1. INTRODUCTION

Methylation is a widely occurring chemical modification in nucleic acids and proteins. Methylating agents, either extracellular or intracellular, can attack vulnerable sites in DNA, which can lead to cytotoxic and/or cancerogenic DNA damages. Methylation also plays critical signaling roles in biology. Using S-adenosylmethionine (SAM) as the most common electrophilic source of methyl groups, various methyltransferases modify DNA, RNA, and proteins to generate different biological methylations that impact gene expression regulation.1,2 Whereas the significance of methylation is widely appreciated, the demethylation process, oxidative demethylation in particular, has received much recent attention due in large part to its cellular regulatory functions. Demethylation, together with methylation, continuously sculpts the methylomes of biomolecules. This review focuses on oxidative demethylation as mediated by a family of mononuclear iron(II)-containing enzymes. The members of this family of enzymes were first discovered as DNA-repair proteins that oxidatively reverse DNA methylation damage. Subsequent research in recent years has revealed much broader and significant roles of these demethylases in controlling gene expression through the demethylation of epigenetic methylations on DNA, RNA, and histones.

N1-Methyladenine (m1A) and N3-methylcytosine (m3C) are major lesions formed in single-stranded DNA (ssDNA) in the presence of SN2-type methylating agents.3,4,6 Methylations in these positions compromise Watson–Crick base pairing during DNA replication, resulting in cytotoxicity.7 Through an unprecedented oxidative demethylation mechanism revealed over 10 years ago, the FeII/α-ketoglutarate-(α-KG-) dependent AlkB family dioxygenases can repair these methylating DNA lesions.8 Since then, human homologues that perform similar repair functions have been identified. Studies of other homologues or proteins belonging to the same general family have uncovered a range of demethylation functions that reverse epigenetic methylations on histones, RNA, and DNA in higher
eukaryotes. These studies have revealed that oxidative demethylation is the primary pathway used to reverse epigenetic methylations in biology.

Methylations in histone proteins are known to be important epigenetic marks that significantly affect gene expression. These post-translational N-methylations occur on the lysines, arginine, and histidine residues of histones and serve as a dynamic control that participates in a wide range of biological development and differentiation processes, as well as cellular response. Decades of effort have shifted the view of histone methylation from a static modification to a dynamic regulatory marker. In recent years, researchers have identified the enzymes responsible for the removal of these histone methylations. The most prevalent class of histone demethylases, the Jumonji C (JmjC) domain-containing histone demethylases (JHDMs), belongs to the FeII/α-KG-dependent dioxygenase family. Containing a conserved JmjC domain, JHDMs adopt a conserved catalytic domain similar to that of the AlkB protein. JHDM proteins catalyze direct removal of histone lysine methylation through the same mechanism of oxidative demethylation as used by the AlkB proteins. The milestone discoveries of histone demethylation indicate that epigenetic methylation marks on other macromolecules could be reversed through the same oxidative demethylation pathway. In this review, we discuss the more recent research advances on oxidative demethylation of RNA and DNA.

As a modification, methylation is widely present in RNA and is thought to fine-tune the structure and function of mature RNA. A significant amount of methylation is present on the nitrogen atoms of bases, such as N7-methylguanosine in messenger RNA (mRNA), N6-methyladenosine in transfer RNA (tRNA), and N2′-methylguanosine in messenger RNA (mRNA). Our work has proposed that RNA modifications can be oxidatively reversed; we also propose that RNA modifications might serve functional roles in gene expression regulation. Our recent discovery of two RNA N6-methyladenosine (m^6A) demethylases, FTO (fat mass and obesity-associated) and ALKBH5 (AlkB homologue 5) confirmed these hypotheses. These two AlkB-family proteins are capable of demethylating m^6A of RNA both in vitro and in vivo. Yet, they play distinct but indispensable roles in mammals, thus strongly supporting the regulatory significance of such reversible RNA methylation.

DNA methylation is one of the most widely recognized methylations in biological systems. In terms of epigenetic regulation, the nucleotide variant 5-methylcytosine (5mC) has long been established as a landmark modification in mammalian genomic DNA. Recognized as the “fifth base”, 5mC encodes another layer of heritable information on the DNA code. DNA methylation occurs primarily at CpG dinucleotides in vertebrates, but it frequently displays a mosaic methylation pattern in invertebrate animals and plants. In plants (e.g., A. thaliana), the Demeter (DME)/repressor of silencing 1 (ROS) family of 5mC glycosylases functions to remove 5mC through the base-excision-repair (BER) pathway. Although the enzymes that catalyze DNA methylation in mammals have been well characterized, the enzymes responsible for demethylation were unknown until the recent ground-breaking discovery of the TET (ten-eleven translocation) family of mononuclear nonheme Fe^II-dependent dioxygenases. As identified, TET proteins can oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and then 5-carboxylcytosine (5caC) through stepwise oxidations; 5fC and 5caC can be further converted to cytosine through BER, which provides the first biochemically confirmed active demethylation pathway in mammalian cells. The TET protein family includes three members, TET1–TET3, which all adopt the conserved dioxygenase motif similar to that of AlkB to catalyze consecutive oxidations in an Fe^II/α-KG-dependent manner.

