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## Mechanisms that regulate the activities of TET proteins

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### Abstract

The ten-eleven translocation (TET) family of dioxygenases consists of three members, TET1, TET2, and TET3. All three TET enzymes have Fe<sup>+2</sup> and  $\alpha$ -ketoglutarate ( $\alpha$ -KG)-dependent dioxygenase activities, catalyzing the 1<sup>st</sup> step of DNA demethylation by converting 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), and further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Gene knockout studies demonstrated that all three TET proteins are involved in the regulation of fetal organ generation during embryonic development and normal tissue generation postnatal. TET proteins play such roles by regulating the expression of key differentiation and fate-determine genes via 1) enzymatic activity-dependent DNA methylation of the promoters and enhancers of target genes; and 2) enzymatic activity-independent regulation of histone modification. Interacting partner proteins and posttranslational regulatory mechanisms regulate the activities of TET proteins. Mutations and dysregulation of TET proteins are involved in the pathogenesis of human diseases, specifically cancers. Here, we summarize the research on the interaction partners and posttranslational modifications of TET proteins. We also discuss the molecular mechanisms by which these partner proteins and

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#### Authors' contributions

Kanak Joshi and Shanhui Liu drafted the first version of this review. All of the authors contributed to the writing of this manuscript. Peter Breslin did the final editing. All authors read and approved the final manuscript.

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modifications regulate TET functioning and target gene expression. Such information will help in the design of medications useful for targeted therapy of *TET*-mutant-related diseases.

## Keywords

TETs; mutations; interaction partners; posttranslational modifications; gene expression

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## Introduction

Lineage commitment and differentiation of tissue stem/progenitor cells are tightly controlled by transcriptional programming<sup>1,2</sup> and are delicately regulated by an ordered, stepwise reconfiguration of the DNA methylome and histone modifications.<sup>3–8</sup> Dysregulation of either transcriptional programming or the epigenetic machinery will cause diseases such as cancers by disrupting cell fate determination and differentiation. Thus, a more complete understanding of how transcriptional programming and epigenetic functioning collaboratively regulate lineage fate and differentiation of stem/progenitor cells will provide information that will improve our understanding of disease pathogenesis and can point the way toward the development of novel medications for the treatment of diseases.

Transcription factors (TFs) regulate target gene expression by binding to specific consensus motifs in their enhancers and promoters.<sup>9</sup> The binding motifs of most TFs contain CpG dinucleotides. Such TFs have different sensitivities to methyl-CpG (mCpG) motifs for DNA binding. Many genes have CpG-rich (CpG islands or CGIs) promoters. Methylation of these promoters is associated with target gene repression due to the condensation of local chromatin.<sup>8,10,11</sup> Removing methyl groups from these promoters is required for TF binding and gene expression. In genes with non-CGI promoters and enhancers, TF-regulated expression of such genes is determined by the methylation status of CpG within the binding motifs.<sup>12–14</sup>

The dynamic methylation of DNA is regulated by a balance of DNA methyltransferases (including DNMT1, DNMT3a, and DNMT3b) and the Ten-eleven translocation (TET) family of dioxygenases (including TET1, TET2, and TET3).<sup>15,16</sup> The methylation state of DNA sequences regulates the accessibility of key TFs to genetic regulatory elements including promoters and enhancers of target genes, which in turn determines cell fate.<sup>8,17</sup> Disruption of the dynamic methylation programming of DNA has been observed in almost all types of hematopoietic malignancies and has emerged as a hallmark of various types of hematological cancers, including myelodysplastic syndromes (MDS), acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), diffuse large B cell lymphomas (DLBCLs) and peripheral T-cell lymphoma (PTCL).<sup>18–42</sup> Consistently, somatic mutations of several key regulators of DNA methylation including *DNMT3A*, *isocitrate dehydrogenase (IDH1)*, *IDH2*, and *TET2* have been detected in almost all types of hematopoietic cancers.<sup>43</sup> Detailed studies demonstrated that somatic mutations of *DNMT3A* and *TET2* are also frequently detected in small clones in the hematopoietic tissue of healthy people, specifically those >50 years old. The frequency of such mutations is increased during aging and has been called age-related clonal hematopoiesis (ARCH).<sup>44–46</sup> The selective acquisition and

expansion of *DNMT3A*- or *TET2*-mutant clones during aging suggest that ARCH might be a consequence of compensatory hematopoiesis against the pressure of aging. In support of such a concept, it was found that hematopoietic stem and progenitor cells (HSPCs) showing either *DNMT3A* or *TET2* mutations display growth advantages in response to treatment with interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), respectively.<sup>47,48</sup> Nevertheless, people with ARCH showed a 10–12 fold increased risk for developing hematopoietic malignancies than age-matched ARCH-negative populations.<sup>49–51</sup> Thus, as is the case with somatic *DNMT3A* mutations, somatic *TET2* mutations are founder mutations for almost all types of hematopoietic malignancies, and occur in hematopoietic stem cells (HSCs) during aging and are selected under the pressure of aging-associated inflammation. Additional genetic mutations are required for the full malignant transformation of *TET2*-mutant HSCs, which drive the abnormal proliferation, lineage commitment, differentiation, and survival of HSPCs. In addition, *TET1* is frequently mutated in B-cell malignancies and *TET3* is down-regulated in HSPCs during aging as well as in the malignant cells of many types of hematopoietic cancers.<sup>52</sup> Thus, all three members of the TET family are involved in the pathogenesis of hematopoietic cancers. In this review, we summarize the research on TET protein interaction partners and translational modifications of three TETs in the regulation of TET function. We also discuss the molecular mechanism by which TET proteins regulate target gene expression.

## 1. The three *TET* genes and their isoforms

The human *TET1* gene is located on chromosome 10q21.3. It expresses two transcriptional isoforms owing to the use of alternate promoters (Figure 1a).<sup>53</sup> Transcription starting from promoter 1a or 1b (distal) produces a 2,136 a.a. full-length TET1 protein (2039 a.a. for the mouse), while transcription starting from promoter 2 (proximal) in front of exon 2 gives rise to a 1465 a.a. short isoform of TET1 (TET1s, 1386 a.a. for the mouse).<sup>53,54</sup> TET1s lacks a large portion of the TET1 N-terminus, including the CXXC (CXXC5) domain. Both TET1 and TET1s have enzymatic activity. In mice, TET1 is primarily expressed in the embryo and is replaced by TET1s in adult tissues<sup>53</sup>.

The human *TET2* gene is located on chromosome 4q24. In contrast to the *TET1* and *TET3* genes, the ancient *TET2* gene was split during evolution into two genes, *IDAX* (also called *CXXC4*) and *TET2*. The *IDAX* gene is located 700 kb upstream of *TET2* and is transcribed in the opposite direction; it encodes the CXXC domain-containing IDAX protein.<sup>55</sup> The *TET2* gene produces three protein isoforms, TET2-a, TET2-b, and TET2-c, because of the alternating use of 3 promoters and associated transcriptional initiation sites (TIS) (Figure 1a). *TET2-b* utilizes the second promoter in front of exon 1b and produces a 2002 a.a. full-length TET2 protein (TET2 hereafter, 1912 a.a. for the mouse). *TET2-a* uses the first promoter, which is localized upstream of the second promoter and produces a truncated 1165 a.a. protein terminating at a poly-A site in the fourth intron, while *TET2-c* utilizes the 3<sup>rd</sup> promoter in front of exon 1c and produces a much shorter truncated protein terminating within the 3<sup>rd</sup> exon. Both TET2-a and TET2-c lack enzymatic activity and might function as dominant-negative forms of TET2. TET2 is most abundant in normal human tissues, while TET2-a is primarily expressed in the human spleen, and TET2-c is weakly expressed in most tissues, with the highest levels observed in human spleen, bone marrow, fetal brain,

and embryoid bodies. The dynamic switching of active promoters and enhancers regulates TET2-a, TET2, and TET2-c expression during cell state transitions between pluripotency and differentiation.<sup>56</sup>

The human *TET3* gene is located on chromosome 2p13.1. The CXXC10–1 ORF is about 13 kb upstream of the annotated TSS of *TET3* with the same orientation as the TET3 ORF. *TET3* gene encodes three isoforms owing to the alternative use of promoters and alternative splicing (Figure 1a). A 1795 a.a. full-length TET3 protein (TET3 hereafter; 1803 a.a. for the mouse) is transcribed starting from promoter 2 in front of exon 1b, and a 1660 a.a. TET3 short isoform (TET-3s; 1668 a.a. for the mouse) is transcribed starting from promoter 3 in front of exon 2. A 1713 a.a. oocyte-specific isoform of TET3 (TET-3o) has been identified in the mouse, which is transcribed starting from promoter 1 in front of exon 1a approximately 5 kb upstream of the start codon with skipping of exon 1b.<sup>57</sup> Human TET-3o has not been reported.

TET1, TET2, and TET3 share a conserved dioxygenase domain at their C-termini (Figure 1b).<sup>58–60</sup> The dioxygenase domain is composed of a cysteine (Cys)-rich domain and a double-stranded  $\beta$ -helix fold (DS $\beta$ H) domain that is compactly arranged to mediate catalytic activity. The DS $\beta$ H domain consists of 3 Fe<sup>2+</sup> binding sites and one  $\alpha$ -ketoglutarate ( $\alpha$ -KG) binding site. In addition, full-length TET1 and TET3 proteins contain N-terminal CXXC-type zinc finger domains. CXXC regulates the recruitment and binding of TET1 and TET3 to DNA sequences and provides a unique regulation of methylation signature for genes associated with embryogenesis, gametogenesis, and neuronal development.<sup>53,61–63</sup> TET2 protein lacks a DNA recognition domain and depends on other DNA binding proteins for interaction with DNA. In addition, the short forms of TET1 and TET3, including TET1s, TET-3s, and TET-3o (an oocyte-specific isoform), all lack CXXC domains.<sup>54,63</sup> Therefore, TET1s, TET-3o, and TET-3s are primarily dependent on the interaction of other DNA binding proteins for DNA binding.

