Biochemical and Biophysical Research Communications 436 (2013) 115–120

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Mini Review

A mechanistic overview of TET-mediated 5-methylcytosine oxidation

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ARTICLE INFO

Article history:
Received 16 May 2013
Available online 29 May 2013

Keywords:
Epigenetics
Oxygenase
DNA demethylase
Gene silencing
Transcription regulation

ABSTRACT

Methylation of DNA at the carbon-5 position of cytosine plays crucial roles in the epigenetic transcriptional silencing during metazoan development. Recent identification of Ten-Eleven Translocation (TET)-family demethylases have added a new dimension to dynamic regulation of 5-methylcytosine (5mC), and thus, inheritable and somatic gene silencing. The interest in hematology was particularly stimulated by the recent discovery of TET2 mutations in myeloid malignancies which were proven to be leukemogenic in murine knockout models. The TET-family enzymes are Fe(II), 2-oxoglutarate-dependent oxygenases and catalyze demethylation of 5mC by iterative oxidation reactions. In the last decade results from numerous studies have established a key role for these enzymes in epigenetic transcriptional regulation in eukaryotes primarily by hydroxylation reactions. The TET catalyzed hydroxylation and dehydration reactions in the mammalian system exemplify the diversity of oxidation reactions catalyzed by Fe(II), 2-oxoglutarate-dependent oxygenases, and suggest an existence of other types of oxidation reactions catalyzed by these enzymes in the eukaryotes, which are so far only documented in prokaryotes. Here, we review the TET-mediated 5mC oxidation in light of the putative reaction mechanism of Fe(II), 2-oxoglutarate-dependent oxygenases.

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1. Introduction

Cytosine methylation at carbon-5 is an important covalent modification of mammalian DNA. Methylation of DNA at cytosine carbon-5 in the CpG dinucleotides is carried by DNA methyltransferases [1]. Due to the critical role played by the human DNA methyltransferases in normal development and disease states, numerous studies were directed toward the identification of 5mC demethylases. Ultimately, using sophisticated bioinformatics tools three putative DNA modifying enzymes, TET1-3, were identified in the mammalian genomes [2]. Biochemical characterization of the newly identified putative DNA modifying enzymes showed that TET1 hydroxylates 5mCs into 5-hydroxymethylcytosines (5hmCs) both *in vitro* and in mammalian cells [3]. A significant genomic presence of 5hmC (~0.6% of all Cs; accounting for ~40% of all 5mCs) was also observed in Purkinje cells from the mouse cerebellum [4]. TET1 is highly expressed in embryonic stem cells (ESCs), and several studies have used high quality TET1 antibodies to characterize TET protein binding and 5hmC deposition across the mESC genomes using parallel DNA sequencing technology [5–8]. These studies have shown that TET1 binds predominantly to CpG islands and TET1-bound promoters show significantly lower levels of 5mC marks, possibly due to TET1-mediated demethylation. Surprisingly, given the established role of 5mC in gene repression, all these studies have shown that the knockdown of TET1 causes more genes to be de-repressed than de-activated.

The TET1 gene was initially identified as a fusion partner of the MLL (myeloid/lymphoid or mixed lineage leukemia) gene in acute myeloid leukemia (AML) [9]. Using cytogenetics and single nucleotide polymorphism arrays, we and others have shown that TET2 is one of the most frequently mutated genes in myelodysplastic syndromes (MDS) [10–12]. The TET2 mutations are also prevalent in a number of myeloid malignancies such as MDS-myeloproliferative neoplasms (MDS-MPN) and acute myeloid leukemia derived from MDS and MDS-MPN (sAML) [12]. Patients with TET2 mutations show low levels of genomic 5hmC in the marrow compared to those with wild-type TET2 [10].

2. Role of TET2 oxygenase in hematopoiesis

In order to determine the function of TET2 in normal hematopoiesis and myeloid transformation, a number of groups have

Abbreviations: TET, Ten-Eleven Translocation; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; 5fC, 5-formylcytosine; 5caC, 5-carboxylcytosine; 2OG, 2-oxoglutarate; KDM, histone lysine demethylase; PHD, Prolyl-hydroxylase-domain; FIH, Factor-inhibiting-HIF.

