

Minireview

Functions of TET Proteins in Hematopoietic Transformation

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DNA methylation is a well-characterized epigenetic modification that plays central roles in mammalian development, genomic imprinting, X-chromosome inactivation and silencing of retrotransposon elements. Aberrant DNA methylation pattern is a characteristic feature of cancers and associated with abnormal expression of oncogenes, tumor suppressor genes or repair genes. Ten-eleven-translocation (TET) proteins are recently characterized dioxygenases that catalyze progressive oxidation of 5-methylcytosine to produce 5-hydroxymethylcytosine and further oxidized derivatives. These oxidized methylcytosines not only potentiate DNA demethylation but also behave as independent epigenetic modifications *per se*. The expression or activity of TET proteins and DNA hydroxymethylation are highly dysregulated in a wide range of cancers including hematologic and non-hematologic malignancies, and accumulating evidence points TET proteins as a novel tumor suppressor in cancers. Here we review DNA demethylation-dependent and -independent functions of TET proteins. We also describe diverse *TET* loss-of-function mutations that are recurrently found in myeloid and lymphoid malignancies and their potential roles in hematopoietic transformation. We discuss consequences of the deficiency of individual *Tet* genes and potential compensation between different *Tet* members in mice. Possible mechanisms underlying facilitated oncogenic transformation of TET-deficient hematopoietic cells are also described. Lastly, we address non-mutational mechanisms that lead to suppression or inactivation of TET proteins in cancers. Strategies to restore normal 5mC oxidation status in cancers by targeting TET proteins may provide new avenues to expedite the development of promising anti-cancer agents.

INTRODUCTION

In mammals, the DNA methylation and hydroxymethylation

status is dynamically regulated by the interplay between DNA methyltransferase (DNMT) and Ten-eleven-translocation (TET) family enzymes (Pastor et al., 2013; Shen et al., 2014; Suzuki and Bird, 2008). DNMTs carry out DNA methylation by transferring a methyl group (CH₃) from S-adenosyl methionine (SAM) to the 5-carbon of cytosine in a CpG dinucleotide to generate 5-methylcytosine (5mC), which takes place predominantly in a symmetrical manner (Fig. 1A) (Ooi et al., 2009). DNMT3A and DNMT3B are called *de novo* DNA methyltransferases because they create the initial methylation marks on unmethylated substrates during the early stage of embryonic development. Replication of the DNA bearing the symmetrically methylated CpG dinucleotides results in hemimethylated DNA strands because new strands are synthesized in the absence of methylation marks. However, DNMT1/UHRF1 complex is preferably targeted to these hemimethylated regions and restores normal symmetrical methylation patterns, a process termed maintenance methylation (Fig. 1A) (Arita et al., 2008; Avvakumov et al., 2008; Bostick et al., 2007; Hashimoto et al., 2008; Sharif et al., 2007). Under conditions where maintenance methylation is blocked by aberrant targeting, expression, or function of the DNMT1/UHRF1 complex, 5mC undergoes progressive, passive dilution following each round of replication (Pastor et al., 2013; Shen et al., 2014).

Enzymes of TET family (TET1, TET2 and TET3) belong to the superfamily of Fe²⁺- and 2-oxoglutarate (2OG)-dependent dioxygenases. All three TET proteins possess a highly conserved carboxy-terminal catalytic region that is composed of a cysteine-rich (Cys-rich) and a double-stranded β-helix (DSBH) domain (Fig. 1B) (Iyer et al., 2009; 2011; Tahiliani et al., 2009). TET proteins modify the methylation status of DNA by catalyzing consecutive oxidation of the methyl group of 5mC to form 5-hydroxymethylcytosine (5hmC), which in turn undergoes further oxidation by TET proteins into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Fig. 1C) (He et al., 2011; Ito et al., 2011; Ko et al., 2010; Tahiliani et al., 2009). These oxidized methylcytosines are collectively termed 'oxi-mCs'. When expressed in cells, the catalytic region alone is capable of oxidizing 5mCs in genome. TET proteins require ferrous iron Fe²⁺ (as an essential cofactor) and 2OG (as an obligatory co-substrate) which bind to the highly conserved 'His-Xaa-Asp-(Xaa)n-His' motif (Xaa refers to any amino acid) and Arg residues within the DSBH domain, respectively (Fig. 1C) (Hu et al., 2013; Tahiliani et al., 2009). Once Fe²⁺ and 2OG are incorporated into their cognate binding motifs in the active site, dioxygen (O₂) binds to Fe²⁺ and oxidizes it to ferric intermediates (Fe³⁺), stimulating the oxidative decarboxylation of 2OG (to succinate and CO₂) and

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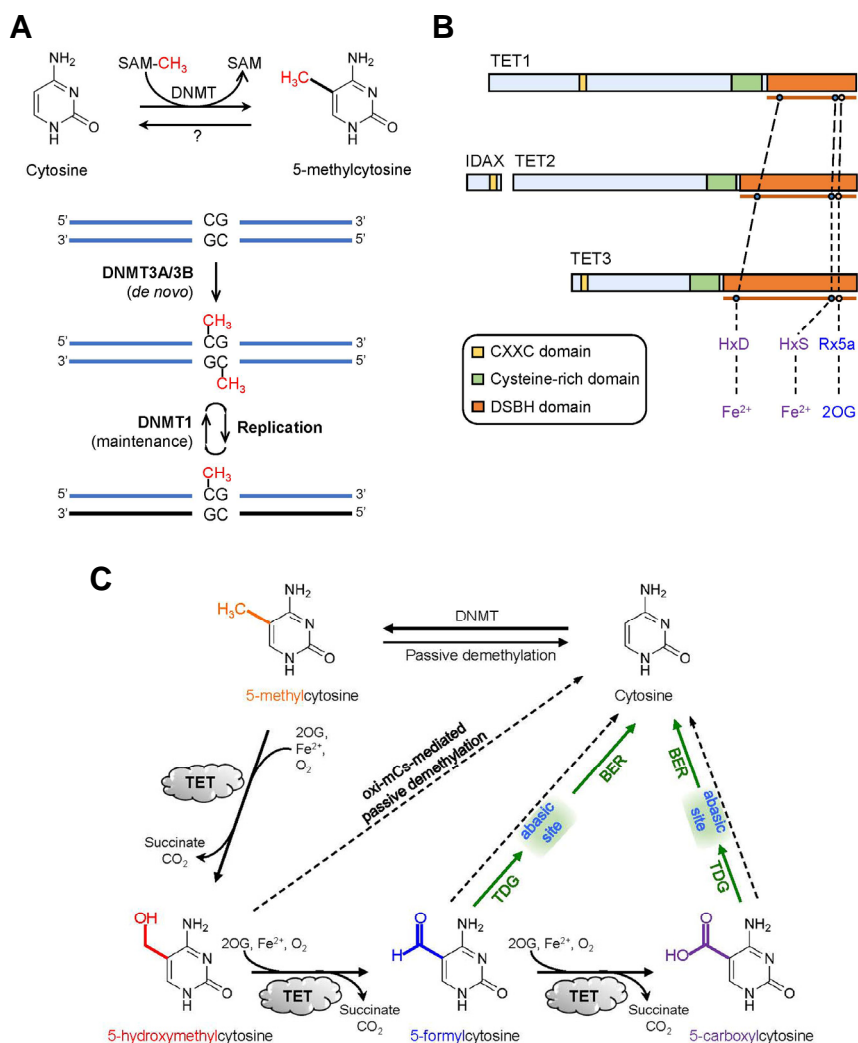