## 2. The mutations and expression of TET genes in the pathogenesis of cancer

**The expression of TET genes in cancers.**—Compared to non-cancerous surrounding tissues, reduced levels of 5-hydroxymethylcytosine (5hmC) have been reported in multiple types of human cancers, such as hematopoietic malignancies, melanoma, lung cancers, pancreatic cancers, hormone-receptor-positive breast cancers, colon cancers, liver cancers, and glioblastoma multiforme, which are all associated with loss of functional TET mutations or decreased levels of TET proteins.<sup>64–74</sup> The reduction of 5hmC results in the aberrant methylation of the tumor suppressor genes that leads to tumor formation, progression, and invasion. Studies suggested that low 5hmC is an important marker for early diagnosis and predicts poor prognosis in some cancer types.<sup>73–80</sup> However, in some other cancer types, including gastric cancers, lung cancers, triple-negative breast cancer, human epidermal growth factor receptor-enriched breast cancers, ovarian cancers, and glioma, levels of TET proteins and 5-hmC are increased.<sup>81–84</sup> TET proteins in such cancers function as oncoproteins, which promote cell proliferation and tumor progression. Thus, the roles of TET proteins in cancer pathogenesis might be tissue- and cell type-specific.<sup>85</sup>

**The mutations of TET genes in cancers.**—Loss-of-function *TET2* mutations are frequently detected in blood cells from healthy individuals over 50 years old; it is referred to as ARCH. *TET2* mutations in ARCH lead to a premalignant condition in hematopoietic tissue, which predisposes to leukemia/lymphoma transformation. *TET2* mutations are commonly detected in almost all types of hematopoietic malignancies including MDS, myeloproliferative neoplasms, AML, PTCL, and DLBCL.<sup>30–42,86</sup> Loss-of-function *TET1* and *TET3* mutations are detected in non-Hodgkin B-cell lymphoma, including DLBCL, and follicular lymphoma.<sup>87–91</sup> In addition, *TET1* is also mutated in 12–15% of T-ALL and 1–5% of AML patients.<sup>92,93</sup> *TET3* mutations are very rarely identified in PTCL<sup>37,94</sup> and chronic lymphocytic leukemia.<sup>95</sup> However, mutations of the *TET1/2/3* genes are infrequent in solid cancers and their significance in such cases is unknown.<sup>66</sup> In prostate cancers, *TET2* mutations are detected in 6% of primary tumors and 20% of metastatic lesions.<sup>96</sup> Whether *TET2* mutations contribute to the metastatic advantage of prostate cancer needs to be determined experimentally.

**Transcriptional regulation of TET1 gene in cancers (Figure 1a).**—In embryonic stem (ES) cells, pluripotent genes *OCT4*, *NANOG*, *MYC*, and *SOX2* are strongly enriched in a super-enhancer upstream of promoter 1 of the *TET1* gene, stimulating the expression of TET1 but not TET1s.<sup>53</sup> During differentiation, TET1 is down-modulated by PRC2 binding of the super-enhancer, localized +40 kb downstream of the *TET1* TIS.<sup>97</sup> HIF-2 $\alpha$  binds –158 to –91 bp upstream of the TIS of *TET1* and induces *TET1* expression in response to conditions of hypoxia.<sup>98</sup> In lung epithelial cells, p53 binds to –192bp/+29 bp of the promoter and represses *TET1* expression.<sup>84</sup> FOXA1 occupies the *TET1* enhancer at +50 kb downstream of TIS and induces *TET1* gene expression.<sup>99</sup> During the prepubertal period, gonadotropin-releasing hormone (GnRH) stimulates luteinizing hormone- $\beta$  polypeptide expression and differentiation of gonadotropic cells by repressing *TET1s* expression. GnRH plays such a role by inactivating a distal enhancer located –20 to 22 kb upstream of the TIS.<sup>100</sup> TET2 binds to this enhancer to maintain *TET1s* expression.<sup>100</sup> TET1 is downregulated in many types of cancers such as breast cancer, pancreas cancer, rectal cancer, oral squamous cell carcinoma, lymphoma, multiple myeloma, bladder cancer, liver cancer, and non-small-cell lung cancer, implying a tumor repressive activity for TET1.<sup>78,85,87,101,102</sup> A CGI has been identified in the *TET1* promoter and exon 1 region. In many types of cancers, downregulation of *TET1* might be mediated by HMG2 and PRC2 via epigenetic methylation of the CGI promoter.<sup>97,103</sup> C/EBP $\alpha$  directly binds to the *TET1* promoter and regulates *TET1* expression<sup>104</sup>. In lung cancer and glioblastoma multiforme, epidermal growth factor receptor and MAPK activation silence *TET1* expression by down-regulating C/EBP $\alpha$ . In basal-like breast cancer, thyroid carcinoma, skin cutaneous melanoma, and lung adenocarcinoma, TNF $\alpha$  stimulates NF- $\kappa$ B activation, which represses *TET1* expression by binding to the *TET1* promoter.<sup>105</sup> In both cellular and animal models, inhibition of EGFR signaling restores *TET1* expression.<sup>104</sup> In colon cancers, BRAF<sup>V600E</sup> downregulates *TET1* and *TET2* expression which results in a hypermethylation phenotype in the cancer cells.<sup>106</sup> *TET1* downregulation is involved in disease initiation and cancer invasiveness/metastasis and is associated with a poor prognosis. In breast cancers, down-regulation of *TET1* results in HOXA9/HOXA7 repression, which leads to breast cancer growth and metastasis.<sup>103</sup> In prostate cancers, TET1 suppresses

cancer invasiveness by activating the tissue inhibitors of metalloproteinases.<sup>107</sup> In rectal cancers, TET1 inhibits the WNT signaling pathway by upregulating WNT inhibitors DKK3 and DKK4. Downregulation of TET1 promotes cancer development due to the activation of WNT signaling. Interestingly, a study suggests that TET1 is overexpressed in 40% of triple-negative breast cancer patients. In these types of cancers, TET1 expression is involved in cancer activation pathways including EGFR, PI3K, and PDGF, and is correlated with cell migration, cancer stemness, tumorigenicity, and poor survival.<sup>108–110</sup> It suggests that TET1 might function as an oncoprotein and a therapeutic target in these types of cancers.<sup>85</sup> Furthermore, TET1s is aberrantly expressed in multiple cancer types including breast, uterine, and glioblastoma. The predominant TET1s activation in cancer cells results in dynamic site-specific demethylation outside of CGIs, which is associated with worse overall survival in breast, uterine and ovarian cancers.<sup>54</sup>

**Transcriptional regulation of TET2 and TET3 genes in cancer.**—Compared to TET1, the transcriptional regulation of *TET2* and *TET3* genes has been studied much less (Figure 1a). In ES cells, OCT4 binds to the promoter at +1,788/+1,795 bp (relative to the TSS) of the *TET2* gene and promotes *TET2* expression.<sup>111</sup> In addition, OCT4-SOX2 binding elements are identified at ~-140 kb and -200 kb of the *TET2* TSS.<sup>112</sup> In response to hypoxia, HIF1 $\alpha$  was found to repress *TET2* expression in melanoma cells.<sup>113</sup> In pancreatic cells, TGF1 $\beta$  induces the expression of TET2 by stimulating SMAD4 binding of an enhancer proximal to the distal 3' region of the *TET2* gene.<sup>70</sup> Decreased TET2 and 5-hmC were found in ovarian carcinoma tissues and colorectal cancer patients, which was associated with high tumor grade, pathologic stage, lymph node metastasis, and vascular thrombosis as well as chemoresistance and poor clinical outcomes.<sup>114–116</sup> GATA6 is a key TF for the differentiation of pancreatic progenitors. In aggressive squamous-like PDAC subtypes, *TET2* is downregulated due to the loss of SMAD4, which is correlated, with a reduction of 5hmC and GATA6. Metformin and Vitamin C restore 5hmC and GATA6 levels by enhancing TET2 stability, reverting squamous-like tumor phenotypes, and WNT-dependence both *in vitro* and *in vivo*.<sup>70</sup>

CGIs have also been identified at the promoter, intron 1, and intron 2 of the *TET3* gene. *TET3* is epigenetically repressed in gliomas due to the methylation of these CGIs.<sup>117</sup> Loss of *TET3* expression was identified in 32 % of GCs and 28 % of CRCs.<sup>118</sup> *TET3* was downregulated in ovarian cancer cells during TGF- $\beta$ 1-induced epithelial-mesenchymal transition (EMT) and was correlated with pathological grade. *TET3* over-expression was found to suppress ovarian cancer by up-regulating miR-30d, which then blocks TGF- $\beta$ 1-induced EMT.<sup>119</sup> However, a study suggested that increased *TET3* levels in ovarian carcinoma are associated with poor clinical-pathological status and poor prognosis.<sup>120</sup>

**MicroRNAs regulate the expression of TET genes.**—The expression of TET proteins is also regulated by microRNA (miR)-mediated posttranscriptional repression.<sup>121</sup> Approximately 30 miRNAs have been identified that repress *TET2* expression, including miR-7, miR-125b, miR-29b/c, miR-26, miR-101, miR142, and Let-7.<sup>122</sup> *TET1* expression is regulated by miR-29 family members including miR-26a, miR-767, miR-494, and miR-520b.<sup>123–126</sup> In hematopoietic tissues, miR-22 promotes HSC self-renewal and

leukemic transformation by repressing TET2.<sup>127</sup> In inflamed mouse epithelial cells, inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  repress the expression of TET proteins by inducing NF- $\kappa$ B signaling-mediated miR20a, miR26b, and miR29c expression.<sup>128</sup> In gastric carcinogenesis, miR-26 represses TET1/2/3 expression.<sup>81</sup> In hepatocellular carcinomas, miR29a promotes SOCS1–MMP9 signaling axis-mediated tumor metastasis by repressing TET proteins.<sup>72</sup> In models of type 1 diabetes, miR142–3p targets TET2 and impairs Treg differentiation and stability.<sup>129</sup> In macrophages, Let-7 promotes IL-6 by repressing Tet2 expression.<sup>130</sup>