We first introduce and review direct oxidative demethylation in DNA damage repair. The discovery of direct RNA demethylation is discussed next. Because 5mC is a form of
carbon methylation, the oxidation and demethylation of 5mC in DNA by the TET proteins are more complex. This review presents the mechanism and functional significance of this unique 5mC oxidation and demethylation. Our goal is to outline the most recent advances and chemical aspects of these biological demethylations.

2. ALKB AND ITS HUMAN HOMOLOGUES

2.1. Oxidative Dealkylation Mediated by AlkB

2.1.1. E. coli AlkB. Endogenous and environmental alkylating chemicals constantly challenge cellular DNAs, resulting in cytotoxic and mutagenic adducts. Accumulation of these alkylation adducts can lead to senescence, cancer, and even cell death. To ensure genomic integrity and the maintenance of proper cellular function, organisms have evolved a variety of housekeeping proteins to efficiently remove alkylation adducts, including DNA glycosylases in base-excision repair, suicidal O6-methylguanine methyltransferases in guanine methylation repair, and AlkB family proteins in direct oxidative repair.7 Although the alkB gene in E. coli was identified in a mutant strain with increased sensitivity to the SN2-type alkylating agent MMS as early as 1983,34 it took researchers two decades to characterize the gene. The gene encodes a protein that belongs to the family of FeII/α-KG-dependent dioxygenases, which has recently emerged as a versatile family of nonheme oxidation enzymes.35

Early evidence suggested that AlkB is capable of protecting cells from lethal effects by repairing MMS-induced DNA damage under alkylation threats,56−58 in a process different from the DNA-repair mechanisms known at the time.59 AlkB protein could be expressed and purified,40,41 however, it was challenging to biochemically determine the activity of AlkB in vitro. Early studies did suggest that AlkB prefers ssDNA and might repair mA and mC to unmethylated bases in DNA through an oxidative demethylation mechanism in the presence of iron(II), α-KG, and dioxygen.59 To date, the substrates of AlkB have been extended to N1-methylguanine (m1G), N3-methylthymine (m3T),43−45 1,N6-Ethenoadenine (EA),46,47−50 3,N4-α-Hydroxypropanocytosine,59 and m6A (Figure 2). Most of these substrates can be classified into three types under physiological pH: positively charged adducts (the most efficient substrates for AlkB), neutral adducts, and cyclic adducts.60 The versatility reveals the capacity of AlkB to operate on a diverse range of substrates. DNA lesions of mA and mC are believed to represent the physiologically relevant substrates for AlkB. AlkB has also been shown to reduce the toxicity of DNA-damaging agents that induce hydroxyethyl, propyl, and hydroxypropyl adducts in bacteria.61

The AlkB protein uses a mononuclear iron(II) center to donate two electrons for the reduction of dioxygen;7,62−65 α-KG serves as a cosubstrate to provide the other two electrons required for the four-electron reduction (Figure 3). This

Figure 2. DNA/RNA lesions that AlkB can repair. Exogenous or endogenous methylating agents can introduce various DNA methylations as shown, which are known substrates of AlkB. Exocyclic DNA adducts of 1,N6-ethenoadenine (EA) and 3,N4-ethenocytosine (εC) are generated by exposure to electrophilic vinyl chloride (VC) metabolites, chloroethylene oxide (CEO), or chloroacetaldehyde (CAA) introduced exogenously or endogenously from lipid peroxidation.54,55 1,N6-Ethenoadenine (EA) is produced by the reaction of adenine with the anticancer agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU).57,58 3,N4-α-Hydroxypropanocytosine is produced by lipid peroxidation.59

Figure 3. View of active-site stereochemistry with ligand coordination [Protein Data Bank (PDB) ID 2FD8]. His131, Asp133, and His187 are ligands to iron.
Catalysis is composed of two phases: the activation of dioxygen and the oxidation of substrates. In the first phase, AlkB incorporates both α-KG and iron(II) to become catalytically ready. The active-site iron(II) then binds and activates a dioxygen molecule that subsequently attacks the bound α-KG. Cleavage of the O—O bond yields a high-valent, high-spin (S = 2) iron(IV)-oxo species and converts α-KG to succinate. It is proposed that the iron(IV)-oxo species then undergoes further oxidation to complete the catalytic cycle.

