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Oxidative Demethylation of DNA and RNA Mediated by Non-Heme Iron-Dependent Dioxygenases

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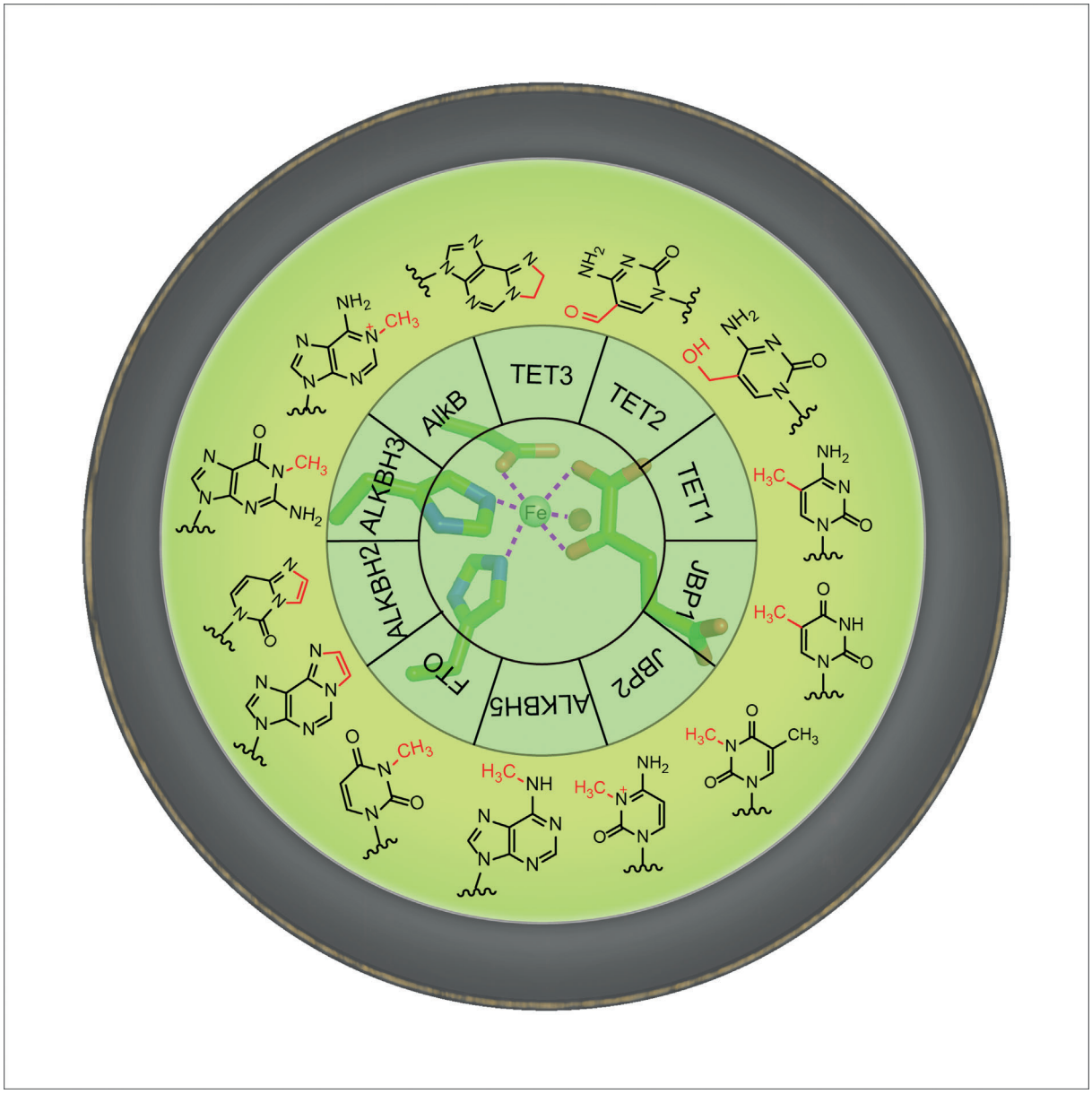

Abstract: DNA/RNA methylation can be generated by methyltransferases and thus plays a critical role in regulating cellular processes; alternatively, nucleic acid methylation can be produced by methylation agents and is cytotoxic/mutagenic if left unrepaired. Oxidative demethylation mediated by non-heme iron-dependent dioxygenases is an efficient way to reverse either the cellular roles of regulatory methylation or the cytotoxic/mutagenic effects of methylation damage. In this Focus Review we summarize recent advances in the study of nucleic acid dioxygenases exemplified by the TET and AlkB family proteins, with an emphasis on chemical insights from the recent literature. Comparison of the chemical mechanisms of these dioxygenases revealed that differences in the mechanism also contribute significantly to their distinct biological functions.

Keywords: demethylases · dioxygenases · DNA methylation · DNA/RNA repair · epigenetics · non-heme iron

Introduction

DNA/RNA methylation is unarguably one of the most important chemical signals in biology. Eukaryotic DNA methylation—namely 5-methylcytosine (5mC)—imposed by DNA methyltransferases, controls the fate of cells without altering the DNA sequence.[1] Enzyme-catalyzed RNA methylation, for instance, N^6-methyladenosine (m^6A) in the messenger RNA (mRNA) of eukaryotic cells, may also function as an "epigenetic marker" by determining the fate of mRNA.[2] In addition to enzyme-mediated modification, DNA/RNA methylation can also be produced by endogenous and/or exogenous alkylating agents, and hence is termed methylation damage/lesion. Such aberrant methylation can cause replication errors, which could be tumorigenic; it may also block replication or transcription, thereby resulting in cell death.[3]