### 3. TETs-TDG-BER system regulates DNA demethylation.

TET1, TET2 and TET3 are Fe<sup>2+</sup> and  $\alpha$ -KG-dependent dioxygenases. TETs catalyze the 1<sup>st</sup> step of demethylation by the hydroxylation of 5-methylcytosine (5mC) to 5hmC, and further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC).<sup>16</sup> To fully complete the demethylation process, 5fC and 5caC, the products of TETs, can be replaced by cysteine via either replication-dependent dilution/passive DNA demethylation or thymine DNA glycosylase (TDG) and base excision repair (BER)-mediated active DNA demethylation.<sup>59,131</sup> Both 5fC and 5caC are substrates for TDG. TDG catalyzes the excision of 5fC and 5caC to generate an apyrimidinic site (AP site). By coordinating with BER enzymes, TDG mediates the replacement of 5fC and 5caC with cysteine. Studies suggest that TDG is essential in protecting CpG-rich promoters from hypermethylation and collaborating with key TFs by actively removing methyl groups from enhancers and promoters of target genes.<sup>132</sup> Thus, active dynamic DNA demethylation is primarily mediated by an IDHs-TETs-TDG-BER-driven cytosine modification system. In addition, it was found that activation-induced cytidine deaminase (AID)/APOBEC mediates an alternative oxidative deamination-demethylation pathway. AID/APOBEC is required for DNA demethylation during reprogramming of somatic cells and B cell maturation.<sup>133,134</sup> AID catalyzes cytidine deaminases primarily at 5hmC sites to generate 5-hydroxymethyluracil (5hmU). 5hmU is subsequently cleaved by TDG, single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1), Nei-like DNA Glycosylase 1 (NEIL1), or methyl-CpG binding protein 4 (MBD4) and can be replaced by cytosine, as mediated by BER enzymes.<sup>135</sup> Thus, AID mediates TET-dependent DNA demethylation.<sup>133,136,137</sup> Furthermore, it was reported that both growth arrest and DNA damage-inducible protein 45a (GADD45a)<sup>138,139</sup> and GADD45b play critical roles in the demethylation of specific promoters,<sup>138–140</sup> and BER plays essential roles in genome-wide active DNA demethylation in primordial germ cells (PGCs).<sup>141</sup> Further study demonstrated that TDG, AID, and GADD45a form a ternary complex in regulating the methylation state of promoters and enhancers within the genome. Thus, it is most likely that GADD45a/b-TDG-AID-BER altogether mediate active DNA demethylation.<sup>132</sup>

AID is a key enzyme that mediates DNA methylation dynamics in germinal center B-cells.<sup>142–144</sup> AID initiates the somatic hypermutation process through deamination of cytidine to uridine in the recombined variable region, followed by removal of the uracil base by uridine DNA glycosylase and DNA repair by several error-prone BER and mismatch-repair enzymes.<sup>136</sup> AID further induces the second step of antibody diversification, class-switch recombination, through deamination of bases in the switch region, causing double-

strand breaks and recombination.<sup>137</sup> AID is a key regulator of myeloid and erythroid differentiation and DNA methylation in HSPCs.<sup>134,145</sup> The demethylation activity of AID is severely impaired in the absence of TET2, without impairment of AID mutability, suggesting that AID is dependent on TET2 for its demethylating capacity. This explains an AID-dependent hyper-mutagenesis feature and tumor development in *TET2*-deficient animals.

#### 4. DNA 5-hmC is an epigenetic mark of gene activation

It should be clarified that 5hmC, 5fC, and 5caC are not only intermediates of passive and active DNA demethylation but also serve as stable epigenetic marks<sup>146,147</sup> and have distinct epigenetic regulatory functions because they are distributed genome-wide and can be recognized by specific reader proteins.<sup>148,149</sup> For example, several selective 5-hmC readers have been identified, such as MeCP2, the MBD3/NURD complex, E3 ubiquitin-protein ligases (UHRF1 and UHRF2), DNA glycosylases (MPG and NEIL3), SALL1/SALL4, Thy28, PRMT1 (CHTOP)-methylome complex, Recql helicase, RBM14, PRP8, RPL26, MSH6, PNKP, and WDR76.<sup>149–153</sup> Only three of them, NP95/UHRF1, MeCP2, and MBD3, have been confirmed in more than one study.<sup>150</sup> These proteins bind to 5hmC-DNA and regulate gene expression by recruiting co-activators or co-repressors. 5hmC is present in high amounts at active enhancers and the gene bodies of highly transcribed genes.<sup>154</sup> 5-hmC is associated with the activating histone marks H3K4me1, H3K4me3, and H3K27ac<sup>154–158</sup>. This explains, in many cases, that gene expression is closely related to the 5hmC/5mC ratio of enhancers and/or promoters.<sup>159–161</sup> In addition, some of these 5-hmC reader proteins bind to 5hmC-DNA and recruit TETs, which further recruit TDG-BER complexes for completing the remaining steps of DNA demethylation.

#### 5. The selective DNA binding of TET proteins.

Although all 3 TET family members and their isoforms have similar catalytic activity as demonstrated by certain levels of functional redundancy,<sup>162,163</sup> the distinct phenotypes of *Tet1*, *Tet2*, and *Tet3* knockout mice, as well as the distinct 5hmC/5mC patterns of *Tet1*, *Tet2*, and *Tet3*-deficient cells, suggest significant non-redundant functions for the Tet proteins.<sup>164–169</sup> Such distinct roles of the three Tets are partially explained by their distinct expression profiles within developmental tissues. For example, *Tet1* and *Tet2* mRNA levels are abundant in ES cells and PGCs,<sup>170,171</sup> while *Tet3* is the only Tet gene expressed at substantial levels in oocytes and zygotes.<sup>172,173</sup> *Tet1* is expressed in fetal heart, lung, and brain, and adult skeletal muscle, thymus, and ovary, but not in adult heart, lung, or brain. *Tet2* is primarily expressed in hematopoietic tissues.<sup>101,174</sup> *Tet3* is highly expressed in neural progenitor cells where it preferentially binds to TSSs and regulates cellular identity and genes associated with the lysosomes, autophagy, and base excision repair pathways.<sup>57,175</sup> The non-redundant functions of the three TETs and their isoforms are also determined by their selective binding to genomic DNA regions. TET1 has a high affinity for a high density of CpG promoters, while TET2 is more commonly located at low CpG density promoters.<sup>169,175</sup> In mouse ES cells, *Tet1* primarily regulates 5hmC levels at gene promoters and TSSs, whereas *Tet2* mainly regulates 5hmC levels in gene bodies and exon boundaries of highly expressed genes and exons, respectively.<sup>166,176</sup> In induced pluripotent stem cells (iPSCs), TET1 and TET2 appear to target different genomic regions and promote



opposing functions in reprogramming-mediated erasure of imprints and naïve pluripotent state transitions. TET1 promotes a primed state of pluripotency, while TET2 regulates a naïve state of pluripotency.<sup>166,177,178</sup>

DNA binding by TET1 and TET3 is primarily mediated by their CXXC domains, while the DNA binding of TET2 and the short isoforms of TET1 and TET3 is mediated by interactions with partner proteins. Both TET1 and TET3 regulate DNA methylation specifically at CpG sites within and around CGIs, appearing to show more flexible substrate specificity.<sup>179,180</sup> The TET1-CXXC domain binds CpG-rich DNA irrespective of methylation status, while the TET3-CXXC domain binds methylated CpG sites with relatively low affinity compared to a non-methylated CpG dinucleotide, with the highest affinity toward 5caC sequences.<sup>61,181,182</sup> In addition, TET1-CXXC also binds to TFs FOXA1 and HIF2 $\alpha$ , selectively mediating active epigenetic modifications at FOXA1 and HIF2 $\alpha$ -dependent enhancers, respectively.<sup>99</sup>

TET1 binds CGI chromatin globally via its CXXC to protect CpG sites within and around CGIs from gaining aberrant methylation,<sup>179</sup> while TET1s preferentially binds to CpG sites at non-CGIs and some targeted CGI chromatin.<sup>54</sup> Due to the selective binding of DNA regulatory regions, the roles of TET1 and TET3 are not always the same as their short isoforms and in many cases are the opposite. For example, compared to neurons, TET1 is highly expressed in glial cells, while TET1s is downregulated. TET1 and TET1s expression have opposing effects on synaptic transmission and hippocampal-dependent memory.<sup>183</sup> In mice, Tet1 is restricted to early embryos, ES cells, and PGCs, whereas Tet1s is preferentially expressed in somatic cells. The expression of Tet1 and Tet1s switches during development and regulates epigenetic memory erasure.<sup>53</sup> TET1s is overexpressed in multiple cancer types including breast, uterine, and glioblastoma, which is associated with worse overall survival.<sup>54</sup>

## 6. The interaction partner proteins of TETs.

Many partner proteins of TET1, TET2, and TET3 have been identified; however, the interaction regions have been defined only for some of them (Figure 2). Based on available information, most of the partner proteins bind to the C-terminal fragment including the DS $\beta$ H enzymatic domain of TETs; only a few of them bind to the N-terminal fragment. However, the details of the interaction sites are only well known for Sin3A on TET1 and TET3, and O-linked GlcNAc transferase (OGT) on TET1. Sin3A interacts with the Sin3-interaction domain (SID) on TET1 (a.a. 889–903) and TET3 (a.a. 257–271). Although SID is absent from TET2 and its dimeric partner, CXXC4 might mediate the Sin3A-TET2 interaction.<sup>184,185</sup> All three TETs interact with OGT through its C-terminal fragment.<sup>186–188</sup> Detailed analysis demonstrated that the last 45 a.a. at the C-terminus (C45) of TET1 mediates the OGT binding.<sup>189</sup> However, the detailed binding sites of OGT on TET2 and TET3 have not been determined.

**Partner proteins for all 3 TETs.**—Among all partner proteins, some of them can interact with all three TETs. For example, CTCF can interact with all three TETs and recruit them to the CTCF-binding sites outside of CGIs, regulating DNA methylation

and gene expression.<sup>190–193</sup> CXXC4 and CXXC5 interact with the catalytic domain of TET2 as well as short isoforms of TET1 and TET3, recruiting TETs to DNA.<sup>194</sup> As is true for the CXXC domain of TET1 and TET3, the CXXC domain in CXXC4 and CXXC5 proteins preferentially bind to unmethylated CGIs in gene promoter regions to maintain hypomethylation of CGIs.<sup>194,195</sup> CXXC5 forms a complex with NANOG, OCT4, TET1, and TET2 and positively regulates the transcription of pluripotency genes and TET enzymes.<sup>196</sup> Interestingly, CXXC4 negatively regulates TET2 activity by promoting caspase-mediated degradation of TET2 protein.<sup>194</sup> WT1 physically interacts with TET2 and selectively regulates TET2-dependent expression of target genes such as RUNX1.<sup>197,198</sup> WT1 also interacts with TET1 and TET3 for target gene expression.<sup>199</sup> In addition, some histone modifiers such as SIRT1, histone deacetylases (HDACs) 1/2, and OGT as well as a variety of factors of the BER-DNA glycosylase pathway, including PARP1, MBD4, NEIL1, NEIL2, NEIL3, TDG, SMUG1, PARP1, LIG3, and XRCC1, also interact with all three TETs.<sup>200–202</sup> All these shared interaction partners might partially explain the overlapping and compensatory functions of the three TET molecules.