Fig. 1. DNA methylation and demethylation processes mediated by DNMTs and TET proteins. (A) DNA methylation refers to the transfer of the methyl group from S-adenosyl methionine (SAM) to the 5-carbon of cytosine to yield 5mC. *De novo* DNA methyltransferases DNMT3A and DNMT3B originally generate 5mC marks on unmethylated CpG dinucleotides. Replication leads to hemimethylated CpG sites that are remethylated by maintenance methyltransferase DNMT1. (B) Domain structure of the TET family proteins. TET proteins contain the catalytic core region that consists of cysteine-rich and double stranded β -helix (DSBH) domain. Within the DSBH domain, key catalytic residues that bind Fe²⁺ and 2-oxoglutarate (2OG) are present. (C) Oxidative reversal of DNA methylation marks in mammals. TET proteins promote both passive and active DNA demethylation. TET proteins oxidize 5mCs to produce oxi-mCs that antagonize DNA binding of DNMTs, promoting passive demethylation. They also facilitate active DNA demethylation because thymine DNA glycosylase (TDG) directly excise 5fC and 5caC, followed by base excision repair (BER) of the abasic site and replacement with an unmodified cytosine.

the oxidation of substrate molecules (Fig. 2) (Shen et al., 2014).

TET1 (also known as CXXC6) and TET3 additionally have the amino-terminal CXXC domain that belongs to the zinc finger type DNA-binding domain. This domain preferably recognizes unmethylated relative to methylated CpG dinucleotides *in vitro* and in cells (Xu et al., 2011b; 2012; Zhang et al., 2010). The *TET2* gene does not contain exons that encode the CXXC domain but studies on the ontogeny of *TET* genes during vertebrate evolution proposed that after an ancestral *TET* gene underwent triplication to give rise to three different *TET* paralogs, a chromosomal inversion occurred in the *TET2* gene, leading to the separation of the segments that encode the CXXC domain and catalytic domain (Iyer et al., 2009; 2011; Ko et al., 2013). Thus, the exon encoding ancestral CXXC domain of *TET2* formed a distinct gene named *IDAX* (also known as *CXXC4*) (Fig. 1B). Similar to the linked CXXC domains of *TET1* and *TET3*, the *IDAX* CXXC domain has a high affinity for unmethylated CpG sequences and thus is preferentially targeted to CpG islands (Ko et al., 2013), suggesting that TET proteins contribute to the maintenance of the hypomethylated status of CpG islands.

The early observation of the chromosomal translocation t(10;11)(q22;q23) that occurs rarely in patients with acute mye-

loid leukemia (AML) and acute lymphocytic leukemia (ALL) revealed that TET proteins may play a role in the pathogenesis of hematopoietic cancers (Lorsbach et al., 2003; Ono et al., 2002). As a result of this translocation, the segment on chromosome 10 that encodes the amino-terminal region of mixed-lineage leukemia (MLL) H3K4 methyltransferase is fused to the segment on chromosome 11 that encodes the DSBH domain of *TET1* (Lorsbach et al., 2003; Ono et al., 2002). Loss-of-function mutations or deletions in *TET2* gene are recurrent in a wide range of myeloid and lymphoid malignancies (Huang and Rao, 2014; Ko et al., 2015), and hypermethylation of the *TET1* promoter leads to a decrease in its expression in B cell lymphoma (Cimmino et al., 2015). Moreover, the expression and function of TET proteins and/or their modulators are frequently dysregulated in a wide variety of cancers (Huang and Rao, 2014; Ko et al., 2015), significantly impairing 5mC oxidation in the genome. Despite incomplete comprehension, various *Tet*-deficient murine models have provided valuable insights into the physiological function of TET proteins in normal hematopoiesis together with their pathogenic functions in malignant hematopoiesis, establishing TET proteins as novel tumor suppressors that directly drive hematopoietic transformation upon dysregulation. In this review, we focus on various loss-of-function *TET* muta-

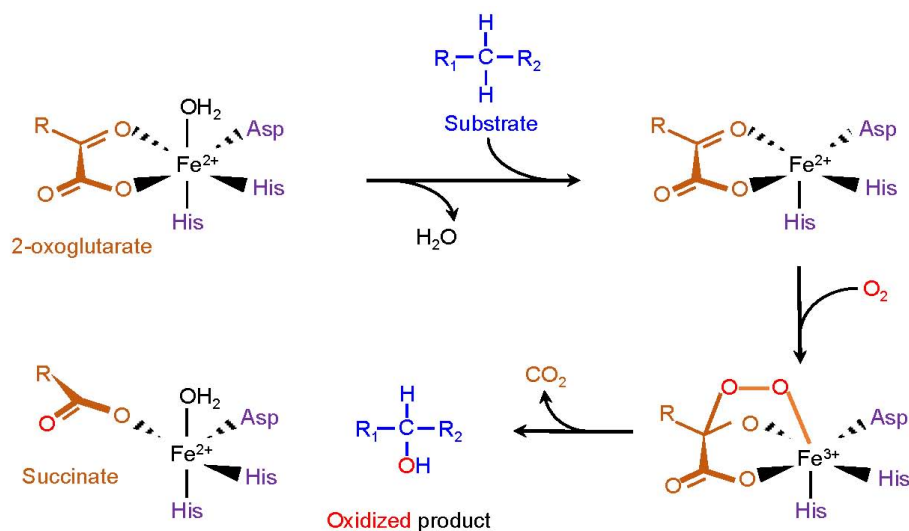


Fig. 2. The catalytic reaction mediated by TET proteins. The catalytic residues within the DSBH domain bind 2OG and Fe²⁺. Incorporation of O₂ yields ferric intermediate Fe³⁺, stimulating the substrate oxidation and oxidative decarboxylation of 2OG. The final products of this process are the oxidized product (oxi-mCs), succinate and CO₂.

