The FEBS Journal

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REVIEW ARTICLE

Oxygen regulation of TET enzymes

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Keywords
5hmC; cancer; hypoxia; immunotherapy; TET; α-KG-dependent enzymes

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(Received 29 October 2020, revised 23 December 2020, accepted 4 January 2021)

doi:10.1111/febs.15695

Hypoxia has a significant impact on many physiological and pathological processes. Over the recent years, its role in modulation of epigenetic remodelling has also become clearer. In cancer, low oxygen environments and aberrant epigenomes often go hand in hand, and changes in DNA methylation are now commonly recognised as potential outcome indicators. TET (ten-eleven translocation) family enzymes are alpha-ketoglutarate-, iron- and oxygen-dependent DNA demethylases and are key players in these processes. Although TETs have historically been considered tumour suppressors, recent studies suggest that their functions in cancer might not be straightforward. Recently, inhibition of TETs has been reported to have positive impact in cancer immunotherapy and vaccination studies. This underlines the current interest in developing targeted pharmaceutical inhibitors of these enzymes. Here, we will survey the complexity of TET roles in cancer, and its hypoxic modulation, as well as highlight the potential of these enzymes as therapeutic targets.

Introduction

In the update to their seminal review of the hallmarks of cancer, Hanahan and Weinberg drew attention to the significance of the tumour microenvironment in tumorigenesis [1]. One central and important aspect of the microenvironment is the hypoxia found in solid tumours.

Hypoxia, or low levels of available oxygen, can be understood as an inability to provide tissues with an adequate oxygen supply relative to demand [2]. However, it is difficult to describe and quantify hypoxia precisely, as oxygen levels vary significantly between different tissues even in physiological conditions, with levels as low as 1.1% in the superficial layers of the skin [3], 4.6% in the brain [4] and 13.2% in some regions in arterial blood [5]. For certain organs, the exact oxygenation demands may depend on their

Abbreviations
α-KG, α-ketoglutarate; 5caC, 5-carboxylcytosine; 5fC, 5-formylcytosine; 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; ALKBH5, AlkB homolog 5; Arg1, arginase 1; As(III), arsenite; BTIC, breast tumour-initiating cell; CAFs, cancer-associated fibroblasts; CAMs, cancer-associated macrophages; CAR, chimeric antigen receptor; CEBPα, CCAAT enhancer-binding protein alpha; CMML, chronic myelomonocytic leukaemia; CXXC, cysteine-X-X-cysteine; DMOG, dimethyloxallyl glycine; DNMTs, DNA methyltransferases; DSBH, double-stranded β helix; EMT, epithelial-to-mesenchymal transition; ESCs, embryonic stem cells; FIH, factor-inhibiting HIF-1; HDACs, histone deacetylases; HIF, hypoxia-inducible factor; HREs, hypoxia-responsive elements; HSPC, haematopoietic stem and progenitor cell; IDH1, isocitrate dehydrogenase 1; IL-1R, interleukin 1 receptor; IL-12b, interleukin 12B; IL-4, interleukin 4; IL-6, interleukin 6; KDMs, Jumonji-type histone lysine demethylases; METTL3/14, RNA methyltransferases like 3/14; Mgl2, macrophage galactose N-acetyl-galactosamine-specific lectin 2; MLL, mixed-lineage leukaemia gene; MSCs, mesenchymal stem cells; Ni(II), nickel; NSUN5, NOP2/Sun RNA methyltransferase 5; p38-MAPK, p38 mitogen-activated protein kinase; PDAC, pancreatic ductal adenocarcinoma; PHDs, propyl-4-hydroxylases; R-2-HG, R-2-hydroxyglutarate; ROS, reactive oxygen species; S-2-HG, S-2-hydroxyglutarate; shRNAs, short hairpin RNAs; siRNA, short interference RNAs; SNIP1, SMAD nuclear-interacting protein 1; TCA, citric acid cycle; TCR, T-cell receptor; TET, ten-eleven translocation; TNFα, tumour necrosis factor-α; VHL, von Hippel-Lindau; WT1, Wilms' tumour 1; YB-1, Y box-binding protein 1.

functional state [5], thus making it even harder to determine a concrete threshold between hypoxia and 'physioxia', that is normal oxygen levels for the tissue in question.

In most solid tumours, oxygenation levels range around 0.3–4.2% oxygen, with a median of 2%, which is notably lower than *in vitro* culturing conditions [6]. Tumour regions may experience either acute (transient) or chronic hypoxia. Acute hypoxia in tumours results from fluctuations in perfusion due to aberrant vasculature, whereas chronic hypoxia is a consequence of tumour angiogenesis failing to keep up with tumour growth, thus reflecting the physical limits of oxygen diffusion [7]. Both of these types of hypoxia have been implicated in malignant progression. Importantly, hypoxic regions show genomic instability and increased invasion and metastatic potential [8]. Hypoxia-driven genomic instability has been attributed partially to replication stress and activation of the DNA damage response pathways and eventually can contribute to resistance to therapy [9,10]. This genomic instability often results in aberrant expression of oncogenes and tumour suppressor genes which helps to explain the increased cell growth and metastasis in hypoxic tumours [8,11].

The HIF (hypoxia-inducible factor) family of transcription factors regulate many cellular responses in hypoxia. HIFs are heterodimeric DNA-binding complexes with a shared constitutively expressed β subunit and one of the oxygen-responsive α subunits (HIF-1α, HIF-2α, HIF-3α) [12]. In the presence of oxygen, the HIF-α subunit gets rapidly degraded [13]. Specifically, PHDs (propyl-4-hydroxylases) hydroxylate the conserved proline residues of the HIF-α subunits, and the VHL (von Hippel-Lindau) E3 ubiquitin ligase complex ubiquitinates the hydroxylated prolines, thus targeting HIF-α subunits for degradation [14–16]. Another pathway of HIF-α oxygen-dependent regulation is through FIH (factor-inhibiting HIF-1)-mediated hydroxylation of an asparagine residue, which impairs HIF ability to recruit transcriptional co-activators to hypoxia-responsive elements (HREs) and inhibits its transcriptional activity [17–19]. Both the PHD dioxygenase superfamily and FIH are α-ketoglutarate (α-KG)-dependent enzymes, which require oxygen and iron as cofactors for their enzymatic activity [20].

Hypoxia-inducible factor targets are involved in a range of biological processes ranging from metabolism, cell cycle regulation and apoptosis to carotid body development and leucocyte activation and response to infection [21–24]. Some of these processes are often the focus of HIF and/or hypoxia-mediated changes in cancer. It has become increasingly clear over the past decade that the responses to hypoxia also involve epigenetic remodelling; for example, changes in the epigenome can predict outcome in various cancers [25]. Epigenetic regulation of gene expression occurs through modifications at the histone and/or DNA level, with methylation being one of the most recognised epigenetic marks. Histone methylation may lead to gene activation or repression, depending on the methylation status of a particular amino acid [26]. Several histone-modifying enzymes are α-KG and oxygen-dependent, including Jumonji-type histone lysine demethylases (KDMs), which have been shown to be induced under hypoxia [27]. Furthermore, histone deacetylases (HDACs) were found to enhance HIF-1α stability and activity by modulating the acetylation levels of its cofactors [28].