**Partner proteins that have been identified for certain TET proteins.—**Many of the partner proteins selectively bind to one or two of the TETs and their isoforms. Several partner proteins for TET1 have been identified; these include MeCP2, EZH2, LSD1, hMOF, and PCNA.<sup>152,157,203–206</sup> TET3 interacts with TFs including REST, ASXL1, MORF4L1, VAX1, and thyroid hormone nuclear receptor (TR), as well as the H3K36 methyltransferases NSD2, NSD3, and SETD2, as determined by immunoprecipitation and LC-MS/MS.<sup>193</sup> Significantly more TET2-interacting partners have been identified, including TFs (C/EBP $\alpha$ , PU.1, Klf4, Tfc211, MBD3, MBD3L2, YBX1, FOXK2, IKZF1, NFIL3, ATRX, CUX1, YY2, WT1, EBF1, SNIP1, PML and I $\kappa$ B $\zeta$ <sup>159,198,207–212</sup>), histone modifiers (SMARCB1, SMARCC2, SMARCE1, P300/CBP, HDAC1, HDAC2, SIN3A, EZH2, HCFC1, NCOR1/2, BAZ1A/B, TOP2A/B, MBD2, PHF2, INO80, SAP30BP, TRRAP, WDHD1, CHD8, MLLT3, UHRF2 and CHAF1A<sup>149,159,208,213–217</sup>), and signaling regulators (AMPK, JAK2, 14–3-3Z/D and 14–3-3E proteins<sup>203</sup>). These selective interacting partners of TETs determine the TETs' functional specificity. For example, Mbd3/NURD recruits TET1 to genomic sites to regulate the expression of 5-hmC-marked genes in ES cells.<sup>152</sup> Lin28A binds to active promoters and recruits TET1 to regulate gene expression.<sup>218</sup> EGR1 recruits TET1s to target genes and selectively regulates the expression of EGR1 target genes by DNA demethylation.<sup>219</sup> In iPSCs, ZFP281 drives TET1 to the promoter of target genes including TET2 to promote primed pluripotency. SNIP1 selectively interacts with TET2 (but not TET1 nor 3), bridging TET2 to TFs, including C-MYC, CDC5L, and BCLAF1.<sup>209</sup> SNIP1 recruits TET2 to C-MYC target genes and regulates C-MYC target gene expression. TET2-SNIP1-cMYC ternary complex regulates target gene expression, playing a crucial role in DNA damage response and cellular apoptosis.<sup>209</sup> REST recruits neuronal TET3 to mediate 5hmC formation and transcriptional activation.<sup>193</sup> TET-3s also interact with NSD2, NSD3, and SETD2 to regulate gene expression by mediating H3K36 trimethylation. In addition, TET3 interacts with TR to stabilize it and enhance its function independent of TET3 catalytic activity.<sup>220</sup> TET3 also interacts with histone variants H3F3A, regulating chromatin modification.<sup>193</sup>

**Functional subgroups of the partner proteins.**—Based on their functions, the partner proteins of TETs can be divided into four groups: TFs, histone modifiers, signaling molecules, and factors of the BER-DNA glycosylase pathway. Most TFs such as NANOG, RUNX1, PU.1, and PPAR $\gamma$  bind to regulatory regions of the target genes and recruit TETs to regulate target gene expression.<sup>204,216,217,221</sup> The binding motifs of ~66% TFs contain CpG dinucleotides. The binding of these TFs may be affected by CpG methylation.<sup>7</sup> Based on the binding affinity of methylated CpG (mCpG) motifs, TFs can be divided into 4 types: MethylPlus TFs (TF1, preferred to bind to mCpG), 5hmC DNA readers (TF2, preferred to bind to 5hmCpG), Methylminus TFs (TF3, preferred to bind to CpG) and methylation insensitive TFs (TF4, little affected by methylation)<sup>7,8</sup> (Figure 3). The TF1 (such as CEBPB, MBD1, MBD3, MeCP2, MBD3L2, GATA3, GATA5, WT1, PRDM14, Nanog, ZFP57/KAP1, OCT4, SOX2, HOXB13, KLF4, FOXA1, EBF1, EGR2) preferentially bind to 5mCpG motifs and function as pioneer factors to recruit TETs for converting 5mC into 5hmC.<sup>9,198,222–230</sup> The TF2 (such as MeCP2, MBD3/NURD complex, UHRF1, UHRF2, MPG, NEIL3, and SALL1/SALL4) preferentially bind to 5hmC DNA sequences to either recruit TET-BER-DNA glycosylase complexes for fully demethylating DNA or recruit co-activators for activation of gene expression,<sup>202</sup> while TF3 (such as AP-1, C-MYC/MAX, N-MYC, ETS-2, C-MYB, NF- $\kappa$ B, PAX5, RUNX1/2/3, NRF1, CTCF, CEBP $\alpha$ , CREB and PU.1) bind to CpG motifs to regulate gene expression by recruiting TET-histone modification complexes.<sup>231,232</sup> On the unmethylated DNA sequences, TETs might also play a role in maintaining the unmethylated state. Interestingly, the IDAX protein binds to unmethylated CpGs and inhibits TET2 binding to the demethylated regions through activation of caspase-mediated degradation, which might help to stop the demethylation process.<sup>194</sup> Thus, it is most likely that the TFs form a hierarchy, which sequentially binds to DNA sequences and cooperates with TET proteins and histone modifiers to regulate target gene expression. Consequently, cell-type-specific TFs mediate a cell-type-specific binding pattern of TET proteins (Figure 3). For example, in mouse ES cells, Tet1 uses its CXXC domain to bind to enhancers with 5mCpG islands and converts 5mC into 5hmC. Sall4a binds to 5hmC at enhancers and facilitates further oxidation of 5hmC at its binding site by recruiting Tet2.<sup>233</sup> MBD3/NURD binds to 5hmC and recruits TET1 to genomic sites to regulate the expression of 5-hmC-marked genes.<sup>152</sup> Such TET protein-associated sequential binding of TFs to DNA sequences is observed in almost all cellular processes by regulating the epigenetic landscape and inducing the expression of fate-determining genes.

Methylation serves as a barrier to reprogramming and differentiation.<sup>234,235</sup> During induced reprogramming of epiblast-like cells to PSCs, PRDM14 induces TET1/2-demethylation-mediated recruitment of OCT3/4 to the enhancers of pluripotent genes such as Klf2.<sup>236</sup> During the specification of PSCs to primordial germ cells, PRDM14, Nanog, and OCT4 are capable of binding to 5mCpG sites to initiate the stepwise epigenetic modification by recruiting TET1/2 proteins and other epigenetic modifiers.<sup>224</sup> During induced reprogramming of B cells or embryonic fibroblasts to generate PSCs, Tet2 is recruited by Klf4 and Tfcp2l1 respectively to drive active enhancer demethylation of chromatin and induce pluripotency-related genes.<sup>210</sup> Thus, most TF1s are fate-instructive pioneer factors that initiate the cellular processes such as lineage commitment and differentiation by establishing epigenetic configurations,<sup>237–241</sup> specifically when they collaborate with non-pioneer TFs.<sup>242–244</sup>

However, such mGpC binding affinity-based sub-classification of TFs is not always accurate because the binding affinity can be influenced by the surrounding sequence context. In addition, many TFs have more than one consensus-binding motif, while methylation only influences the binding of TFs to certain motifs. Thus, many TF3s can also function as pioneer factors, especially in collaboration with other TFs. For example, during the differentiation of fibroblasts to adipocytes, C/EBP $\alpha$  and CREB heterodimerize and bind half-CRE (CGTCA) and half-C/EBP (CGCAA) sequences of the tissue-specific methylated promoters to initiate DNA demethylation by recruiting TET2.<sup>245</sup> This allows the binding of other TF3s (such as CEBP $\alpha/\beta$ , c-Jun, JunD, ATF2 or PU.1) for transcriptional activation.<sup>7</sup> During differentiation of pro-B progenitors to pre-B progenitors, PU.1 and E2A bind to the 5mC enhancers of target genes and recruit TET2 and TET3 for stepwise DNA methylation. This is followed by the binding of other key B-cell-specific TFs to turn on the B-cell differentiation process.<sup>215</sup> During induced pre-B-cell-to-macrophage trans-differentiation, CEBP $\alpha$  collaborates with PU.1 to induce the myeloid cell fate by regulating two types of enhancers on myeloid genes, pre-existing ones, and *de novo* ones. The pre-existing enhancers are primed by PU.1, which maintains chromatin accessibility for the binding of CEBP $\alpha$ . In *de novo* promoters, CEBP $\alpha$  acts as a pioneer factor to initiate TET2-mediated de-methylation followed by PU.1 recruitment.<sup>210,246</sup> Therefore, the functional identification of pioneer factor(s) for different cellular processes is needed to elucidate transcriptional-epigenetic regulatory mechanisms for each cellular process.

Among the histone modifiers, most of them are negative transcriptional regulators such as the SIN3A complex, the NuRD complex, HDAC1, HDAC2, and EZH2, which mediate target gene repression,<sup>157,208,247</sup> while some others are positive transcriptional regulators, including CBP, hMOF, NSD2, NSD3 and SETD2 that promote gene expression via modulating H3K27<sup>ac</sup>, H4K16<sup>ac</sup> and H3K36<sup>Me</sup> on the promoters.<sup>248</sup> Such TET-related histone modification is independent of the catalytic activity of TETs. Furthermore, some of the partner proteins such as OGT, PARP1, and VprBP regulate the functions of TETs through post-transcriptional modifications (see the following section).

## 7. Post-translational regulation of TET proteins

The N-terminal sequence of the TET proteins plays a critical role in regulating TET activity by interacting with their catalytic domains. Mammalian TETs undergo a plethora of post-translational modifications (PTMs). However, the functional significance of some of these modifications is not yet well understood. Some of the well-known PTMs that are commonly found on TETs are GlcNAc, phosphorylation, ubiquitylation, acetylation, and proteolysis<sup>213,249–253</sup> (Figure 4).