tions and mechanisms leading to the dysregulation of TET expression and function in hematopoietic cancers. We also discuss the functional redundancy among different TET proteins, their association with other mutations, and the potential mechanisms by which TET loss-of-function promotes malignant transformation.

TET PROTEINS FACILITATE DNA DEMETHYLATION IN MAMMALS

DNA demethylation refers to the erasure of the methyl group from 5mC in DNA. This process occurs through at least two pathways, the passive or active demethylation pathways (Pastor et al., 2013; Shen et al., 2014; Wu and Zhang, 2014). As mentioned earlier, 5mC can be removed passively if newly synthesized DNA strands fail to be methylated again following replication when DNMT1 is absent or prevented from being recruited to these hemimethylated sites. TET proteins potentiate passive demethylation because all oxi-mCs are capable of interfering with maintenance methylation by antagonizing DNA binding of the DNMT1/UHRF1 complex, which is termed 'oxi-mC-facilitated passive demethylation' (Fig. 1C) (Hashimoto et al., 2012; Valinluck and Sowers, 2007).

It has long been known that the methyl group of 5mC can also be removed regardless of replication, particularly in the zygotic paternal genome or primordial germ cells, a process denoted active demethylation (Pastor et al., 2013; Shen et al., 2014). However, the mechanism that underlies the active demethylation of the mammalian genome has remained enigmatic until the TET enzyme function was identified recently (Tahiliani et al., 2009). TET proteins facilitate this replication-independent active demethylation in conjunction with DNA repair machinery (Fig. 1C); TET proteins iteratively oxidize 5mC to generate oxi-mCs, of which 5fC and 5caC are subject to removal by DNA repair enzyme thymine-DNA glycosylase (TDG), which triggers base excision repair that ultimately substitutes the resulting abasic sites with unmodified cytosines (Hashimoto et al., 2013; He et al., 2011; Maiti and Drohat, 2011; Zhang et al., 2012). TDG typically removes the mispaired thymine (generated as a result of 5mC deamination) from T:G mismatch but recent studies demonstrated that it also has affinity for 5fC and 5caC that are normally base-paired with G (He

et al., 2011; Maiti and Drohat, 2011; Zhang et al., 2012). Additional mechanisms that are potentially implicated in the DNA demethylation but are relatively less characterized include decarboxylation of 5caC by an unidentified enzyme (Schiesser et al., 2012), 5mC removal by DNMT (Chen et al., 2012), and 5hmC deamination by AID (activation-induced deaminase)/APOBEC (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide) family of cytidine deaminases followed by base excision repair (Guo et al., 2011).

DNA DEMETHYLATION-INDEPENDENT FUNCTIONS OF TET PROTEINS

Increasing evidence suggests that TET-generated oxi-mCs are not simple intermediates in DNA demethylation pathways. Instead, they have direct regulatory roles in gene expression by behaving as independent epigenetic modifications (Fig. 3). The extensive genome-wide mapping of oxi-mCs in various cell types revealed that these modified bases are strongly enriched at enhancers, promoters and gene bodies, with distinct effects on gene expression (Pastor et al., 2013; Wu and Zhang, 2014). In addition, 5hmC and 5fC are fairly stable bases that are diluted mainly through cell proliferation (Bachman et al., 2014; 2015). Compared with unmodified cytosine, the presence of 5hmC increased the thermodynamic stability of double-stranded DNA (Raiber et al., 2015). In contrast, 5fC did not affect thermodynamic stability but induced the conformational change of a DNA double helix, leading to an unusual structure (designated as 'F-DNA' by the authors) with characteristic helical underwinding (Raiber et al., 2015), raising the possibility that 5fC may affect local DNA supercoiling and packaging and hence modify the DNA's capacity to accommodate proteins. Also, RNA polymerase II specifically recognized the 5-carboxyl or 5-carbonyl moieties of 5caC or 5fC, respectively, by forming a hydrogen bond (Wang et al., 2015a). This interaction induced transient Pol II pausing or stalling and reduced the rate of Pol II transcription elongation on gene bodies (Kellinger et al., 2012; Wang et al., 2015a). Moreover, all oxi-mCs were shown to be recognized by their unique protein readers including transcription factors, splicing factors, DNA repair enzymes and chromatin remodelers (Hashimoto et al., 2014; Iurlaro et al., 2013; Spruijt et al., 2013) and TET proteins were also shown to interact with a