Another level of epigenetic gene expression regulation is achieved by modifying DNA itself. For example, aberrant demethylation of CpG islands in the HRE in HIF-1α promoters leads to auto-transactivation of HIF-1α and activation of HIF target genes in colon cancer [29]. Aberrant levels of DNA methylation are commonly reported in a range of tumours. These DNA modifications are primarily regulated through the DNA methyltransferases (DNMTs) and DNA demethylases (ten-eleven translocation (TET) family proteins), both of which have been reported to show altered activity in hypoxic conditions [30].

Much less explored in this context is the emerging field of RNA epitranscriptomics. Studies suggest that RNA post-transcriptional modifications might have functions beyond their roles in fine-tuning RNA structures and increased mRNA methylation of specific targets has been reported under hypoxic conditions [31,32]. One of such RNA-modifying enzymes is the AlkB homolog 5 (ALKBH5) RNA demethylase, which has been reported to be a HIF-1a target, and its induction under hypoxic conditions leads to stabilisation of *NANOG* mRNA, which ultimately contributes to a breast cancer stem cell phenotype [33]. On the other end, knockdown of the RNA methyltransferases like 3/14 (METTL3/14) revealed the importance of RNA methylation in the recovery of translational efficiency after hypoxic stress [32] and silencing of the NOP2/Sun RNA methyltransferase 5 (NSUN5) in gliomas led to an adaptive translational programme for survival under oxidative stress [34]. In addition, multiple RNA-binding proteins have been shown to regulate the stabilisation and translation of HIF-1α mRNA, contributing as such to the hypoxic response (reviewed in ref. [35]). For example, the Y box-binding protein 1 (YB-1) has been shown to interact directly with the 5′ UTR of HIF-1α mRNA and promote HIF-1α

translation under hypoxic conditions [36,37]. Not surprisingly, YB-1 overexpression has been reported in numerous cancers, including colon, lung, gastric, oesophageal and glioblastoma [38–40].

Taken together, these examples highlight the wide-ranging effects that hypoxia has on epigenetic and epitranscriptomic modifications and, consequently, regulation of gene and protein expression. For the purposes of this review, we will focus on the effects of hypoxia on DNA demethylases and specifically on the TET family proteins, as the effects of hypoxia on histone modifiers have already been extensively reviewed [41].

TET proteins

Ten-eleven translocation proteins, like PHDs, KDMs and ALKBHs, are α-KG-, oxygen-, and iron-dependent dioxygenases [42,43]. They catalyse oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Mammalian TET proteins—TET1, TET2 and TET3—are known to play significant roles in a wide range of processes such as embryonic development, cell stemness and cancer. However, the precise mechanisms of their function are yet to be fully understood.

TET protein structure

Structurally, the three TET family proteins—TET1, TET2 and TET3—share a common catalytic domain. Located at the C terminus of the protein, it consists of a double-stranded β-helix (DSBH) domain and a cysteine-rich domain [43,44]. In contrast, the N terminus is less conserved. TET1 and TET3 have a cysteine-X-X-cysteine (CXXC) domain which modulates interactions with methylated and unmethylated CpG regions on the DNA [45,46], while TET2 lacks this domain and has to partner with an independent CXXC-containing protein (CXXC finger protein 4, also known as IDAX) instead [47]. A study investigating *Xenopus* eye development showed that loss of the CXXC domain leads to loss of TET3 binding to the promoters of target genes [46]. This domain (IDAX in case of TET2) appears to be highly conserved among vertebrates [45–47]. The relationship between TET protein sequence motifs and protein function, as well as their phylogenetic relationships, has been recently reviewed in ref. [48].

Considering TET2 does not contain a specific DNA-binding domain, proteins other than IDAX have also been reported to mediate its interactions with the DNA. For example, transcription factor WT1 (Wilms' tumour 1) physically interacts with TET2 to recruit it to the target genes and induce their expression [49]. Similarly, through the interactions with transcriptional co-activator SNIP1 (SMAD nuclear-interacting protein 1), TET2 can associate with sequence-specific DNA-binding proteins such as c-MYC, which reveals a potential pathway of TET2 involvement in DNA damage response [50]. Taken together, these studies suggest that CXXC domain—either within the actual protein in case of TET1 and TET3 or through partner interactions in case of TET2—has a role in targeting the TET proteins to specific sites within the genome to limit their unspecific activity [46,47,49,50].

Interestingly, IDAX also appears to regulate TET2 activity by targeting it for degradation through the caspase pathway [47]. During differentiation of embryonic stem cells (ESCs), IDAX is upregulated and seems to account for downregulation of TET2 observed during this process given that short hairpin RNAs (shRNAs) against *Idax* prevented TET2 protein downregulation in differentiating mouse ESCs [47]. Similar interactions are also observed between the CXXC domain of TET3 and its catalytic domain, suggesting that the CXXC domain might be responsible for TET3 autoregulation via a comparable mechanism, as cells with mutant TET3 CXXC domain showed higher 5hmC levels compared with those found in wild-type cells [47]. Another pathway of TET turnover regulation is known to be mediated by a family of calcium-dependent proteases—calpains, with calpain 1 regulating TET1 and TET2 levels in mouse ESCs and calpain 2 controlling TET3 turnover in differentiated cells by mediating enzyme degradation [51]. The degradation of TET enzymes could also be regulated post-translationally [52,53], highlighting the intricacy of both auto- and targeted-regulation of TET activity and function.

TET expression and development

A number of roles in development and stem cell differentiation have been attributed to TET family enzymes (recently reviewed in ref. [54]). This broad functional range can in part be achieved by utilising different transcripts through a range of regulatory and promoter sequences. Cells in developing embryos tend to be exposed to oxygen gradients [55], which likely affects TET activity, thus contributing to alterations in cell fate and differentiation. This aspect of TET modulation is discussed in later sections of this review.

In terms of a general expression pattern, *TET3* is the primarily expressed *TET* in oocytes and fertilised zygotes, with expression of *TET1* and *TET2* taking over during early development [56,57]. Several studies

have attempted to characterise *TET* expression patterns in different stem cell populations. High expression of *Tet1* and *Tet2* is detectable in the inner cell mass of mouse preimplantation embryos [58]. Subsequently, *Tet1* remains expressed at the early postimplantation stage, while *Tet2* expression is below detectable levels at that stage, whereas, at later stages of development, expression of all three *Tets* can be detected in the tissues forming the developing brain [59].