**Phosphorylation regulates the activities of TET proteins.**—Mass spectrometric analysis identified over 10–20 residues that can be phosphorylated in each of the TET proteins.<sup>254</sup> However, the role of phosphorylation has only been functionally studied for a few of these residues. During DNA damage repair, ATM phosphorylates TET1 on S116, S262, and S546, regulating DNA repair.<sup>255</sup> The energy sensor, AMPK (AMP-activated protein kinase), phosphorylates human TET2 on Ser99 (murine Tet2, Ser97), protecting TET2 protein from calpain-mediated degradation. Thus, active AMPK promotes TET2

stability and facilitates its tumor-suppressive function.<sup>252,256,257</sup> Several members of the 14–3–3 group of adaptor proteins bind to Ser99 phosphorylated TET2 and protect it from phosphatase 2A (PP2A)-mediated dephosphorylation.<sup>258,259</sup> The association of 14–3–3 proteins is impaired in some leukemia-related TET2-mutants (around residue Ser99), explaining the reduced protein stability of these mutant TET2 proteins.<sup>258</sup> In diabetic mice, high glucose levels impede the tumor-suppressive activity of TET2 and accelerate tumor development by blocking AMPK-mediated phosphorylation of TET2 and reducing TET2 protein levels as demonstrated in xenograft tumor models.<sup>252</sup> This explains why diabetic patients have an increased risk for cancer and cancer patients with diabetes have a poor prognosis as observed by epidemiological studies.<sup>260,261</sup> The anti-diabetic drug metformin and other AMPK activators such as A769662 display antitumor activity by activating AMPK-mediated phosphorylation of TET2 Ser99 and increasing 5hmC levels. Diabetes risk reduction diets improve the survival of cancer patients.<sup>262</sup> In addition, in erythroid progenitor cells, hematopoietic cytokines such as erythropoietin (EPO) stimulate JAK2-mediated phosphorylation of TET2 on Tyr1939 and Tyr1964 residues, which enhances TET2 binding of the TF KLF1 and increases TET2 activity for the proliferation and differentiation of erythroid progenitor cells.<sup>263</sup> Consistently, in primary samples from patients with myeloproliferative neoplasms, JAK2<sup>V617F</sup> increases TET2 activity and 5-hmC with genome-wide loss of cytosine methylation, leading to increased expression of several oncogenic transcripts, such as *MEIS1* and *HOXA9*.<sup>263</sup> In hepatocellular carcinoma patients, FGFR3<sup>7–9</sup>, a splicing mutant of FGFR3, directly interacts with TET2 and phosphorylates TET2 on its Y1902 site, leading to the ubiquitination and proteasome-mediated degradation of TET2.<sup>264</sup> Such phosphorylation-related downregulation of TET2 enhances cancer cell proliferation through repression of PTEN and upregulation of AKT signaling. Interestingly, in CML cell lines, the BCR-ABL fusion protein interacts with TET2 and sequesters the latter by cytoplasmic compartmentalization in a complex tethered by FOXO3a.<sup>265</sup> Imatinib treatment releases TET2 from the complex and imports TET2 into the nucleus together with FOXO3a to activate *BIM* expression by binding to the *BIM* promoter.<sup>265</sup> Whether TET2 is phosphorylated by BCR-ABL kinase needs to be determined.

During neuronal differentiation, CDK5 phosphorylates TET3 on residues Ser1310 and Ser1379 (Ser1318 and Ser1387 for the mouse) within its catalytic domain, changing its dioxygenase activity.<sup>253</sup> Phosphorylated TET3 promotes the expression of the neuron-specific TF BRN2, as well as neuronal differentiation, through enhancing the enrichment of 5hmC and H2A.Z occupancy at the promoter of the *BRN2* gene. Non-phosphorylated TET3 promotes the expression of genes that are linked to metabolic processes.<sup>253</sup> In response to DNA damage, ataxia-telangiectasia and Rad3-related kinase (ATR) phosphorylates TET3 on residues Ser42, 637, and 1426. TET3 phosphorylated in this way mediates DNA oxidation which promotes the ATR-dependent DNA damage response.<sup>266</sup>

**GlcNAc regulates the activity of TET proteins.**—All three TETs interact with OGT.<sup>186–188</sup> OGT regulates their stability and their activities by catalyzing GlcNAc and thereby regulating the phosphorylation of the proteins at their N-termini and low-complexity insert regions.<sup>254,267,268</sup> OGT is also involved in the regulation of the binding of TETs to some genomic sites.<sup>269</sup> At least 8 GlcNAc sites have been reported for TET1 and up

to 20 such have been identified for both TET2 and TET3.<sup>186,254,267,269</sup> Many of these GlcNAc sites, such as Ser97 and Ser374 of TET2, Ser362 and Ser557 of TET3, and Ser950 and Ser2016 of TET1, could also be phosphorylated. Thus, GlcNAc represses the phosphorylation of the corresponding sites and regulates the binding of TETs with other partners.<sup>254</sup> In addition, the GlcNAc site Thr535 on TET1 enhances this protein's stability,<sup>269</sup> while GlcNAc of TET3 promotes its cytoplasmic relocation,<sup>268</sup> and GlcNAc of TET2 reduces its enzymatic activity by enhancing its nuclear export.<sup>188</sup> Furthermore, OGT regulates the expression of TET target genes by GlcNAc and several other epigenetic modifiers and histones (see the following section).

**Ubiquitination regulates the activities of the TET proteins.**—VprBP binds the cysteine-rich, dioxygenase domain of all three proteins, exerting a critical regulatory function on TET dioxygenases in normal tissue development and tumor suppression. VprBP induces CRL4VprBP (VprBP-DDB1-CUL4-ROC1) E3 ubiquitin ligase-mediated monoubiquitylation of TET1 on Lys1589 (Lys1537 in the mouse), of TET2 on Lys1299 (Lys1212 in the mouse), and of TET3 on Lys994 (Lys983 in the mouse). Such monoubiquitylation facilitates TET binding to chromatin and enhances 5hmC in corresponding genomic regions.<sup>213</sup> *TET2* mutations in leukemic cells on either Lys1299 or residues essential for VprBP binding result in reduced chromatin binding and activity of TET2.<sup>213</sup> In addition, mutation of Lys983 of TET3, but of neither TET1 nor TET2, also alters the enzyme's subcellular localization from almost exclusively nuclear to mostly cytoplasmic. Whether monoubiquitylation selectively regulates the subcellular localization of TET3 needs to be determined. Interestingly, during HIV infection, the viral protein Vpr induces CRL4VprBP-mediated poly-ubiquitination of TET2 Lys1299, inducing the degradation of TET2 to sustain IL-6 expression and enhance HIV-1 replication.<sup>213,270</sup>

**Acetylation regulates the activity of TET proteins.**—During oxidative stress, transcriptional co-activator p300 acetylates TET2 on Lys110/111 residues to enhance the enzymatic activity of TET2 and to protect the protein against proteasomal degradation by the inhibition of TET2 ubiquitination on certain residues in the C-terminal DSBH domain.<sup>211</sup> TET2 acetylation enhances DNMT1 binding to promote protein stability. Consequently, TET2, along with TDG, is recruited to chromatin by DNMT1 to prevent abnormal DNA methylation. TET2 Lys110/111 deacetylation is mediated by HDAC1/2.<sup>208</sup> TET1 and TET3 are also acetylated by p300; however, the detailed sites for such modifications have not been determined.<sup>211</sup> In MDS, SIRT1 interacts with the TET2 C-terminal domain (a.a. CD1129–2002) and deacetylates it on Lys1472, 1473, and 1478 in CD34<sup>+</sup> HSPCs, regulating the stability and function of TET2 protein. *SIRT1*-deficient MDS HSPCs exhibit enhanced cell growth and self-renewal due to the reduction of TET2 levels.<sup>271</sup> The SIRT1 activator SRT1720 inhibits colony formation in MDS HSPCs and *in vivo* engraftment in NSGS mice by enhancing the tumor repressive activity of TET2.

**Other post-translational modifications of TET proteins.**—The stability of TET proteins is regulated by calpains.<sup>272</sup> TET1 and TET2 are degraded by calpain 1 in mouse ES cells; whereas TET3 is degraded by calpain 2 during ES cell differentiation.<sup>272</sup> TET1 interacts with PARP1/ARTD1 and is targeted by both noncovalent and covalent PARylation

in TET1's catalytic domain. The noncovalent binding of ADP-ribose polymers decreases TET1's hydroxylase activity, while covalent PARylation stabilizes the TET1 enzyme and enhances its activity.<sup>273–275</sup> In addition, PARP1 also promotes *TET1* gene expression by regulating DNA and histone modifications on the TET1 promoter.<sup>274</sup>

**Metabolic regulation of TET protein activity.**—Fe<sup>2+</sup> and  $\alpha$ -KG, together with O<sup>-2</sup> and vitamin C, function as TET co-factors and are required for their dioxygenase activity.<sup>276–279</sup> Both Fe<sup>2+</sup> and  $\alpha$ -KG bind to the catalytic domain of TETs facilitating the insertion of 5mC into their catalytic pocket and providing accommodation to the oxidized derivatives of 5mC including 5hmC, 5fC, and 5caC.<sup>180,280,281</sup> Thus, the dioxygenase activity of TET proteins is dependent on the availability of  $\alpha$ -KG, Fe<sup>2+</sup>, and O<sub>2</sub>.  $\alpha$ -KG is a product of IDHs, a family of metabolic enzymes. The IDHs, IDH1, IDH2, and IDH3, catalyze the oxidative decarboxylation of isocitrate to  $\alpha$ -KG, which is an essential step in the tricarboxylic acid cycle. IDH1/2 mutations are commonly detected in gliomas and hematopoietic malignancies which lead to the production of the oncometabolite 2-hydroxyglutarate (2HG). Mutations in other genes encoding for the metabolic enzymes succinate dehydrogenase (SDH) and fumarate hydratase (FH) are prevalent in gliomas, cholangiocarcinomas, renal cell carcinomas, and acute myeloid leukemias, among others. SDH and FH mutations lead to the production of the oncometabolite succinate and fumarate, respectively.<sup>282–284</sup> An overabundance of these oncometabolites influences the catalytic activities of TET1/2/3 by competitive inhibition of their  $\alpha$ -KG binding site. In activated macrophages, itaconic acid, a metabolic product of the IRG1 enzyme, also inhibits the catalytic activity of TET2 via inhibition of TET2/ $\alpha$ -KG binding.<sup>285</sup> Thus TET-mediated DNA demethylation is tightly regulated by glucose metabolism. Reactive oxygen species (ROS) and metal chelators can impede TETs' oxidizing activities by reducing the Fe<sup>2+</sup> availability.<sup>286</sup> Vitamin C converts inactive Fe<sup>3+</sup> to active Fe<sup>2+</sup> which promotes TET1/2/3 enzymatic activity.<sup>287</sup> Thus, in addition to its antioxidant properties, Vitamin C regulates gene expression and genomic stability by increasing TET-mediated 5hmC formation and promoting DNA demethylation.<sup>277,288–290</sup>

## 8. TETs regulate gene expression by both enzyme-dependent and -independent mechanisms.

TETs play critical roles in organ generation during embryonic development and tissue regeneration during postnatal life.<sup>162,176</sup> TETs play such roles by regulating the timed expression of the key genes that determine cell identity and control cell differentiation. TETs and lineage-specific TFs cooperate to influence chromatin accessibility and regulate gene expression by 1) promoting site-specific DNA demethylation (mainly enhancers and CGI-rich promoter elements), and enzymatic-dependent activity;<sup>16,58,157,173,176,280</sup> and 2) regulating histone modifications via an enzymatic-independent activity by forming chromatin regulatory complexes with OGT, HDACs and/or histone acetyltransferases (HATs)<sup>188,208,291,292</sup> Figure 5.