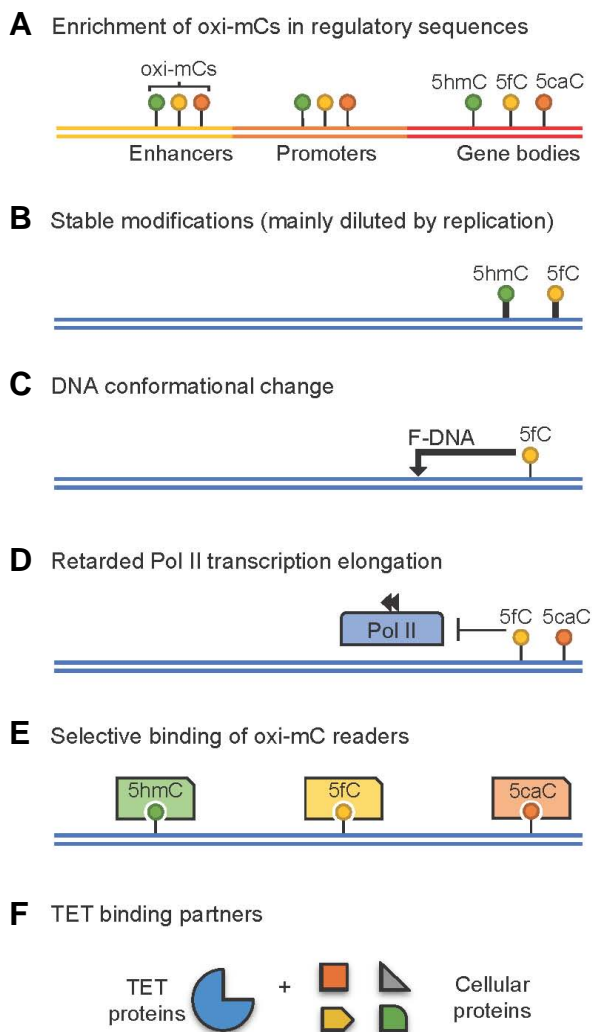


Fig. 3. Oxidized methylcytosines(oxi-mCs) can function as independent epigenetic modifications. (A) Oxi-mCs are enriched at DNA sequences associated with gene expression control. (B) 5hmC and 5fC are suggested as stable modifications that are mainly diluted through cell proliferation. (C) 5fC induces conformational change of DNA to 'F-DNA'. (D) 5fC and 5caC interact with RNA polymerase II and induce a pausing or retardation during transcription elongation. (E-F) Physical association of oxi-mCs (E) and TET proteins (F) with unique cellular proteins.

number of cellular proteins (Ko et al., 2015; Shen et al., 2014). These results collectively indicate that oxi-mCs potentially function as independent epigenetic marks regardless of DNA demethylation, and that the loss of these marks *per se* can directly affect expression of genes that are implicated in various aspects of cellular development and functions.

TET LOSS-OF-FUNCTION IN HEMATOPOIETIC TRANSFORMATION

TET1: friend or foe?

The potential function of TET1 in oncogenesis was first suggested in early studies in which *TET1* was discovered as a

partner gene that undergoes fusion with the *MLL* gene in rare cases of AML/ALL (Lorsbach et al., 2003; Ono et al., 2002). However, because cancer genome sequencing analyses rarely identified mutations in the *TET1* (as well as *TET3*) coding sequences in hematologic cancers (Huang and Rao, 2014; Ko et al., 2015), its exact function in normal and malignant hematopoiesis remained unexplored until recent studies reported key roles of TET1 in hematopoietic transformation (Cimmino et al., 2015; Huang et al., 2013). Interestingly, TET1 seems to have distinct roles depending on the types of cancers. In MLL-rearranged leukemia, TET1 acts as an oncogene that facilitates leukemogenesis by being directly activated by MLL fusion proteins and upregulating the expression of key oncogenic target genes such as *Hoxa9*, *Meis1* and *Pbx3* (Huang et al., 2013).

By contrast, another study using mice with germline deletion of *Tet1* showed that Tet1 may function as a tumor suppressor in the pathogenesis of B cell lymphoma (Cimmino et al., 2015). Ablation of Tet1 in mice led to lymphocytosis, enlargement of lymph nodes, splenomegaly, hepatomegaly, disrupted splenic architecture and infiltration of atypical lymphocytes into various organs such as the liver, lung and kidney, ultimately resulting in transplantable B cell lymphoma albeit at advanced age (Cimmino et al., 2015). Tet1 deficiency conferred a significant advantage on the ability of hematopoietic stem cells (HSCs) to reconstitute hematopoietic lineages in a cell-intrinsic manner, with a slight bias toward the B cell lineage. *TET1* promoters tended to be hypermethylated and transcriptionally silenced in human non-Hodgkin B cell lymphoma (B-NHL). Tet1 deficiency significantly decreased the frequency of long-term HSCs in the bone marrow and induced skewing toward the B cell lineage at early stage as shown by an increased frequency of lymphoid-primed multipotent progenitors (LMPPs) (Cimmino et al., 2015). Loss of Tet1 in hematopoietic stem/progenitor cells (HSPCs) altered transcriptional programs particularly for B lineage specification, chromosome maintenance, transcription and DNA repair. Tet1-deficient pro-B cells were able to self-renew more efficiently in semi-solid medium, compared with wild-type cells. They also displayed accumulation of DNA damage (γ H2AX staining) presumably due to reduced expression of genes implicated in DNA damage repair (Cimmino et al., 2015). Whole-exome sequencing of the tumors that developed in Tet1-deficient mice identified numerous co-existing mutations that are also recurrently found in patients with B cell lymphoma (Cimmino et al., 2015). However, how Tet1 plays distinct roles in the development of myeloid or lymphoid malignancies; how Tet1 loss induces alterations in the differentiation and function of HSCs, gene expression and DNA repair; and how these phenotypes are linked to oncogenic transformation remain to be determined.

High prevalence of *TET2* mutations in hematopoietic malignancies

In contrast to *TET1* and *TET3*, *TET2* undergoes somatic mutations very frequently in hematopoietic malignancies (Huang and Rao, 2014; Ko et al., 2015). Early studies showed that the chromosome 4q24 recurrently undergoes microscopic deletions and copy number-neutral loss-of-heterozygosity (also referred to as uniparental disomy) in patients with various myeloid malignancies such as myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN), acute myeloid leukemia (AML) and chronic myelomonocytic leukemia (CMML) (Delhommeau et al., 2009; Langemeijer et al., 2009; Viguie et al., 2005). *TET2* was initially described as a tumor suppressor because the deleted regions on 4q24 in these patients contained only *TET2* gene. Somatic mutations in the *TET2* coding

regions were very common in patients with either a heterozygous deletion or a copy number-neutral loss of heterozygosity (Delhommeau et al., 2009; Langemeijer et al., 2009). Following these reports, extensive whole-exome sequencing was performed in a wide range of myeloid malignancies and as a result, *TET2* was established as one of the most frequently mutated genes in myeloid malignancies (Huang and Rao, 2014; Ko et al., 2015). However, the prognostic value of *TET2* mutations in myeloid malignancies is still not clearly defined (Huang and Rao, 2014; Ko et al., 2015). Most of the *TET2* missense mutations tend to be clustered in two highly conserved regions within the catalytic domain, some of which are directly targeting key catalytic residues that interact with Fe²⁺ and 2OG and impair the normal enzymatic activity of TET2 (Ko et al., 2010). *TET2* mutations in clinical samples also correlate well with a profound reduction in the global level of 5hmC (Ko et al., 2010; Konstandin et al., 2011; Pronier et al., 2011). These results collectively indicate that *TET2* undergoes inactivating loss-of-function mutations during malignant myeloid transformation. Depending on the mutational status of *TET2*, myeloid cancer patients can be classified into two groups (i.e. 5hmC^{hi} vs. 5hmC^{low} groups) at the molecular level (Ko et al., 2010). Future studies will resolve whether these groups display any differences in disease progression or therapeutic responses, and whether 5hmC levels can be used as a novel diagnostic or prognostic marker.