TET expression is not limited to development, and Tet proteins are detected in a range of adult tissues. However, different isoforms usually predominate in different tissues. For example, the dominant TET1 form in differentiated tissues is shorter and lacks the N terminus, including CXXC domain [60]. It remains functionally active, although its chromatin-binding capacity is reduced. Similarly, unlike a unique isoform found in oocytes, *TET3* expressed in neurons can be detected in a number of different forms, resulting from alternative splicing and promoter usage [61]. Conversely, *TET2* is encoded by a single transcript but utilises alternative regulatory sequences in cell-specific contexts. For example, in mouse ESCs, *Tet2* has been reported to have a distal intragenic ESC-specific enhancer, which is downregulated in differentiating cells [62]. The stem cell-specific enhancer is required because *Tet2* expression is regulated by a promoter without pluripotency-dependent activity [62]. In more differentiated cells, control of expression is achieved through regulatory sequence interactions with cell-specific regulatory factors, such as CEBPα (CCAAT enhancer-binding protein alpha). It has been shown that CEBPα interaction with TET2 drives pre-B-cell transdifferentiation into macrophages [63].

To investigate the functional significance of TET proteins, several knockout models have been generated. While able to develop all three embryonic germ layers, the triple knockout progeny resulting from a cross of mice with adult germline-specific deletion of all three *Tets* failed to develop beyond gastrulation [64]. Similar results were observed in a zebrafish triple *tet* depletion model: the knockdown animals generated using morpholinos generally did not survive past gastrulation [65]. This shows that TET proteins play an essential role during early development. However, the effects of specific *Tet* gene deletion can be quite varied. Constitutive *Tet3* knockouts appear to have the most severe phenotype, resulting in perinatal lethality [66,67], while deletions of *Tet1* [68] or *Tet2* [69] do not appear to have significant developmental phenotypes, but show impairments at later stages of life. Considering this in line with the expression patterns, TET3 activity appears to be essential in the oocytes, in fertilised zygotes, as well as throughout development, and cannot be compensated for by TET1 and/or TET2. When *Tet1* and *Tet2* double knockout animals were studied, a wide range of phenotypes was observed. On one end of the phenotypic spectrum, *Tet1* and *Tet2* double knockout animals appear to experience severe developmental defects and perinatal lethality. However, some of these animals are able to survive into adulthood with relatively few defects [70], showing the complicated interplay between the different TET isoforms.

TETs and oxygen

Ten-eleven translocations require oxygen for catalytic function and, as a result, their enzymatic activity can be affected by oxygen's availability and/or redox-linked mechanisms. Interestingly, these enzymes are known to be catalytically active in physiologically hypoxic environments and the oxygen K_M for the purified catalytic domains of TET1 and TET2 has been reported to be around 30 μM, in contrast to 230 μM for PHD1 and PHD3 enzymes [71], or 158 μM for KDM4C enzyme [72]. Such a low oxygen K_M implies that the TETs should remain at least partially active in low oxygen conditions. However, when investigating the purified protein activity *in vitro* in a range of decreasing oxygen levels, significant activity reductions—45% for TET1 and 52% for TET2—were noted in 0.5% O_2, suggesting that oxygen availability modulates TET's enzymatic activity [73]. At the moment of writing, no studies investigating TET3's enzymatic activity in a range of oxygen levels were available. An overview of oxygen effects on *TET* expression and/or protein activity is provided in Table 1.

It has been shown that TET1 activity is differentially regulated by different oxygen concentrations during both embryonic development and stem cell differentiation [74]. In comparison with the other *Tets*, *Tet1* expression is the highest on day 3 of mouse ESC (mESC) differentiation *in vitro*, which coincides with the peak levels of 5hmC in the genome. These 5hmC levels are markedly decreased in cells with shRNA-mediated knockdown of *Tet1* as well as by culturing cells in 1% O_2, suggesting that low oxygen conditions may decrease TET1 activity, which subsequently leads to inhibition of mESC differentiation. Interestingly, TET3 appears to be a downstream target of the 5hmC burst, with its expression increasing as a consequence of promoter demethylation. While *Tet3* expression levels do not significantly change at day 3 of mESC differentiation in conditions of 1% and 3% O_2, its

Table 1. Overview of oxygen effects on TET expression and protein activity.

Condition/treatment	TET/5hmC levels	HIF involvement	Cell type/model	Functional effects	Reference
0.2 % oxygen for 24/48/72 h	↑ 5hmC; ↑ *TET1/TET3*	HIF-1α/2α	Glioblastoma	Induction of pluripotency genes, neurosphere formation	Prasad *et al.* [89]
0.5 % oxygen for 24 h	↓ 5hmC in 11 cell lines; ↓ activity by ~ 45 % in TET1 and ↓ activity ~ 52 % in TET2 reduction *in vitro*	HIF-2α in some cell lines	HepG2, HT-1080, MCF10A, H358, MCF7, Hep3B, LCC, N2a, A549, mouse ESCs (WT and *Tet1* knockout)	Hypermethylation due to reduced TET activity	Thienpoint *et al.* [73]
1 % oxygen for 17 h	↑ *TET1*; changes in 5hmC levels at specific loci	HIF-1α/2α	H1299 lung cancer cell line and FADU head and neck cancer cell line	*INSIG1* induction; induction of EMT	Tsai *et al.* [93]
1 % oxygen for 24 h	↑ *TET1/TET3*	HIF-1α	Human breast cancer cell lines and primary breast cancer samples	Induction of breast tumour-initiating properties through upregulation of TNFα and p38-MAPK pathway	Wu *et al.* [91]
1 % oxygen for 48 h	↑ 5hmC; ↑ *TET1*	HIF-1α	N-type neuroblastoma	Increased 5hmC at hypoxia-responsive elements, hypoxia-responsive gene induction through facilitated HIF-1α	Mariani *et al.* [90]
1 % oxygen for 48 h	↑ 5hmC; ↑ *TET2/TET3*	HIF-1α	AML cells lines and primary samples	Control/induction of *WT1* expression, linked to AML progression	McCarty *et al.* [88]
1 % oxygen for 7 days	↓ 5hmC, ↓ TET1 activity	not investigated	Mouse ESCs	Impaired mESC differentiation	Burr *et al.* [74]
3 % oxygen	↑ *TET1*	HIF-1α	Early pregnancy chorion villi/ JEG3	Upregulated migration and invasion	Zhu *et al.* [130]
5/1 % oxygen for 24 h	↑ 5hmC; ↑ *TET1/TET2* at 5 %; ↑↑ *TET1/TET2/ TET3* at 1 %	HIF-1α	Hepatoma (HepG2)	Hypoxia-induced *TET* expression; *TET1* and *TET2* more sensitive to hypoxia than *TET3*	Lin *et al.* [92]
5 % oxygen for 4 h (zebrafish); 10 % oxygen for 2 h and 8 % oxygen for 4 h (mice)	↑ *Tet1/Tet2/Tet3* in WT animals	HIF-1α/2α	Zebrafish, mice	*Tet1* knockout zebrafish and mice die in prolonged hypoxia and have lower expression of hypoxia-inducible genes; Tet1 facilitates HIF-1α/2α stabilisation	Wang *et al.* [131]
$CoCl_2$ treatment	↑ *TET2/TET1*	HIF-1α/2α	Retinal pigmental epithelium	Decreased TET1/TET2 promoter methylation, VEGF upregulation	Alivand *et al.* [75]
Normoxia and vitamin C treatment	↑ 5hmC and ↑*TET2* in HIF-1α knockdowns	HIF-1α	Melanoma (WM9) and glioblastoma (T98G)	HIF aberrantly stabilised in cancer cell lines, *TET2* expression upregulated in HIF-1α knockdowns and TET2 activity increase with vitamin C treatment	Fischer and Miles [87]
Atmospheric levels	↓ *TET1/TET3* at blastocyst stage	Not investigated	Bovine embryos	High oxygen detrimental to embryonic development, upregulated expression of antioxidant response genes, reduced cleavage and blastocyst formation rates	Skiles *et al.* [132]

expression is highly reduced at day 7 compared to cells cultured at atmospheric oxygen levels. This suggests that oxygen levels mediate *Tet3* expression through TET1 activity [74]. Conversely, when exposing retinal pigment epithelial cells to $CoCl_2$ (a treatment that stabilises HIF), an increase in *TET1* and *TET2* expression was observed, compared to levels seen in untreated cells [75]. It is believed that this upregulation is due to decreased promoter methylation in the *TET* genes, as a similar response was observed by using