**Figure 4.** Proposed mechanisms of AlkB-mediated oxidative dealkylation toward (A) mA and 1,N⁶-ethenoadenine (εA) and (B) 1,N⁶-ethanoadenine (EA). The EA demethylation involves two steps of oxidation, one at N¹ and a second at N⁶, as well as further hydrolysis to completely restore the lesion back to adenosine.
conformational reorientation upon the release of CO2 from the active site.64,68–70 The FeIV=O double bond is reoriented to the trans position to His187, which draws the FeIV=O oxygen closer to the target methyl carbon.71 In the second phase of the proposed mechanism, the highly oxidative FeIV=O species could abstract an H atom from the methyl group of the aberrant methyl adduct to yield an FeIII=OH species and a carbon radical. The iron-associated OH can rebound to the carbon radical, yielding a hydroxylated intermediate that dissociates from the active site and subsequently decomposes in water to afford the final demethylated product (Figure 4).

Replacing iron(II) with nickel or other metals eradicates this enzymatic activity.72,73 In the absence of substrates, this reaction has a modest α-KG turnover, and the produced iron(IV)-oxo species can hydroxylate the side chain of Trp178 of AlkB, leading to irreversible modification of the protein itself.75 The α-KG turnover could be significantly stimulated by the addition of methylated DNA, suggesting that binding of the methyl group primes the protein to be catalytically ready.8,9,74 Analogues of α-KG were found to inhibit AlkB with high specificity.74 Fluorescence-based assays have been developed to characterize oxidation kinetics and screen inhibitors of its human homologues.75–79 Because DNA-damage-induced apoptosis is one of the major mechanisms for cytotoxic anticancer drugs, inhibitors of oxidative demethylases might have the potential to improve the efficacy of certain chemotherapies.

With exocyclic DNA adducts such as εA and εC,49,53 epoxide intermediates were observed in AlkB-mediated oxidation through the use of mass spectrometry, thus providing evidence of the direct reversal mechanism. Recently, a chemical cross-linking strategy coupled with in crystallo reaction was employed to stabilize and characterize the hydroxylated intermediates glycol (from εA), hemiaminal (from m3T), and a zwitterionic intermediate (from m3C) (Figure 5).60 These intermediates were generated from representatives of all three types of AlkB substrates, thus confirming the mechanism of oxidative demethylation. In addition, the positively charged zwitterion intermediate from m3C contains a better leaving group than the neutral hemiaminal thymine derived from m3T at physiological pH; therefore, m3C undergoes a decomposition more quickly than m3T to liberate the intact base. This might partially explain the higher repair rate of AlkB to m3C and m1A than to m3T and m1G.60

2.1.3. Substrate Site Specificity. Substrate recognition of AlkB has been investigated by kinetic analyses and crystallographic studies. AlkB preferentially repairs positively charged lesions.52,53 These positively charged substrates could be
favorably positioned in the active-site pocket through interaction with the negatively charged side chain of Asp135 in the enzyme (Figure 6).59 A polynucleotide structure is not essential, but a nucleotide 5' phosphate group is required for AlkB to effectively repair the substrate.61 AlkB creates an electropositive binding groove (Thr51 to Tyr55, Ser129, and Lys127) to anchor the phosphodiester backbone of the substrate. Trp69 and His131 stabilize the base by π-π stacking in the active-site pocket. Tyr78, Lys134, Asp135, and Glu136 coordinate to recognize the in the active-site pocket. Tyr78, Lys134, Asp135, and Glu136

### 2.2. AlkB Homologues

Bioinformatics and functional analyses reveal that AlkB homologues are widely expressed in many organisms, from bacteria to humans, and carry out diverse biological functions.42,95–103 The majority of bacterial AlkB homologues are DNA-repair proteins.100,103 Two AlkB homologues have been found in the genome of fission yeast S. pombe.101,104 One of them, Ofd2, has been characterized as an FeII/α-KG-dependent dioxygenase that interacts with histones. In mammals, nine homologues of AlkB have been identified so far, termed ALKBH1–ALKBH8 and FTO.95,97,105,106 All of them contain a double-stranded β-helix (DSBH) catalytic core that is conserved for this family of dioxygenases,107 including an HXDXnH motif for iron binding, as well as a RXXXXXR motif for the α-KG binding (Figure 7).7,106,108 Among these proteins, ALKBH2 and ALKBH3 are the most similar to AlkB. They function as DNA-repair proteins to protect the genomic integrity of mammalian cells.