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0006-291X/$ - see front matter Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2013.05.077

generated TET2-knockout mouse models [13–16]. These studies showed that TET2 was highly expressed in hematopoietic stem cell (HSC) and other progenitor cells, and that ablation of TET2 dramatically diminished the levels of genomic 5hmC in bone marrow cells. Importantly, in contrast to TET1-knockout mouse model [17], which shows some reduction in the cellular 5hmC levels but are viable, fertile and appear to develop normally, TET2-knockout mice developed diverse myeloid malignancies. Although TET2-knockout mice are viable and grossly normal, as they age, they start dying from different hematopoietic malignancies.

Results from studies of TET2-knockout mice suggest that wild-type TET2 promotes hematopoietic differentiation. TET2 loss resulted in an expansion of the HSC and other myeloid progenitor cell numbers. TET2-deficient HSC and progenitor cells also showed progressively diminished hematopoiesis. $TET2^{-/-}$ and $TET2^{+/-}$ HSCs had a higher ability to self-renew, providing a competitive advantage to these cells compared to wild-type HSCs for repopulating hematopoietic lineages. Further, consistent with a high percentage of patients with heterozygous TET2 alterations, heterozygous cells from $TET2^{+/-}$ mice behaved similar to $TET2^{-/-}$ cells and developed diverse myeloid malignancies. $TET2^{+/-}$ cells also exhibited a competitive advantage over wild-type TET2 cells in transplantation assays, although this occurred over a longer period compared to $TET2^{-/-}$ cells. These results suggest that TET2 haploinsufficiency is sufficient to change the properties of HSCs leading to induction of myeloid malignancies.

Other genes also play a role in the maintenance of 5hmC levels in cells (Fig. 1). For example, the normal function of isocitrate dehydrogenases (IDH1/2) is to convert isocitrate into 2-oxoglutarate (2OG). In some AML and glioblastoma patients mutations in IDH-1/2 create neomorphic variants of the enzymes, which produce 2-hydroxyglutarate (2HG) instead of 2OG [18]. Due to the structural similarity with 2OG, 2HG binds in the 2OG binding pocket in the active site of dioxygenases, which impairs their function. Thus, 2HG acts as a competitive inhibitor for a number of 2OG-dependent dioxygenases like histone lysine demethylases (KDMs), prolyl hydroxylases and DNA demethylases like TET2 [19,20]. Inhibition of dioxygenases such as KDMs and TET2 leads to an increase in the histone lysine methylation and a decrease in the 5-hydroxymethylcytosine levels in cells. Thus, clinical features of some IDH1/2 gain-of-function mutations are similar to TET2 mutations in AML patients.

Other key TCA cycle enzymes (*e.g.*, succinate dehydrogenase, SDH and fumarate hydratase, FH) are also mutated in various cancers [21,22]. Mutations in these enzymes lead to cellular accumulation of succinate and fumarate, respectively. Due to structural similarity with 2OG, these TCA cycle intermediates inhibit the activity of dioxygenases like prolyl-hydroxylase-domains (PHDs) resulting in pseudo-hypoxic environment and activation of hypoxia-inducible-factor (HIF) pathway leading to aberrant cellular proliferation [23] (Fig. 1). It would be interesting to check the status of these enzymes in leukemia patients and evaluate their effects on TET dioxygenases.