5-azacytidine treatment, which inhibits the methyltransferase DNMT and thus prevents promoter methylation [75]. Taken together, the above observations suggest that TET expression in hypoxic conditions may depend on the specific cell type and could in part be mediated through promoter methylation status. As far as enzymatic activity is concerned, given their low oxygen K_M it is reasonable to expect that TET enzymes would remain at least partially catalytically active in low oxygen environments.

In addition to limited oxygen availability, hypoxia can also result in increased production of reactive oxygen species (ROS) [76]. The effects of redox mechanisms on TETs have not been investigated as extensively. However, as these enzymes require ferrous iron for their catalysis, it is intuitive to expect some level of redox-mediated control of their function.

One of most striking examples of redox-linked alterations to TET function is the effects of addition of ascorbate (vitamin C) to cellular 5hmC levels. Treating mouse embryonic fibroblasts with a range of ascorbate concentrations led to rapid increase in 5hmC levels with the effect observed within as little time as 1 h after treatment [77]. Such rapid effect suggests that ascorbate is able to enhance the activity of the enzymes instead of promoting their *de novo* protein synthesis. TET involvement after ascorbate treatment was demonstrated by attenuated 5hmC levels in cells transfected with short interference RNA (siRNA) for all three *Tet*s compared to the control cells, although ascorbate was still able to increase 5hmC in the knockdown cells compared to no ascorbate treatment [77]. Another study has also shown that 72 h of vitamin C treatment leads to increase in 5hmC levels [78]. Mechanistically, *in vitro* work showed that vitamin C binds TET2 and acts as an electron donor to restore Fe^{2+} state, thus increasing TET2 catalytic capacity up to eightfold [79].

TETs in cancer

Aside from their roles in development discussed above, TET involvement in cancer has been a focus of a number of studies. Even the initial discovery of this DNA demethylase family is linked to cancer: identification of the gene of the first member of the family—*TET1*—came from studies of cases with acute myeloid and lymphocytic leukaemias with ten-eleven translocation t (10;11)(q22;q23) where it is fused with mixed-lineage leukaemia gene (*MLL*) on chromosome 10q22 [42,80]. In particular, loss of TET family activity could partially contribute to increased 5mC levels observed in various cancer types [81]. However, some recent studies appear to implicate *TET* expression in driving tumour progression [82], suggesting that the overall picture is not as straightforward as one might initially assume. A general overview of the roles that TETs may play in tumour development and cancer progression is provided in Table 2.

Tumour suppressor activity of TETs

In the first examinations of Tet function, *TET*s appeared to be tumour suppressor genes. A look through The Cancer Genome Atlas (cancergenome.nih.gov) shows that loss-of-function mutations of all three *TET*s have been identified in a range of cancer types, with *TET2* being among most commonly mutated genes in myeloid cancers [83]. Similarly, in the Catalogue of Somatic Mutations in Cancer (COSMIC, cancer.sanger.ac.uk), both *TET1* and *TET2* are identified as cancer census genes—that is genes that have documented evidence of their relevance to cancer. COSMIC also shows that the largest proportion of *TET* family gene mutations identified in tumours are either missense or nonsense substitutions [84].

Key lines of evidence supporting a role for *TET*s in tumour suppression come from findings of decreased 5hmC levels in various cancers. A study investigating 5hmC in normal and cancerous human tissues, including liver, lung, pancreas, breast, prostate and others, found that 5hmC levels were reduced in all tumour samples, regardless of specific cancer type [85]. Furthermore, such reductions could also be observed in genetically engineered mouse tumour models. Supporting the idea that *TET* loss results in 5hmC reduction, a study has also showed decreased expression of all three *TET*s in human breast and liver tumours, with *TET1* expression being the most significantly reduced, followed by *TET2* and *TET3* [85]. Another study investigating metastatic melanomas also showed significant reduction of 5hmC in malignant tissue, and, more importantly, *TET2* overexpression in human melanoma cells re-established the 5hmC landscape and led to smaller, less invasive tumours when grafted into mice [86].

Considering both that a large proportion of solid tumours are hypoxic or have poorly oxygenated regions [6] and that TETs are functionally dependent on oxygen, hypoxic effects on TET activity can provide another avenue to illuminate the mechanisms behind the 5hmC reduction in cancer. Thienpont *et al.* [73] demonstrated that hypoxia leads to an approximately 44% decrease in 5hmC levels in investigated cell lines, and showed that both *TET* expression and

Table 2. Overview of TET effects in cancer.

TET involvement	Changes in TET/5hmC	Cancer type	Functional effects	Reference
Tumour suppressive effects				
TET1/ TET2/ TET3	↓ 5hmC, ↓ *TET1*, *TET2*, *TET3*	Breast, liver	Tumour development associated with decreased *TET* expression and 5hmC levels	Yang *et al.* [85]
TET1	*Tet1* knockout	B-cell lymphoma	B-like lymphoblast cell expansion in lymph nodes, spleen and peripheral blood	Cimmino *et al.* [97]
TET2	↓ 5hmC	Melanoma	*TET2* overexpression restores 5hmC; grafted tumours smaller and less invasive	Lian *et al.* [76]
TET2	*Tet2* knockout	Chronic myelomonocytic leukaemia-like	Monocytosis and granulocytosis in the spleen	Ko *et al.*; Moran-Crusio *et al.* [69,99]
TET2/ TET3	*Tet2* knockout with acute *Tet3* deletion	AML	Development of aggressive myeloid leukaemia	An *et al.* [101]
Tumour induction/promotion effects				
TET1	↑ *TET1* under hypoxia	Lung	Promotes EMT, *in vitro* invasion and migration	Tsai *et al.* [93]
TET1	↑ *TET1* under hypoxia	Colon	*TET1* upregulates hypoxia-responsive gene expression and promotes tumour cell migration activity	Ma *et al.* [82]
TET1, TET3	↑ 5hmC around *OCT4* and *NANOG*, *TET1* and *TET3* induced under hypoxia	Glioblastoma	*TET1* and *TET3* knockdown downregulates pluripotency genes and reduces neurosphere formation	Prasad *et al.* [89]
TET1, TET3	↑ *TET1* and *TET3* under hypoxia	Breast	Upregulates TNFα and p38-MAPK pathway, promotes breast tumour-initiating cell formation	Wu *et al.* [91]
TET2	↑ 5hmC	Pancreatic ductal adenocarcinoma	*Tet2* knockout MSCs show slower tumour growth *in vivo* and have fewer CAFs	Bhagat *et al.* [94]
TET2	↑ *TET2*	Melanoma	TET2 mediates upregulation of immunosuppressive genes in CAMs and promotes cancer evasion	Pan *et al.* [95]
TET2, TET3	↑ *TET2* and *TET3* under hypoxia	Myeloid leukaemia	Demethylation of lncRNA promoter to induce *WT1* expression—linked to myeloid leukaemia progression	McCarty *et al.* [88]

