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TET proteins and 5-methylcytosine oxidation in hematological cancers

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Summary

DNA methylation has pivotal regulatory roles in mammalian development, retrotransposon silencing, genomic imprinting and X-chromosome inactivation. Cancer cells display highly dysregulated DNA methylation profiles characterized by global hypomethylation in conjunction with hypermethylation of promoter CpG islands (CGIs) that presumably lead to genome instability and aberrant expression of tumor suppressor genes or oncogenes. The recent discovery of Ten-Eleven-Translocation (TET) family dioxygenases that oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) in DNA has led to profound progress in understanding the mechanism underlying DNA demethylation. Among the three *TET* genes, *TET2* recurrently undergoes inactivating mutations in a wide range of myeloid and lymphoid malignancies. *TET2* functions as a *bona fide* tumor suppressor particularly in the pathogenesis of myeloid malignancies resembling chronic myelomonocytic leukemia (CMML) and myelodysplastic syndromes (MDS) in human. Here we review diverse functions of TET proteins and the novel epigenetic marks that they generate in DNA methylation/demethylation dynamics and normal and malignant hematopoietic differentiation. The impact of *TET2* inactivation in hematopoiesis and various mechanisms modulating the expression or activity of TET proteins are also discussed. Furthermore, we also present evidence that *TET2* and *TET3* collaborate to suppress aberrant hematopoiesis and hematopoietic transformation. A detailed understanding of the normal and pathological functions of TET proteins may provide new avenues to develop novel epigenetic therapies for treating hematological malignancies.

Keywords

TET proteins; 5-methylcytosine oxidation; hematopoiesis; malignant transformation; myeloid malignancies; lymphoid malignancies

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Introduction

Methylation of cytosine in DNA is one of the most extensively studied epigenetic modifications in the mammalian genome (1). In most somatic cells, DNA methylation typically takes place symmetrically on the 5-position of cytosine in the context of CpG dinucleotides (Fig. 1A). The *de novo* DNA methyltransferases DNMT3A and DNMT3B create the initial patterns of DNA methylation during embryonic development. Subsequently, the maintenance methyltransferase DNMT1 faithfully maintains the methylation patterns of the parental DNA strands. During DNA replication, DNMT1 is targeted to hemi-methylated DNA by its obligate partner protein UHRF1, which binds hemi-methylated CpGs, and adds methyl groups to the newly-replicated DNA strands to restore the symmetrical DNA methylation pattern.

DNA methylation was long considered a relatively stable epigenetic mark, but this view has been reversed by our recent discovery of the enzymatic function of Ten-eleven-translocation (TET)-family proteins as 5-methylcytosine oxidases (2, 3) (Fig. 1A). The *TET1* gene was the first member of this family to be identified, as a fusion partner in rare cases of acute myeloid and lymphocytic leukemias bearing the Ten-Eleven chromosomal translocation t(10;11)(q22;q23) which results in fusion of the *TET1* gene on chromosome 10q22 with the mixed-lineage leukemia gene (*MLL*; also known as *KMT2A*) on human chromosome 11q23 (4, 5). Based on their sequence homology with *TET1*, two additional *TET* genes, *TET2* and *TET3* were identified (5). All three mammalian TET proteins catalyze the successive oxidation of 5mC to yield 5hmC, 5fC and 5caC (hereafter collectively referred to as oximC)(2) (6–8). TET proteins utilize Fe(II) and α -ketoglutarate (α KG; also known as 2-oxoglutarate) as cofactors to activate molecular oxygen, then decarboxylation of α KG is coupled to the oxidation of TET substrates, 5mC and its two intermediate oxidized derivatives, 5hmC and 5fC (9); the final oxidation product is 5caC. Consistent with their roles in modifying DNA methylation status, TET orthologues are strictly restricted to metazoan organisms that utilize cytosine methylation (3, 10).

DNA methylation is dynamically regulated along the pathway of hematopoietic differentiation (11) and individual DNMTs play important roles in normal hematopoiesis (12–15). In early studies, *de novo* (Dnmt3a and Dnmt3b) and maintenance (Dnmt1) methyltransferases were both shown to be necessary for the self-renewal of hematopoietic stem cells (HSCs) (12, 14, 15). More recently, Dnmt3a-deficient HSCs were demonstrated to show augmented self-renewal capacity in serial transplantation assays by upregulating multipotency genes (13). DNA methylation is also involved in fate decisions of hematopoietic stem/progenitor cells (HSPCs) by directly regulating lineage priming (16) through transcriptional regulation (11, 17). In early progenitors that are undergoing differentiation towards a specific lineage, the regulatory regions of lineage-related genes are demethylated and expressed, whereas the regulatory regions of genes that specify alternative lineages are silenced by robust methylation (17). Compared with myeloid progenitors, lymphoid progenitors show a greater dependence on DNA methylation for efficient suppression of alternate-lineage (i.e. myeloerythroid) genes (11, 17). Consistently, lymphoid, but not myeloerythroid, genes were strikingly suppressed in HSPCs from *Dnmt1* hypomorphic mice, resulting in developmental skewing toward myeloerythroid lineages

with impaired B lymphopoiesis (12). Similar myeloid skewing was also induced by treatment with Dnmt1 inhibitors (11). *Dnmt1* hypomorphic mice were shown to develop aggressive T cell lymphoma possibly by increasing genome instability (18). Consistent with the high frequency of *DNMT3A* mutations in myeloid malignancies such as acute myeloid leukemias (AMLs; 20~30%) (19–21), myelodysplastic syndromes (MDS; 10~15%) (22) or myeloproliferative neoplasms (MPN) (23), mice reconstituted with HSPCs overexpressing *DNMT3A*^{R882H} mutant were recently shown to develop chronic myelomonocytic leukemia (CMML)-like disease (24).

In contrast, it is well documented that the *TET2* gene undergoes frequent somatic mutations in a wide spectrum of hematopoietic cancers including myeloid and lymphoid malignancies (25–27). In this review, we discuss *TET2* mutational profiles in hematopoietic cancers and the consequences of *TET2* loss-of-function, emphasizing its impact on normal and malignant hematopoiesis. Furthermore, we provide evidence for potential functional redundancy between Tet2 and Tet3 in the mouse hematopoietic system and document how Tet3 deficiency affects hematopoietic development in mice.