Interestingly, both regulatory and aberrant methylation can be oxidatively reversed by a non-heme iron-dependent dioxygenase superfamily.[4] *Escherichia coli* AlkB is the first such dioxygenase that has been shown to carry out oxidative demethylation reactions on nucleic acids. It was first identified by isolation of an *E. coli* mutant sensitive to MMS (methyl methane sulfonate, an S_N2-type methylation agent) but not to UV light.[5] AlkB effectively defences against the cytotoxic effects of S_N2-type alkylating agents; thus it was hypothesized to protect bacteria from the lethal effects of alkylation damage such as N^1-methyladenosine (m^1A) and N^3-methylcytosine (m^3C).[6] Bioinformatics studies placed AlkB into the Fe^{II}- and α-ketoglutarate (α-KG)-dependent dioxygenase family, in which Fe^{II} and α-KG are utilized to activate dioxygen and perform various biochemical oxidation reactions.[7] One year later, two groups independently reported the oxidative demethylation activities of AlkB and hence opened up the field of oxidative nucleic acid demethylation.[7,8]

AlkB is conserved from bacteria to humans, and nine human homologues of AlkB (ALKBH1-8, and FTO) have been identified so far.[7,9] ALKBH2 and ALKBH3 are repair proteins (like AlkB) that demethylate a similar spectrum of methylated DNA damage; the nucleus-localized ALKBH2 accumulates in the nucleoli but is present diffusely throughout the nucleoplasm, whereas ALKBH3 is found diffusely in the nucleus and is present to a lesser extent in the cytoplasm.[10] The *FTO* (fat-mass and obesity-associated) gene was found to be associated with the body mass index (BMI) by a genome-wide association study;[11] subsequently, it was shown to encode an Fe^{II}- and α-KG-dependent dioxygenase.[9c] FTO displays weak activities towards N^3-methyluracil (m^3U) in RNA and N^3-methylthymine (m^3T) in DNA,[12] and it was found recently to demethylate m^6A with high efficiency.[13] Interestingly, FTO also partially colocalizes with nuclear speckles, thus supporting the notion that m^6A in nuclear RNA is a major physiological substrate of FTO.[13] ALKBH5 is the second mammalian RNA demethylase that erases the m^6A modification in mRNA; it also localizes to the nucleus and its demethylation activity affects nuclear RNA export and metabolism, gene expression, and mouse fertility.[14] Additionally, ALKBH5 is a direct transcriptional target of hypoxia-inducible factor-1 and is induced by hypoxia in a range of cell types.[14b] ALKBH1, localizing mainly to the nucleus,[15] was first shown to demethylate m^3C in vitro.[16] Later on, its histone H2A demethylase activity[15] and AP-lyase activity[17] were reported, of which the latter was shown to be independent of Fe^{II} or α-KG.[17a] ALKBH4 was found through yeast two-hybrid screens to bind proteins associated with chromatin and/or to be involved in transcription; yet it was found both in the nucleus and in the cytoplasm.[18] Recently ALKBH4 was found to demethylate K84me1 of actin, thereby regulating actomyosin function.[19] ALKBH8, localizing mainly to the cytoplasm,[20] is a tRNA hypermodification enzyme that is required for the biogenesis of 5-methoxycarbonylmethyluridine (mcm^5U) and (*S*)-6-methyoxycarbonylhydroxymethyluridine ((*S*)-$mchm^5U$).[20,21] ALKBH7 is nuclear-encoded but imported into mitochondria; it is essential for pro-

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grammed necrosis induced by alkylation and oxidation,[22] and deletion of mouse ALKBH7 results in increased body weight.[23] The biological functions of ALKBH6 remain unclear at this moment. In this Focus Review, we mainly focus on the DNA/RNA demethylases AlkB, ALKBH2, ALKBH3, ALKBH5, and FTO.

In recent years, another family of Fe^{II}- and α-KG-dependent dioxygenases called the ten-eleven translocation (TET)/J-binding protein (JBP) family proteins (TET1, TET2, and TET3; JBP1 and JBP2) have also caught the interest of scientists. Based on their similarity to the AlkB family proteins,[24] JBP1/2 were shown to be the thymidine hydroxylases (THs) in the thymine oxidation step of β-D-glucosyl-hydroxymethyluracil (base J) biogenesis in kinetoplastid flagellates like *Trypanosoma brucei*,[25] which causes sleeping sickness in Africa. Subsequent bioinformatics screens of JBP homologs led to the prediction that TET proteins are Fe^{II}- and α-KG dependent dioxygenases.[26] Actually, *TET1* had already been discovered as a fusion gene in rare cases of acute myeloid leukemia (AML).[27] In 2009, two groups reported the presence of 5-hydroxymethylcytosine (5hmC) in the mammalian genome;[28] in one of these studies, Rao and colleagues also reported the major breakthrough that the TET1 protein is capable of oxidizing 5mC to 5hmC,[28b] opening up the possibility of active demethylation mediated by oxidation. In a subsequent work, 5hmC was shown to be particularly prevalent in the DNA from the central nervous system;[29a] yet it was found to be strongly reduced in brain tumor tissues.[29b,c] Despite of discovery of 5hmC as an oxidation product of 5mC, initial attempts to identify further oxidation products of 5hmC, namely 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), were unsuccessful when studies were carried out using brain tissues.[30] In continuation of the work, Carell and colleagues first demonstrated the existence of 5fC in embryonic stem cell DNA.[31] Later in 2011, two studies simultaneously showed that TET proteins can oxidize 5mC further to 5fC and 5caC.[32] With the help of the DNA glycosylase TDG that excises 5fC and 5caC from DNA,[32b,33] a complete active demethylation pathway was thus established in mam-

Abstract in Chinese:

DNA/RNA 甲基化可以由甲基转移酶催化产生并在调节细胞进程中起到重要作用；也可以由内源或外源的甲基化试剂造成，如不及时修复将产生细胞毒性或突变损伤。由非血红素-铁依赖型双加氧酶催化的氧化去甲基化过程是细胞内维护甲基化平衡的有效途径：去甲基化反应既可以调控甲基化的细胞功能，又可以抵抗甲基化造成的细胞毒性或突变损伤。本综述主要从氧化去甲基化的化学机理出发，介绍两类作用于核酸的双加氧酶家族：TET 家族蛋白和 AlkB 家族蛋白。我们比较了各个成员在化学机理上的差异，以期更好地理解这些酶的不同生物学功能。

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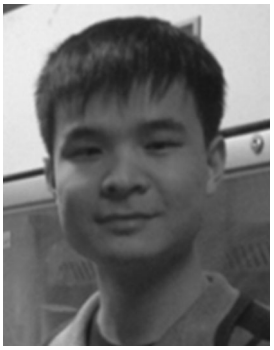

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malian cells. Whether an alternative demethylation pathway exists remains to be investigated.[34]

As the biological aspects of the AlkB and TET/JBP family dioxygenases have been extensively reviewed,[2a,3a,4a,b,d,25a,35] this review focuses primarily on the chemical mechanisms of oxidation reactions mediated by these dioxygenases. Specifically, we will discuss the metal binding sites and dioxygen activation strategy, compare substrate recognition and oxidation reactions, and survey the possibility of inhibition or enhancement of this activity using small molecules.

