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TET proteins in natural and induced differentiation

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Abstract

The ten-eleven-translocation (TET) proteins oxidize 5-methylcytosine in DNA. Alterations in TET protein function have been linked to cancer, but TETs have also been observed to influence many cell differentiation processes. Here we review recent work assessing the contribution of TET proteins to natural and induced differentiation. Altogether these analyses have helped characterize how TETs and their enzymatic products influence DNA methylation patterns, regulatory element activity, DNA binding protein specificity and gene expression.

Introduction

Methylation of cytosines in eukaryotic DNA is a widely studied epigenetic modification with important roles in genomic stability, X-chromosome inactivation, chromatin accessibility, nucleosome positioning, and gene expression [1]. DNA methylation is controlled by the enzymes of the DNA methyltransferase family (DNMT) and the distribution of 5-methylcytosine (5mC) in the genome varies between lineages during development and in cancer [2,3]. A new mechanism to actively influence DNA methylation patterns was identified by the discovery of the Ten-eleven-translocation (TET) proteins [4,5].

The TET proteins were identified as encoding a family of Fe(II) and 2-oxoglutarate (2OG)-dependent dioxygenases that could sequentially oxidize 5mC to oxidized methylcytosines (oxi-mC) including 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxycytosine (5caC), thus providing a potential mechanism to directly alter DNA methylation ([5,6], and reviewed in [7]). At the same time the function of TET proteins was identified, high levels of their enzymatic product, 5hmC, was found in embryonic stem (ES) cells [5] and Purkinje neurons [8], accounting for ~10% and ~40% of 5mC, respectively. 5fC and 5caC modifications in DNA have been observed to be targeted by thymine DNA glycosylase for active removal and replacement with unmodified bases, but oxi-mC may also alter maintenance DNA methylation and passive demethylation after replication [7].

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Conflict of interest statement

Nothing declared.

Despite altered levels of 5hmC in the absence of TET proteins, *Tet*-deficient ES cells maintained self-renewing potential with some altered differentiation phenotypes (reviewed in [7,9,10]). Subsequently, several studies have demonstrated that TET proteins influence a variety of cell differentiation processes, providing new insights into the function of TET proteins in gene expression, transcription, and enhancer activity. Here we review recent work describing the functions of TET proteins and their effects on natural and induced differentiation.

TET proteins during natural differentiation

Loss of *Tet* gene expression or altered TET enzymatic activity is associated with many human cancers [3]. In particular, mutations altering the enzymatic activity of TET2 have been associated with myelodysplastic syndromes [11] and characterized by the expansion of immature myeloid cells. Studies characterizing *Tet2*-deficient mouse strains recapitulated these findings and found impaired differentiation, altered self-renewal, and expansion of bone marrow progenitor cells [12,13]. A consistent myeloid differentiation bias was apparent among hematopoietic precursors which could progress to myeloid leukemia-like disease, and was associated with altered DNA methylation at distal regulatory elements [12–14].

Lineage-specific deletion of *Tet* genes in lymphoid cells also resulted in more specific effects on their differentiation, that were frequently associated with malignant transformation. After deletion of *Tet2* and *Tet3* genes broadly in all T cells, a subset of innate-like and glycolipid-CD1d specific T cells called NKT cells displayed an altered differentiation pattern and were expanded and could transfer lymphoma-like disease [15•]. NKT subset specific transcription factor expression was altered in these mice and the population of NKT cells was biased to a normally rare ROR γ t-dependent subset called NKT17 [15•]. This phenotype was associated with reduced chromatin accessibility at distal regulatory elements that were normally enriched for 5hmC in wild-type NKT cells [15•].

Deletion of *Tet2* and *Tet3* in the B lineage resulted in a developmental block that was associated with impaired rearrangement of gene segments encoding the κ light chain of the B cell antigen receptor [16•,17•]. This defect was mapped to distal enhancers which were hypermethylated in the absence of TETs, and associated with low levels of germline transcription [16•], which is required to enable rearrangement [18]. Chromatin accessibility at an array of distal regulatory elements was reduced in the absence of TETs, with an associated increase in DNA methylation [16•]. TET1 was also identified as a suppressor of lymphomagenesis from the B cell lineage [19,20], similar to observations after loss of *Tet2* and *Tet3* in myeloid and T lineages [14,15•].