Structure and function of TET proteins

TET proteins typically possess a CXXC domain at the amino-terminal region and a catalytic domain consisting of a cysteine-rich and a double-stranded β -helix (DSBH) domain at the carboxy-terminal region (2, 3, 10). Structural analysis showed that the DSBH domain consists of eight conserved anti-parallel β -strands with a highly conserved Hix-Xaa-Asp-(Xaa)n-His motif (where Xaa means any amino acid) and conserved Arg residues that bind Fe(II) and α KG, respectively (28). The DSBH and Cys-rich domains are brought together by two zinc fingers to constitute the compact catalytic core (28). In jawed vertebrates, triplication of a common ancestral *TET* gene led to three distinct *TET* paralogues, and a subsequent chromosomal inversion split *TET2* gene into two segments encoding catalytic domain and CXXC domain, respectively (3, 10, 29). Thus, the ancestral CXXC domain of *TET2* is now separately encoded by a neighboring gene named *IDAX* (also known as *CXXC4*); *IDAX* and *TET2* are separated by ~650 kb in humans and ~800 kb in mice and are transcribed in opposite orientations.

TET proteins are clearly implicated in the pathway of DNA demethylation (reviewed in refs (30) and (31)) (Fig. 1A). First, under conditions where DNMT1 is absent or inhibited from access to newly replicated DNA, 5mC is diluted passively during DNA replication. Second, 5hmC and presumably other oxi-mC interfere with maintenance methylation by inhibiting DNA binding of the UHRF1/DNMT1 complex (32, 33), so TET proteins *per se* have the potential to facilitate passive DNA demethylation. The paternal genome of preimplantation embryos (i.e. zygotes) (34, 35) and germ cell precursors (called primordial germ cells) (36, 37) adopt both strategies to achieve genome-wide DNA demethylation. Third, TET proteins also mediate active demethylation, a replication-independent process that erases 5mC indirectly: a DNA repair enzyme thymine DNA glycosylase (TDG) directly excises the TET-generated 5mC oxidation products 5fC and 5caC, after which the resulting abasic sites are repaired by the base excision repair pathway to produce unmethylated cytosines (38–40). Additional demethylation mechanisms have been suggested but their physiological

significance requires further validation (30, 31). Notably, loss of TET proteins is not always linked to increased 5mC by impaired DNA demethylation (41), suggesting that oxi-mC intermediates can behave as stable epigenetic marks regardless of their roles in the DNA demethylation pathway, presumably by displacing methyl CpG-binding proteins, recruiting unique oxi-mC-interacting proteins or influencing the epigenetic modification of histones.

The CXXC domain of TET proteins is implicated in their recruitment to DNA, especially to the CpG-rich genomic regions known as CpG islands (42). The CXXC domain is a Zn finger type DNA-binding domain that recognizes CpG dinucleotides in DNA, and is found in several chromatin-associated proteins implicated in the epigenetic modification of DNA (DNMT1(43), MBD1(44)) and histones (MLL (45, 46), CFP1 (47–49), KDM2A (50, 51)). The preferred binding of CXXC domains to unmethylated CpG sites enables diverse CXXC domain-containing epigenetic enzymes to contribute to the unique epigenetic status of chromatin at CpG islands (CGIs) (42, 52). For example, CFP1 (also known as CXXC1) confers H3K4me3 by recruiting H3K4me3 methyltransferases MLL or SETD1 (48). KDM2A is also recruited to CpG islands via its CXXC domain, leading to depletion of H3K36me2 (50, 51).

The linked CXXC domain of TET1 and TET3 and the separated CXXC domain of TET2 (i.e. IDAX CXXC domain) preferably bind unmethylated compared with methylated CpG DNA (29, 53–55). Thus, TET proteins also contribute to the unique features of CpG islands by maintaining CpG island DNA in a hypomethylated state (Fig. 1B). In murine ES cells (ESCs), Tet1 preferentially binds CpG islands through its CXXC domain (53, 56). Tet1-bound CpG islands become hypermethylated upon Tet1 depletion. In HEK293T cells, ectopically expressed TET1 is selectively enriched within unmethylated CpG islands and generates 5hmC marks at their edges (57). In contrast, depletion of TET1 induced methylation spreading into the normally hypomethylated CpG islands, leading to the contraction of unmethylated regions at the edges of the CpG islands. Recently, genome-wide mapping of 5mC in HSCs led to a discovery of a novel genomic feature called 'canyons' (58), extended genomic regions with low methylation that are conserved among various cell types and species. Canyons are different from CpG islands in that they are ~10 times larger (i.e. ~3.5 kb in length); moreover, ~10% of canyons does not contain CpG islands. Genes differentially expressed in leukemias are significantly enriched for canyon-associated genes. Interestingly, canyon borders are demarked by 5hmCs. In the absence of Dnmt3a, canyon borders become eroded, leading to the expansion of canyons. These results suggest that Dnmt3a and Tet proteins collaborate to control canyon size, which is essential for normal hematopoiesis as well as suppression of malignant transformation.

Mutations in *TET* genes in human hematopoietic cancers

The aforementioned t(10;11)(q22;q23) translocation, which results in fusion of the N-terminal region of MLL H3K4 methyltransferase with the DSBH domain of TET1, has been reported in only a handful of acute myeloid/lymphoid leukemias (4, 5, 59–61). The molecular consequences of this fusion have yet to be characterized in detail, but the chimeric proteins may not retain 5mC oxidase activity because they lack the Cys-rich domain of TET1 that appears to be essential for enzymatic activity (2). Instead, the MLL-TET1 fusion

protein may exert its oncogenic effects by recruiting unknown factors, which physically associate with the TET1 DSBH domain, to MLL target genes. A recent study showed that TET1 has essential oncogenic roles in the development of MLL-rearranged leukemia (62). TET1 was aberrantly overexpressed by various MLL fusion proteins and promoted leukemogenesis by upregulating the expression of oncogenic target genes such as *Hoxa9*, *Meis1* and *Pbx3* (62).