1. The 2-His-1-carboxylate Facial Triad and the Mononuclear Non-Heme Fe^{II} Site

Comprehensive studies, including extensive crystallographic experiments, have been performed over the past 20 years in order to advance our understanding of the metal binding site of mononuclear non-heme Fe^{II} enzymes.[36] The Fe^{II} is ligated by a conserved "two His and one Asp/Glu" structural motif in the center of these enzymes. This HxD/E···H coordination motif, constituting one face of an octahedron is referred to as the 2-His-1-carboxylate facial triad.[37] To constitute the remaining face of an octahedron, a subset of these enzymes utilize α-KG as a cofactor, and therefore these dioxygenases are both Fe^{II}- and α-KG-dependent. α-KG occupies two of the three remaining octahedral coordination sites in a bidentate manner opposite to the triad face. The last site requires a dioxygen molecule in order to catalyze oxidative reactions that incorporate one oxygen atom into their substrates, which are methylated DNA/RNA in the case of TET proteins and several AlkB members. Observations of the detailed geometry of the metal cores were enabled by X-ray crystallography for AlkB, ALKBH2, ALKBH3, FTO, ALKBH5, ALKBH8, NgTET1, and TET2.[38] Since previous studies have shown that Co^{II} or Mn^{II} can be used to replace Fe^{II} without affecting the geometry of the octahedral coordination,[38a] many of such structures were solved with a Co^{II} or Mn^{II} ion in the active site. Representative metal-coordinating geometries determined by crystallography are presented in Figure 1.

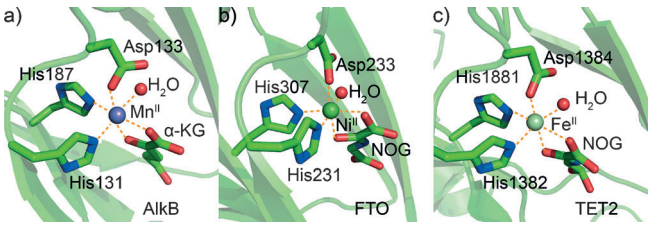


Figure 1. The 2-His-1-carboxylate facial triads conserved in Fe^{II}- and α-KG-dependent dioxygenases. The octahedral coordination of the divalent metal is occupied by two His residues and one Asp residue, and α-KG (or NOG, *N*-oxalylglycine). The crystallographic water is replaced by an O_2 molecule during initiation of the oxidation reactions. PDB accession numbers: AlkB 2FDH, FTO 4IDZ, and TET2 4M6.

In addition to crystallographic evidence, spectroscopic studies have also provided valuable insights into the metal binding site of these dioxygenases. *E.coli* AlkB, the prototype of these nucleic acid dioxygenase, is the best characterized member:[8,10b,38b,e,g–l,39] Matthews et al. succeeded in the first preparation of a Cu^{II}-reconstituted form of AlkB in various complexes; using advanced electron paramagnetic resonance (EPR) spectroscopy, they classified the Cu^{II} site as a type 2 site, which also allows AlkB to fold correctly.[40] They further showed, using nuclear magnetic resonance spectroscopy, that α-KG functions in maintaining a fully folded conformation of AlkB by keeping the necessary interactions between the large β-sheet and the Fe^{II} site.[41] Oxidative decarboxylation of α-KG to succinate induces an enhanced dynamic state, which allows for the replenishment of α-KG and probably for an effective release of demethylated DNA.[42] Recently, Matthews and colleagues reported a comprehensive structural analysis using NMR, fluorescence, and CD spectroscopies: they demonstrated that different folding states exist for AlkB upon binding to α-KG, Fe^{II}, and the substrate. These findings provide the first insights into the protein folding of AlkB.[43] While recombinant AlkB with added metal ions were used in most studies, He and coworkers successfully isolated the native Fe^{II}-AlkB (also with a bound α-KG) directly from *E. coli*. The characteristic absorption peak at 560 nm proved the existence of a bidentate α-KG bound to an Fe^{II}; a 9 nm shift occurred when excess amounts of single-stranded DNA (ssDNA) was added, thus indicating that a geometry change of the active site is induced by DNA binding.[44] Without binding of substrate, AlkB processes an aberrant reaction to the side chain of its endogenous amino acid residue. Hausinger et al. reported that a blue chromophore was generated by the coordination of Fe^{III} with a hydroxytryptophan at position 178 of the AlkB protein.[45] Cui et al. used a simple +2 point charge to replace the non-heme Fe^{II} site in their computational study of the AlkB family proteins and also achieved a satisfactory description of the structural properties of the Fe^{II} site in AlkB enzymes.[46] Andersson et al. also applied EPR and UV/Vis spectroscopies to study ALKBH4 and confirmed that it is Fe^{II}/α-KG-dependent.[47]

In comparison with AlkB, the metal binding sites of other AlkB family dioxygenases are relatively less characterized. One may argue that many of the features revealed from the studies of AlkB are perhaps shared by other members within the AlkB family. Yet, considerable differences exist, both in terms of the primary amino acid sequences around the metal and substrate binding sites and the three-dimensional folding and organization of these closely related homologue proteins. Concerning the TET family proteins, characterizations of their metal sites are even scarcer, presumably due to the difficulty to express these proteins (full-length TET proteins are normally large in length, with about 1600–2100 amino acids). Very recently, the structures of a catalytically competent truncation of TET2 and a Naegleria Tet-like dioxygenase have been solved.[38k,l] Compared to the AlkB family proteins, major differences have already

been revealed from these structures. Hence, such soluble truncations will provide great opportunities for future investigations of their metal binding sites.

