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TET2 in Normal and Malignant Hematopoiesis

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The ten-eleven translocation (TET) family of enzymes were originally cloned from the translocation breakpoint of t(10;11) in infant acute myeloid leukemia (AML) with subsequent genomic analyses revealing somatic mutations and suppressed expression of TET family members across a range of malignancies, particularly enriched in hematological neoplasms. The TET family of enzymes is responsible for the hydroxylation of 5-methylcytosines (5-mC) to 5-hydroxymethylcytosine (5-hmC), followed by active and passive mechanisms leading to DNA demethylation. Given the complexity and importance of DNA methylation events in cellular proliferation and differentiation, it comes as no surprise that the TET family of enzymes is intricately regulated by both small molecules and regulatory cooperating proteins. Here, we review the structure and function of TET2, its interactions with cooperating mutations and small molecules, and its role in aberrant hematopoiesis.

Although often thought to be a stable epigenetic mark, recent research has revealed DNA methylation to be dynamic modification capable of regulating critical features of cellular proliferation, differentiation, and gene expression. Integral to this regulatory function are the enzymes necessary for both addition of the DNA-methyl mark and subsequent removal. Amongst these enzymes, the ten-eleven translocation (TET) family of proteins has emerged as critical regulators of the oxidation of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC). Since its recent identification in 2009, an explosion of studies has interrogated the roles of TET2 in malignancies of the blood and brain, developmental processes, and roles in inflammation. Genetic and biochemical studies in both human tumor specimen and animal models of disease have revealed TET2 as a critical node linking alterations in tumor metabolism to alterations in DNA methylation and modified chromatin. These features require a refined understanding of how to classify a cancer-associated gene that fits neither rigid definitions of an oncogene nor a tumor suppressor.

In this review, we will review the initial studies in hematologic malignancy that led to the discovery of TET2, the function and structure of the enzyme, its interactions with cooperating mutations and small molecules, and a perspective into other diseases in which TET2 mutations have been identified.

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MUTATIONS IN HEMATOLOGIC DISEASE

In 2009, a series of papers identified somatic mutations in *TET2* in multiple hematologic malignancies (Delhommeau et al. 2009; Jankowska et al. 2009; Langemeijer et al. 2009; Tefferi et al. 2009a,b). Mapping of minimal regions of deletion in the 4q24 cytoband revealed *TET2* loss of heterozygosity (LOH) and somatic mutations in as many as 30%–50% of myelodysplastic syndrome (MDS) and myeloproliferative neoplasia (MPN) patients, whereas 32% of secondary acute myeloid leukemia (AML) patients harbored *TET2* mutations (Jankowska et al. 2009). Further genomic studies in MPN patients revealed the presence of *TET2* mutations in both *JAK2-V617F*-positive and -negative patients, with relatively equal distribution across essential thrombocytosis (ET), polycythemia vera (PV), and myelofibrosis (MF) (Tefferi et al. 2009b). In each of these studies, deletions as well as nonsense and missense mutations were found across multiple exons. Interestingly, most AML patients with *TET2* mutations retain expression of the wild-type allele with only 10% of patients possessing biallelic mutations (Delhommeau et al. 2009). Although the function of TET2 was not known at the time, these data were suggestive of a tumor suppressor role and potentially haploinsufficient loss-of-function role in *TET2* mutants.

TET2 mutations are present in multiple lymphoid and myeloid lineages, as well as $CD34^{+}$ progenitor cells, suggestive of an early clonal mutation in the stem cell compartment (Smith et al. 2010). In line with this early mutation designation, *TET2* mutants have continually been found at high allele frequency indicating that they are often the “first hit” in the multihit model of leukemogenesis (Smith et al. 2010; Papaemmanuil et al. 2016). These findings are reinforced by genetic studies identifying somatic *TET2* mutations in asymptomatic, healthy adults with clonal hematopoiesis (Smith et al. 2010; Busque et al. 2012). This, however, does not appear to always be the case, as two studies have shown that in MPN patients with *JAK2-V617F* mutations *TET2* can either present as the first hit or the second hit based on mutant allele frequency (Abdel-Wahab et al. 2010; Ortmann et al. 2015). Interestingly, “JAK2-first” patients presented with significantly worse overall survival compared with “TET2-first” patients (Ortmann et al. 2015). In addition to the co-occurrence with *JAK2* mutations in MPN mentioned above, mutations in *TET2* have been shown to co-occur with mutations in *ASXL1, SRSF2, SF3B1, U2AF1,* and *CALR* (Rampal et al. 2014a). In AML, *TET2* shows comutational patterns with *NPM1, FLT3* and *DNMT3a* (Papaemmanuil et al. 2016). How these mutations cooperate in leukemogenesis remains an area of intense investigation and will be discussed later in this review.