It is generally accepted that *TET1* and *TET3* mutations are very infrequent in hematologic malignancies (63). Thus far, only a few *TET1* mutations have been reported in chronic lymphocytic leukemia (CLL) (64), AML (65) and T-cell acute lymphoblastic leukemia (T-ALL) (66). Similarly, *TET3* mutations are occasionally, but very rarely, identified in peripheral T cell lymphomas (PTCL) (67) and CLL (64). Because most mutational analyses (e.g. exome sequencing) have assessed only coding sequences of *TET* family genes in adult patients, it remains unknown whether *TET1* or *TET3* mutations occur more frequently in early embryonic or fetal cancers and also whether regulatory sequences such as promoters, enhancers or splicing sites are more susceptible to alterations relative to exons.

Among the TET family genes, *TET2* is most commonly mutated in hematopoietic cancers. In 2009, there were two reports showing that the chromosomal region 4q24 in human containing *TET2* gene frequently undergoes microdeletions and copy number-neutral loss-of-heterozygosity (also named uniparental disomy) in myeloid cancers (68, 69). *TET2* mutations were identified only in myeloid cancer cells but not in the other cells isolated from the same patients, indicating that they were somatically acquired. Following these reports, many groups have sequenced the *TET2* coding region in a wide range of myeloid malignancies classified as MDS, MPN, CMML, AML, secondary AML, and so on (63, 68–75) and have established *TET2* as one of highly mutated genes in myeloid malignancies. There are no mutational hotspots in *TET2*. Instead, mutations are distributed throughout its coding regions. Various types of mutations are observed including missense and nonsense mutations, out-of-frame insertions/deletions and splice site mutations, with missense mutations being relatively enriched in the core catalytic domain (27).

Intriguingly, *TET2* mutations are also recurrently found in various lymphoid malignancies. *TET2* is more frequently mutated in T cell lymphoma (~11.9% of total cases) than in B cell lymphoma (~2% of total cases) (76). The highest incidence of *TET2* alterations was observed among patients with peripheral T-cell lymphoma (PTCL), particularly in subsets of 33–76% of angioimmunoblastic T cell lymphoma (AITL) and 20–38% PTCL, not otherwise specified (PTCL-NOS) (76–78). AITL is thought to be derived from T follicular helper (T_{FH}) cells, and in AITL-NOS, *TET2* mutations were more common in a subgroup expressing T_{FH}-like features, so these mutational profiles suggest that *TET2* mutations in PTCL correlate with T_{FH} derivation (77). In PTCL (particularly AITL), *RHOA*, *DNMT3A* and *IDH2* genes are also mutated at very high frequency (~67, ~33 and ~20%, respectively) (67, 78, 79). Interestingly, *TET2* mutations co-exist with *RHOA*^{G17V} and *DNMT3A* mutations in a significant proportion of patients (67, 78–80), suggesting that cooperation of *TET2* mutations with these mutations may drive the development of AITL. Moreover, contrary to the mutually exclusive nature of *IDH* and *TET2* mutations commonly observed in myeloid malignancies, a significant proportion of (~88.2%) patients harbor both *IDH2*

and TET2 mutations at the same time (77, 78). The cooperative interactions among these mutations are being studied now.

The nature of leukemia-associated *TET2* mutations

Many *TET2* missense mutations present in leukemia patients, including those targeting catalytic residues of TET2, impair the catalytic activity of TET2 (8, 81, 82). No evidence of a dominant-negative effect has been reported yet. Determination of *TET2* mutational status of cells from patient with MDS, MPN, CMML and AML, and quantification of global 5hmC levels by DNA dot blot or mass spectrometry, have shown that *TET2* mutations correlate with a significant reduction in the levels of genomic 5hmC (8, 81). CD34⁺ stem cells from MPN patients with *TET2* mutations reconstituted hematopoiesis in NOD/SCID mice and displayed skewed differentiation toward myeloid lineages at the expense of lymphoid lineages (68). In addition, shRNA-mediated Tet2 knockdown led to an accumulation of Lineage-negative (Lin⁻) Sca-1⁺ c-Kit⁺ (LSK) fraction in which HSCs are enriched (83, 84) and skewed the differentiation of murine or human progenitor cells toward monocyte/macrophage lineages over lymphoid or erythroid lineages *in vitro* (8, 81, 84). Accumulation of progenitor cells was also observed when *Tet2*-disrupted fetal liver cells are cultured *in vitro* (85). This developmental bias is in line with the high prevalence of *TET2* mutations in CMML, whose defining feature is augmented monocytosis. These results suggest that *TET2* mutations promote leukemogenesis by interfering with 5mC oxidation and/or subsequent changes in DNA methylation status; these processes are critical for the homeostasis and differentiation of early HSPCs and interference with them may promote HSPC expansion and clonal hematopoiesis with differentiation bias (86).

Because TET2 converts 5mC to 5hmC, one would expect that inactivating mutations of *TET2* would cause accumulation of 5mC in the genome. However, how *TET2* mutations are linked to DNA methylation is still controversial. Figueroa *et al.* observed that patients with *TET2*-mutated *de novo* AMLs display a small number of hypermethylated *HpaII* sites (129) compared with CD34⁺ bone marrow cells from healthy controls (83). However, in our analyses, low 5hmC in patients with various myeloid malignancies was predominantly associated with mild DNA hypomethylation at most differentially-methylated CpG sites (8). We have discussed possible reasons for this discrepancy elsewhere (84, 87). Subsequent methylation profiling in CMML patients also showed that patient with *TET2* mutations contain more hypomethylated regions at differentially methylated CpG sites (88). In another study, *AIM2* and *SP140*, the only two genes that were hypermethylated in low 5hmC samples in our analysis, were hypermethylated in patients with *TET2*-mutated CMML (89). Furthermore, Tet2 knockdown in murine ESCs also did not lead to widespread hypermethylation (in fact, there were more regions of hypomethylation than hypermethylation), most likely due to compensation by Tet1, the other major Tet protein in ESCs (41). Further studies, including base resolution whole-genome bisulfite sequencing (90) and oxidative bisulfite sequencing (91) of patient genomes, will clarify this issue. Because *TET1* and *TET3* are intact in *TET2*-mutated myeloid malignancies, whether the other TET proteins compensate for loss of TET2 function or whether the expression or activity of DNMTs is aberrantly controlled by *TET2* mutations warrants further investigation.