**TETs regulate gene expression by enzymatic activity-dependent site-specific DNA demethylation.**—TET proteins collaborate with lineage-specifying TFs in cells, promoting the expression of cell type-specific genes by demethylation of enhancers of

target genes. ChIP-seq assays demonstrated that the top enriched binding motifs of TET proteins in DNA are enriched with binding sites for lineage-specifying TFs of the respective cell types, maintaining 5hmC and demethylation state in the active enhancers of target genes in an enzyme-dependent fashion.<sup>12,30,176,223,293,294</sup> For example, in ES cells, TET1/2 together with master self-renewal TFs including SOX2, KLF4, ESRRG, POU5F1, and NANOG bind enhancers of the target genes that are essential for the maintenance of self-renewal.<sup>14</sup> In myeloid cells, TET2, together with key myeloid differentiation TFs such as ERG, RUNX1, CEBPA, and GATA1, bind enhancers of genes that are necessary for myeloid lineage commitment and differentiation.<sup>14</sup> Loss of TET2 causes down-regulation of cell-type-specific genes due to the widespread reduction of 5hmC and increased methylation of their enhancers, altering cell fate.<sup>14,295</sup>

In many types of cancers, TET proteins function as tumor suppressors by activating the expression of tumor repressive genes. For example, in gastric cancer, TET1 inhibits the AKT and FAK signaling pathways by demethylation of the PTEN gene promoter.<sup>68</sup> In colon cancer, TETs suppress the proliferation of cancer cells by demethylating DKK gene promoters, inhibiting the Wnt signaling cascade.<sup>296</sup> In pancreatic cancer, TET1 restricts the cell cycle of cancer cells by upregulating negative cell cycle regulators such as p16.<sup>69</sup> In these types of cancers, restoration of the enzymatic activity of TETs is a potent treatment strategy. However, in some other types of cancers, TET proteins function as putative oncoproteins and promote stemness in the cancer stem-like subpopulation that drives aggressiveness and chemoresistance. For example, in ovarian cancers, TET1 induces the expression of cancer stem cell genes, which reprograms epithelial cancer cells into a cancer stem-like state.<sup>82</sup> In gliomas, TET1 and TET3 promote stemness and self-renewal of tumor cells by regulating the expression of core stem cell genes.<sup>297,298</sup> In breast cancers, TET1 and TET3 cooperatively induce cancer stem-like cells by activating the TNF $\alpha$ -p38-MAPK signaling axis.<sup>299</sup> In such types of cancers, high levels of TET proteins promote a subpopulation of the slow-growing chemoresistant stem-like cells that are associated with disease relapse and poor prognosis.<sup>300</sup> In addition, TET proteins also regulate EMT and cancer metastasis in a context-dependent manner.<sup>98,119,299,301</sup> Thus, targeting TET enzymes for cancer therapy should be also strategized for context-dependence.

**TETs regulate gene expression via enzymatic activity-independent histone modifications.**—Through their enzymatic-independent activities, TET proteins primarily repress gene expression. For example, TET proteins recruit OGT to histones at the promoters of target genes and regulate target gene expression by mediating OGT-dependent GlcNAc of TFs, epigenetic regulators, and histones.<sup>89,90,302</sup> GlcNAc of H2B on Ser112 is required for subsequent Lys120 monoubiquitination and PRC1-mediated gene silencing.<sup>303</sup> GlcNAc of RING1B on Ser278 and Thr250/Ser251 residues promotes the binding of RING1B to CBX7 and RYBP to form PRC1, resulting in H2B<sup>K119</sup> ubiquitination and silencing of a specific subset of genes.<sup>304</sup> In the PRC2 complex, EZH2 is modified by GlcNAc on Ser75, which results in its being stabilized, thus negatively regulating tumor suppressor genes.<sup>305</sup> In addition, TET proteins recruit Sin3A/HDAC1/2 to target genes, repressing target gene expression by deacetylation of histone H3K27.<sup>206,208</sup> However, TET proteins also promote some target gene expression via enzymatic-independent activity. For



example, via OGT-mediated GlcNAc of HCF1 (Tyr17, 19, 21, and 22), the key component of H3K4 methyltransferase SET1/COMPASS complexes, TET proteins promote chromatin binding and H3K4<sup>me3</sup>, inducing target gene expression.<sup>186–188,267,269,306,307</sup> TET1 also upregulates the expression of proliferation and DNA damage repair genes by recruiting the HAT protein MOF to promoters, acetylating H4K16.<sup>248</sup> TET3 promotes transcriptional activation in neurons by recruiting NSD2, NSD3, and SETD2, thus mediating H4K36 tri-methylation.

## Prospective

The activity of TET proteins shapes the local chromatin environment at enhancers and promoters to facilitate the binding of TFs and affect gene expression patterns.<sup>14</sup> Mutations or dysregulation of TET proteins lead to abnormal DNA methylation patterns and epigenetic chromatin modifications, driving disease development. Although TET1 and TET3 have their DNA binding CXXC-domains which mediate a region-specific DNA binding and demethylation, TET2 and the short forms of TET1 and TET3, rely on interactions with TFs for DNA binding. Thus, the regions of DNA binding of these TETs must conform to cell-type specificity, which is controlled by cell-type-specific TFs.

Based on their binding affinity to methylated DNA, TFs form a hierarchy in DNA binding, chromatin modification, and gene regulation. First, the methylation-insensitive pioneer TFs bind to methylated DNA to initiate DNA demethylation by recruiting TET proteins to convert 5mC to 5hmC.<sup>308</sup> Next, the secondary level of TFs (5hmC readers) bind to 5hmC-DNA to activate gene expression by recruiting co-activators or to further complete DNA demethylation by recruiting a TET-TDG-BER complex. Finally, the third level TFs (methylation-sensitive) occupy the unmethylated DNA to control gene expression by recruiting co-activators or co-repressors. Such sequential and cooperative binding of TFs and TETs leads to a relatively large open region of chromatin, which forms a super-enhancer in the target genes to determine the fate of cells.<sup>232</sup> Many TFs can bind TET proteins. However, the hierarchy of these TFs has not yet been well characterized. Thus the manner in which these TFs cooperate with TET proteins in the regulation of target gene expression needs to be better elucidated in the future.

Both positive and negative regulatory effects of TET proteins on target gene expression have been reported. TET proteins activate the expression of target genes primarily by enzymatic activity mediated DNA demethylation and/or by OGT-regulated SET1/COMPAS-mediated H3K4 trimethylation, while they repress the expression of target genes by recruiting SIN3A/HDAC1/2 or OGT-regulated PRC1. It is still unknown how such positive and negative regulatory mechanisms are coordinated in regulating target gene expression. Furthermore, the activity of TET proteins is regulated by many types of posttranslational modifications. Detailed study of the molecular mechanisms by which the activities of TET proteins are regulated and how TET proteins selectively regulate target gene expression will provide useful information for designing medications for a new generation of TET-related disease treatment.

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## Abbreviations used

<b>TET</b>	Ten-eleven translocation
<b><math>\alpha</math>-KG</b>	$\alpha$ -ketoglutarate
<b>5mC</b>	5-methylcytosine
<b>5hmC</b>	5-hydroxymethylcytosine
<b>5fC</b>	5-formylcytosine
<b>5caC</b>	5-carboxylcytosine
<b>TFs</b>	Transcription factors
<b>ARCH</b>	Age-related clonal hematopoiesis
<b>MDS</b>	Myelodysplastic syndromes
<b>AML</b>	Acute myeloid leukemia
<b>ALL</b>	Acute lymphoblastic leukemia
<b>DLBCLs</b>	Diffuse large B cell lymphomas
<b>PTCL</b>	Peripheral T-cell lymphoma
<b>IFN-<math>\gamma</math></b>	Interferon- $\gamma$
<b>TNF-<math>\alpha</math></b>	Tumor necrosis factor- $\alpha$
<b>TIS</b>	Transcriptional initiation sites
<b>DSBH</b>	Double-stranded beta-helix domain
<b>TDG</b>	Thymine DNA glycosylase
<b>BER</b>	Base excision repair
<b>ES</b>	Embryonic stem cells
<b>IDHs</b>	Isocitrate dehydrogenases
<b>AP site</b>	Apyrimidinic site
<b>AID</b>	Activation-induced cytidine deaminase
<b>5hmU</b>	5-hydroxymethyluracil
<b>GC</b>	Germinal center

<b>CSR</b>	Class-switch recombination
<b>DSBs</b>	Double-strand breaks
<b>PGCs</b>	Primordial germ cells
<b>CGIs</b>	CpG islands
<b>OGT</b>	O-linked GlcNAc transferase
<b>SID</b>	Sin3A interacts with the Sin3-interaction domain
<b>CoA</b>	Coactivators
<b>PTM</b>	Post-translational modifications
<b>AMPK</b>	AMP-activated protein kinase
<b>C/EBP<math>\alpha</math></b>	CCAAT/enhancer-binding protein alpha
<b>KLF4</b>	Kruppel-like factor-4
<b>TFCP211</b>	Transcription factor CP2 like-1
<b>YBX1</b>	Y box-binding protein-1
<b>FOXK2</b>	Forkhead box protein K-2
<b>KZF1</b>	DNA-binding protein Ikaros 1
<b>NFIL3</b>	Nuclear factor interleukin-3-regulated protein
<b>ATRX</b>	Alpha-thalassemia/mental retardation syndrome X-linked transcriptional regulator
<b>CUX1</b>	Homeobox protein cut-like-1
<b>YY2</b>	Yin and yang-2 transcription factor
<b>I<math>\kappa</math>B<math>\zeta</math></b>	Inhibitory-kappa-B-zeta