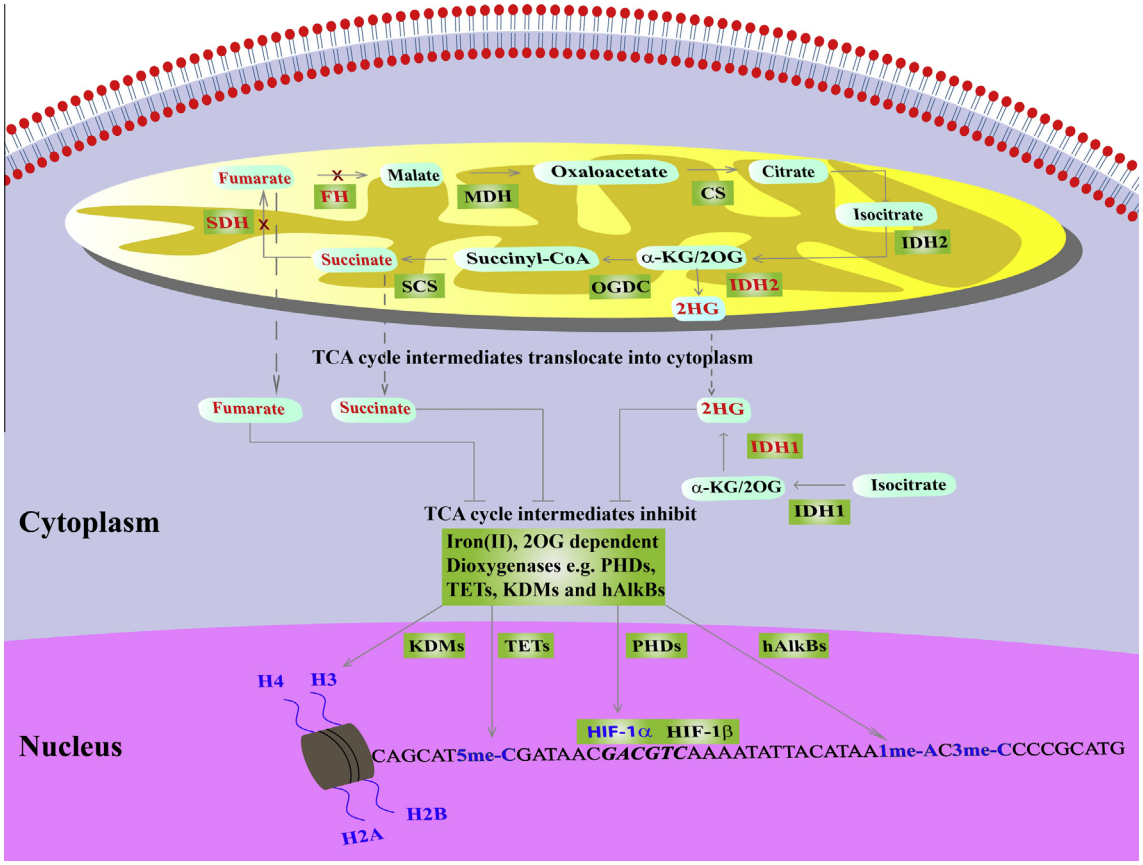

Fig. 1. Mutations in the enzymes of TCA cycle can influence the activity of Fe(II), 2OG-dependent dioxygenases involved in epigenetics causing cancers. Normally IDH1/2 converts isocitrate to 2OG, while in subsequent steps succinate is converted to fumarate by succinate dehydrogenase (SDH) and fumarate to malate by fumarate hydratase (FH). Mutations in the SDH and FH genes lead to accumulation of succinate and fumarate, respectively, while mutations in IDH1/2 gene allow neomorphic enzymes to convert isocitrate into 2HG. Accumulated succinate, fumarate or 2HG, due to structural similarity with 2OG, act as competitive inhibitors of 2OG-dependent dioxygenases leading to changes in the epigenetic landscape. Mutated enzymes and their altered substrates or products are represented in red font. The substrates of Fe(II), 2OG-dependent dioxygenases involved in epigenetics are shown in bold blue font. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. The Fe(II), 2OG-dependent oxygenases and their substrate specificity

Since our initial identification of the first oxygenase involved in transcription regulation [24], results from numerous studies have established a key role for these enzymes in epigenetic transcriptional regulation in eukaryotes (Figs. 1 and 2). Every 2OG-dependent oxygenase characterized to date requires Fe(II) for catalysis and oxidize prime substrate (*e.g.*, 5mC into 5hmC) with coupled oxidation of 2OG cosubstrate into succinate and CO_2. During this process one atom of oxygen gets incorporated into the carboxylic acid group of succinate, while the fate of other oxygen atom of dioxygen molecule depends on the type of oxidation reaction catalyzed. In the case of dehydration and desaturation reactions, the other oxygen is assimilated into water. However, in epoxidation and hydroxylation reactions, incorporation of oxygen occurs into the epoxide or alcohol products, respectively. Since these oxygenases incorporate both the atoms of molecular oxygen into the products (*e.g.*, succinate and 5hmC), they are also known as dioxygenases.