hypoxia are inversely correlated with hypermethylation. While a global increase in 5mC levels was not observed in this study, a site-specific investigation revealed increased methylation at gene promoter and enhancer sites, especially for tumour suppressor genes, suggesting that reduced TET activity in hypoxic conditions might contribute to tumour progression. Similarly, metastatic melanoma cells with aberrantly overexpressed HIF-1α display reduced 5hmC levels [87]. This can be rescued by treating cells with ascorbate, which acts as an enhancer of TET2 catalytic activity, and/or by HIF-1α knockdown, which leads to increased *TET2* expression and protein levels in metastatic melanoma and glioblastoma cell lines [87]. Such findings highlight the importance of hypoxia-mediated changes in tumour epigenetic landscape. However, as discussed below, the effect of hypoxia on TET expression and activity might be tumour and tissue/cell line specific, as there are contradictory data on hypoxic TET regulation and its consequences. Further investigations are needed to better understand how TET function is altered by decreased oxygen availability and HIF stabilisation.

TET upregulation in cancer

While loss of TET function in tumours described above contributes to their reputation as tumour suppressors, some studies describe an increased *TET* expression and/or protein activity in malignant tissues, especially in hypoxic conditions.

Conversely to what was shown in metastatic melanoma cells [87], in myeloid leukaemia cells it has been observed a hypoxia-specific upregulation of *TET2* and *TET3*, which leads to demethylation of a specific CpG island and to induction of WT1 mRNA expression

[88]. WT1 is a transcription factor expressed in hematopoietic stem cells and is upregulated in myeloid leukaemia [88]. These results highlight a hypoxia-mediated epigenetic regulatory mechanism which is likely to affect proliferation and differentiation of hematopoietic stem cells, potentially resulting in aberrant growth.

Similarly, in glioblastoma, hypoxia leads to induction of pluripotency genes by 5hmC enrichment around *OCT4* and *NANOG* promoter loci [89]. Hypoxia both induces *TET1* and *TET3* in this model but also enhances their binding to promoter regions of pluripotency genes. How essential *TETs* are to maintain pluripotency networks is highlighted by the fact that *TET1* and *TET3* knockdown leads to downregulation of pluripotency genes and reduction in neurosphere (3D clusters of neural stem cells) formation. This observation could explain how cancer stem cells are maintained in the hypoxic tumour core, thus contributing to its therapeutic resistance.

TET1 upregulation under hypoxia is also seen in several types of cancer, including N-type neuroblastoma [90], breast tumours [91], hepatoblastoma [92] and colon cancer [82]. Hypoxia upregulation of TETs may also contribute to additional adaptation mechanisms to those previously discussed. For example, in a lung cancer cell line (H1299), hypoxia leads to induction of epithelial-to-mesenchymal transition (EMT), while *TET1* knockdown abolishes mesenchymal gene expression and decreases *in vitro* migration and invasion activity. Interestingly, the hypoxia-induced EMT phenotype can be rescued by catalytically inactive *TET1* mutants [93]. Similarly, in breast tumours, hypoxia-induced *TET1* and *TET3* upregulate TNFα (tumour necrosis factor-α) and p38-MAPK (p38 mitogen-activated protein kinase) pathways, which are involved in breast tumour-initiating cell (BTIC) formation. In this model, HIF-1α knockdown reduced hypoxic activation of *TET1* and *TET3*, which led to decreased expression of hypoxia-induced stemness markers, as well as reduced EMT and BTICs. However, in this case catalytically inactive mutants were not sufficient to restore the phenotype [91], potentially implicating additional pathways. Interestingly, while *TET*s are upregulated in both of these cases, they might play a different mechanistic role, depending on whether their catalytic activity is essential or not.

Hypoxic (up)regulation of TETs may be necessary throughout development as discussed previously and under certain physiological conditions. However, in specific cancers increased TET activity may lead to hypomethylation of pluripotency genes, upregulation of angiogenesis and EMT, which may confer advantages against therapeutic interventions.

TETs and tumour microenvironment

Aside from TET functions that are specific to tumour suppression and/or progression, these proteins have also been implicated in shaping the tumour microenvironment, especially through their effects on cancer-associated fibroblasts (CAFs) and cancer-associated macrophages (CAMs).

A recent study on human pancreatic ductal adenocarcinoma (PDAC) showed that CAFs derived either from patient samples or *in vitro* generated by mesenchymal stem cells (MSCs) show loss of cytosine methylation [94]. Lactate, produced by neoplastic cells, subsequently get incorporated by MSCs in the culture and converted into pyruvate and Krebs cycle intermediates, including α-KG, which leads to TET activation and increased active demethylation. These increases in 5hmC levels appear to underlie the MSC conversion to CAFs. Furthermore, co-injection of murine PDAC tumour model cells with *Tet2* knockout MSCs led to slower tumour growth rates *in vivo*, and their histological examination revealed fewer cells with a CAF phenotype, indicating less efficient conversion from MSCs [94].

Similarly, CAMs were reported to have increased *Tet2* expression in melanoma patient samples as well as in mouse melanoma models [95]. Consistent with increased *Tet2* expression, global 5hmC levels in CAMs were also increased. Such increase appears to be induced through interleukin-1 receptor (IL-1R) pathway and results in TET2-mediated upregulation of immunosuppressive genes, including *Arg1* (arginase 1), *Mgl2* (macrophage galactose N-acetyl-galactosamine-specific lectin 2) and *Il4* (interleukin 4). In contrast, mice with a targeted *Tet2* deletion in the myeloid compartment exhibited slower tumour growth and bore smaller tumours, compared to wild-types. On molecular level, CAM samples from *Tet2* myeloid knockout cells showed increased mRNA levels of proinflammatory cytokines, such as *Il6* (interleukin 6), *Il12b* (interleukin 12B) and *Tnfa* [95].

A link between *TET1* expression and tumour immunity has also been demonstrated in a study investigating basal-like breast cancer [96]. In this case, the authors observed that high *TET1* expression correlates with low immune cell infiltration and expression of immune markers. Further investigations showed that *TET1* downregulation is achieved through NF-κβ activation via p65 binding to a consensus sequence in *TET1* promoter both *in vitro* and *in vivo*. These *TET1*

downregulation mechanisms are also seen in other cancer types, including thyroid carcinoma, skin cutaneous melanoma, lung adenocarcinoma, ovarian cancer and others [96].