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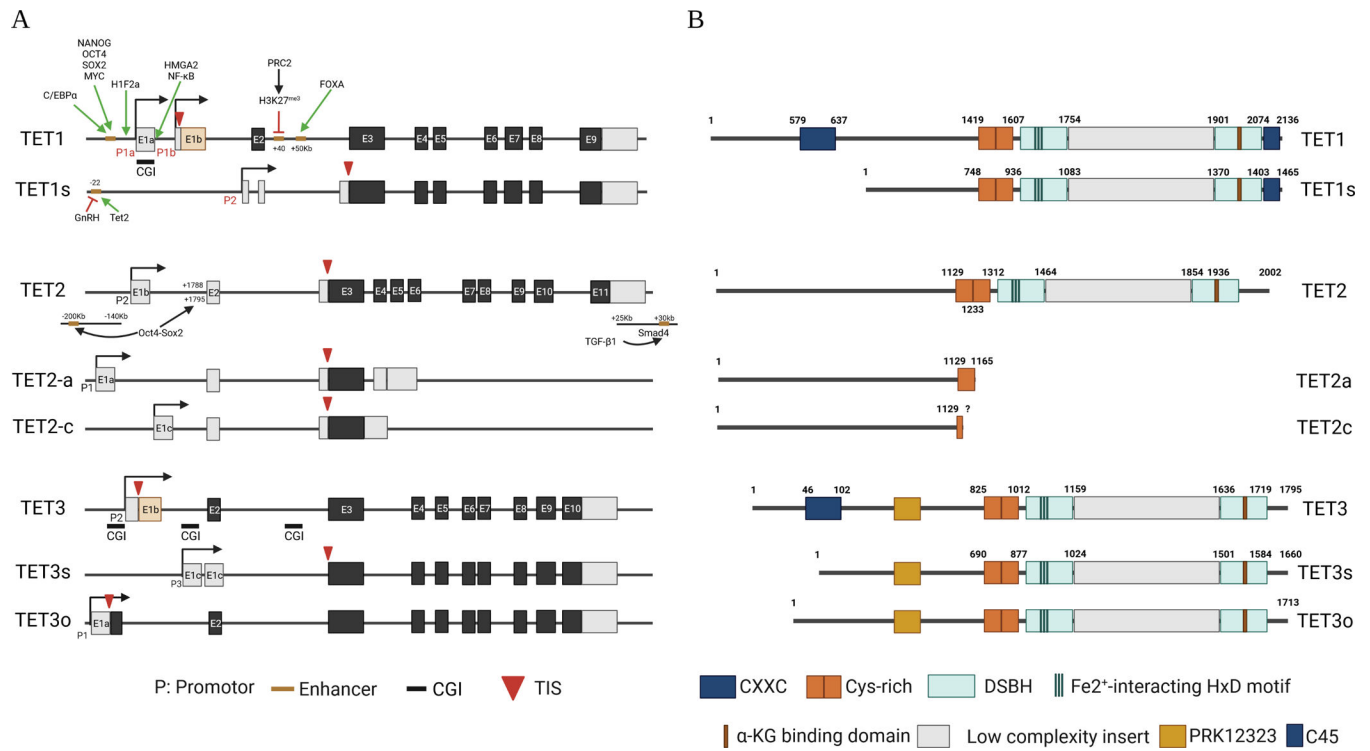
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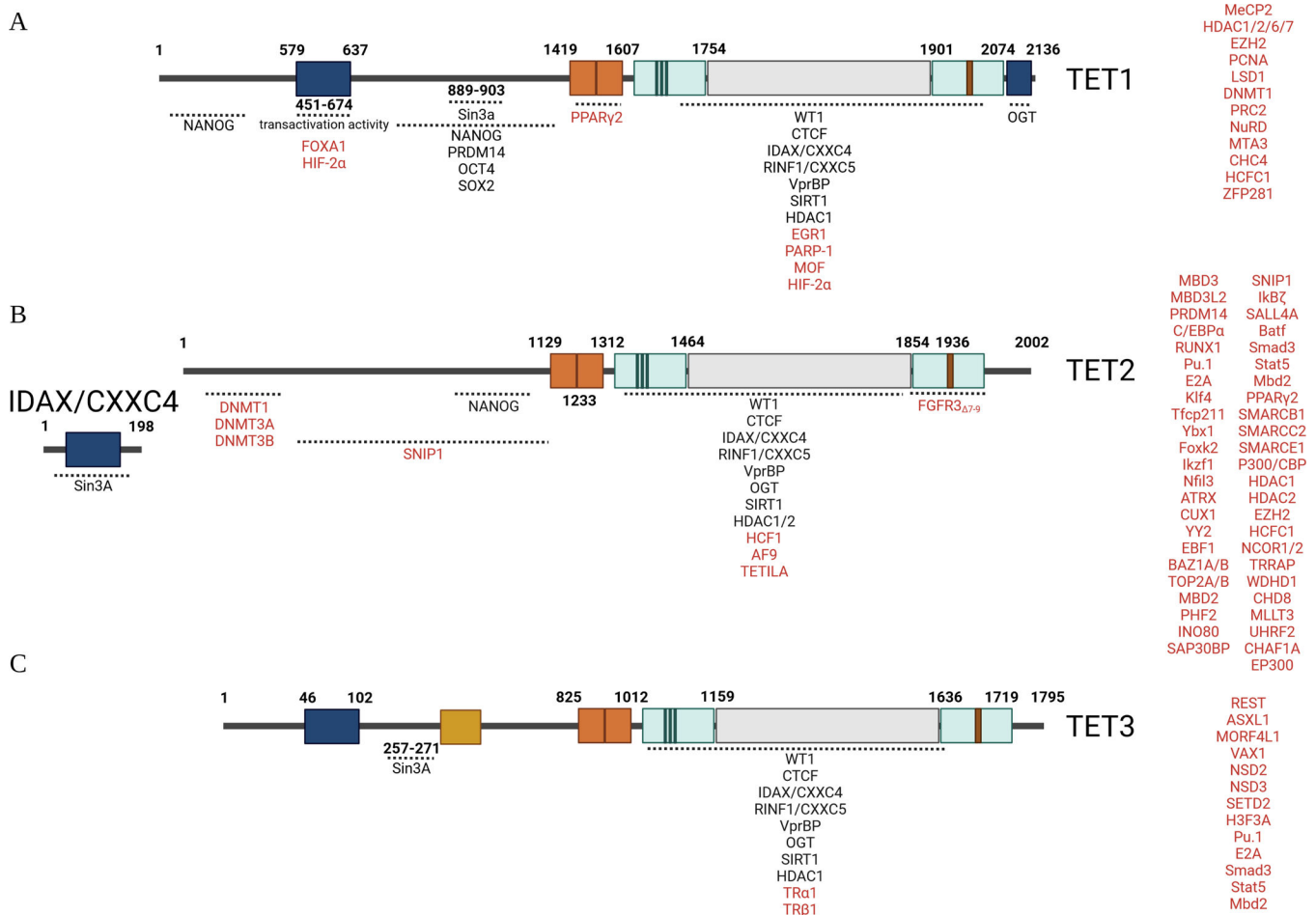
**Key points:**

1. TET proteins play a key role in regulating both normal and disease tissue regeneration;
2. TET proteins regulate the dynamic differentiation and fate determination of tissue stem and progenitor cells;
3. TET proteins regulate target gene expression by demethylation of enhancers/promoters and modification of histones of the target genes;
4. The function of TET proteins is regulated by interacting partners and posttranslational modifications.



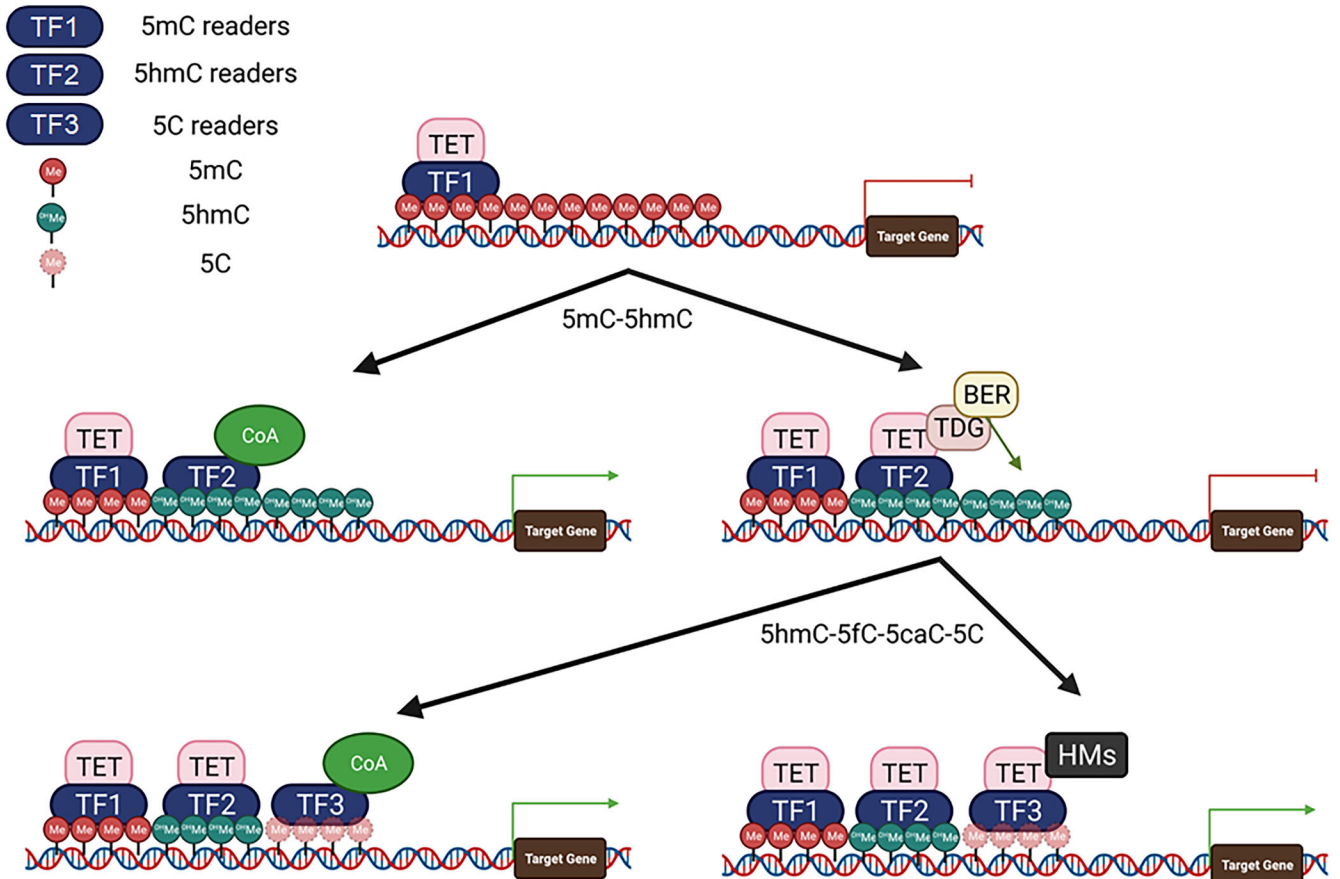
**Figure 1. TET genes and TET proteins.**

**A.** *TET1*, *TET2*, and *TET3* have 2, 3, and 3 transcriptional products respectively due to the alternative use of promoters, which are regulated by the alternative activation of enhancers. The green arrow indicates induction of expression; the red cross depicts inhibition of expression. **B.** The corresponding protein isoforms of *TET1*, *TET2*, and *TET3*. The structural domains of the proteins are indicated.