The enrichment of oxi-mC at enhancers has been frequently observed (reviewed in [7,10]) and analysis of TET-deficient cells has revealed TET-dependent developmentally related enhancers. In ES cells, *Tet* deficiency resulted in loss of 5hmC at enhancers, a process accompanied by increased DNA methylation, reduced acetylation of Histone 3 lysine 27 (H3K27ac) and impaired gene expression during differentiation [21]. The distribution of 5hmC within enhancers is somewhat variable, having been reported to occur either at or

adjacent to the center of the enhancer [21,22]. The presence of oxi-mC adjacent to enhancers may alter regulatory element activity, since the presence of oxi-mC can alter the stability and flexibility of DNA [23–25] and stabilize the interactions with nucleosomes [25]. High-resolution analyses of modified bases within specific regulatory elements near lineage-defining transcription factors has provided additional information about progressive changes during induction of these genes. Absence or alteration of TET protein function was observed to impair the maintenance of regulatory T cells (Treg), a subset of T cells with immunomodulatory functions [26], by controlling the expression of the lineage-defining transcription factors *Foxp3* [27,28••]. Stable expression of *Foxp3* is critically dependent on the conserved intronic regulatory element called CNS2 [26]. Yue *et al.* observed progressive TET-dependent demethylation of 11 CpGs within CNS2 [28••]. This was associated with transient increases in 5hmC at individual CpGs in intermediate cell types along the differentiation pathway. Most importantly, TET-mediated epigenetic changes at CNS2 were important for stable *Foxp3* expression, which is critical to maintain the Treg lineage.

The modification of DNA bases may be a mechanism to influence transcription factor binding, thereby controlling regulatory element activity. Some transcription factors interact poorly with methylated DNA [29] and thus may not bind to an inactive regulatory element (Figure 1). Pioneer transcription factors that are not sensitive to DNA modifications or nucleosome occupancy may be able to bind to these sites and recruit TET proteins to generate oxi-mC (Figure 1), with subsequent effects on other transcription factor binding such as basic helix-loop-helix (bHLH) family members binding E-box sites. Some E-box motifs contain a CpG and hemi-modified sequences containing C on one strand and 5caC on the other augmented the TF/DNA interaction [30]. At CpG containing sites, DNA containing symmetrical 5mC or 5hmC modifications impaired binding, while hemi-modified sequences were permitted [30,31]. In this scenario, a single base modification followed by DNA replication could allow binding at this site in both daughter cells. 5caC has been observed to augment the interaction between basic helix-loop-helix (bHLH) family members and E-box sites. In other cases, the presence of 5caC at positions just flanking an E-box motif without a CpG substantially increased affinity of bHLH family members for this sequence [32,33], perhaps by altering DNA shape [34]. In other cases, modification of bases within the recognition sequence could promote binding or alter binding preference compared to unmodified DNA. Members of the basic region-leucine zipper (bZIP) family can bind to CpG-containing sequences and, in the case of, C/EBP α , 5mC and oxi-mC promoted interaction with non-canonical sequences [35,36]. In this model (Figure 1), TET activity with stable and/or transient oxi-mC control transcription factor binding, nucleosome positioning or accessibility, and enhancer activity.

Other than at enhancers, oxi-mC is consistently found enriched over the gene bodies of transcribed genes (reviewed in [7,10]), where it may promote transcriptional consistency. Single cell RNA-seq experiments found high variability of gene expression among individual cells in 8-cell embryos of TET-deficient compared to wild-type mice [37]. Similarly, Treg cells with impaired TET function show increased variability of *Foxp3* expression compared to wild-type Treg cells [28••]. 5hmC has also been found to be enriched at promoters, with differential contributions between family members (reviewed in [7,10]). The contributions of TETs to transcription may be linked to recruitment of TET-

interacting proteins such O-linked β -D-N-acetylglucosamine transferase (OGT) [38,39] to promoters, which may influence transcription as OGT activity on histone H2B and components of the COMPASS H3K4 methyltransferase complex are positively associated with transcription.

These influences of TETs may also be directly related to RNA polymerization, as the structure of RNA polymerase II bound to DNA containing oxi-mC revealed direct contacts between the modified base and the DNA recognition loop of the enzyme which was linked to impaired PolII elongation *in vivo* [40]. Additionally, methylated and hydroxymethylated cytosines have been associated with variable splicing by altering CTCF binding and controlling the rate of RNA polymerization. In the *Ptprc* locus, methylation of an intragenic CTCF site altered the rate of RNA polymerization and promoted exclusion of exon 5 [41], while the presence of 5hmC or 5caC at this site enabled exon inclusion [42].