There is evidently a great degree of crosstalk between tumour microenvironment and TET proteins, which may significantly impact tumour progression and outcome. To an extent, it also challenges the dominant view of *TET*s as tumour suppressors, considering that *TET* activation within cells in the tumour microenvironment has been demonstrated to aid tumour progression and its capacity to evade the immune system [94–96].

TETs in the haematopoietic and immune system

Prevalence of *TET2* mutations in haematopoietic malignancies has led to a number of studies of the TET family in blood cell development and differentiation. For example, *Tet1* knockout in mice has been shown to promote development of B-cell lymphoma after an extended latency period [97]. In this study, *Tet1* knockout mice appeared relatively healthy but had a significantly decreased survival after ageing a year, when compared to wild-type mice. These *Tet1* mutant mice were found to have enlarged peripheral lymph nodes, hepato-splenomegaly, disrupted spleen architecture as well as mature B lymphomas as revealed by immunostaining of cells in the peripheral blood, lymph nodes or spleen [97]. Furthermore, analysis of 1- to 3-month-old *Tet1* knockout mouse bone marrow and spleen cells showed biased haematopoietic stem and progenitor cell (HSPC) differentiation in the lymphoid compartment, despite a later B-cell maturation block [97].

Tet2 loss in mouse models leads to an increase in HSPCs and myeloid progenitor cells, with enhanced self-renewal capacity, resulting in enhanced haematopoiesis in both lineages [69,98,99]. Some of these animals subsequently develop chronic myelomonocytic leukaemia (CMML)-like malignancies. However, as in the case of *Tet1* knockouts, disease onset was also relatively delayed for *Tet2*-deficient animals. Interestingly, *Tet2* loss-induced leukaemia progression is reversible upon restoration of *Tet2* expression, or with vitamin C treatment to enhance the catalytic capacity of endogenous TET2 [78]. In addition to functions associated with its enzymatic activity, TET2 appears to be important in HSPC development and differentiation in a noncatalytic way. A comparison between *Tet2* knockout and *Tet2* catalytic mutant mice showed that *Tet2* mutant mice predominantly developed myeloid-lineage disorders, whereas the knockouts displayed both myeloid and lymphoid malignancies [100]. Although both groups had similar global 5hmC levels, the HSPCs of each group had distinct gene expression profiles. In particular, these findings suggest that noncatalytic *Tet2* functions might be significant for lymphopoiesis, as lymphoid malignancies were only present in null mutants, but not in the catalytic region mutant animals.

Conversely, in a study where *Tet2* knockout mice were acutely deleted for *Tet3*, the animals developed a fully penetrant, aggressive myeloid leukaemia within a few weeks [101]. Furthermore, these animals display almost complete loss of 5hmC in bone marrow and spleen. Such results suggest a degree of redundancy between *Tet2* and *Tet3* tumour suppressive functions within the haematopoietic compartment.

While loss of *TET2* during haematopoiesis appears linked to progression of malignant phenotypes, studies of cancer immunotherapy trials and vaccination models suggest that *TET2* inactivation may have therapeutic benefits in the context of adoptive cell transfer, which will be discussed below.

TET2 and T-cell differentiation

Upon encountering an antigen, naïve CD8+ T cells start their differentiation programme to effector and memory CD8+ T cells [102]. While effector T cells die off upon resolution of the immune response, memory cells persist, thus enabling a rapid enhanced response upon subsequent encounters with the same antigen.

Upon activation of T-cell receptor (TCR), CD8+ T cells stabilise HIF-1α and HIF-2α in an oxygen-independent manner [103,104]. HIF stabilisation results in a metabolic switch towards glycolysis, which supports proliferation and effector differentiation [103–106]. The switch from oxidative phosphorylation to glycolysis, as well as other metabolic adaptations T cells undergo over the course of their differentiation, has been subject to several reviews [107,108]. There have also been emerging reports of the effects of various metabolites on TET protein activity, both in the immune context and beyond.

Fumarate and succinate are the most commonly reported competitive inhibitors of α-KG-dependent dioxygenases (Figure 1) [109–112]. Like α-KG, they are intermediates of the citric acid (TCA) cycle and substrates of fumarate hydratase and succinate dehydrogenase, respectively. Mutations in these enzymes result in accumulation of fumarate and/or succinate in tumours, inhibiting α-KG-dependent enzymes, including TETs and PHDs [113]. It has been reported that

both fumarate and succinate cause global decreases in 5hmC levels [109–111] as well as downregulation of HIF target genes involved in glycolytic pathways, mostly through inhibition of TET1 and TET3 [110]. Furthermore, fumarate-mediated TET inhibition was demonstrated to promote expression of EMT-related transcription factors and enhanced cell migratory properties [112].

Mutations in another TCA cycle enzyme—isocitrate dehydrogenase 1 (IDH1)—results in accumulation of R-2-hydroxyglutarate (R-2-HG), whereas hypoxia and specifically HIF-1a can induce accumulation of the enantiomer of R-2-HG, S-2-hydroxyglutarate (S-2-HG) [114,115]. Both enantiomers of 2-HG are structurally similar to α-KG and as such can also act as competitive inhibitors of α-KG-dependent enzymes, including TETs (Figure 1) [116]. Interestingly, mouse CD8+ T cells accumulate S-2-HG in response to TCR activation and HIF stabilisation [117]. Among other effects, S-2-HG treatment alters the methylation profile of CD8+ T cells, biasing them towards a memory-like state [117]. The development of memory-like phenotypes after S-2-HG treatment is attributed—at least in part—to TET inhibition, as these cells show reduced 5hmC levels [117]. This feature of S-2-HG is preserved in human CD8+ T cells and it has been shown that it can be beneficial in adoptive cell transfer studies [118]. Specifically, S-2-HG treated chimeric antigen receptor (CAR) T cells (genetically modified T cells expressing tumour-specific CARs) showed increased memory-like phenotypes *in vivo* and demonstrated superior antitumour efficacy compared to control CAR-Ts [118].

Similar results are also seen in CAR-T therapy and vaccination studies, further supporting the idea that *TET2* loss and/or inhibition maintains T cells in a memory-like state (Figure 2). An interesting example of this is an accidental finding from a patient with chronic lymphocytic leukaemia treated with CAR-T immunotherapy [119]. In this patient, successful treatment for leukaemia was achieved through an expansion of a single CAR-T cell clone, where the lentiviral CAR vector was by chance integrated into one of the *TET2* alleles; the other allele had a missense mutation. T cells from this CAR clone had more accessible chromatin on genes linked to cell cycle and T-cell receptor signalling pathways, and less accessible chromatin in T-cell terminal differentiation or exhaustion genes [119]. In line with their less-differentiated state, most of the CAR-T cells from this patient had a central memory phenotype, in contrast to more effector memory and effector T cells in CAR populations of other patients who responded less well to CAR immunotherapy [119]. Generally, in therapeutic contexts, such as adoptive cell transfer therapy, effector T cells have been reported to be less competent for *in vivo* tumour clearance [120]. In contrast, T central memory and T stem cell memory cells convey enhanced antitumour activity (Figure 2), and the efforts to promote their