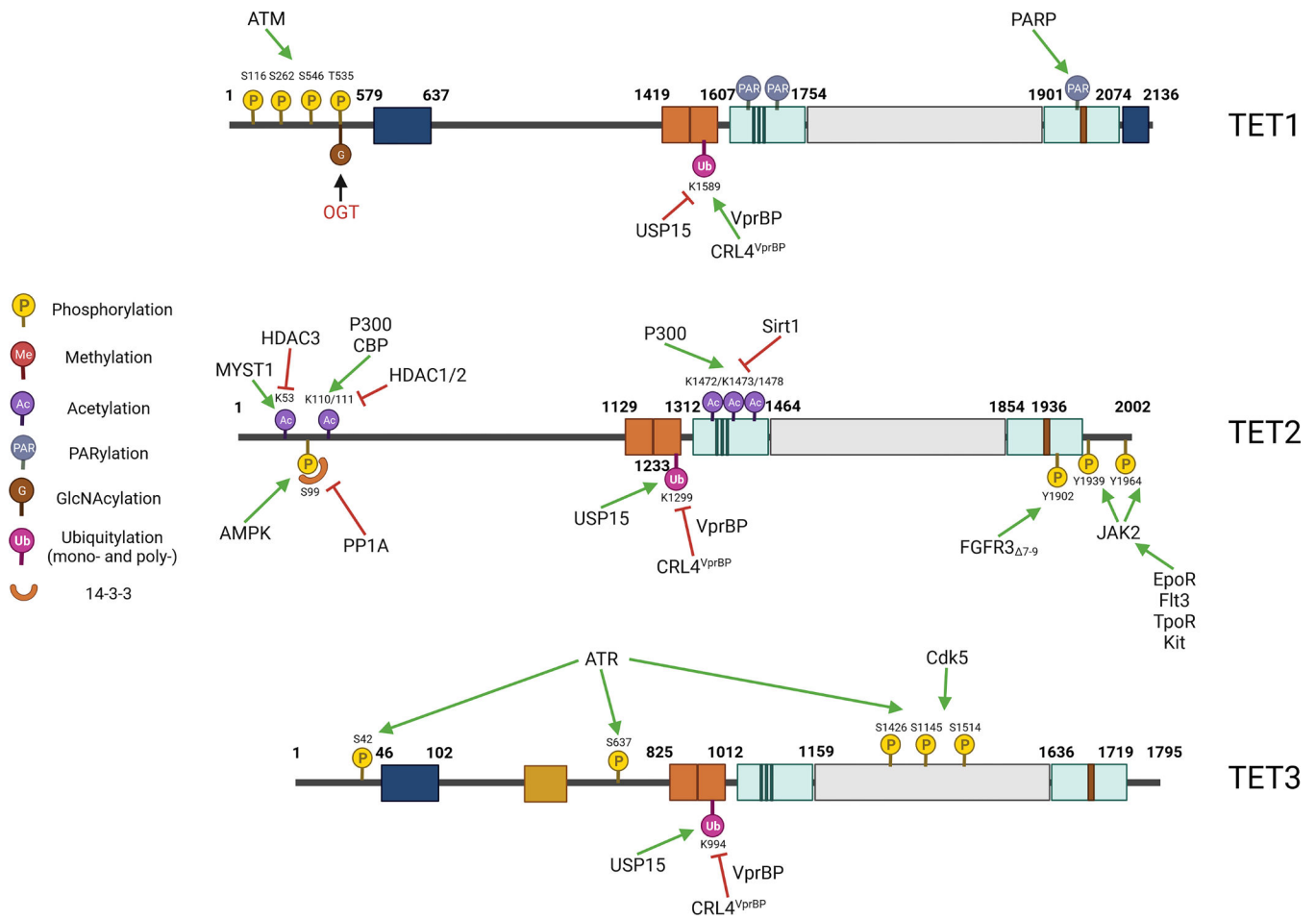
**Figure 2. Interaction partner proteins of TET proteins.**

The interacting partner proteins of TET1, TET2, and TET3 are listed in **a**, **b**, and **c**, respectively. The partner proteins for which the interaction regions have been identified are listed under each TET at the corresponding regions. The partner proteins for which the interaction regions have not yet been defined are listed on the right side of each TET. The partners that are shared by all three TETs are listed in black font, while the partners that are specific for one or 2 TETs are listed in red font.



**Figure 3. Subgroups of TET-interacting TFs.**

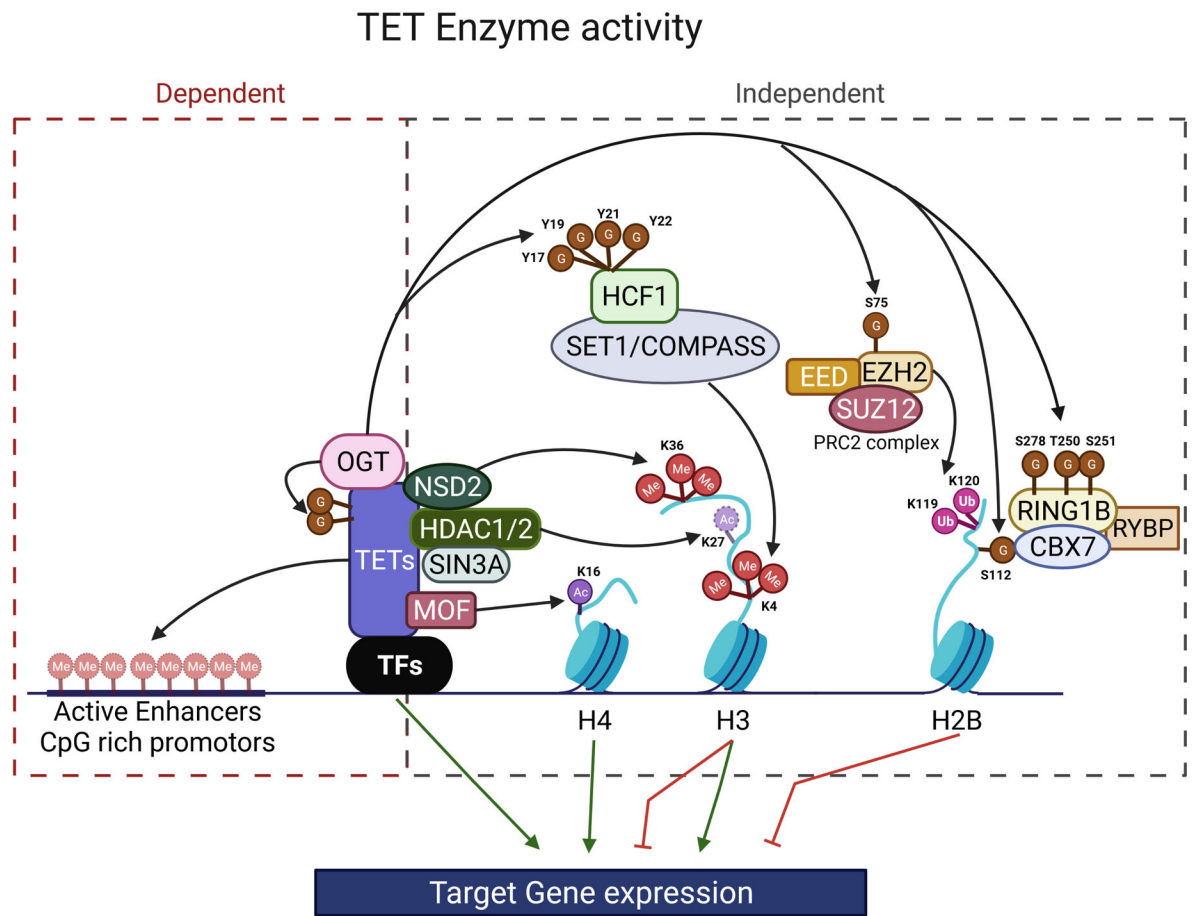
The TET-interacting TFs can be divided into TF1, TF2, and TF3 based on their binding affinity for 5mC, 5hmC, and 5C, respectively. TF1 can bind 5mC DNA and recruits TET to initiate the first step of DNA demethylation by converting 5mC to 5hmC. TF2 can bind to 5hmC promoters/enhancers to turn on gene expression by recruiting coactivators (CoA) or to further complete the DNA demethylation elements by recruiting TET-TDG-BER complexes. TF3 binds 5C promoters/enhancers to promote gene expression by recruiting CoA or regulating gene expression by recruiting TET-histone modifiers (HMs).



**Figure 4. Post-translational modifications of TET proteins.**

The post-translational modifications of TET1, TET2, and TET3 are listed in a, b, and c, respectively. The green arrow depicts the addition of modifications. The Red Cross indicates removal modifications.





**Figure 5. TETs regulate gene expression by both enzyme-dependent and -independent mechanisms.**

After binding to DNA regulator regions, TETs regulate target gene expression by 1) enzymatic demethylation of 5mC; and 2) recruiting histone modifiers, including OGT, Sin3A/HDACs, or HATs.