Outside of the hematopoietic system, TET proteins have been associated with a broad range of differentiation processes. *Tet*-deficient embryos had gastrulation phenotypes associated with hyperactive Nodal and Wnt signaling, low levels of *Lefty* gene expression owing to hypermethylation of *Lefty* regulatory elements, and skewed differentiation of neuromesodermal progenitors into mesoderm at the expense of neuroectoderm [43]. *Xenopus* eye and neural development were impaired and associated with altered expression and DNA methylation patterns at developmental genes in the absence of *Tet3* [44]. In neurons, TET1 maintained expression of genes related to neuronal activity, affecting learning and memory [45–47]. 5hmC was also observed at distal regulatory elements during brain development, in particular at enhancers that changed activation state between fetal and adult brains, which was at least partially dependent on *Tet2* expression [48•]. Mouse ES cells lacking TETs poorly differentiated to neural fate, where TET3 was observed to directly regulate Wnt/ β -catenin pathway genes, with low expression and altered DNA modification at the *Sfip4* locus, an inhibitor of Wnt signaling [49]. Altogether, these findings indicate that TET proteins support a variety of natural differentiation processes, with a consistent influence on distal regulatory element activity and gene expression.

TET proteins during induced differentiation and reprogramming

Because TETs are associated with a variety of natural differentiation processes, it was reasonable to hypothesize that they would be involved in experimentally induced differentiation as well. Reprogramming of fibroblasts to induced pluripotent stem (iPS) cells by the ‘four-factor’ combination of *Oct4*, *Sox2*, *Klf4*, and *Myc* (OSKM) is accompanied by changes in DNA methylation at the promoters and enhancers of pluripotency genes [50,51]. TET2 was also observed to influence OSKM activity in fibroblasts, by altering methylation around the *Nanog* promoter [48•]. In another experiment starting with neural stem cells, NANOG was observed to interact with TETs and both *Nanog* and *Tet1* were expressed at higher levels in iPS cells compared to intermediates. Reprogramming could be enhanced by *Tet* expression in a manner dependent on catalytic activity [52•] and *Tet* could replace some transcription factors such as *Oct4* [53] or *Sox2*, *Klf4*, and *Myc* [54] during iPS cell reprogramming.

A role for TET proteins has also been identified in directed differentiation from one lineage to another (often referred to as transdifferentiation). For example, expression of *Tet2* was found to be directly induced by C/EBP α during B cell to macrophage transdifferentiation, and knockdown of *Tet2* reduced the expression of myeloid markers during this process [55]. This C/EBP α pulse was also found to efficiently prime cells for subsequent reprogramming to iPS cells [56,57]. During the initial pulse, C/EBP α was found to induce TET2 binding and oxidation of 5mC [57] and to promote active chromatin states at superenhancers near several pluripotency genes [58]. As with other methods, the efficiency of reprogramming in this system was impaired by knockdown of *Tet2* with short hairpin RNAs [58].

The differentiation of CD4⁺ T helper cells (Th) to functionally distinct subsets, associated with the emergence of characteristic patterns of cytokine and transcription factor expression, can be induced in culture by supplementing media with specific ‘polarising’ cytokines while blocking others [59]. DNA modification patterns around genes encoding key transcriptional regulators and cytokines were altered during T helper lineage differentiation. For example, three regulatory regions near the *IL17* locus, that were associated with enhancer activity, gained 5hmC during differentiation to Th17, and Th17 differentiation was impaired in the absence of Tet2 [60]. Human Th cell subset differentiation was also associated with dynamic changes in 5hmC and corresponding alterations of 5mC at regulatory elements that frequently over-lapped polymorphisms associated with several autoimmune diseases [61].

Modulation of TET activity to influence cell differentiation and reprogramming

The contributions of TET proteins to natural and induced differentiation suggested that differentiation could be influenced by modulation of TET activity by the availability of co-factors. In particular, TET dioxygenases utilize 2-oxoglutarate (2OG) generated by isocitrate dehydrogenase (IDH) enzymes as an essential cofactor, but mutant IDH1 and IDH2 enzymes associated with some cancers produce the R enantiomer of 2-hydroxyglutarate (2HG) instead of 2OG. Although the S enantiomer of 2HG is a far more potent inhibitor of TET and other dioxygenases such as histone lysine demethylases, the R enantiomer is produced at up to millimolar levels and is associated with diminished TET function and 5hmC [62]. In activated T cells, the S enantiomer of 2HG is preferentially produced in CD8⁺ T cells in hypoxic conditions and the presence of S-2HG altered the distribution of 5mC and 5hmC around the *Sei* gene [63].