Consequences of *Tet2* disruption in mice

Biochemical and in vitro studies clearly indicate that loss of TET2 function strongly correlates with myeloid transformation. To address directly the role of Tet2 in hematopoietic development and cellular transformation, many groups have generated *Tet2*-disrupted mice (Table 1). Mice with Cre-mediated hematopoietic specific-deletion of exon 11 (76) or exon 3 (92) or systemic deletion of exons 8–10 of the *Tet2* locus (84) were born at Mendelian ratios. However, *Tet2* deficiency differentially affects survival in certain gene-trap mice: specifically, a gene-trap strain in which a *LacZ-neomycin* cassette (β -geo) was introduced into intron 2 of the *Tet2* locus displayed perinatal lethality (85, 93, 94). However, other gene-trap strains with insertion at exon 3 (6 bp upstream of start codon) (95) or intron 9 (76) did not show lethality. In all of these mice, *Tet2* mRNA was significantly diminished with no compensatory upregulation of *Tet1* and *Tet3* mRNA, and 5hmC levels were reduced (76, 84, 85, 92, 94, 95); thus the reason for the discrepancy is unknown.

In general, the different *Tet2*-deficient mice showed very similar hematopoietic phenotypes. In the pre-leukemic state, *Tet2* deficiency in adult mice augmented the size of the LSK and LK ($\text{Lin}^- \text{c-Kit}^+ \text{Sca-1}^-$) compartments that are enriched in HSC and myeloid progenitors, respectively. *Tet2* deficiency also conferred a significant advantage on the ability of bone marrow cells to reconstitute heterogeneous hematopoietic lineages in a cell-autonomous manner (76, 84, 92, 95). *Tet2*-deficient LSK cells were highly proliferative and displayed enhanced colony formation in vitro (76, 92, 95). Cells obtained after serial methylcellulose cultures in vitro showed expression profiles similar to common myeloid progenitors (CMPs), with increased expression of genes related to self-renewal such as c-Kit, Meis1 and Evi1 but decreased expression of myeloid-specific genes, suggesting that these cells acquire stem cell-like features with reduced differentiation potentials (92). Transplantation experiments showed that Tet2 deficiency in fetal liver progenitor cells also resulted in LSK cell expansion and enhanced self-renewal and repopulating capacity (85, 94, 96), suggesting that Tet2 normally restricts expansion and function of HSCs in the fetus as well as in adults. By performing a more rigorous test in which wildtype or *Tet2*-deficient CD150⁺ LSK cells in fetal livers were serially transplanted into lethally irradiated recipients, it was recently confirmed that Tet2 deficiency increases HSC self-renewal in a cell-intrinsic manner (97). Notably, Tet2-deficient CMPs also displayed aberrant serial replating capacity in vitro but failed to self-renew in vivo.

Certain strains of *Tet2*-deficient mice developed diverse myeloid malignancies, predominantly CMML-like diseases characterized by splenomegaly associated with myeloproliferation, extramedullary hematopoiesis and peripheral blood leukocytosis associated with monocytosis and neutrophilia (76, 92, 95). Normal splenic architecture in these mice was disrupted by massive infiltration of myeloid or erythroid cells. MDS with expansion of erythroid progenitors and MPN-like myeloid leukemias were also induced upon Tet2 deficiency (95). Importantly, these myeloid leukemias were transplantable to secondary recipients, confirming that Tet2 deficiency induced aggressive leukemia in a cell-intrinsic manner (76, 95). Consistent with the high frequency of heterozygous *TET2* mutations in patients, *Tet2*^{+/-} mice also developed MPN- or CMML-like leukemias (76, 92, 95).

It appears clear that Tet2 deficiency can function as a driver in the pathogenesis of myeloid malignancies. However, the disease latency in Tet2-deficient mice is very long and its penetrance is very low: only 20–30% of the Tet2-deficient mice develop myeloid malignancies within 1 year after birth. Furthermore, for reasons that are not clearly understood, Tet2 inactivation did not reliably result in oncogenic transformation in every mouse model (76). Despite the high incidence of *TET2* mutations in lymphoid malignancies, lymphoma formation was rare in Tet2-deficient mice. In addition, although somatic *TET2* mutations analogous to the inactivating *TET2* mutations in myeloid malignancies accumulate with age in elderly individuals with age-related myeloid skewing, they do not appear to induce overt hematopoietic malignancies (98). Collectively, these results suggest that *TET2* mutation alone is not enough to induce malignancy: rather, cooperation with additional genetic lesions is necessary to cause full-blown disease. Indeed, *TET2* mutations often co-exist with mutations in *NRAS*, *KRAS*, *ASXL1*, *DNMT3A*, *EZH2*, *JAK2* and *SRSF2* (99, 100) and Tet2 deficiency was recently shown to cooperate with concurrent loss of other tumor suppressors such as *Asx1* (101), *Ezh2* (96) and *Ncstn* (102) or the presence of *KIT*^{D816V} mutations (103, 104) to accelerate development of a more advanced disease phenotype with markedly reduced latency. Depending on the types of cooperating mutations, the rate and fate of oncogenic transformation might be affected.

Consequences of *Tet3* disruption in mice

Except for the expansion of HSPCs, overall hematopoiesis takes place normally in *Tet2*-deficient mice. The development of myeloid (Gr-1⁺/Mac-1⁺), B lymphoid (B220⁺CD19⁺), or erythroid (Ter-119⁺) lineage cells in the bone marrow was largely unchanged, and T-cell development was normal in the thymus and periphery. This observation raised the possibility that other TET family members might compensate for the loss of Tet2. Although 5hmC is ubiquitous in tissues and organs (8, 105–109), its levels are differentially controlled depending on cell context and developmental stage, mostly through differential regulation of TET expression or activity. Murine HSPCs show high expression of *Tet2* and *Tet3* mRNA relative to *Tet1* mRNA, and TET expression levels are dynamically controlled during subsequent hematopoietic differentiation (8, 92), with erythroid cells displaying the lowest levels. Tet2 protein expression is also similarly controlled in hematopoietic lineages, as assessed by expression of a GFP reporter inserted into the endogenous *Tet2* locus in Tet2-GFP knock-in mice (95). Thus, Tet2 and Tet3 may play overlapping functions in hematopoietic tissues and compensate for one another. Tet3 may also suppress rapid malignant transformation in the Tet2-deficient mice.

