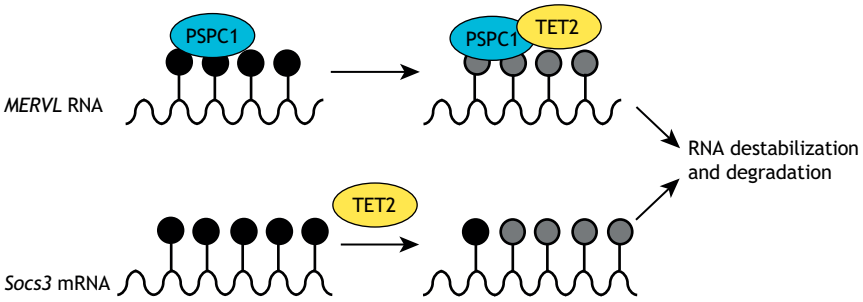

Fig. 6. TET proteins and RNA modification. The DNA-/RNA-binding protein PSPC1 recruits TET2, which deposits hm5C marks on *MERVL* RNA. TET2 can also carry out m5C/hm5C modifications on *Socs3* mRNA (Shen et al., 2018). Both lead to RNA destabilization and further degradation.

in mESCs (Legrand et al., 2017). It is therefore possible that TET-mediated RNA m5C/hm5C modifications make modified RNA highly unstable and, hence, difficult to be detected.

Conclusions and perspectives

In summary, TET protein functions, including TET-mediated DNA demethylation as well as functional interplay with other protein complexes and non-coding RNAs, dynamically manifest in a balance between maintenance of pluripotency and lineage development. TET-mediated 5mC oxidation at promoter and enhancer sites modulates the expression of cell fate-determining genes to further facilitate the transcription of these genes during lineage commitment; as such, a lack of 5mC oxidation (e.g. in the case of *Tet* TKO) impairs proper differentiation of ESCs. In contrast, *Tet* TKO cells display no obvious effects of the maintenance of ESCs. The developmental defects that occur during gastrulation in TKO mice are probably due to impaired lineage specification.

It is also clear that TET proteins exhibit non-catalytic functions, interacting with many other proteins as well as being part of large protein complexes. Unbiased protein-protein interaction screening of TET-involved complexes has revealed that both TET1 and TET2 directly interact with the SIN3A/HDAC complex (Williams et al., 2011), OGT (Shi et al., 2013; Vella et al., 2013) and NANOG (Costa et al., 2013), and that TET2 also interacts with PSPC1 in ESCs (Guallar et al., 2018). TET1 may also interact with PRC2, although purification of the PRC2 complex failed to recover TET1 as an associated component, suggesting that the interaction between TET1 and PRC2 may be transient and difficult to capture (Wu et al., 2011). Alternatively, the PRC2-TET1 interaction could be developmental stage dependent, as TET1 recruits PRC2 to complexes at the promoters of lineage-specific genes in ESCs and, upon differentiation, PRC2 is released from these complexes. Indeed, TET1 also interacts with EZH2 and SUZ12, two PRC2 components, in mESCs but not in somatic cells (Neri et al., 2013). It is well-recognized that the PRC2 complex has RNA-binding capacity and can interact with a wide range of RNAs (Zhao et al., 2010), including nascent coding RNAs or non-coding RNAs (Kaneko et al., 2014, 2013). Therefore, it is possible that RNA-mediated interactions between TETs and other partner proteins can be achieved through either direct RNA-binding regions of TETs on their C termini (He et al., 2016), or indirectly through other RBPs such as PSPC1 (Guallar et al., 2018). Both direct and indirect interactions of TETs with other proteins and RNAs, as well as their functional roles are yet to be determined.

Another aspect of TET protein function that is poorly understood is their regulatory activities on RNAs, particularly with regard to establishment of the hm5C mark on mammalian mRNAs. However, it should be noted that a similar function of oxidizing m5C to hm5C is well-established for dTet in *Drosophila* (Delatte et al., 2016). In this context, dTet is the only conserved Tet ortholog in *Drosophila*, and depletion of dTet in S2 cells decreases the formation of hydroxymethylcytosine in RNA. Because m5C on tRNAs promotes tRNA stability and protein synthesis (Tuorto et al., 2012), it is possible that oxidizing m5C to hm5C on mRNAs may destabilize target RNAs and promote degradation, as has been observed for MERVL (Guallar et al., 2018). Thus, although the RNA m6A decoration, including its writers, erasers, readers and functions in RNA metabolism, is well-established (for a review, see Yang et al., 2018), the dynamics and functions of RNA m5C/hm5C, especially on mRNAs, await further investigation.

In closing, although steady progress has been made in understanding TET functions in stem cells and development, more studies are needed to reveal the mechanisms that underlie TET functions in balancing pluripotency and differentiation, controlling embryonic development and regulating RNA modification and gene expression.

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Competing interests

The authors declare no competing or financial interests.

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