Prognosis

TET2 mutational status has been found to be a variable prognostic indicator. One of the earliest studies of a cohort of 48 patients with systemic mastocytosis found no prognostic association with *TET2* mutational status (Tefferi et al. 2009a). Similarly, no survival association was found in a cohort of 63 patients with AML, chronic myelomonocytic leukemia (CMML), or MPN/MDS (Jankowska et al. 2009). Meanwhile, other studies showed a significant association with poor prognosis in AML (Abdel-Wahab et al. 2009) and a favorable prognostic association in MDS (Kosmider et al. 2009). In the largest cohorts to date by The Cancer Genome Atlas (TCGA) (Cancer Genome Atlas Research Network 2013) and by a group at the Sanger Institute (Papaemmanuil et al. 2016), there was no independent association with survival for *TET2* mutations. Interestingly, in a cohort of 211 MDS patients, there was an association in response to the hypomethylating agents decitabine and azacitidine, in which patients with *TET2* mutant AML were more likely to respond to therapy than those without the mutation, a finding that was more pronounced when the comutational partner *ASXL1* was not mutated (Bejar et al. 2014). Further studies into the functional role of *TET2* in disease progression and response to different therapeutic regiments may help clarify the prognostic value

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of *TET2* mutations in various hematologic malignancies.

TET2 FUNCTION AND STRUCTURE

When mutations in *TET2* were first discovered through mapping of the 4q24 region of loss/LOH, the functions of this protein remained unknown. Shortly after, homology searches for the trypanosome proteins JBP1 and JBP2, enzymes known to oxidize methyl-thymine, identified the mammalian TET family as 2-oxoglutarate (2-OG) and Fe(II)-dependent enzymes (Tahiliani et al. 2009). These studies revealed that TET1 possessed enzymatic activity for converting 5-mC to 5-hmC, and follow-up studies soon confirmed similar enzymatic activity for TET2 and TET3 (Ito et al. 2010; Ko et al. 2010). Subsequent studies would reveal that TET proteins are capable of generating iterative cytosine alterations leading to the formation of 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) (Fig. 1) (Ito et al. 2011). These intermediates were further shown to be substrates for thymine-DNA glycosylase (TDG)-mediated base excision repair (BER), converting the modified cytosine residue back to the unmethylated cytosine base (He et al. 2011; Maiti and Drohat 2011). Alternative demethylating mechanisms involve the APOBEC family mem-