Thus, in order to understand the function of TET proteins in hematopoietic development, we generated Tet3-deficient mice (Fig. 2). Because Tet3 deficiency induced perinatal lethality as reported previously (110), we explored the hematopoietic consequences of *Tet3* deficiency by generating mice carrying *LoxP*-flanked *Tet3* alleles and a hematopoietic-specific *Vav-iCre* transgene (*Tet3*^{fl/fl} *Vav-iCre*⁺) (Fig. 2). As expected, ablation of either *Tet2* or *Tet3* led to a modest decrease in 5hmC levels in the bone marrow (84) (data not shown). *Tet3* deficiency also did not significantly alter the frequency and numbers of myeloid, B lymphoid, or erythroid cells in the bone marrow (Fig. 3A,C), but led to a minor increase in the frequency of LSK cells and a decrease in the frequency and absolute number

of HSCs (LSK CD150⁺ CD48⁻) (111) in the bone marrow (Fig. 3B,D,E). Despite the decrease in HSCs, *Tet3* deficiency augmented the repopulating capacity of HSPCs in competitive engraftment assays (Fig. 4). Collectively, these data suggest that Tet2 and Tet3 play redundant roles in the hematopoietic system and can compensate for one another. Whether combined deficiency of Tet2 and Tet3 accelerates the development of myeloid malignancies is being investigated.

Regulation of TET protein expression and activity

A significant proportion of tumor samples from patients with myeloid malignancies had low levels of 5hmC even though there were no mutations in the *TET2* coding sequence (8, 81, 112), suggesting that 5mC oxidation can be impaired by additional factors that affect the expression or activity of TET enzymes. Multiple regulatory mechanisms have been directly or indirectly implicated in the modulation of TET expression or activity. Because impaired 5mC oxidation seems to promote malignant transformation, it is worth testing whether restoration of 5mC oxidation status by manipulating TET modulators is therapeutically beneficial for the treatment of cancers with low level of 5hmC.

Transcriptional and post-transcriptional control

The expression of *TET* genes can be directly regulated at the transcriptional level. In ESCs, pluripotency factors such as Oct4 and Sox2 directly activate *Tet1* expression by binding to conserved non-coding regions resembling consensus Oct4-Sox2 composite sites (113). Multiple microRNAs (miRNA) are reported to control TET expression post-transcriptionally and several miRNAs coordinately regulate expression of all the *TET* genes. For example, the oncogenic miR-22 was reported to repress *TET2* expression by directly binding its 3' untranslated region (3'UTR) in breast cancers and hematopoietic stem cells (114, 115). Both human and mouse *TET2* genes have conserved elements that are predicted to bind miR-22 at their 3'UTRs. Expression of miR-22 in transgenic mice mimics phenotypes observed in Tet2-deficient mice: augmented HSC self-renewal and transformation, which can be rescued by ectopic expression of Tet2 with mutations in the miR-22 binding sites. In addition, miR-26a (in pancreatic cells) and miR-29a were also shown to directly target TET and TDG enzymes and downregulate 5hmC levels (116, 117). In a subsequent high-throughput screen, it was validated that *TET2* expression is under the control of multiple miRNAs in hematopoietic cells such as miR-125b, miR-29b, miR-29c, miR-101, and miR-7 (118). These *TET2*-targeting miRNAs induce abnormal hematopoiesis including myeloid expansion, which can be rescued by co-expression of Tet2 cDNA lacking 3'UTR. Additionally, although there was a report that CpG islands at the *TET2* promoter are hypermethylated in a very small fraction (4.4%) of patients with MPN (119), it appears that *TET2* expression is not mainly controlled by promoter hypermethylation (63, 70).

Proteolysis by caspases and calpains

Levels of genomic oxi-mCs can be also maintained by regulating TET turnover at the protein level. In addition to its role in recruiting Tet2 to chromatin, IDAX at high levels induces degradation of Tet2 protein by causing caspase activation through an unknown mechanism (29). Intriguingly, this process requires the DNA-binding activity of Idax

because a DNA-binding mutant of Idax does not activate caspases or affect Tet2 protein levels. Like Tet2 deficiency, enforced expression of wildtype Idax, but not its DNA-binding mutant, skewed the differentiation of HSPCs toward the monocyte/macrophage lineage *in vitro*. Furthermore, calpains, a family of calcium-dependent proteases, have been reported to control the steady-state levels of all Tet proteins (120). Specifically, calpain1 regulated Tet1 and Tet2 protein levels in undifferentiated ESCs whereas calpain2 regulated Tet3 levels in differentiated cells.

Control by metabolic intermediates

Identification of frequent genetic aberrations in genes encoding proteins with key roles in intermediary metabolism – the isocitrate dehydrogenases (IDH1 and IDH2), succinate dehydrogenase (SDH), fumarate hydratase (FH) and phosphoglycerate dehydrogenase (PHGDH) – has led to a resurgence of interest in the potential causal link between altered metabolism and cancer development. Recent data suggest that one of the most plausible pathways by which metabolic changes induce cell transformation is to modulate various α KG-dependent enzymes that are responsible for chromatin modification or hypoxia response. α KG is produced as a key intermediate in the tricarboxylic acid (TCA) cycle through oxidative decarboxylation of isocitrate by IDH enzymes. Inactivating mutations in *succinate dehydrogenase* (SDH) and *fumarate hydratase* (FH) genes that encode TCA cycle enzymes lead to the accumulation of succinate and fumarate, respectively, which competitively inhibit α KG-dependent enzymes (121, 122). Cells also can produce α KG anaplerotically via oxidative deamination of glutamate by glutamate dehydrogenase or as a product in serine biosynthesis. PHGDH catalyzes the first step of serine biosynthesis and *PHGDH* is recurrently amplified in certain human cancers, leading to accumulation of α KG (123, 124). It seems likely that *PHGDH* amplification affects α KG-dependent enzymes to promote maintenance of cancer cells.