To date two Fe(II), 2OG-dependent dioxygenase superfamilies catalyzing the oxidative modifications of nucleic acid bases have been identified; the AlkB- and the TET/JBP-family. The AlkB-family dioxygenases protect *Escherichia coli* against the cytotoxic effects of methylating substances by directly dealkylating bases (1 mA, 3mC, 1mG and 3mT) in DNA [25,26]. In humans, at least nine potential homologues of *E. coli* AlkB have been identified. Interestingly, similar to *E. coli* AlkB, some human homologs (*e.g.*, ABH3 and FTO) can efficiently demethylate bases in single-stranded DNAs (ssDNA) and RNAs along with double-stranded DNA (dsDNA) [27,28]. It is not known whether the TET-family of dioxygenases prefer 5mC demethylation in dsDNAs, ssDNAs or even in RNAs. Interestingly, 5mC is also a major RNA modification without much known functional significance [29].

Although most 5mC modifications are present in the CpG dinucleotides in the genome, for an active 5mC demethylation, TET1 enzyme is not dependent on +1 residue in substrates (*i.e.*, it can demethylate mCpG, mCpC, mCpA and mCpT with equal efficiency) [30]. However, bisulfite sequencing revealed that: (i) TET1-mediated 5mC demethylation is coupled with transcription because analysis of promoter truncated, transcriptionally inactive construct showed significantly less demethylation; (ii) demethylation is highly selective with few DNA strands being extensively demethylated while others barely demethylated; (iii) of the two DNA strands in the transcribed region, 5mC demethylation is much more efficient on the non-transcribed (sense) strand than on the transcribed (antisense) strand [30]. All these observations suggest that the lax substrate specificity of TET1 is regulated by its interacting proteins which target it to specific sites of transcription initiation/elongation complexes.