2. Diverse DNA/RNA Substrates Recognized by Dioxygenases

AlkB has been shown to be a very promiscuous enzyme. m^1A and m^3C in both single- and double-stranded DNA are AlkB substrates.[8] In addition, alkylated RNA lesions were also shown to be reversed by AlkB soon after, hence becoming the first demonstration of RNA repair.[10b,48] Moreover, AlkB can repair N^1-methylguanine (m^1G) and m^3T with lower activity,[49] as well as exocyclic adducts 1,N^6-ethenoadenine (εA), 1,N^6-ehanoadenine, and 3,N^4-ethenocytosine (εC).[50] Its human homologue ALKBH2 is a primary housekeeping enzyme repairing endogenous m^1A lesions in double-stranded DNA in mammalian cells.[51] Furthermore, ALKBH2 can also demethylate m^3C[51] as well as reverse etheno adducts εA[52] and εC.[53] ALKBH3 has also been shown to directly repair m^1A and m^3C in both DNA and RNA, and εA in DNA.[10a,b,53] FTO was first shown to demethylate m^3T in ssDNA and m^3U in ssRNA; subsequently, this enzyme was reported to demethylate m^6A in RNA with much higher activity.[13] As for ALKBH5, only m^6A demethylation activity has been reported.[14a] Chemical structures of the substrates of AlkB family proteins are presented in Figure 2.

Several key residues in the active site of these enzymes are important for their substrate specificity. In AlkB, for example, His131 and Trp69 stack with the flipped base, and a hydrogen-bond is formed between Asp135 and the N^6-amino group of the positively charged m^1A (Figure 3a).[38a–e] ALKBH2 is another AlkB member whose structure with bound substrates has been reported.[38b,f] In comparison with AlkB, the binding of m^1A by ALKBH2 seems tighter: besides similar stacking interactions formed by side chains of His171 and Phe124, both the N^6- and N^7-positions of m^1A are recognized with specific hydrogen bonds (Figure 3b). The structure of catalytically active ALKBH3 has been solved;[38g] however, an ALKBH3 structure with a bound substrate is needed in order to allow examinations of base recognition. The structure of FTO with an m^3T mononucleotide has also been reported;[38h] yet a complex structure with its best substrate m^6A in the context of an oligo RNA would be highly desired. Very recently, two independent groups reported the protein structure of ALKBH5;[38i,j] again, protein/RNA complexes would be necessary to reveal the binding mode of m^6A in the active site.

Despite extensive crystallographic studies on the AlkB family demethylases (particularly AlkB and ALKBH2), the mechanism of selective substrate recognition remains poorly understood. We recently showed that the divergent amino acid sequences around the active sites of these demethylases are critical for determining the substrate specificity of AlkB, ALKBH5, and FTO.[54] More specifically, we discovered that the flexible loops (135 to 139 in AlkB, corresponding to 175–180 in ALKBH2, 209–241 in ALKBH5, and 235–240 in FTO) within these demethylases contribute to the different recognition of methylated substrates; by exchanging just one or two key amino acids within these loops, we were able to partially switch the demethylation preference of AlkB, FTO, and ALKBH5. Further investigations combining both X-ray crystallography and enzyme kinetics revealed that the divergent amino acid compositions within the loop sequences affect mainly substrate binding rather than causing a dramatic change in the catalytic ability of the demethylases. Therefore, such divergent amino acid sequences not only present an elegant solution to achieve selective substrate recognition by these closely related homologue proteins but also offer opportunities for the design of selective inhibitors

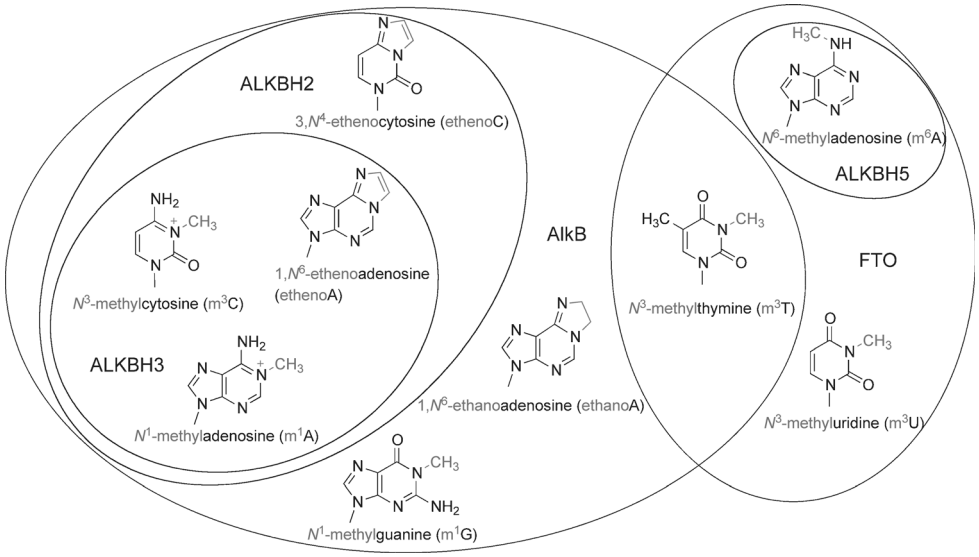


Figure 2. Substrate diversity of AlkB family proteins. The methylated (or alkylated) bases in each circle are recognized by each corresponding protein.