Importantly, somatic heterozygous mutations in *IDH1* and *IDH2* genes are recurrently identified in a majority (~75%) of low-grade gliomas and secondary glioblastomas and a subset (~20%) of hematopoietic malignancies such as AML, sAML, MDS, MPN, AITL and ALL (125–132). Other solid cancers including chondrosarcoma, intrahepatic cholangiocarcinoma, osteosarcoma and melanoma also harbor *IDH* mutations. Remarkably, most *IDH* mutations are missense mutations that almost exclusively occur at certain active site residues (R132 in IDH1 and R140 and R172 in IDH2), resulting in increased binding affinity for NADPH relative to isocitrate or NADP⁺ (133). As a result, the resulting mutant enzymes acquire the neomorphic ability to reduce α KG to 2-hydroxyglutarate (2HG) while oxidizing NADPH to NADP⁺ (133). All IDH mutants tested so far produce only the (*D*), but not the (*L*), enantiomer of 2HG, which accumulates up to the low mM level (83, 129, 134). In patients, elevated levels of D-2HG in serum or urine could be a faithful biomarker for the presence of *IDH* mutations, which can be exploited clinically to determine *IDH* mutational status (129, 135–138).

In addition to cancers, 2HG accumulation had earlier been reported in D- or L-2-hydroxyglutaric aciduria, a rare neurodegenerative disorder associated with mutations in *D*- or *L*-2HG dehydrogenases. However, it remains controversial whether and how 2HG

enantiomers play a pathogenic role. Both 2HG enantiomers competitively inhibit a variety of α KG-dependent enzymes, with L-2HG displaying a more potent inhibitory effect (139–141). For example, mutant IDH enzymes that produce D-2HG inhibit several JmjC domain-containing histone demethylases and TET proteins, resulting in hypermethylation of histone and DNA (139, 142–145); L-2HG inhibits the same enzymes but at considerably lower concentrations. 2HG and mutant IDH enzymes also regulate the activity of prolyl 4-hydroxylase (PHD or EGLN) enzymes – also Fe(II) and α KG-dependent dioxygenases – that hydroxylate hypoxia-inducible factors (HIFs) under normoxic conditions and target them for proteasomal degradation. Initially, mutant IDH proteins were shown to stabilize HIF-1 α proteins and induce HIF downstream target genes in cells (139, 140) or mice (146). However, later studies found no alterations or reduction in HIF levels (141, 147, 148). Chowdhury et al. showed that 2HG, particularly cancer-associated D-2HG, is a very weak inhibitor of EGLN1 (also known as PHD2) prolyl 4-hydroxylase (141). In contrast, Koivunen et al. showed that D-2HG, but not L-2HG, stimulates EGLN1 activity in vitro after being oxidized to α KG by EGLN (148). A recent study has called these data into question: the authors showed that EGLN1 does not convert D-2HG into α KG although both enantiomers could be oxidized into α KG non-enzymatically (149). Clearly, further studies are necessary to define precisely how IDH mutations and 2HG affect cellular HIF levels. Notably, the expression of HIF target genes was not significantly altered in cells from mice expressing mutant IDH1 (150) or IDH2 (151), indicating that alteration of HIF levels is unlikely to be a major cause of the pathological effects of IDH mutations.

Forced expression of mutant IDH1 promotes proliferation and soft agar colony formation by primary human astrocytes (148). A specific inhibitor (AGI-5198) that inhibits 2HG production by mutant IDH1 inhibited the growth of gliomas in vitro and in vivo and induced histone demethylation and expression of differentiation-related genes with no significant changes in genome-wide DNA methylation (152). Similarly, overexpression of mutant IDH or TET2 depletion promoted cytokine-independent growth of hematopoietic cells and partially blocked terminal differentiation of TF-1 human erythroleukemic cells (153, 154). Intriguingly, even though both D- and L-2HG are capable of inhibiting TET2 activity albeit at vastly different concentrations, only D-2HG promoted cytokine independence and differentiation block by activating EGLN1 (153). Importantly, the differentiation block was relieved upon removal of exogenous D-2HG or treatment with mutant IDH inhibitors (AGX-891 or AGI-6780) (153, 154). Collectively, these data suggest that IDH mutations drive cellular transformation in vitro in a reversible manner. Thus, targeted inhibition of mutant IDH or EGLN enzymes would be beneficial for the differentiation therapy of cancers harboring IDH or TET2 mutations.

Do IDH mutations drive leukemia initiation or progression in vivo? To address this question, several knock-in or transgenic mice expressing mutant IDH1 (IDH1^{R132H}) or IDH2 (IDH2^{R140Q} or IDH2^{R172K}) have been generated (150, 155). Also, chimeric mice reconstituted with bone marrow progenitors in which mutant IDH proteins (IDH1^{R132C} or IDH2^{R140Q} or IDH2^{R172K}) were retrovirally overexpressed were generated (156, 157). These models show that mutant IDH proteins results in aberrant hematopoietic phenotypes characterized by an expansion of HSPCs, reduced cellularity in bone marrow, splenomegaly,

anemia and extramedullary hematopoiesis. However, unlike Tet2 deficiency, *IDH* mutants did not affect myeloid differentiation from bone marrow precursors in vitro and also did not provide any advantage over control cells to repopulate hematopoietic lineages in competitive engraftment assay (150, 155). Interestingly, none of these mouse models developed leukemia, indicating that *IDH* mutations alone are not sufficient to induce leukemogenesis. However, when combined with other oncogenes such as HoxA9, Meis1, and Flt3-ITD, they promoted development of aggressive myeloid leukemia with significantly reduced latency. Notably, suppression of mutant IDH expression or pharmacological inhibition of 2HG production could reverse or sometimes eradicate the pathogenic features by inducing differentiation of leukemic cells, suggesting that ongoing production of 2HG by mutant IDH proteins is necessary for the maintenance of leukemic cells (155, 157).

Ascorbic acid (vitamin C)

Vitamin C (ascorbate) was shown to enhance the catalytic activity of TET proteins in vitro and in vivo (158–160). In wildtype ESCs, vitamin C induced a significant increase in the levels of all oxi-mCs, particularly 5fC and 5caC, followed by a concomitant decrease in 5mC by ~40% (158, 160). Tet1 and Tet2 mediated this change because the effect of vitamin C was abrogated in the absence of Tet1 and Tet2 (158, 160). Interestingly, vitamin C induced 5mC loss in regions that are methylated in ESCs compared with blastocyst (i.e. regions that gain methylation post-implantation in vivo) (160). The mechanism by which vitamin C enhances TET activity is not entirely understood but a study suggested that it directly activates the catalytic activity of TET proteins by binding to the core catalytic domain (158).