#### 2.2.1. ALKBH2

ALKBH2 has been characterized as a bona fide DNA-repair enzyme that guards the mammalian genome. It displays robust repair activity against cytotoxic m1A and m3C in vitro and in vivo,82,109,110 but reduced activity toward m3Ci n dsDNA.43 ALKBH2 also protects the mammalian genome against εa and εC through direct oxidative dealkylation, a process complementary to that of DNA glycosylase, which repairs the same lesions through the BER pathway.13,111,112 Knockdown of ALKBH2 in 239T cells has resulted in globally increased single-stranded and double-stranded DNA breaks, especially among the highly transcribed rRNA genes, thereby suggesting that ALKBH2 is involved in DNA repair in humans.113 In mice, ALKBH2 serves as the primary oxidative demethylase for repairing m1A and m3C lesions in DNA,114 thus protecting the mouse genome when exogenous methylating agents threaten primary mouse embryonic fibroblasts.115 In addition, the homologue of ALKBH2 in A. thaliana also acts as an important enzyme for protecting A. thaliana against DNA methylation damage.116 These results suggest that the homologues of ALKBH2 in other organisms share a similar DNA-repair function.
In the same oxidative dealkylation mechanism, ALKBH2 reverses DNA damage by using an active iron center as AlkB (Figure 3).\textsuperscript{117,118} ALKBH2 prefers to repair damage in duplex DNA over ssDNA, however.\textsuperscript{82,83,92} Crystallographic studies have revealed that, unlike AlkB, ALKBH2 makes extensive contact with both strands of duplex DNA.\textsuperscript{93} Unlike certain glycosylases, ALKBH2 does not contain a damage-checking site.\textsuperscript{119} Rather, ALKBH2 appears to detect damaged bases by probing their base-pair stability.\textsuperscript{120} Consider m\(^1\)A as an example: It primarily adopts a syn conformation to pair with the opposite T in a Hoogsteen base pair, which exhibits lower base-pairing stability compared to the normal A\textsuperscript{−}T base pair.\textsuperscript{121} This reduced stability facilitates the recognition and repair of m\(^1\)A by ALKBH2. Compared to AlkB, ALKBH2 contains a unique short hydrophobic \(\beta\)-hairpin in proximity to the active site; this hairpin is significant to the preference of ALKBH2 for double-stranded DNA substrates (Figure 8A).\textsuperscript{122,123} More specifically, the aromatic finger residue, Phe102, intercalates into the duplex stack to facilitate the base flipping. Phe124,
His171, and Glu175 coordinate with other protein residues to recognize and flip the damaged base.

ALKBH2 is frequently down-regulated in gastric cancer and is also involved in the growth of brain tumor cells, glioblastoma, colorectal cancer, and bladder cancer. Knockdown of ALKBH2 increases the sensitivity of cancer therapies, such as photodynamic therapy (PDT) mediated by Photofrin and chemotherapy with cisplatin. Based on these observations, ALKBH2, together with its repair partners, might serve as a biological marker for cancer monitoring, as well as a potential target for therapy.

2.2.2. ALKBH3. ALKBH3 is a close homologue of ALKBH2 and was identified at the same time as ALKBH2. ALKBH3 demethylates both m\(^1\)A and m\(^3\)C (residues 230–243), with lowered activity toward m\(^1\)T and m\(^3\)A. Although these two proteins work on similar substrates, ALKBH2 prefers double-stranded substrates, whereas ALKBH3 favors single-stranded nucleic acid substrates. ALKBH3 can also demethylate m\(^3\)A and m\(^3\)C in RNA, suggesting the possibility of repairing RNA lesions.

Recently, ALKBH3 was found to maintain genomic integrity by coordinating with ASCC3 (activating signal cointegrator 1 complex subunit 3) in a cell-line-specific manner. ASCC3 encodes a 3′–5′ DNA helicase that unwinds duplex DNA to generate ssDNA and exposes DNA lesions, thus providing access for ALKBH3-mediated repair. Loss of ALKBH3 or ASCC3 abrogates cells’ tolerance toward DNA damage, which implies their significance in guarding genomic integrity. Interestingly, only one of these two proteins, either ALKBH2 or ALKBH3, functions in a specific cell line to resist alkylation, suggesting the potential reciprocal nature of these two repair pathways.