One of the prominent features of *TET2* loss-of-function mutations is their ability to cause developmental skewing during differentiation of HSPCs. CD34⁺ HSPCs derived from MPN patients bearing *TET2* mutations efficiently reconstituted the hematopoietic system in nonobese diabetic mice with severe combined immunodeficiency (NOD-SCID), with a bias toward myeloid cells at the expense of lymphoid cells (Delhommeau et al., 2009). Similarly, murine and human HSPCs depleted of *TET2* also preferentially differentiated toward monocyte/macrophage lineages over the other lineages *in vitro* (Chang et al., 2014; Ko et al., 2010; 2011; Pronier et al., 2011). However, the molecular basis behind this biased differentiation is still ambiguous.

In addition to myeloid malignancies, *TET2* mutations are also common in lymphoid malignancies, particularly among patients with peripheral T cell lymphoma (PTCL) such as angioimmunoblastic T cell lymphoma (AITL: 33~76%) and peripheral T cell lymphoma, not otherwise specified (PTCL-NOS: 20~38%) (Lemonnier et al., 2012; Odejide et al., 2014; Quivoron et al., 2011). These patients frequently harbor *TET2* mutations together with mutations in *RHOA*, *DNMT3A* and *IDH2* genes (Couronne et al., 2012; Lemonnier et al., 2012; Odejide et al., 2014; Palomero et al., 2014; Sakata-Yanagimoto et al., 2014). *TET2* mutations correlate with derivation of T follicular helper cells and are associated with advanced-stage diseases and poorer clinical outcomes (Lemonnier et al., 2012). However, little is known about whether *TET2* mutations have any prognostic value in lymphoma. Given the rare occurrence of lymphomas in Tet-deficient murine models, acquisition of additional mutations may be important to drive lymphomagenesis. The pathophysiologic function of *TET2* mutations in lymphoma development and the outcome of their genetic interactions with other mutations warrant further investigation.

Lessons from murine models with individual deficiency of *TET* genes

In order to model a human disease containing *TET2* loss-of-function mutation, various groups have generated a variety of

murine models by either conditionally deleting critical exons of *Tet2* or adopting insertional mutagenesis [reviewed in (Ko et al., 2015)]. Despite distinct gene targeting strategies, all the Tet2-disrupted mice exhibited very similar hematopoietic phenotypes *in vivo*. Deficiency of Tet2 in adult mice as well as fetal livers resulted in amplification of the cell fraction (known as LSK, Lin⁻c-Kit⁺Sca-1⁺) that is enriched in HSCs (Ko et al., 2011; Kunimoto et al., 2012; 2014; Li et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011; Shide et al., 2012). Under competitive conditions, cells isolated from the bone marrow or fetal liver in Tet2-deficient mice were more efficient in reconstituting hematopoietic lineages in a cell-autonomous manner, compared with wild-type cells (Ko et al., 2011; Kunimoto et al., 2012; Li et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011). Similarly, serial transplantation of Tet2-deficient LSK CD150⁺ fetal liver cells showed augmented hematopoietic repopulation capacity (Kunimoto et al., 2014). Tet2 ablation seemed to increase serial replating capacity of HSPCs in colony formation assays *in vitro* (Kunimoto et al., 2014; Moran-Crusio et al., 2011; Quivoron et al., 2011). Taken together, these results imply that Tet2 normally restricts the expansion, differentiation and function of HSCs. Several studies have argued that Tet2 deficiency increases HSC self-renewal. However, the term 'self-renewal' is often misinterpreted in a manner that sometimes can be confused with 'serial repopulating capacity'. Strictly speaking, HSC 'self-renewal' refers to the process by which HSCs undergo divisions to give rise to identical HSCs to perpetuate themselves throughout life. Therefore, the impact of Tet2 deficiency on HSC self-renewal has yet to be directly tested. More rigorous test that directly quantifies amplified HSCs, instead of measuring peripheral blood chimerism, after serial transplantation of purified HSCs is required, as exemplified by a recent study that confirmed an augmented HSC self-renewal upon loss of Dnmt3a (Challen et al., 2012).

Notably, both homozygous and heterozygous deletion of *Tet2* gene can predispose mice to the development of CMML-like myeloid disease in several models, characterized by myeloid hyperplasia, leukocytosis associated monocytosis and neutrophilia, extramedullary hematopoiesis and enlargement of the spleen and liver (Li et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011). However, the mechanistic links between Tet2 loss and myeloid transformation are still lacking. Considering the increase of HSC number and repopulating capacity upon Tet2 deficiency, additional studies including limiting dilution assays might be of significant help to determine whether Tet2 deficiency promotes leukemogenesis by conferring a stem cell advantage on leukemia initiating cells.