Vitamin C is also reported to enhance somatic cell reprogramming through activation of histone demethylases (161, 162). Depending on the presence or absence of vitamin C, TET1 differentially regulates somatic cell reprogramming (163). In the presence of vitamin C, TET1 impairs reprogramming by modulating the obligatory mesenchymal-to-epithelial transition (MET). However, in the absence of vitamin C, TET1 promotes somatic cell reprogramming independent of MET. Consistently, TET1 generates 5hmC marks at loci critical for MET in a vitamin C–dependent manner. Because TET1 and TET3 remain intact in myeloid cancers, it would be interesting to assess whether vitamin C shows any clinical efficacy by enhancing TET1/3 activity in *TET2*-mutated cancers. Further studies including small library screens will identify more activators of TET proteins and test their usefulness in epigenetic cancer therapy.

Control of chromatin accessibility

After fertilization, 5mCs in the paternal genome in zygotes are quickly oxidized to oxi-mCs by Tet3, followed by passive dilution during replication (30, 31). However, 5mCs in the maternal genome are simply diluted after each round of cell division. Primordial Germ Cell 7 (PGC7; also known as STELLA or DPPA3) has been suggested as one of the key factors that dictate this epigenetic asymmetry in zygotes (164–166). PGC7 and TET3 proteins are present in both maternal and paternal pronuclei in zygotes. However, PGC7 recognizes dimethylated histone H3 lysine 9 (H3K9me2) that is present only in maternal chromatin (and at certain imprinted loci in the paternal genome), where it apparently interferes with

tight binding of TET3 to chromatin (166–169). In the absence of PGC7, 5mCs in both pronuclei undergo TET3-mediated oxidation. Thus, PGC7 selectively protects the maternal genome against TET3-mediated oxidation. This is a good example of a scenario in which asymmetry of histone modification ensures asymmetry of DNA methylation. However, it is unclear whether PGC7 plays similar roles in other somatic tissues including the hematopoietic system. Recently, a study showed that the DNA-binding domain of PGC7 physically associates with the catalytic domain of TET2 and TET3, but not TET1 in HEK293T cells, resulting in suppression of the hydroxylase activities of TET proteins although the underlying mechanism is unknown (170). The CpG islands surrounding the PGC7-binding motifs remain hypermethylated. Since recombinant PGC7 impairs TET activities in vitro and reduction of H3K9me2 only slightly affects the chromatin binding of PGC7, PGC7 seems to differentially control TET activity in differentiated cells, compared with zygotes.

Control by physical association with other proteins

In addition to IDAX and PGC7, the enzyme O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT) has also been identified as a key TET-interacting protein (171–175). OGT transfers a single O-GlcNAc moiety to serine/threonine residues in numerous proteins. TET proteins co-localize with OGT in the genome primarily at GC-rich promoters and transcriptional start sites that contain CpG islands. The TET-OGT interaction enhances the O-GlcNAc'ylation of H2B Ser112 (171) and host cell factor 1, a key component of the SET1/COMPASS H3K4 methyltransferase complex (172). Thus, the regions co-occupied by TET and OGT proteins also show high levels of H2B Ser O-GlcNAc and H3K4me3 marks, suggesting that the TET-OGT interaction contributes to gene activation. In Tet2-deficient mice, the global levels of O-GlcNAc'ylation and H3K4me3 are reduced (172). However, there are several inconsistencies in previous studies. First, it is unclear if TET1 is a genuine interacting partner of OGT. Also, the consequences of TET-OGT interaction are also still controversial. One study showed that recruitment of Ogt to chromatin is strongly dependent on its interaction with Tet2 in murine ESCs, as demonstrated by impaired Ogt chromatin association upon Tet2 depletion (171). However, in another study, Tet2 deficiency did not significantly affect OGT chromatin association but Tet1 played a major role in recruiting OGT to chromatin (173). Furthermore, whereas initial reports suggested that OGT does not affect TET activity (171, 172), a recent study claimed that O-GlcNAc'ylation of TET1 by OGT increased 5hmC production by stabilizing TET1 proteins (174). Mutations in the potential O-GlcNAc'ylation site resulted in decreased levels of TET1 protein. Moreover, OGT was also shown to impair the enzymatic activity of TET2 by promoting its nuclear export (175).

Conclusions and perspectives

During the last five years, there has been remarkable progress in understanding the role of TET enzymes in the regulation of DNA methylation/demethylation, embryogenesis and normal and malignant cellular function. By mediating sequential oxidation of 5mC, TET enzymes may promote DNA demethylation in collaboration with DNA repair enzymes. Extensive studies have established TET2 as a frequently mutated gene in hematologic

cancers, and fostered the idea that aberrant 5mC oxidation occurring as a result of loss-of-function mutations/deletions of *TET2* predispose to the development of hematological malignancies of myeloid and lymphoid origin, particularly in combination with mutations in specific other cellular proteins (second hits). However, the fundamental question still remains unanswered – how does *TET2* loss-of-function contribute to malignant transformation? Future studies are necessary to elucidate the underlying molecular mechanisms.

In addition to the hematopoietic system, malignant transformation seems to be generally associated with a decrease in genomic 5hmC levels in different tissues (176–180). Therefore, it would be also of great importance to assess the relation between *TET* mutations (or dysregulation) and the development of non-hematopoietic cancers. Furthermore, it would be also interesting to determine whether 5hmC quantification is useful as a diagnostic or prognostic tool in cancer and whether manipulation of TET activity by small molecules or other modulators might be applicable to cancer therapy.

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Figure 1. Functions of TET proteins

(A) TET proteins control DNA methylation-demethylation dynamics. DNA methyltransferases (DNMTs) add a methyl group to the 5 position of cytosine in the context of CpG dinucleotides to produce 5-methylcytosine (5mC). TET proteins are Fe(II)/ α -ketoglutarate-dependent dioxygenases that successively oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). 5mC and all its oxidized derivatives (ox-mCs) are passively diluted and converted into cytosines when maintenance methylation is blocked during DNA replication (*dashed lines*).

Furthermore, 5fC and 5caC are directly excised by TDG (thymine DNA glycosylase), then replaced with unmethylated cytosine via the base excision repair (BER) pathway.