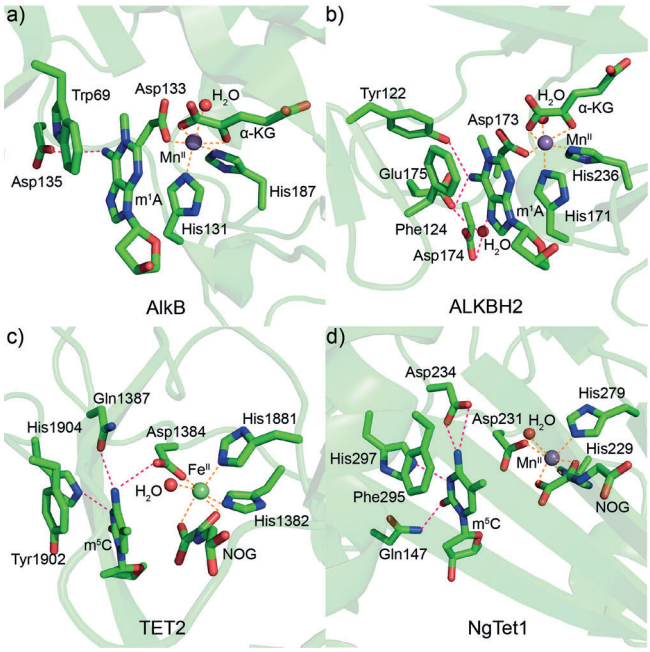
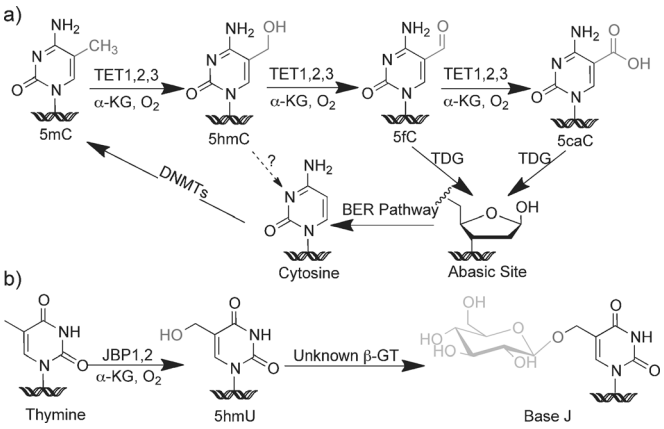


Figure 3. Substrate recognition by: a) AlkB (PDB 3BIE), b) ALKBH2 (PDB 3BUC), c) hTET2 (PDB 4M6), and d) NgTet1 (PDB 4LT5). The hydrogen bonds formed between the base and the side-chain of the protein are indicated by magenta dashed lines, and the coordination bonds formed with the metal center are indicated by orange dashed lines.

that are capable of discriminating between these demethylases.

In difference to the AlkB family proteins which catalyze the oxidation of *N*-alkylation of DNA and RNA, TET/JBP family dioxygenases are responsible for oxidizing the *C*-methyl group at the 5′ position of 5 mC or thymine. The TET proteins are capable of oxidizing 5mC to 5hmC, 5fC, and 5caC (Scheme 1 a).[32] Recent structural studies of human TET2 (hTET2, Figure 3 c)[38l] and of the *Naegleria gruberi* TET1 homologue (NgTET1, Figure 3 d)[38k] with 5mC-containing DNA revealed that TET proteins recognize CpG-containing DNA from the minor groove with a 5′-methyl group-independent oxidation model. Similar to the AlkB family, 5mC is flipped into the catalytic core, with the methyl group headed into the Fe^{II} catalytic center (Figure 3 c and 3 d). Notably, the 5′-methyl group of 5mC occupies a somewhat vacant spot in the active site of both structures, consistent with the fact that TET proteins can also oxidize 5hmC and 5fC. The hTET2 and NgTET1 structures both reveal that the Watson–Crick base pair edge of 5mC in TET is stabilized by nearby amino acids: the 3-nitrogen atom of 5mC forms a hydrogen bond with His (hTET2 or NgTET1), while the 4-amino group is recognized by Asn (hTET2, Figure 3 c) or Asp (NgTET1) (Figure 3 d). Together, these data explain the selectivity of TET proteins towards cytosine over thymine.

JBP1 and JBP2 oxidize thymine to 5hmU in repeat sequences like telomeres;[55] 5hmU is then modified with a glucose by an unknown β-glucosyltransferase (β-GT) to generate the base J (Scheme 1 b). JBP1 but not JBP2 contains a J-binding domain, which selectively binds to J-containing DNA. So far, only one crystallographic study on JBP proteins has been reported,[56] and preliminary results suggest that the JBP1 DNA-binding domain selectively binds to J-containing DNA but not to normal DNA containing thymine, with an about 10000-fold preference. The strong affinity of the J-binding domain offers a glimpse into its selectivity, which potentially is largely determined by the 5′ glucose moiety. Interestingly, JBP1 has been used to selectively pull down glucose-labeled 5hmC DNA fragments as a genomic distribution profiling method for 5hmC,[57] further suggesting its feasibility of selective substrate binding.

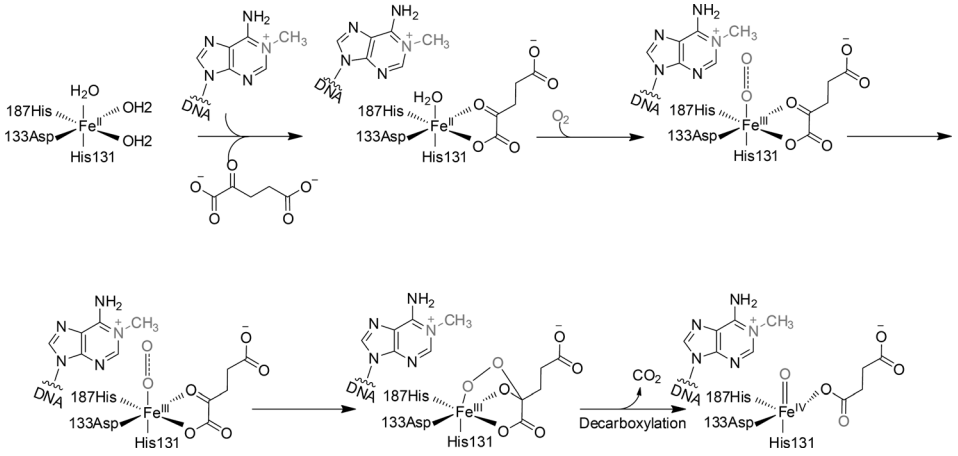


Scheme 1. Substrates of TET and JBP proteins and related biological pathways. a) 5mC can be oxidized to 5hmC and then to 5fC and 5caC, both of which can be glycosylated by TDG protein in a BER pathway; 5hmC might be deaminated to 5hmU to enter the BER pathway of TDG or SMUG; however, the reported results were contradictory.[34a,b,d] b) JBP1 and JBP2 oxidize thymine to 5hmU, which is the precursor of the base J.