It is now generally accepted that Tet2 loss-of-function facilitates myeloid transformation. However, for reasons that are not entirely clear, only part of the Tet2-deficient mice strains sporadically developed full-blown malignancy with a very long latency and incomplete penetrance (Huang and Rao, 2014; Ko et al., 2015). Some strains of Tet2-deficient mice did not develop disease, implying that the deletion of Tet2 alone is not sufficient to drive hematopoietic malignancies (Quivoron et al., 2011). Furthermore, *TET2* mutations observed in elderly people with clonal hematopoiesis were not always associated with an overt hematological malignancy (Busque et al., 2012). Considering that *TET2* mutations frequently occur at an early phase of oncogenesis in MDS, CMML and MPN and seem to contribute to clonal expansion of leukemic cells (Delhommeau et al., 2009; Haferlach et al., 2014; Itzykson et al., 2013; Papaemmanuil et al., 2013), *TET2* mutations are likely to represent an early oncogenic event that facilitates the initiation of transformation, and

then the resulting *TET2*-mutated pre-malignant cells acquire additional mutations in order to progress to a more malignant phenotype (Huang and Rao, 2014; Ko et al., 2015), in line with the 'second hit' hypothesis in cancer development. In support of this hypothesis, analyses of the mutational landscape in human cancers showed that *TET2* mutations are indeed frequently found in conjunction with other mutations such as mutations in splicing factors *SRSF2*, *SF3B1* and *ZRSR2*, and the polycomb proteins *EZH2* and *ASXL1* in MDS and CMML (Gelsi-Boyer et al., 2010; Haferlach et al., 2014; Hanssens et al., 2014; Meggendorfer et al., 2012; Muto et al., 2013). They also co-exist with *KIT*^{D816V} or *FLT3*^{TD} (ITD refers to internal tandem duplication) mutations in systemic mastocytosis (Tefferi et al., 2009; Traina et al., 2012) or AML (Patel et al., 2012), respectively. In murine models, concurrent depletion of *Tet2* together with either *Asx1* or *Ezh2* hastened the development of myeloid disease, recapitulating characteristics of human MDS (Abdel-Wahab et al., 2013; Muto et al., 2013), and the co-existence of *KIT*^{D816V} or *Flt3*^{TD} led to a more aggressive form of systemic mastocytosis (De Vita et al., 2014) or AML (Shih et al., 2015), respectively. Thus, accumulation of secondary mutations in a *TET2*-deficient background may affect the disease type, progression and latency of tumorigenesis.

Other than the expansion of HSPCs and sporadic occurrences of full-blown malignancy, *Tet2* deficiency in mice results in relatively mild hematopoietic phenotypes and all hematopoietic lineages differentiate normally. In murine hematopoietic cells, *Tet2* and *Tet3* mRNAs are both highly expressed and their expression levels are dynamically regulated during differentiation (Ko et al., 2010; Li et al., 2011; Moran-Crusio et al., 2011). Loss of either *Tet2* or *Tet3* results in merely a modest decrease in levels of genomic 5hmC (Ko et al., 2011; 2015). Like *Tet2* deficiency, conditional ablation of *Tet3* with a hematopoietic-specific *Vav-iCre* transgene leads to an alteration in the frequency of HSCs and augments their repopulating capacity under competitive condition (Ko et al., 2015). However, the overall hematopoiesis takes place normally even in the absence of *Tet3* (Ko et al., 2015). These results suggest that *Tet2* and *Tet3* may have overlapping functions and can compensate for one another. Further studies involving simultaneous loss of *Tet2* and *Tet3* in the hematopoietic system will resolve this issue.

POTENTIAL MECHANISMS UNDERLYING ONCOGENESIS DRIVEN BY TET DYSREGULATION

So far, numerous studies strongly suggest that *TET2* is a bona-fide tumor suppressor in the pathogenesis of myeloid cancers. However, most of these studies are descriptive with no detailed explanation about the underlying mechanisms. It is clear that *Tet2* deficiency not only alters homeostasis, differentiation and function of the HSCs, but also elevates 5mC levels at specific genomic loci by impairing 5mC consumption. However, whether these aberrations contribute to oncogenic transformation still remains unclear. Also, the impact of *TET* loss on changes of DNA methylation status might be more complex than we expect because the oxi-mCs can function independent of DNA demethylation as described earlier.

Aberrant gene expression and function are hallmarks of cancers and it was proposed that altered DNA methylation in *TET*-deficient cells would disrupt transcriptional programs that have pivotal regulatory roles in normal hematopoiesis. However, recent global approaches that involve genome-wide mapping of 5mC-marked regions combined with global gene expression profiles of wild-type and *Tet*-deficient embryonic stem cells or hematopoietic cells indicate that the changes in DNA methyla-

tion upon *Tet* loss do not directly account for altered patterns in gene expression (Challen et al., 2012; Hon et al., 2014; Huang et al., 2014; Lu et al., 2014). The conventional view regarding the abnormal DNA methylation patterns in cancers (global hypomethylation and localized CpG island hypermethylation) was proposed mainly based on the bisulfite sequencing, which does not discriminate between 5mC and 5hmC (Huang et al., 2010; Pastor et al., 2013). Considering a substantial loss of 5hmC in cancers, the apparent global hypomethylation in cancers may reflect reduced levels of 5hmC rather than 5mC. Thus, the cancer-associated DNA methylation patterns and their correlation with gene transcription need to be reevaluated using more advanced techniques that allows for genome-wide mapping of individual cytosine derivatives. Furthermore, the question regarding whether loss of *TET* catalytic activity, *TET* protein scaffolding function or both are responsible for hematopoietic transformation is also not clearly resolved, so there remains a possibility that the *TET* catalytic activity-independent mechanisms could also contribute to oncogenic transformation by altering chromatin architecture and gene expression through interactions with various cellular proteins, which will be discussed in more detail in a later section. Since the identification of factors leading to *TET* dysfunction and oncogenesis remains critical for the development of more safe and effective cancer therapy, the molecular basis of oncogenesis upon loss of *TET* function deserves further exploration.

NON-MUTATIONAL MECHANISMS THAT INACTIVATE TET PROTEINS

The mechanisms of *TET* protein regulation are of intense interest as *TET* protein expression and the global level of their primary catalytic product, 5hmC, is significantly diminished in many cancers (Huang and Rao, 2014; Ko et al., 2015). Given that *TET* proteins interact with a variety of proteins and that they are subject to regulation by various modulators, it is not surprising that the expression and activity of *TET* proteins can be targeted by non-mutational mechanisms during the onset and/or progression of cancers (Fig. 4). Indeed, a significant proportion of myeloid cancer patients who do not have somatic mutations in any *TET* genes had substantially reduced levels of 5hmC (Ko et al., 2010; Konstandin et al., 2011; Pronier et al., 2011), illustrating that wild-type *TET* proteins can become non-functional in these cancers. Even though mutations in *TET1* and *TET3* coding sequences are infrequent in hematopoietic malignancies, this does not necessarily mean that they are functional. Understanding of the various mechanisms that lead to the silencing of *TET* proteins in cancers will increase the options for manipulating *TET* activity or expression, and provide a more solid basis to restore normal levels of *TET* proteins and oxi-mCs in cancers, which may be beneficial for cancer therapy.