(B) TET proteins maintain CpG islands in a hypomethylated state. TET proteins are preferentially targeted to CpG islands or Canyons which refer to short (~1–2 kb) or large (~3.5 kb) stretches rich in unmethylated CpG dinucleotides across vertebrate genome, respectively, via the linked CXXC domains of TET1 and TET3, or the separated CXXC domain (i.e. IDAX) of TET2. The borders of CpG islands or Canyons are demarcated by oxi-mCs, reflecting TET activity. Expression of IDAX proteins results in caspase activation and degradation of TET2, restricting further oxidation and production of oxi-mCs.

Figure 2. Generation of a targeted allele for conditional ablation of *Tet3*

(A) Targeting strategy for site-specific insertion of *LoxP* sites in exon 2 of the endogenous *Tet3* locus. Locations of restriction enzymes used for Southern blotting are indicated.

(B) Southern blot analysis was performed after digestion of genomic DNA from selected embryonic stem cells with KpnI (left) or HindIII (right) to identify the cell lines carrying the correctly targeted *Tet3* locus. Asterisks indicate targeted allele.

(C) Expression of *Tet3* in sorted LSK and myeloid progenitor cells. Cells were isolated from bone marrow of $Tet3^{fl/fl}$ or $Tet3^{fl/fl} Vav-iCre^+$ mice by flow cytometry, and quantitative RT-

PCR was performed. The relative levels of *Tet3* mRNAs after normalization to the level of *Gapdh* mRNA in the same cell population are shown, with the amount in the control LSK cells arbitrarily set to 1. *N.D.*, not detected.

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Figure 3. Hematopoietic phenotypes of *Tet3*-deficient mice

(A) *Tet3* deficiency does not significantly perturb hematopoietic development. Flow cytometric analysis was performed to assess major hematopoietic subpopulations in the bone marrow of 6- to 12-wk-old *Tet3^{fl/fl}* or *Tet3^{fl/fl} Vav-iCre⁺* mice (n = 5~11 mice per genotype). *Top*, myeloid cells; *middle*, erythroid cells; *bottom*, B cells.

(B) Control of hematopoietic stem/progenitor pool by *Tet3*. The frequency of LSK and LK was assessed by discriminating lineage-negative cells (Lineage: Gr-1, Mac-1, Ter-119, CD3 ϵ , B220) based on the expression of c-Kit and Sca-1 (*middle*). SLAM (CD150)-enriched HSCs were assessed by surface expression of CD48 and CD150 after gating LSK cells (*bottom*) (n = 10~11 mice per genotype).

(C-E) Summary of percentage of cells shown in a and b. ***P* < 0.005 (Student's *t* test).

Figure 4. Augmented hematopoietic repopulation capacity of Tet3-deficient bone marrow precursors

(A) Competitive repopulation assay. CD45.2⁺ bone marrow cells from $Tet3^{fl/fl}$ or $Tet3^{fl/fl} Vav-iCre^{+}$ mice were mixed with an equal number of CD45.1⁺ competitor cells and transplanted into lethally irradiated CD45.1⁺ congenic mice. At 4, 8 and 16 weeks after transplantation, peripheral blood was examined for donor/competitor chimerism at the indicated time. A representative flow cytometry plot (left) and percentage of CD45.2⁺ donor chimerism (right) at each time point are shown. ** $P < 0.005$, *** $P < 0.0005$ (Student's t test).

(B) Percentage of CD45.2⁺ donor chimerism in hematopoietic lineages in peripheral blood after transplantation. The percentage of CD45.2⁺ cells was calculated after gating on T-cell (CD3 ϵ^{+} , left), B-cell (CD19⁺, middle) and myeloid (Mac-1⁺, right) populations. ** $P < 0.005$, *** $P < 0.0005$ (Student's t test).

Table 1

Phenotypes of Tet2-deficient mice.

Genetic modification (Cre or insertion)	Targeted region	Phenotype	Reference
Conditional deletion (<i>Mx1-Cre</i> and <i>Vav-iCre</i>)	Exon 3	Increased LSK/LK number, Increased serial replating in vitro, increased repopulation in vivo, extramedullary hematopoiesis (myeloproliferation), splenomegaly, leukocytosis, development of CMML-like disease	Moran-Crusio <i>et al.</i> (Ref #92)
Conditional deletion (<i>Mx1-Cre</i>)	Exon 11	Increased LSK/LK number, increased replating in vitro, increased repopulation in vivo, impaired lymphopoiesis, extramedullary hematopoiesis (myeloid and erythroid hyperplasia), splenomegaly, hepatomegaly, no prominent hematopoietic malignancies	Quivoron <i>et al.</i> (Ref #76)
Systemic deletion (<i>CMV-Cre</i>)	Exons 8–10	Increased LSK/LK number, increased repopulation in vivo, resistant to differentiation signal in vitro, increased monocyte development in vitro, splenomegaly	Ko <i>et al.</i> (Ref #84)
Gene-trap (<i>nLacZ/nGFP</i>)	Exon 3 (6 bp upstream of start codon)	Increased LSK number, increased replating in vitro, increased repopulation in vivo, extramedullary hematopoiesis (myeloid and erythroid hyperplasia), leukocytosis, splenomegaly, hepatomegaly, development of MDS-, MPD- or CMML-like diseases	Li <i>et al.</i> (Ref #95)
Gene-trap (β -geo)	Intron 9	Increased LSK/LK number, increased replating in vitro, increased repopulation in vivo, impaired lymphopoiesis, extramedullary hematopoiesis (myeloid and erythroid hyperplasia), leukocytosis, anemia, thrombocytopenia, splenomegaly, hepatomegaly, development of CMML-like disease	Quivoron <i>et al.</i> (Ref #76)
Gene-trap (β -geo)	Intron 2	Perinatal lethality, increased LSK number in fetal livers, resistant to differentiation signal, increased myelopoiesis in vitro, mild splenomegaly, monocytosis, increased HSC self-renewal upon transplantation of purified HSCs, increased serial repopulation in vivo and extracellular hematopoiesis upon transplantation of FL cells	Tang <i>et al.</i> (#93) Kunimoto <i>et al.</i> (#85, 97) Shide <i>et al.</i> (#94)