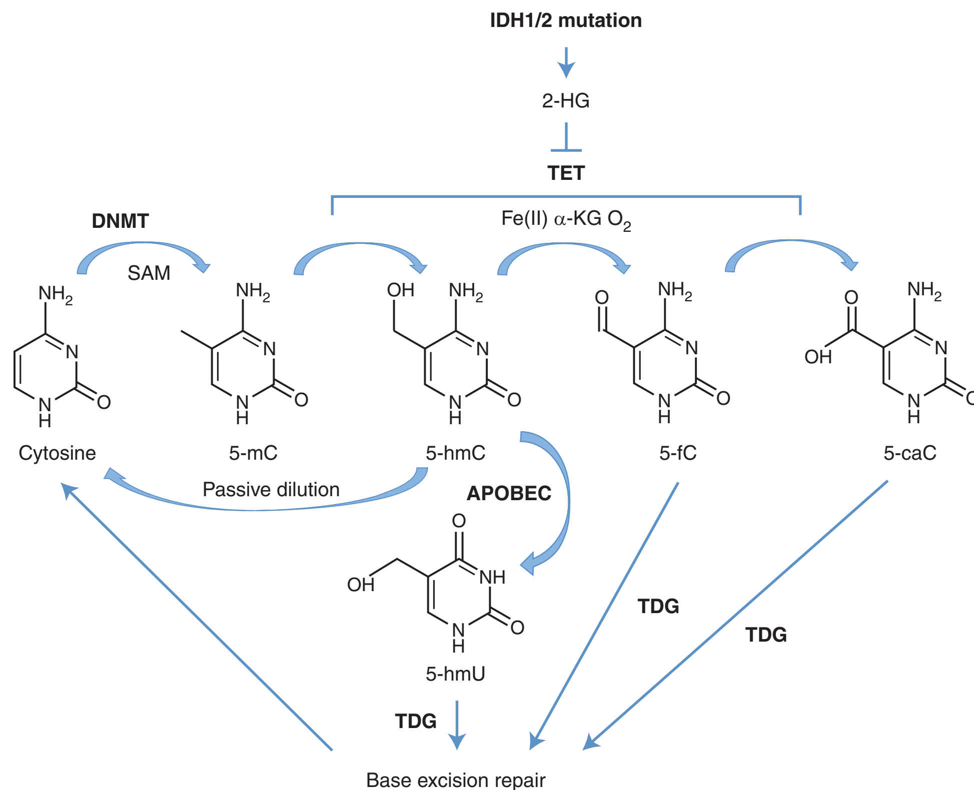

Figure 1. Reactions involved in TET-mediated oxidation of 5-methylcytosine (5-mC). Depicted here is cytosine-mediated methylation by the family of DNA methyltransferases (DNMT) with the substrate *S*-adenosyl methionine (SAM) leading to the formation of 5-mC. TET family members are then capable of mediating the iterative oxidation of 5-mC to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC) in an Fe(II), O_2, and α-ketoglutarate (α-KG)-dependent reaction. These α-KG-dependent reactions can be inhibited by the oncometabolite 2-hydroxyglutarate (2-HG), which is a neomorphic by-product of mutant *IDH1* and *IDH2*. Each downstream product (5-hmC, 5-fC, and 5-caC) can serve as substrates for thymine DNA glycosylase (TDG) leading to base excision repair (BER) and eventual return to unmodified cytosine.

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bers deaminating 5-hmC into 5-hydroxymethyluracil (5-hmU), which presents as a target for TDG and selective monofunctional uracil-DNA glycosylase 1 (SMUG1)-mediated BER (Bhutani et al. 2011). In addition to these active processes of DNA demehtylation, TET2 has been implicated in passive DNA demethylation, as 5-hmC serves as a poor substrate for the cell cycle–regulated DNMT1 leading to dilution of the 5-mC mark with each round of DNA replication and cell division (Hashimoto et al. 2012). It is important to note that the relative role of these different pathways in the ultimate removal of DNA modifications back to unmethylated cytosine remains to be fully delineated.

Recent biochemical studies have identified the structural components of TET2 that mediate these catalytic functions (Hu et al. 2013). Human *TET2* encodes a 2002–amino acid, 223-kDa protein with a carboxy-terminal catalytic domain and poorly conserved amino-terminal domain. Biochemical studies on truncation variants were capable of reconstituting the enzymatic functions of TET2 within an 807–amino acid stretch (1129–1936) containing cysteine rich regions and a double-stranded β helix (DSBH) separated by an unstructured linker. This fragment was subsequently crystalized in complex with methylated DNA revealing coordination of a catalytic core by two zinc finger domains. These studies further revealed a cavity allowing for recognition of various modifications on the 5-mC base in the catalytic core. Subsequent studies showed that 5-hmC and 5-fC substrates possessed enzymatically unfavorable coordination of hydrogen bonds in the catalytic cavity, offering a potential explanation for the preferred substrate specificity of TET2 for 5-mC over that of 5-hmC and 5-fC (Hu et al. 2015), as well as the stability of 5-hmC in vivo (Ito et al. 2011).