Regulation of *TET* mRNA or protein expression

The expression of *TET* proteins is regulated at multiple levels (Fig. 4). Mutations that lead to changes in the key regulatory sequences of *TET* genes such as promoters or enhancers can alter transcription by differentially regulating the recruitment of transcription regulators. Similarly, dysregulation of genes that encode essential upstream regulators can also affect *TET* expression. For instance, depletion of the high mobility group AT-hook 2 (HMGA2), a chromatin remodeling factor that is highly expressed in some cancers but not in most normal cells, upregulated *TET1* expression in breast cancer cells (Sun et al., 2013). Thereafter, *TET1* stimulated the expression of its downstream target *homeobox A* (*HOXA*) genes by being directly

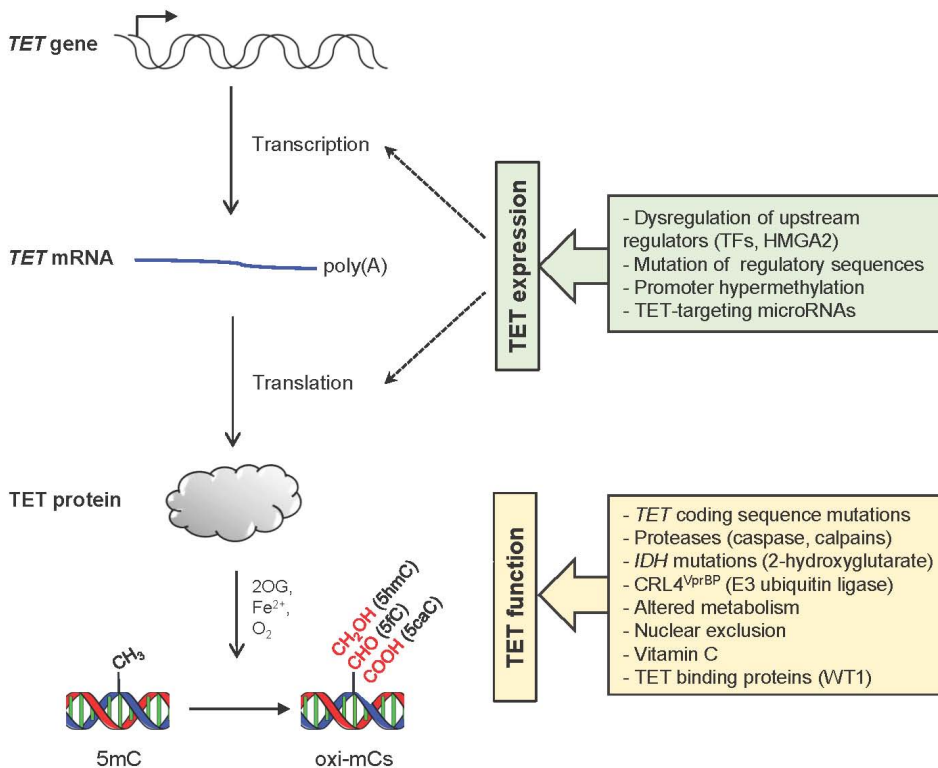


Fig. 4. Non-mutational modulation of TET expression and function. The expression and function of TET proteins are regulated on many levels. For details, please refer to the text.

targeted to their promoters to induce demethylation, preventing breast tumor growth, invasion and metastasis. Promoter CpG hypermethylation can also affect TET expression as reported in the *TET1* promoter in B cell lymphomas (Cimmino et al., 2015) or the *TET2* promoter in some gliomas and MPN (Chim et al., 2010; Kim et al., 2011).

Furthermore, a number of microRNAs repressed TET expression at the post-transcriptional level. One representative example is the oncogenic microRNA miR-22 that is highly expressed in myeloid malignancies (Song et al., 2013a; 2013b). Mimicking Tet2 deficiency, ectopic expression of miR-22 in mice led to HSC expansion and enhanced their repopulating activity and serial replating capacity, ultimately resulting in MDS-like hematopoietic malignancies that progress to myeloid leukemia (Song et al., 2013a). The disease phenotype was transplantable when miR-22-expressing LSK cells were transferred into wild-type recipient mice. The aberrant hematopoietic phenotypes and transformation induced by miR-22 expression were reversed by enforced expression of Tet2, demonstrating that Tet2 is a central downstream target of miR-22. MiR-22 also reduced genomic 5hmC levels in breast cancer cells by directly binding to the 3' untranslated region (3'UTR) of all *TET* mRNAs (Song et al., 2013b). Interestingly, miR-22 promoted the mesenchymal-epithelial transition (MET), mammary tumor development, and metastasis by downregulating TET expression, which in turn led to promoter methylation and suppression of the downstream miR-200 family members that are known to control various genes implicated in MET and metastasis (Song et al., 2013b). In another study utilizing high-throughput screening, more than 30 miRNAs including miR-125b, miR-29a, miR-29b, miR-26a, miR-26b, miR-101 and miR-7 were shown to diminish TET levels (Cheng et al., 2013). Enforced expression of Tet2-targeting miRNAs mimicked Tet2 loss as it induced the

expansion of myeloid lineage cells and CMML-like phenotypes, which was rescued by expressing Tet2 that lacks 3'UTR. Repression of TET expression by miR-26a was also shown to promote pancreatic differentiation (Fu et al., 2013).

TET expression is also controlled at the post-translational level. For example, a recent study showed that TET2 undergoes monoubiquitylation by the CRL4^{VprBP} complex, which increases the DNA binding activity of TET2 (Nakagawa et al., 2015). Several leukemia-associated *TET2* mutations were shown to impair CRL4^{VprBP}-mediated monoubiquitylation. Additionally, calpains, a family of Ca²⁺-dependent cysteine proteases, were shown to control the steady state level of TET proteins in embryonic stem cells (Wang and Zhang, 2014). Calpain 1 (encoded by *Capn1*) catalyzed the proteolysis of Tet1 and Tet2 proteins in undifferentiated embryonic stem cells, and calpain 2 (encoded by *Capn2*) degraded Tet3 in differentiated cells (Wang and Zhang, 2014). Interestingly, the expression of calpains is often aberrantly regulated in cancers. Similarly, caspases, another cysteine protease similar to calpains, were also reported to cleave Tet proteins (Ko et al., 2013).