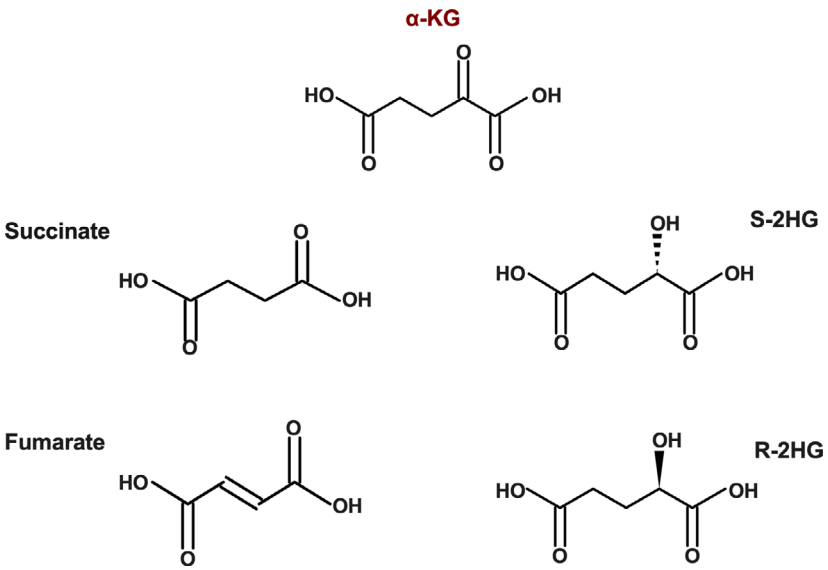

Fig. 1. Chemical structures of metabolites acting as α-KG competitive inhibitors. Overview of chemical structures of α-ketoglutarate (α-KG) and other intracellular metabolites that act as competitive inhibitors for α-KG-dependent dioxygenases: succinate and S-2-hydroxyglutarate (S-2HG) (top row); fumarate and R-2-hydroyglutarate (R-2HG) (bottom row).

abundance either pharmacologically or through direct reprogramming of terminally differentiated T cells have been a point of discussion in the field [121].

Delayed disease progression as well as tumour size reduction were also seen in a melanoma mouse model injected with *Tet2* knockout OT1 CD8+ T cells compared to WT-OT1 CD8+ cells [122]. Analysis of the *Tet2* knockout T-cell populations showed reduction of exhaustion and reduced expression of immunosuppressive genes [122]. A similar CD8+ T-cell differentiation phenotype has been seen in a mouse model with T cell-specific *Tet2* knockout [123]. While these animals do not show specific haematopoietic phenotypes, upon acute viral infection their CD8+ T cells preferentially adopt a memory phenotype. They also demonstrate better response and infection clearance following pathogen rechallenge compared to wild-types (Figure 2) [123]. Again, the methylation changes identified in this study were linked with genes involved in regulation of T-cell differentiation [123].

Together these findings show that loss of *TET2* promotes maintenance of less-differentiated CD8+ T-cell memory state. Such phenotypes confer improved CAR-T efficacy and more efficient response to immune challenges.

Pharmacological TET inhibition

In addition to naturally occurring metabolites which act as TET inhibitors, some effort has gone into development of pharmaceutical drugs targeting these enzymes (Figure 3). For example, nonspecific α-KG analogues such as dimethyloxallyl glycine (DMOG) have been used as TET inhibitors. In a study on bovine embryo development, 1 mM DMOG treatment led to increased 5mC levels, decreases in the mRNA levels of the pluripotency markers *NANOG* and *OCT4* and a decline in blastocyst formation [124], which appears to be in line with the expected effect of TET inhibitors. However, considering the wide range of DMOG targets, it is very likely that the reported phenotype is shaped by inhibition of multiple α-KG-dependent dioxygenases.

When it comes to more targeted approaches, it is often attempted to mimic the substrate of an enzyme; thus, in the case of TETs—5-methylcytosine. One such

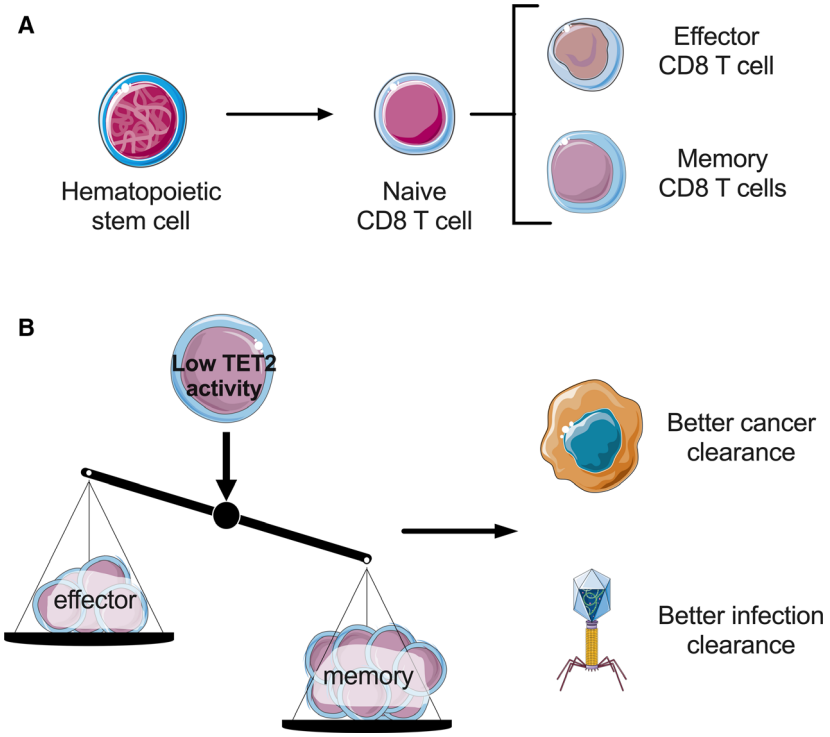

Fig. 2. Effects of TET2 on CD8 T-cell differentiation. (A) Hematopoietic stem cells give rise to naïve CD8 T cells, which differentiate into effector or memory T cells upon antigen presentation. (B) Low TET2 activity biases CD8 T-cell differentiation towards a memory-like state which has been demonstrated to show better infection clearance in vaccination studies and improved cancer clearance in adoptive cell transfer immunotherapy studies.