REGULATION OF TET2 FUNCTION

Inhibition by IDH1/2 Mutant-Derived 2-Hydroxyglutarate

In addition to the genetic and biochemical studies above, one of the most important clues to understanding TET2 biology was the identification of mutually exclusive mutations in the metabolic enzymes isocitrate dehydrogenase 1 (IDH1) and IDH2, placing these enzymes in a putative genetic pathway (Abdel-Wahab et al. 2010). In a broader biological context, the identification of mutation in *IDH1* and *IDH2* was of fundamental importance in linking of altered cellular metabolism to the genomic age of cancer research. Although the altered glycolysis was a long appreciated hallmark of tumorigenesis (Hanahan and Weinberg 2011), it was not immediately clear how mutations in enzymes canonically involved in the citric acid cycle might impact tumorigenesis. The mechanistic role of these metabolic mutations began to take shape on the discovery that R132H mutant IDH1 was capable of producing 2-HG through an NADPH-dependent reduction of α-ketoglutarate (α-KG) (Dang et al. 2009). Soon after, these findings would be extended to the more common leukemic mutations of IDH2-R172K and IDH2-R140K (Ward et al. 2010). As IDH1/2 mutations were enriched in diseases with a relatively undifferentiated phenotype, low-grade glioma and leukemia, it was hypothesized that 2-HG might block differentiation albeit through unknown mechanisms. Definitive evidence would come later that year with the discovery that IDH1/2 mutant-derived 2-HG was capable of blocking differentiation and inhibiting the α-KG dependent enzyme TET2 (Figueroa et al. 2010). Critically, these studies revealed that IDH1/2 mutant AML patients displayed a hypermethylated phenotype (Figueroa et al. 2010), linking TET2 inhibition by 2-HG with the demethylating functions of TET proteins in development (Ito et al. 2011; Ko et al. 2011). The inhibitory capacity of 2-HG would later be extended to most α-KG-dependent enzymes (Xu et al. 2011), suggesting that IDH1/2 mutations might possess TET2-independent functions. Indeed, IDH1 mutant mice have been shown to down-regulate the DNA damage sensor *ATM* through altered histone methylation (Inoue et al. 2016). Further work will aim to identify therapeutic vulnerabilities that are shared between TET2 and IDH1/2 mutant AML, as well as those that are specifically rele-

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vant to the pleiotropic features of IDH1/2 mutant disease.

Although 2-HG has received much attention as a mutant IDH1/2 neometabolite, its production is not limited to these mutations. Importantly, 2-HG is a chiral molecule with the D-enantiomer being produced by mutant IDH1/2. Recent work has identified that under hypoxic conditions the L enantiomer of 2-HG is produced as a promiscuous bioproduct of lactate dehydrogenase A (LDHA)-mediated and malate dehydrogenase 1 (MDH1)-mediated reduction of α-KG (Intlekofer et al. 2015; Oldham et al. 2015). L2-HG was shown to function as a competitive inhibitor of the EGLN prolyl hydroxylase promoting hypoxia-inducible factor 1-α (HIF1-α) stability, whereas D2-HG served a substrate leading to HIF1-α degradation (Intlekofer et al. 2015). Meanwhile, both enantiomers are capable of inhibiting TET2 (Figueroa et al. 2010; Shim et al. 2014). These studies lead to the possibility that physiological production of L2-HG might play a role in modulating TET2 function in homeostasis, especially in the context of the hypoxic hematopoietic stem cell niche (Spencer et al. 2014).

TET2 Binding Proteins

The amino terminus of TET1 and TET3 contain a well-conserved CXXC domain that has been shown to mediate binding to unmethylated CpG residues (Xu et al. 2012); however, no such domain is present in TET2. Interestingly, a CXXC domain–containing protein, IDAX (CXXC4), is encoded 5′ of the *TET2* genomic locus, suggestive of evolutionary splitting of the original TET2-CXXC gene into two separate genes (Ko et al. 2013). Biochemical studies revealed that IDAX is capable of binding both the amino terminus and catalytic domain of TET2, in which binding was associated with caspase-mediated TET2 degradation. This negative regulation of TET2 may present an additional mechanism for affecting 5-mC levels in malignancy, independent of genomic alterations to TET2 or mutations in IDH1/2. Indeed, IDAX has found to be overexpressed in villous adenomas in the colon (Nguyen et al. 2010). In addition to its interaction with TET2, IDAX is a known inhibitor of WNT signaling (Hino et al. 2001), suggesting a potential source of cross talk between these pathways. Another interacting partner with WNT signaling, WT1, has also been shown to bind TET2 and TET3 (Rampal et al. 2014b), acting as a guide for TET2 to specific genomic loci associated with proliferation (Wang et al. 2015). In support of this observation, WT1 is mutated in AML, in a mutually exclusive pattern with *TET2*, and *WT1* loss was further shown to phenocopy *TET2* loss in hematopoiesis (Rampal et al. 2014b). In addition to these factors, the CRL4-VprBP complex has been shown to stabilize TET family members through monoubiquitination, increasing TET family members binding to DNA (Yu et al. 2013). Mutation at, or around, the TET2 monoubiquitination site at residue K1299 have been identified in several leukemia cell lines, offering another plausible mechanism for TET2 dysfunction in cancer (Nakagawa et al. 2015).