In ES cells, vitamin C was observed to induce a substantial loss of DNA methylation patterns [64] and promote the reprogramming of fibroblasts to iPS cells [65]. These phenomena were later associated with TET function, since ascorbate was found to enhance TET-mediated generation of 5hmC [66]. Indeed, vitamin C treatment of ES cells increased TET-dependent generation of 5hmC and expression of germline genes [67]. Vitamin C was observed to modulate TET effects on reprogramming, which enhanced the influence of exogenous TET2 but limited the effect of TET1 [68]. While the TET proteins have similar enzymatic activity, TET1 and TET3 contain a CXXC domain which is absent in TET2 [69]. These differences may account for some of the distinct contributions of TET1 and TET2.

For example, TET1 and TET2 were associated with differential localization of 5hmC in ES cells [70] and this may be influenced by additional cooperating factors. For example, 5hmC (via TET1) promoted SALL4A enhancer occupancy which increased 5fC and 5caC from TET2 activity [71]. Similarly, TET1, but not TET2 was observed to interact with ZFP281, which differentially influenced primed versus naïve pluripotent states [72].

Tregs can be induced from cultured naïve T cells in the presence of TGF β and/or retinoic acid [26] and this differentiation is accompanied by a loss of 5hmC compared to naïve cells. Differentiation in the presence of vitamin C increased total 5hmC, *Foxp3* expression, and loss of 5mC at Treg-specific regulatory regions that was dependent on TET proteins [28•, 73]. Similarly, *Tet2* deficiency altered mast cell differentiation, with associated changes in 5hmC at distal regulatory regions [74]. The differentiation and associated changes in gene expression could be restored by addition of vitamin C, at least partially due to its effects on TET3 function. Together these findings suggest that vitamin C can promote TET activity and differentiation, even under conditions where TET expression or cofactors are limiting.

Conclusion

Altogether, many studies in different systems have revealed diverse roles for TET proteins in cell differentiation including enhancer activation, gene expression, and transcriptional stability. TET activity can influence lineage specificity by controlling the induction of determining transcription factors such as *Foxp3* in Tregs, as well as by modulating binding sites for transcription factors at regulatory elements via altered DNA methylation patterns and chromatin accessibility. These activities are also useful mechanisms by which cell fate and function can be ‘tuned’ during directed differentiation and reprogramming, by altering *Tet* gene expression or activity. The balance of TET versus DNMT activity is critical to maintain stable cell identity and disruption of either has been associated with poor differentiation and malignant transformation. Further studies characterizing the regulation of DNA methylation will help separate differentiation from transformation phenotypes.

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Figure 1.

Model for enhancer regulation by TET proteins. **(a)** Prior to activation, enhancer DNA is tightly associated with nucleosomes and is not accessible to most transcription factors (TFs) except for pioneer TFs that are relatively insensitive to the presence of nucleosomes and DNA methylation and can recruit TET proteins to oxidize 5mC in the vicinity into 5hmC and other oxi-mC, resulting in DNA demethylation. 5hmC deposition and DNA demethylation are associated with increased accessibility of enhancers and acetylation of H3 lysine 27. By increasing enhancer accessibility and altering DNA modification landscapes, TET proteins assist the binding of additional TFs such as those that prefer unmodified C (5mC-sensitive) or 5hmC (5hmC-dependent). In addition, 5hmC may be recognized by specific reader(s), whose function may depend on the local chromatin environment. **(b)** Schematic representation of 5mC and 5hmC dynamics during enhancer activation. The 5mC (black) and 5hmC (green) levels are shown for the corresponding stages of enhancer activation/development/maturation in **(a)**. *Top*, enhancer DNA is fully methylated before activation. *Middle*, TET proteins recruited by pioneer TFs convert 5mC into 5hmC and other oxi-mC. *Bottom*, Prolonged TET activity results in complete cytosine demethylation and depletion of both 5mC and 5hmC at the center of enhancers.