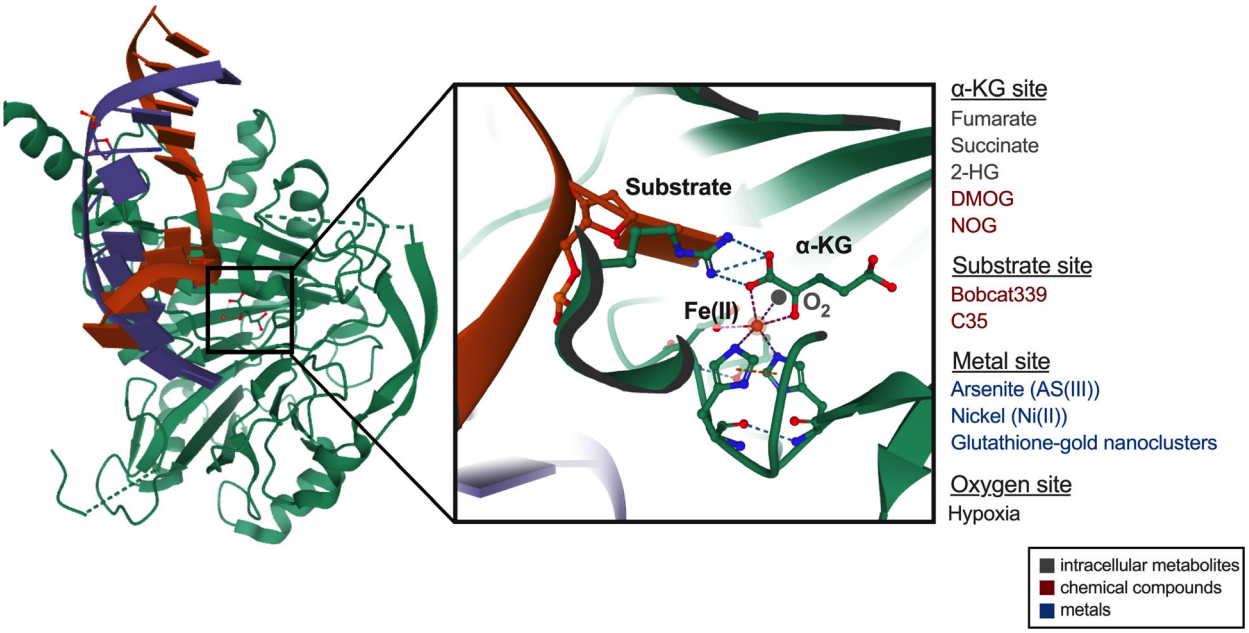

Fig. 3. Inhibitors of TETs and their site of action. The illustration shows a TET2-DNA complex crystal structure. Zoomed in box illustrates the active site of the enzyme. For illustrative purposes, the figure was adapted from the original structure to illustrate the positions of α-KG and oxygen. The list on the right shows inhibitors of TET enzymes and their site of action. The RCSB Protein Data Bank (PDB) (rcsb.org) was used to generate the image of the TET structure and was adapted from PDB ID: 4NM6 [133–135].

TET inhibitor is Bobcat339 [125]. It is cytosine-based and has mid-μM potency against TET1 and TET2 [125]. Another recent study identified a different small molecule which targets the catalytic core of TET enzymes, C35 [126]. Its IC_{50} against TET1, TET2 and TET3 was 3.48, 1.2 and 2.31 μM, respectively, while competitive α-KG-binding assay and *in silico* analysis indicated that this molecule targets the TET catalytic core [126].

There have also been reports of metal-mediated TET inhibition. One study showed that arsenite (As (III)) binds directly to cysteine residues in TET protein zinc finger domains, resulting in decreased catalytic activity *in vitro* as well as dose-dependent 5hmC decreases in arsenite-treated cells [127]. Similarly, nickel (Ni(II)) had a concentration and time-dependent inhibitory effect on TETs by directly displacing the enzyme cofactor Fe^{2+} [128]. 5hmC reduction was also observed in cells treated with glutathione-based gold nanoclusters [129]. The authors reported that nanoparticles induce TET protein aggregation and subsequently affect their enzymatic activity through ROS production [129]. Although the findings of these particular studies are more relevant in the context of metal-induced cytotoxicity, they do provide insight into functionally important enzyme sites and highlight their cofactor and oxygen requirements.

Conclusions and future perspectives

The importance of TET enzymes in development, physiology and disease is well established. Aberrant epigenome changes are also widely accepted as important prognostic biomarkers in cancer. TET family DNA demethylases significantly contribute to shaping the epigenetic tumour landscape. Although a lot of studies have been focused on the effect of TET activity directly on cancer cells, it is now clear that TET involvement in the tumour microenvironment is equally important for the subsequent development of tumour growth. Recent studies have highlighted the effect of TETs in the development and formation of CAFs, CAMs and immunosuppression—key factors of a tumour microenvironment [94,95]. However, as the tumour microenvironment is very heterogeneous between cancers, we should also expect that TET activity might play unique roles depending on the degree of hypoxia, acidosis and availability of nutrients.

In this review, we attempted to highlight the complicated role of hypoxia on TET enzymes. It is well

established that TET activity is modulated by oxygen availability and it is often linked or in some cases even directly regulated by HIF. However, further investigations are needed to fully understand in what direction TET function is altered by hypoxia and HIF stabilisation given the contradictory data reported [73,87–89]. Far less is known about the effect of tumour acidosis and availability of nutrients on TET activity. It is feasible that both of these factors could modulate TET function given its requirement of ferrous iron and α-KG (a TCA cycle metabolite) for enzymatic activity. Studies focused on the effect of the local pH on the radical formation, and the effect of redox mechanisms on TETs would greatly enhance our understanding of TET function. More work is also needed to elucidate the effect of nutrient availability and cell metabolic reprogramming on TETs. A nutrient-deprived environment could affect both the metabolic state and the gene expression patterns of malignant and nonmalignant cells. These changes might be particularly significant in the case of TETs as they are dependent on α-KG, and simultaneously TCA cycle by-products can act as their enzymatic inhibitors. Thus, investigating the metabolic profile of specific cancers would also advance our understanding of TET role.

In conclusion, TETs' involvement in progression or inhibition of cancer development is still far from being elucidated. Although commonly considered to be tumour suppressors, emerging evidence suggests that TET role in cancer development is far more complex. TET inhibition could on one hand decrease the expression of tumour suppressor genes in cancer cells [73] but could also lead to a less aggressive and more immunosusceptive tumour microenvironment [95,96]. TET inhibition can also affect the immune system, specifically by biasing T cells towards a less-differentiated memory-like state—a favourable characteristic in the context of adoptive cell transfer. Therefore, targeted and transient TET inhibition may have benefits in the clinic, especially in immunotherapy. As more studies emerge and the mechanisms behind TET roles in biological processes are illuminated, these proteins will surely gain increased interest as pharmaceutical targets.

Acknowledgements

This work was supported by Apollo Therapeutics, Barncancerfonden (The Swedish Childhood Cancer Fund), Cancerfonden (The Swedish Cancer Society), Vetenskapsrådet (The Swedish Research Council), the Knut and Alice Wallenberg Foundation, and Wellcome Trust Principal Research Fellowship awards to RSJ; the Evelyn Trust, Cambridge (Patrick Sisson's Research Fellowship), and the Jonas Söderquist Fellowship (Karolinska Institutet) awarded to IPF.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

RM wrote the majority of the manuscript and generated the tables and figures. PPC generated/edited figures and provided feedback on the manuscript. RSJ edited and provided feedback on the manuscript. IPF wrote/edited the manuscript, generated figures and provided direction for the manuscript.

Peer Review

The peer review history for this article is available at https://publons.com/publon/10.1111/febs.15695.

[Correction added on 17 March 2021, after first online publication: URL for peer review history has been corrected.]

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