Vitamin C

Vitamin C has been shown to induce TET activity in embryonic stem (ES) cells, and to induce a global increase in 5-hmC content (Blaschke et al. 2013). Although this activity appeared to be specific to vitamin C and no other reducing agents, vitamin C affected both TET1 and TET2, the only TET family members expressed in ES cells. Interestingly, in this study, the investigators found that not all methylation marks were equally sensitive to subsequent demethylation. Indeed 5-hmC levels were most robustly affected at the promoters of genes, whereas methylation of retro elements remained unchanged. Vitamin C has previously been shown to regulate the activity of several iron-dependent dioxygenases; however, in this setting its effects did not appear to depend on either iron availability or α-KG concentration. In contrast, Hore et al. (2016) found in ES cells that vitamin C increased iron recycling and did not function as a cofactor. Although the details of the regulation may diverge, the capacity for vitamin C to induce TET2 activity is robust,

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with consistent effects in ES cells, mouse embryonic fibroblasts, T regulatory cells (Nair et al. 2016; Yue et al. 2016), and melanoma cells (Gustafson et al. 2015). Interestingly, in 2009, a single-arm clinical trial on 16 AML patients revealed a subset of patients that showed a clinical response following vitamin C deprivation (Park et al. 2009), highlighting the clinical relevance of vitamin C to leukemia biology. In addition to vitamin C, vitamin A has also been shown to play a role in inducing TET activity through the direct transcriptional regulation of both *TET2* and *TET3* (Hore et al. 2016). Understanding the mechanisms of both vitamin A and vitamin C activities on TET function could provide key insights into therapeutic options for both IDH and DNMT mutant cancers.

MECHANISMS OF CONTRIBUTION TO LEUKEMOGENESIS

The cancer genetics and biochemical studies discussed above provided substantial insight into the function of *TET2* in DNA methylation, yet understanding the cellular manifestations of these activities was made possible through the development of genetic mouse models. Conditional loss of TET2 activity by Vav:Cre-mediated removal of exon 3 led to an expansion of the lineage negative Sca.1^{+} cKit^{+} (LSK) cells in vivo and an increase in replating potential in a colony forming unit assay in vitro (Moran-Crusio et al. 2011). These studies further showed that *TET2*$^{KO/KO}$ bone marrow was capable of outcompeting *TET2*$^{WT/WT}$ bone marrow in competitive transplant assays and showed increased stem cell function and self-renewal. Finally, aged *TET2*$^{KO/KO}$ mice developed a CMML-like syndrome with expansion of the monocytes, increased spleen weight, and proliferative growth in the bone marrow, spleen, liver, and lung. Multiple studies published in the same year confirmed these findings (Ko et al. 2011; Li et al. 2011; Quivoron et al. 2011; Shide et al. 2012), with many studies revealing decreased 5-hmC levels in the LSK population. The expansion of the LSK and hematopoietic stem cell (HSC) populations in these mice mirror the findings in patient samples in which clonal *TET2* mutations were found in healthy individuals with clonal hematopoiesis (Busque et al. 2012).

In AML and myeloproliferative disease, *TET2* mutations are typically present in concert with other mutations. Mutations in the fms related tyrosine kinase 3 (*FLT3*) are among the most common events in AML with point mutations in the tyrosine kinase domain, and internal tandem duplications (ITDs) near the juxtamembrane domain, leading to autoactivation of the kinase (Levis and Small 2003). Interestingly, when *TET2* loss was combined with a *FLT3-ITD* mutation there was a distinct set of genomic loci that underwent hypermethylation compared with either mutation alone (Shih et al. 2015). Among these loci, hypermethylation of the *GATA2* promoter led to a reduction in expression, blockade in differentiation and the development of a transplantable leukemia derived from the LSK progenitor compartment. Interestingly, in addition to the hypermethylated regions, there were more than 500 hypomethylated regions in the combined TET2-FLT3-ITD mutants that were not present in either mutant alone. These, at first paradoxical, findings may be partially explained by FLT3's role in the commitment to the myeloid lineage and thus hypomethylation of genes necessary for that engagement. Future studies on DNA methylation and 5-hmC will be of interest to determine if the loci-specific effects are indeed specific to this model or representative of a more general leukemic transformation phenotype. Additional models of mutational cooperation with TET2 loss include expression of a mutant c-KIT in mast cells (Soucie et al. 2012), expression of *AML-ETO* (Hatlen et al. 2016), and loss of Notch signaling (Lobry et al. 2013).