3. Oxidative Demethylation: Reductive Activation of Dioxygen

Dioxygen has a high potential reactivity held in check by its molecular structure and thus it is an attractive reagent for use in biochemical reactions. The triplet ground state of dioxygen with the presence of two unpaired electrons in degenerate molecular orbitals makes it impossible to undergo a direct reaction with the most potential reaction partners, singlet molecules.[58] Making dioxygen react at ambient temperature while retaining high specificity is very important for biological systems. In fact, dioxygen activation has been previously studied for many other oxygenases: Lipscomb et al. proposed the catalytic mechanism of protocatechuate 4,5-dioxygenase, which catalyzes the cleavage of O_2 and, in turn, inserts both oxygen atoms into the ring of its aromatic substrates.[59] Andersson et al. proposed a mechanism of dioxygen activation for deacetoxycephalosporin C synthase (DAOCS), a Fe^{II}- and α-KG dependent dioxygenase.[60] AlkB family proteins share a similar mechanism with other enzymes within the non-heme iron protein family.[4c] In the

presence of both methylated substrate and α-KG, dioxygen binds to the enzyme and replaces the water that coordinates to the Fe^{II} core, thereby forming the initial Fe^{III}-$[O_2]^-$ species (Scheme 2).[35] The superoxide radical anion in turn attacks the α-keto carbon of α-KG, and one oxygen atom from the dioxygen cofactor is incorporated into α-KG. The co-substrate α-KG then releases one molecule of carbon dioxide and decomposes to succinic acid. The dioxygen is activated as a putative high-valent Fe^{IV}=O intermediate (Scheme 2).[4c,36a,37b,61]



Scheme 2. Proposed mechanism of the reductive activation of dioxygen by AlkB.

While direct observations of such reactive intermediates remain experimentally challenging, results from several theoretical studies have provided support to the dioxygen activation mechanism mentioned above for AlkB. A DFT study by Liu and co-workers indicated that dioxygen activation through formation of an Fe^{IV}=O intermediate is thermodynamically preferred over activation through formation of an Fe^{III}–O· intermediate.[62] Besides, an initial isomerization of the Fe^{IV}=O intermediate in the catalytic cycle is needed to promote the subsequent rate-limiting hydrogen-atom abstraction step. Visser et al. used quantum mechanics/molecular mechanics (QM/MM) to study the hydroxylation step of m^1A demethylation by AlkB and found that the isomerization is assisted by an Arg residue in the substrate binding pocket, which in turn deduces the distance between the $Fe^{IV}=O$ and the methyl group of the substrate DNA.[63] The transition state between dioxygen activation and hydrogen-atom abstraction has also been studied: Cisneros et al. used hybrid ab initio QM/MM methods to study this step in AlkB: Fe^{III}–O· with an intermediate-spin Fe ($S=3/2$) ferromagnetically coupled to the oxyl radical was found; two conserved residues (in AlkB, ALKBH2, and ALKBH3), Y76 and T51, provide electrostatic stabilization of the transition states in this reaction step.[64] A similar mechanism might be shared by other AlkB family proteins, but direct evidence has yet to be provided.