Regulation of TET protein function

IDH1 and IDH2 are essential components of cellular metabolism that catalyze oxidative decarboxylation of isocitrate in the tricarboxylic acid (TCA) cycle to produce 2OG, a key intermediate in cellular energy metabolism. Genes encoding these proteins frequently undergo somatic heterozygous mutations in a majority of glioblastomas (~75%) and a subset of myeloid leukemias (~20%) or solid cancers (Abbas et al., 2010; Cairns et al., 2012; Gross et al., 2010; Parsons et al., 2008; Thol et al., 2010; Yan et al., 2009). Interestingly, specific arginine residues in the active site of IDH enzymes (R132 in IDH1 and R140 or R172 in IDH2 proteins) are frequently mutated, and the result-

ing mutant IDH enzymes gain a novel enzymatic function to reduce 2OG to oncometabolite 2-hydroxyglutarate (2HG) (Dang et al., 2009; Figueroa et al., 2010; Ward et al., 2010). 2HG functions as a potent inhibitor of TET proteins that competes with 2OG *in vitro* and *in vivo* (Xu et al., 2011a). Mice expressing mutant *Idh1* or *Idh2* showed aberrant hematopoietic phenotypes such as the expansion of HSPCs, splenomegaly, anemia and extramedullary hematopoiesis, although *IDH* mutations alone were not enough to drive full-blown leukemias unless they were combined with other mutations such as *HoxA9*, *Meis1* or *Ft3^{TD}* mutations (Kats et al., 2014; Sasaki et al., 2012). Intriguingly, continuous production of 2HG by mutant IDH proteins seemed to be critical for the maintenance of leukemic cells as suppression of mutant IDH expression or pharmacological inhibition of 2HG production eliminated the pathogenic features by promoting differentiation of leukemic cells. Additionally, because TET proteins are critically dependent on the presence of co-substrates 2OG and O₂ as well as a cofactor Fe²⁺, the activity of TET proteins is sensitive to alterations in the levels of energy metabolites (succinate, fumarate and 2HG), redox status, oxygen availability and iron homeostasis. Ascorbate (vitamin C) is also another cofactor that is presumed to help maintain Fe²⁺ in its reduced form for full TET catalytic activity (Blaschke et al., 2013; Minor et al., 2013; Yin et al., 2013).

TET protein function is also controlled by physical associations with other cellular proteins. A variety of proteins were shown to bind to TET proteins including WT1, IDAX, PGC7 and IκBζ (Ko et al., 2015; Shen et al., 2014). Dysregulation of these binding proteins can potentially lead to inactivation of TET proteins by impairing their chromatin targeting. For instance, WT1 was identified as one of key interacting partners for TET proteins (Rampal et al., 2014; Wang et al., 2015b). WT1 recruited TET2 to specific chromatin regions, particularly promoters, to activate TET2 target genes and thus *WT1* mutations led to functional inactivation of TET2, impairing TET2 target gene expression (Rampal et al., 2014). WT1 was also necessary for TET2 to suppress the growth and the colony formation of leukemic cells (Wang et al., 2015b). In line with these observations, *WT1* mutations were mutually exclusive with mutations in *TET2* and *IDH* genes in leukemias.

TET2 protein levels can also be regulated through association with another interaction partner, IDAX (Ko et al., 2013). IDAX was originally described as an inhibitor of the canonical Wnt/β-catenin signaling (Hino et al., 2001). Through a direct interaction, IDAX directly bound to the catalytic domain of TET2 via its CXXC domain and seemed to recruit TET2 to chromatin regions with high to medium density of unmethylated CpG dinucleotides, such as promoters and CpG islands. Intriguingly, high level of IDAX led to the activation of caspases that degraded TET2 proteins (Ko et al., 2013). IDAX is reported to be dysregulated in cancers; *IDAX* overexpression was reported in villous adenomas of the colon where it would contribute to the depletion of 5hmCs by promoting TET2 degradation (Paez et al., 2014). By contrast, homozygous deletion of *IDAX* was reported in an aggressive renal cell carcinoma (Kojima et al., 2009). In this case, the loss of IDAX would disrupt normal genomic targeting of TET2, resulting in aberrant expression of TET2 target genes.

In innate immune cells such as dendritic cells and macrophages, Tet2 was shown to repress *Il6* transcription in the late phase of the lipopolysaccharide induced inflammatory response (Zhang et al., 2015). Interestingly, Tet2 was recruited to the *Il6* promoter by a transcription factor IκBζ, which in turn additionally recruited Hdac2 to induce *Il6* transrepression through histone deacetylation. In addition to these regulatory

mechanisms, TET3 can be localized in the cytoplasm as well as in the nucleus (Arioka et al., 2012; Zhang et al., 2014). Therefore, it is likely to be subjected to regulation by signals that impair nuclear translocation. However, it is unclear whether subcellular localization of TET3 is altered in cancers, and therefore future studies are required to resolve this point.

CONCLUSION

Epigenetic modifications are generated and erased in a highly dynamic and reversible manner. Therefore, genetic aberrations leading to epimutations that are directly implicated in the initiation and progression of cancers represent an attractive target for cancer therapy. It is well documented that there is a direct causal link between TET dysregulation/mutations, impaired hematopoiesis and malignant hematopoietic transformation. Thus, it would be of great importance to assess whether manipulation of 5mC oxidation status by targeting TET enzymes displays clinical efficacy in cancer patients. In order for these trials to succeed, we essentially need comprehensive understanding of the mechanisms that lead to the dysregulation of TET protein expression and activity during oncogenic transformation. Moreover, further identification of proteins or small molecules that physically or functionally interact with TET enzymes, together with detailed dissection of the molecular basis of their actions are necessary. The knowledge gained through these research will allow us to devise more sophisticated strategies to specifically manipulate TET activities, thereby facilitating the development of more efficient, clinically promising therapeutic avenues to treat human cancers. Since the inactivation of TET function is presumed to be a general molecular basis implicated in the development of diverse solid cancers including cancers of lung, breast, colon, intestine, prostate, liver, brain and skin, an anti-cancer therapy targeting TET activity will be applicable over a broad spectrum of human cancers.

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