MUTATIONS IN OTHER MALIGNANCIES

T-Cell Lymphoma

In addition to myeloid malignancies detailed above, *TET2* mutations have also been identified in patients with T-cell lymphoma (Quivoron et al. 2011). One study found *TET2* mutations in 47% of angioimmunoblastic T-cell lymphomas (ATLs) and in 38% of peripheral T-cell lym-

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phomas not otherwise specified (PCTL-NOS) (Lemonnier et al. 2012). Subsequent studies have identified *TET2* mutations in upward of 75% of ATL patients (Odejide et al. 2014). Interestingly, this study found multiple subclonal mutations in *TET2* within individual patients, all of which resulted in truncation or disruption of the final gene product. Unlike the myeloid leukemia setting, few mutations were found in $CD34^+$ progenitors or subsequent myeloid colonies derived from this population (Odejide et al. 2014). Collectively, these results place TET2 loss as a recurrent driver in ATL with acquisition of the mutation in a lineage committed stage, contrasting sharply with the myeloid malignancies. A related contrast was identified when patients presented with both *TET2* and *IDH1* mutations (Odejide et al. 2014), events that are largely mutually exclusive in myeloid malignancy. This may reflect a different role for subclonal TET2 loss of function in ATL versus the presumed expansion of a preleukemic clone in AML.

Melanoma

TET2 mutations have been predominantly associated with hematologic malignancies; however, whole-genome analyses through TCGA have identified additional mutations in melanoma and cutaneous squamous cell carcinoma (Cancer Genome Atlas Network 2015). Consistent with these genomic findings, loss of 5-hmC has been proposed to be a prevalent, epigenetic hallmark, of melanoma (Lian et al. 2012). Indeed, epigenetic silencing of *TET2* and *TET3* has been shown to drive TGF-β-dependent invasion and acquisition of EMT-like features (Gong et al. 2016). Subsequent in vivo studies showed that overexpression of TET2 blunted tumor growth and metastasis. Given the prominent role of dedifferentiation in metastatic melanoma, it will be interesting to investigate the potentially parallel roles of TET2 in hematopoietic and melanocyte differentiation.

Glioma

CpG-island hypermethylator phenotypes (CIMPs) have also been identified in low-grade gliomas, as well as some glioblastoma patients. Although *TET2* promoter methylation has been identified in glioma (Kim et al. 2011), the predominant mechanism appears to be driven by mutations in *IDH1*, with few loss-of-function mutations present in *TET2* (Kraus et al. 2015). This is in stark contrast to MPN and AML studies in which TET2, IDH1, and IDH2 mutations are all present. These studies support a growing literature showing mutant IDH1 and IDH2 elicit functions outside of 2-HG-mediated TET2 inhibition, including inhibition of histone demethylases (Lu et al. 2012), alteration of DNA damage repair (Inoue et al. 2016), and alterations in branched chain amino acid metabolism (Tonjes et al. 2013). This apparent tissue-specific distinction in mutational patterns may also be the result of tissue-specific gene expression levels of *TET2*, which is substantially more highly expressed in AML than in the gliomas (Fig. 2).

Other Diseases

In addition to melanoma, TET2 has been shown to be down-regulated in an androgen-dependent manner in prostate cancer, with lower expression conferring worse prognosis for patients (Nickerson et al. 2016). In colorectal cancer, TET2 has been shown to be excluded from the nucleus (Huang et al. 2016), with similar findings for TET1 in glioma (Waha et al. 2012).

CONCLUDING REMARKS

In sum, TET2 is a critical regulator of DNA methylation in development and malignancy. Genomic alterations of *TET2*, in addition to modulation of binding partners, lead to alterations in 5-hmC levels and downstream outputs on proliferation and maintenance of stem cells. Although mutations are enriched in hematologic neoplasms, TET2 loss of function has been observed in solid tumors as well. Collectively, these studies have provided insight into how TET2 contributes to disease and may provide clues for identifying specific therapeutic avenues for patients harboring these mutations.

Figure 2. *TET2* expression across malignancy. Normalized RNA-Seq counts (log2) are shown for the indicated malignancies ranked from lowest to highest mean expression of *TET2*. Samples with frameshift mutations are denoted with an inverted triangle, nonsense mutations are denoted as a diamond, and in-frame mutations are shown with a square. Data was collected and graphed using the cBioPortal (see cbioportal.org/) (Gao et al. 2013).

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