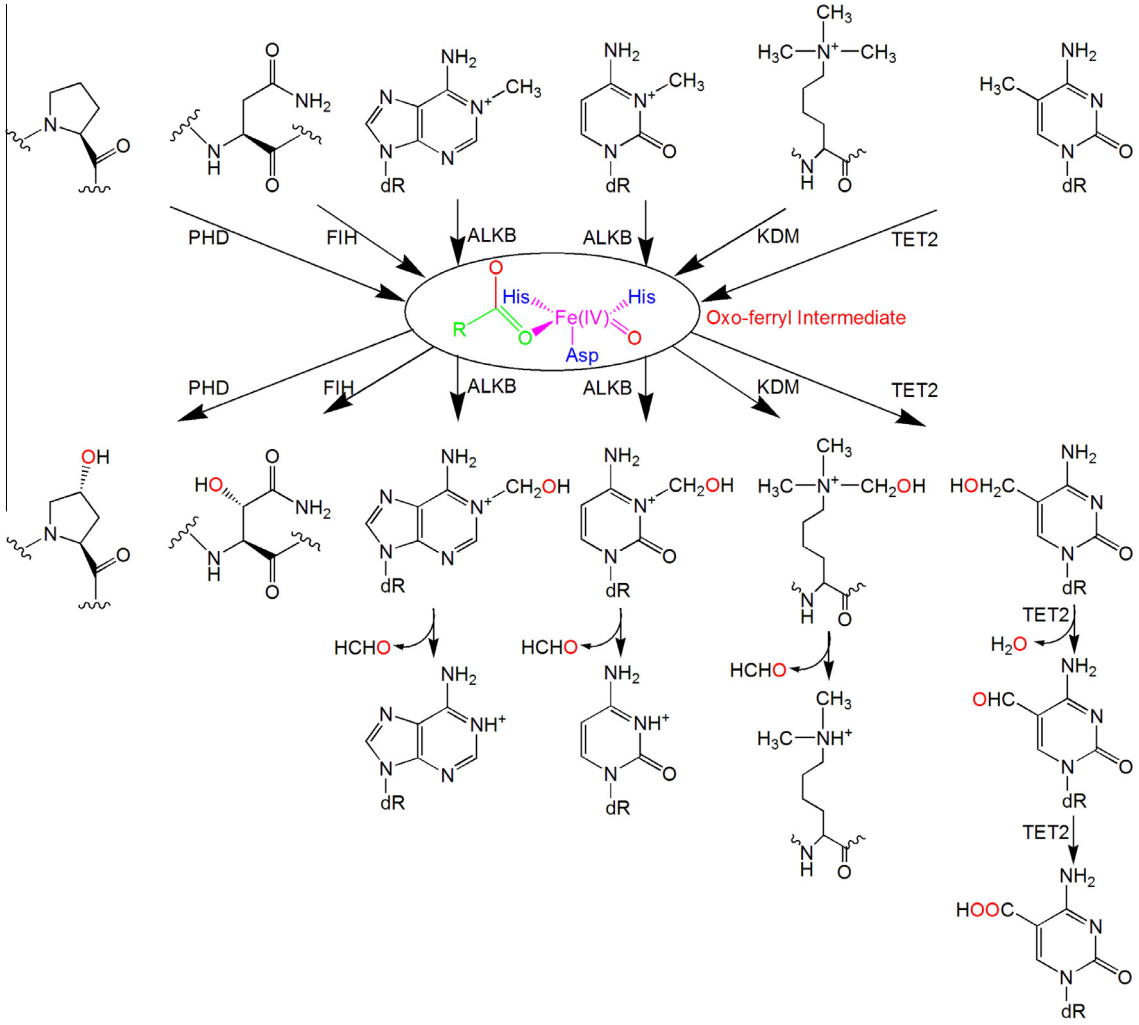

Fig. 2. An overview of Fe(II), 2OG-dependent dioxygenases mediated oxidation of substrates involved in epigenetic regulation via a reactive oxo-ferryl [Fe(IV)=O] intermediate.

4. Three-dimensional topology of TET dioxygenases

A comparison of primary sequences of a number of Fe(II), 2OG-dependent dioxygenases shows that while some conserved motifs (*e.g.*, iron and 2OG binding) can be found, there is often little primary sequence similarity among different subfamilies [31]. Yet, the reported crystal structures of Fe(II), 2OG-dependent dioxygenases have demonstrated an emergence of a common structural platform among different subfamilies. The results from these studies have revealed a β-strand core containing eight anti-parallel β-strands folded into a "Jelly-roll" motif. This characteristic motif harbors the dioxygenase active site consisting of three residues (two histidine and one aspartate or glutamate residues known as the "2-His-1-carboxylate triad") occupying one side of the Fe(II) coordination sphere [32]. The residues which constitute the 2-His-1-carboxylate triad are generally present on or near the relatively rigid β-strand core, indicating a divergent evolution within the 2OG-dependent dioxygenase subfamilies.

Bioinformatics analyses followed by secondary structure predictions suggest the existence of a similar Jelly-roll motif containing the 2-His-1-carboxylate triad (His-1672 × Asp-1674…His-2028 in TET1; His-1382 × Asp-1384…His-1881 in TET2; His-942 × Asp-944…His-1538 in TET3) in the active site of TET dioxygenases (Fig. 3). This 2-His-1-carboxylate triad forms a common Fe(II) binding platform, which allows dioxygenases to carry out a range of oxidation reactions [31]. The Fe(II) metal center, locked in the 2-His-1-carboxylate triad, binds three exogenous ligands (*i.e.*, 2OG and O_2) on the other side. Thus, oxygen binds to the metal center *trans* to any residue constituting the 2-His-1-carboxylate triad. The remaining two Fe(II) coordinates are occupied by the carboxyl and keto oxygen atoms of 2OG (Fig. 4). The 5-carboxylate group of 2OG binds to either an arginine (present on the eighth β-sheet of the Jelly-roll) or a lysine (present on the fourth β-sheet of the Jelly-roll) residue from the enzyme. Thus, based on the binding of 5-carboxylate group of 2OG two subfamilies of Fe(II), 2OG-dependent dioxygenases have been identified. In TET dioxygenases, the 5-carboxylate group of 2OG binds an Arg residue. In some dioxygenases where the 5-carboxylate group of 2OG binds an Arg residue, a characteristic Arg × Ser motif is found where the Ser residue forms an additional hydrogen bond with the 5-carboxylate group of 2OG (Arg-2043 × Ser-2045 in TET1, Arg-1896 × Ser-1898 in TET2, Arg-1553 × Ser-1555 in TET3).