4. Oxidative Demethylation: Oxidation of Substrates

4.1. AlkB, ALKBH2, and ALKBH3: one cycle of oxidation

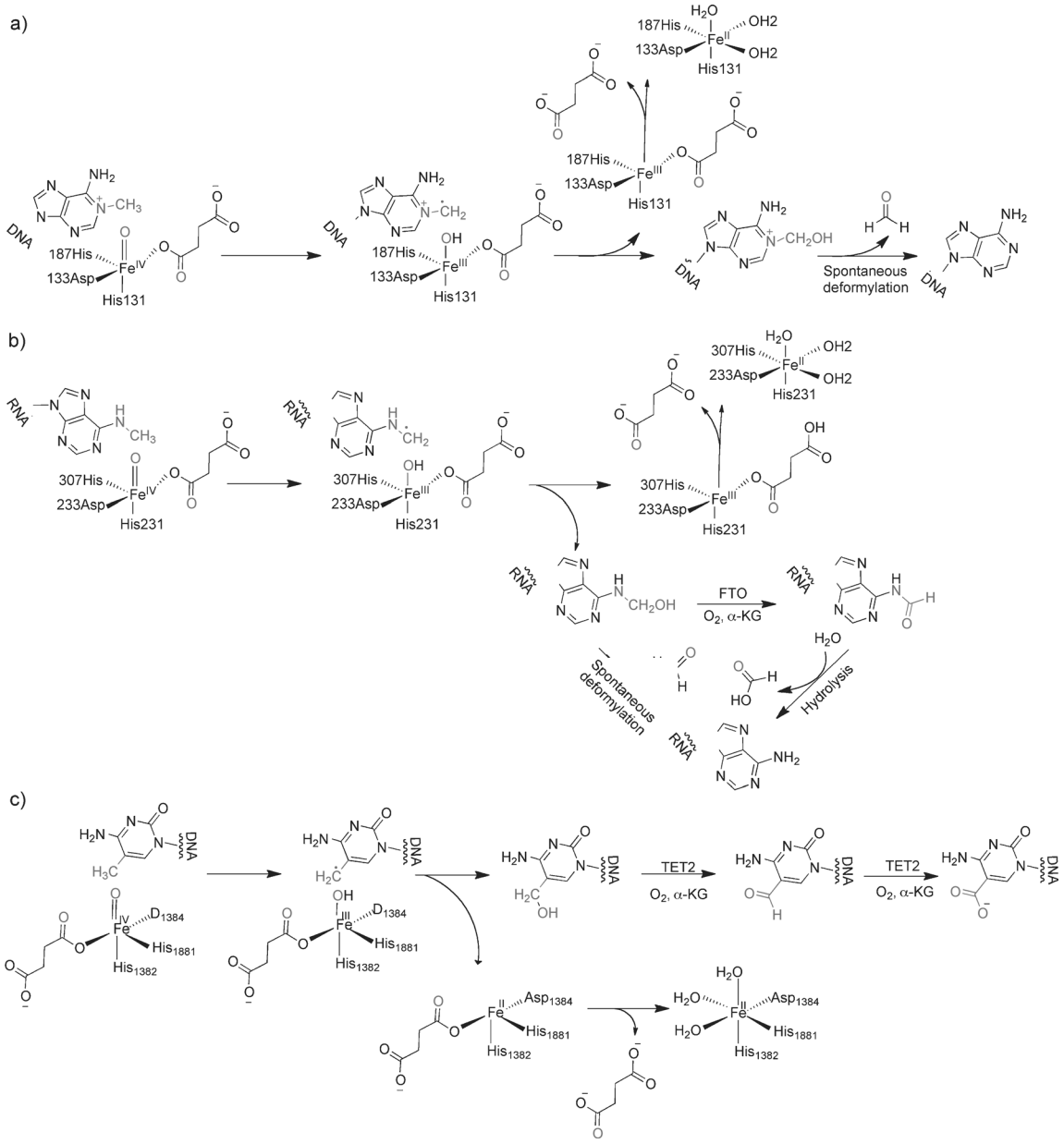
After reductive activation of dioxygen, the high-energy Fe^{IV}=O intermediate then attacks the substrate by abstraction of a hydrogen atom in the methyl group; and the oxygen atom in the Fe^{IV}=O intermediate now becomes the oxygen atom in the hydroxylated product (Scheme 3). In the case of AlkB, hydroxylation at the methyl group on the N^1-position of m^1A or the N^3-position of m^3C is thought to be followed immediately by heterocleavage of the C–N bond, thus leading to spontaneous deformylation, which gives the unmodified base and formaldehyde. A new round of oxidative demethylation will not occur until α-KG and methylated substrates are replenished (Scheme 3a).[3a,4c,38e]

Through the use of a chemical cross-linking strategy and computational analysis, He et al. first reported a direct observation of several unprecedented oxidation demethylation intermediates.[38e] Their strategy involved the stabilization and crystallization of anaerobic complexes of AlkB–dsDNA containing εA, m^3T, and m^3C, respectively, all of which are AlkB substrates. They then exposed these crystals to air in order to initiate in crystallo oxidation reactions. A glycol (from εA), a hemiaminal (from m^3T), and a zwitterionic intermediate (from m^3C) were captured, respectively. This study also depicts a general mechanistic view of how a methyl group can be oxidatively removed from different biological substrates.

4.2. FTO: from m^6A to hm^6A, f^6A, and A

FTO oxidizes its substrates in a slightly different way compared to AlkB. After hydroxylation of the methyl group at the exocyclic N^6-position to give N^6-hydroxymethyladenosine (hm^6A), spontaneous deformylation of hm^6A may occur, yielding a regular adenosine. Interestingly, FTO can potentially recognize hm^6A and process a second round of oxidation to yield N^6-formyladenosine (f^6A). Both *meta*-stable bases (with an estimated half-life of about 3 h) hm^6A and f^6A could be released from the enzyme as an alternative pathway. Hydrolysis of f^6A occurs under physiological conditions and yields regular adenosine as well (Scheme 3b).[65]

m^6A in DNA can also be oxidized to hm^6A as an oxidation intermediate in vitro by AlkB, but no further oxidation product was observed.[66] Thus, the second oxidation process of the *meta*-stable hm^6A to form an unprecedented f^6A is



Scheme 3. Proposed mechanisms of substrates oxidation for a) AlkB, b) FTO, and c) TET2. The metal-bound succinate is a result of the dioxygen activation process presented in Scheme 2.

a unique activity of FTO.[65] ALKBH5 is the second enzyme that can process m^6A in RNA; it appears, though, that hm^6A and f^6A are not generated by ALKBH5-mediated oxidation demethylation.[38j]

In eukaryotes, m^6A is crucial to cell survival and development.[67] One mechanism could be that molecular interactions and structures of RNA can be affected by RNA modifications. For instance, the binding affinity of YTHDF2 (a known m^6A-containing RNA-binding protein[68]) to hm^6A and f^6A is reduced to a similar level to that of regular adenosines, thus indicating that these two RNA modifications might have different cellular roles as compared to m^6A. Consistent with this hypothesis, their intrinsic degradation kinetics indicate that they might serve as markers for nascent RNAs. These new modifications perhaps provide further dynamical tuning of the function and status of mRNA.[65]

4.3. TETs: iterative rounds of substrate oxidation

The substrate oxidation process is also different for the TET proteins in that as many as three rounds of iterative oxidation reactions can occur. Compared to the oxidation products of the AlkB family proteins, the 5mC oxidation derivatives are much more stable chemically.[69] For instance, oxidation intermediates of AlkB were observed in crystals;[38e]

hm^{6}A and f^{6}A[65] from m^{6}A oxidation by FTO could be observed with freshly digested nucleotides.[65] The stability of the C–C bond in *C*-methyl, *C*-hydroxymethyl, *C*-formyl, or *C*-carboxyl groups is believed to be the primary reason.[69]

Both 5fC and 5caC are substrates of TDG,[32b,33,70] which is a DNA glycosylase with a relatively broad substrate specificity. Compared to the methyl group of 5mC and the hydroxylmethyl group of 5hmC, the strong electron-withdrawing effect of the formyl and carboxyl group may weaken the glycosidic bonds of 5fC and 5caC. Early in 2006, Drohat and co-workers already reported the excision specificity of TDG by systematically comparing the glycosylase activity of TDG towards a series of 5-position-modified uracil and cytosine derivatives. The study showed that the substrate specificity of TDG depends on the stability of the *N*-glycosidic bond of the nucleosides.[71] Such valuable chemical insights also led to the hallmark discovery by the same group in 2011 that TDG can excise 5fC and 5caC from DNA.[33] Together with the major discoveries made independently by Zhang et al. and Xu et al.,[32] these important findings finally complete the studies on the active demethylation pathway in mammalian cells. Recently, Drohat, Lee and colleagues further showed in an elegant study that TDG uses divergent mechanisms to enzymatically remove 5fC and 5caC: the activity on 5fC was found to be independent of pH, while 5caC excision was observed to be acid-catalyzed.[72] In addition, they also found a mutant TDG (N191A-TDG) that is inactive towards 5caC while retaining full excision activity towards 5fC. This interesting mutant will be useful for studying DNA demethylation in cells.

Although 5fC and 5caC can be enzymatically removed by TDG, all the oxidation products of 5 mC produced by TETs are chemically stable. Such chemical stability also suggests that these 5 mC oxidation derivatives could have potential biological roles in epigenetic regulation, instead of being just demethylation intermediates. For example, 5 hmC contributes to up to around 40 % of total 5 mC,[4a,b,d,28] thus suggesting that 5 hmC may act as a very important epigenetic marker.[2a,73] Studies have shown that 5 hmC-marked genes may recruit specific "reader" proteins to function in the dynamic regulation of downstream genes.[74] In addition, results from the group of Wang suggest that such modified cytosines may reduce the rate and fidelity of RNA polymerase II.[75] Vermeulen, Carell and colleagues reported the "reader" proteins of 5 mC oxidation derivatives.[76] Taken together, these findings suggest that the unique iterative oxidation reactions catalyzed by TET proteins have a profound impact on biological processes.

5. Inhibition and Activation of Nucleic Acid Demethylases

Considering the biological importance of AlkB and TET/JBP family proteins, small molecules that could function to modulate their oxidative demethylation activities are also of particular interest. In fact, developing small-molecule inhibitors for Fe^{II}- and α-KG-dependent dioxygenases has been a major effort in the scientific community.[77] The largest group of inhibitors are α-KG mimics: natural molecules including 2-hydroxyglutarate (2-HG), fumarate, and succinate can all act as antagonists of α-KG (Figure 4).[78] Accumulation of these small molecules will lead to widespread inhibition of TETs[78b,c,79] and histone demethylases,[78c,80] thereby causing significant epigenetic changes to host cells. In addition, synthetic molecules like *N*-oxalylglycine (NOG, Figure 4) can also act as an α-KG competitor to prevent oxidation. Systematic screening of *N*-oxalyl derivatives (NOX, Figure 4) facilitates the selective inhibition of various dioxygenases because bulky chemical moieties attached to NOX may fit at the same time into the substrate binding pocket to achieve selective inhibition.[77,78,81] A second group of natural products, namely flavonoids, also inhibit Fe^{II}-dependent dioxygenases non-specifically (Figure 4). Quercetin, a flavonoid compound, was reported to inhibit AlkB through metal chelation.[78a] Recently, Yang et al. reported a series of FTO inhibitors, among which rhein showed good selectivity.[82] Schofield and colleagues also reported inhibitors of FTO; interestingly, some compounds seem to function by acting as substrate competitors, which is different from classical α-KG mimics or iron chelators.[83] As we have shown in our recent study, the active sites of AlkB family proteins have noticeable differences, which contribute to their substrate specificity.[54] Such information could also aid the design of selective competitive inhibitors that target the active sites.

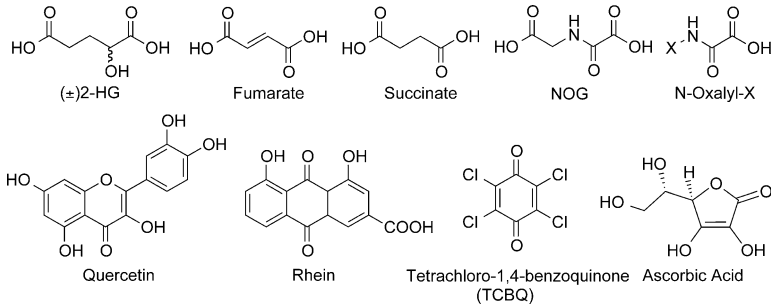


Figure 4. Representative inhibitors and activators of discussed dioxygenases. α-KG analogues include 2-HG, fumarate, succinate, NOG, and NOG derivatives, and natural products like quercetin and rhein. TCBQ and ascorbic acid are the reported activators of TET proteins.

Small molecules that can enhance the enzymatic activity of these dioxygenases have also been reported. For example, redox-active quinones have been shown to induce changes in the methylation status on a genome-wide scale (Figure 4); such chemical-mediated enhancement in demethylation activities is both iron-regulated and Tet-dependent.[84] At pres-

ent, ascorbic acid (vitamin C) seems to be the small molecule that enhances the enzymatic activity of TETs the most, thus promoting the generation of 5 hmC and leading to significant epigenetic changes.[85] Different from AlkB,[78a] such an enhancing effect cannot be achieved with other reducing agents like glutathione,[85b,c] thus suggesting that ascorbic acid acts more than just as a reducing agent for iron. More recent results indicate that ascorbic acid may be involved in somatic cell reprogramming,[86] but the detailed mechanism awaits further investigations.

Conclusions

In summary, we reviewed recent progress in the study of AlkB and TET/JBP family dioxygenases, mainly from the perspective of the chemical mechanisms used by these dioxygenases. As detailed above, the simple oxidative demethylation chemistry has already made a huge impact on various fields in biology, including DNA damage and repair, DNA epigenetics, and the newly termed field of "RNA epigenetics".[2b,87] These exciting findings have allowed to glimpse into the mystery of oxidation-based biological regulation involving DNA and RNA; yet many open questions still remain to be answered. We hope that the chemical insights gathered in this Focus Review will facilitate more thrilling discoveries to be made in the near future.

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