5. Structure–function analysis of TET2 mutations in leukemia

The clinically described TET2 mutations include frame-shift, nonsense and missense mutations. Since the dioxygenase domain is present toward the TET2 C-terminus with the critical 2OG binding motif located toward the very end, most frame-shift and nonsense mutations upstream to it will result in an inactive

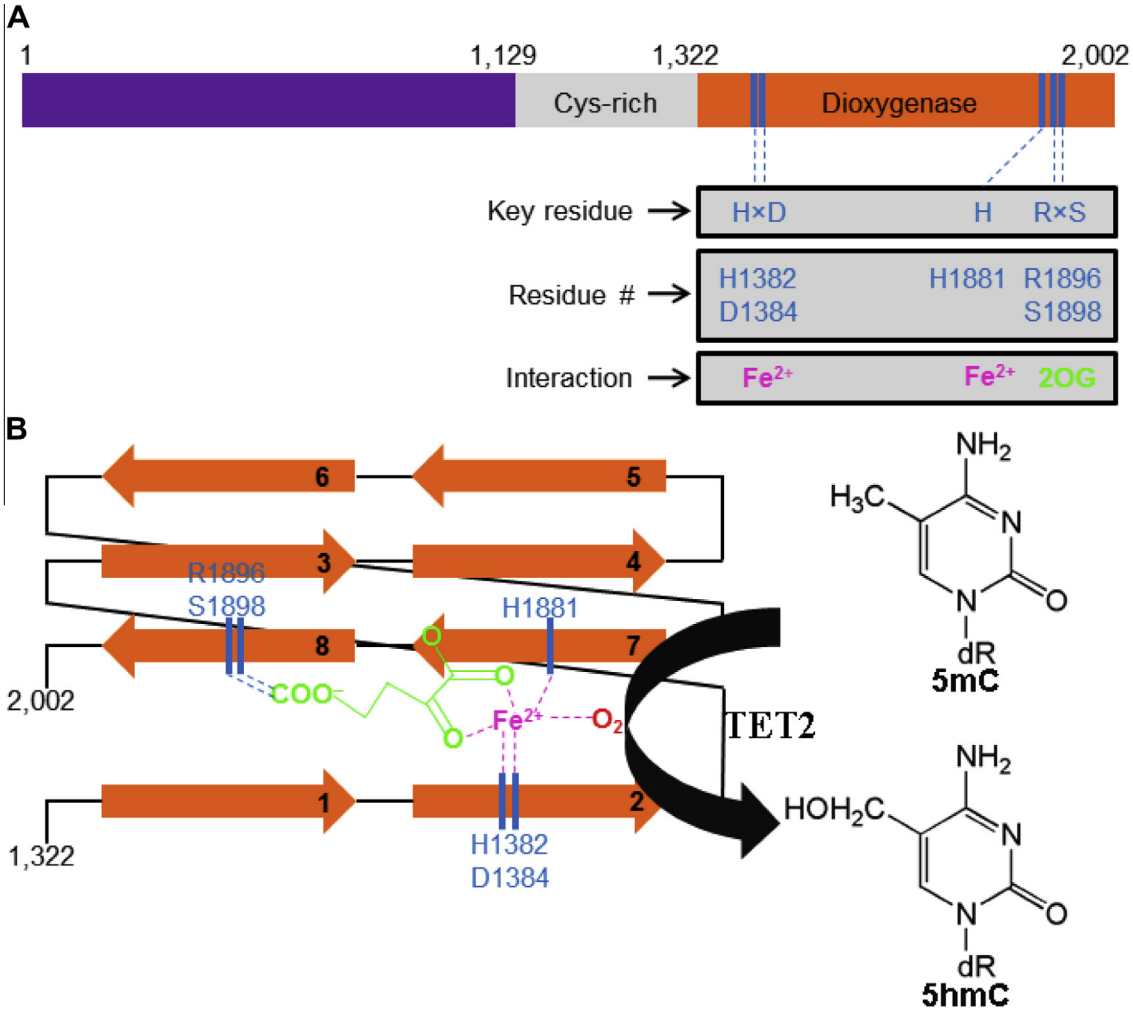

Fig. 3. A graphical representation of the full-length TET2 showing the cysteine rich domain and catalytic dioxygenase domain (A). An active site representation of TET2 showing the eight antiparallel β-sheets constituting the Jelly-roll motif (B). The Jelly-roll motif also represents the bound cosubstrate, 2OG, and cofactors, Fe(II) and O_2, to the active site catalysing the conversion of 5mC into 5hmC.

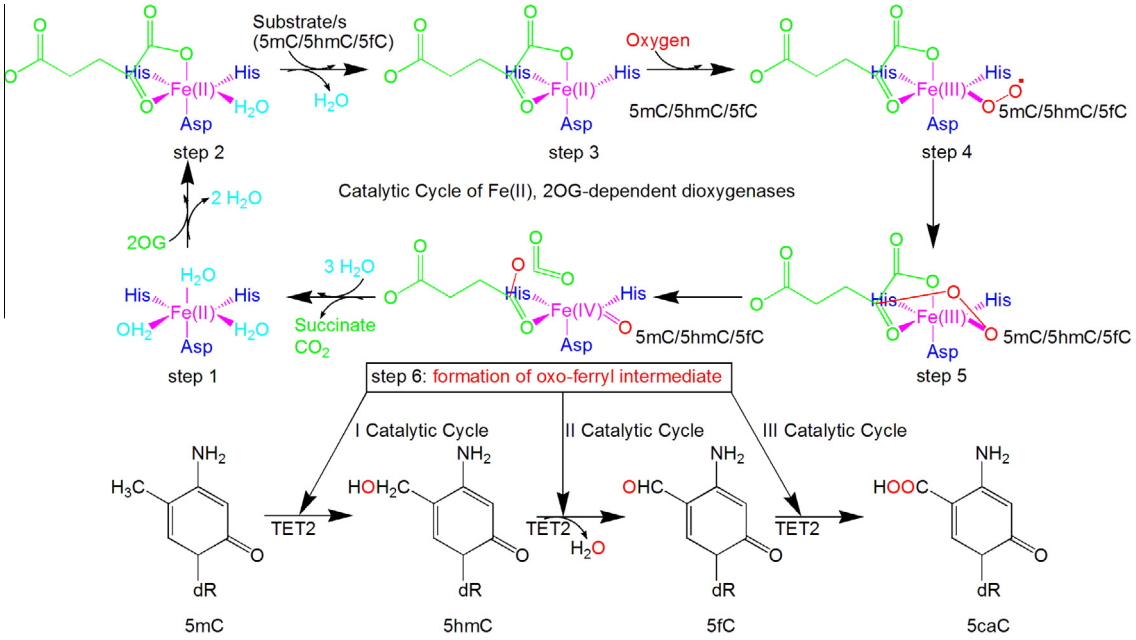

Fig. 4. A catalytic cycle of Fe(II), 2OG-dependent dioxygenases showing sequential binding of the cofactors and substrates leading to the formation of reactive oxo-ferryl [Fe(IV)=O] intermediate. The two histidines and one aspartate residues, which form the active site core, is called the 2-His-1-carboxylate facial triad. The conversion of 5mC to 5caC is mediated by three iterative catalytic/oxidation cycles by TET family of dioxygenases generating 5hmC and 5fC as intermediates.

enzyme. In addition to the frame-shift and nonsense mutations, most of the identified TET2 missense mutations are clustered in the dioxygenase domain (Fig. 1). Interestingly, some of the most frequently mutated residues (Asp-1384Val, His-1881Gln/Arg/Ala) and (Arg-1896Met/Ser/Gly, Ser-1898Phe) form the Fe(II) (His-1382 × Asp1384. . .His-1881) and 2OG (Arg-1896 × Ser-1898) binding motif in the TET2 active site, respectively. Since it has been shown that inactivation of TET2 alone can cause myeloid malignancies in the mouse model, some of the observed clinical heterogeneity in patients with TET2 mutations could be due to the type of the enzymatic defects.

6. Mechanism of Fe(II) and 2OG-dependent dioxygenases

Using a number of techniques, a detailed catalytic mechanism of Fe(II), 2OG-dependent dioxygenases has been proposed (Fig. 4) [33]. The TET-family dioxygenases convert 5mC into 5hmC, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by iterative oxidation steps [34]. Demethylation of 5mC takes place by the excision of 5caC, the final product in the TET-mediated oxidation reaction, by thymine-DNA glycosylase, a base excision repair enzyme [34]. Although, it seems that TET dioxygenases prefer 5mC as substrate over 5hmC and 5fC [35], which may explain the low genomic level of 5fC and 5caC compared to 5hmC [34,35]. It is interesting to note that during the first and third TET-mediated oxidation reactions (from 5mC to 5hmC and then from 5fC to 5caC), the oxo-ferryl intermediate possibly abstracts a hydrogen atom from the substrates (*e.g.*, 5mC and 5fC) and then hydroxylates it in a rebound mechanism. While during the conversion of 5hmC into 5fC, the second TET-mediated oxidation reaction, hydroxylation is proceeded by a dehydration reaction producing water and 5fC. Thus TET enzymes are the first example of a bifunctional Fe(II), 2OG-dependent dioxygenase in the mammalian system.

7. Conclusion and future perspectives

5-methylcytosine plays critical role in gene expression and its dynamic regulation by TET dioxygenases have opened a new era in epigenetic regulation of transcription. More interestingly, discovery of TET dioxygenases have helped identify three additional cytosine modifications (5hmC, 5fC and 5caC), with the possibility of each playing a distinct role in epigenetics. Recent studies have established that HIF pathway plays critical roles in the homeostasis of HSCs, which reside within hypoxic regions of the bone marrow [36]. HIF pathway itself is regulated by and regulates a number of dioxygenases [24,37]. For example, isozymes of PHD and factor-inhibiting-HIF (FIH) dioxygenases negatively regulate the HIF pathway in response to increasing oxygen concentration. The apparent K_M values of PHDs and FIH for oxygen are in the range of 65–240 μM depending on the substrate and enzyme used [38–40]. Since the K_M values of PHDs and FIH for oxygen are higher than the observed physiological oxygen concentration of 30–60 μM, these enzymes are the cellular oxygen sensors. Further, we and others have shown that hypoxia-mediated activation of the HIF pathway leading to the induction of iron(II), 2OG-dependent KDMs is a conserved process [37,41]. Recent studies have shown that the apparent K_M of Jmjd2 family KDMs for oxygen ranges between 50 and 200 μM, suggesting that KDMs may also function as cellular oxygen sensors [42]. Since dioxygenases, including TETs, have an absolute requirement of oxygen, it will be interesting to see if oxygen availability plays a role in the regulation of TET2 activity during hematopoiesis. Our unpublished data indicates differential expression of TET isoforms under hypoxic conditions in cell lineages from diverse origin. However, it is not known if TET isoforms are targets of the HIF pathway. Finally, a recent study has shown that in some hypoxic cells 2HG accumulates even in the presence of wild-type IDH1/2 [43]. Although the amount of 2HG in these cells are much lower than that is observed in IDH-1/2 mutated AML and glioblastoma patients, whether these levels of 2HG are enough to inhibit TET2 activity, and thereby, keep HSCs undifferentiated under hypoxic conditions remains to be seen.

Acknowledgments

MM is supported by funds from AA & MDS International Foundation. JPM is supported by the National Institutes of Health (grants RO1HL-082983, U54 RR019391, and K24 HL-077522), AA & MDS International Foundation, and the Robert Duggan Charitable Fund.

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