Analogously to ALKBH2, ALKBH3 also contains a flexible hairpin that is thought to be involved in base flipping and distinguishing single-stranded versus double-stranded substrates. However, the hairpin in ALKBH3 is quite hydrophilic with heavily charged amino acids. When these two loops are swapped, the ssDNA/dsDNA substrate preference of the proteins is switched, this phenomenon might provide hints about differences in substrate recognition. Despite these findings, a crystal structure of substrate-bounded ALKBH3 complex is highly desirable to interpret the features required for substrate recognition.

As a contributor to DNA repair, ALKBH3 not only guards the genomic integrity in normal cells, but also impacts cancer cell survival and invasion. ALKBH3 is overexpressed in various cancer cells and exhibits a potential role in brain tumors, lung cancer, rectal carcinoma, papillary thyroid cancer, colorectal cancer, prostate cancer, pancreatic cancer, and urothelial carcinoma. An understanding of ALKBH3 in mammalian cells could provide potential therapeutic approaches for the treatment of certain cancers.

3. RNA DEMETHYLASES

Based on the AlkB-mediated DNA demethylation mechanism, we proposed and devoted our efforts to the search for reversible RNA methylation. The discovery that Alkb human homologues FTO and ALKBH5 mediate RNA demethylation represents an exciting breakthrough. FTO and ALKBH5 are the first two RNA demethylases ever to be discovered. FTO shows a strong correlation with obesity in humans, whereas ALKBH5 participates in spermatogenesis in mice. The demethylation function of m\(^3\)A in mRNA and other RNAs by these two enzymes revealed a previously uncharacterized, reversible regulatory mechanism present in mammals. Analogous to the methylation of DNA and histones, reversible RNA methylation might also contribute to gene expression regulation, thus attracting broad attention from the research community.

3.1. FTO

The FTO gene was first described as one of the six genes deleted in a fused-toe (Ft) mutant mouse. Several genome-wide association studies (GWASs) then found FTO to be associated with human fat mass and obesity in 2007. The Ft knockout mice showed multiple phenotypes, including the increased possibility of postnatal lethality, postnatal growth retardation, and reduced fat mass. FTO has the highest expression in brain tissues. FTO has also been linked to food intake, development, cancer, and other emerging functions. Studies indicated that FTO is homologous to the Alkb family dioxygenases and exhibits weak demethylation activity toward m\(^1\)T in ssDNA and N\(^3\)-methyluracil (m\(^3\)U) in ssRNA. Mutation of an amino acid in the conserved active site eradicates its catalytic activity (Figure 7) and leads to human postnatal growth retardation, facial dysmorphism, and certain brain malformations. The crystal structure of FTO supports the preference of FTO for ssRNA; in this crystal structure, an extra loop collides with the

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**Figure 9.** Crystal structures of RNA demethylases: (A) Human FTO (PDB ID 3LFM), where the N-terminal AlkB-like domain (residues 32–326) and C-terminal domain (residues 327–498) are colored in blue and yellow, respectively; the extra loop for single-stranded substrate recognition is highlighted in red; iron(II) is shown in orange; and Mn(II) (orange) replaces iron(II) in the structure. (B) Human ALKBH5 (PDB ID 4NJ4), where the AlkB-like domain is labeled in blue with the single-stranded substrate recognition loop labeled in red.
complementary strand of a potential duplex substrate (Figure 9A).\textsuperscript{153} FTO is characterized by a N-terminal AlkB-like domain and a C-terminal domain with a novel fold composed mainly of α-helices.\textsuperscript{53} In 2011, our group discovered FTO as the first RNA demethylase that reverses mA methylation in mammalian mRNA and other nuclear RNAs.\textsuperscript{23}

The mA level is quite low in the genomic DNA of higher eukaryotes,\textsuperscript{24,154} yet, it is the most prevalent internal modification in mRNA for higher eukaryotes, at a frequency of approximately three sites on average per each mRNA.\textsuperscript{19} mA is also present in tRNA, rRNA, small nuclear RNA (snRNA), and long noncoding RNA (lncRNA).\textsuperscript{155}–157 This methylation in mRNA is installed by mRNA mA methyltransferases at a consensus sequence of Pu[G > A]m6AC[A/C/U].\textsuperscript{157–161} Transcriptome-wide profiling of mA reveals that this modification is present in all regions of mRNA, but is highly enriched around the stop codon region of the mRNA.\textsuperscript{157,159} At present, however, the exact function of this enrichment is unclear.

FTO forms discrete granules in the cell nucleus and partially colocalizes with nuclear speckles where mRNA methylation and splicing take place. Inhibition of transcription using Actinomycin D enhances this colocalization pattern, providing further support for RNA as a direct substrate of FTO in vivo.\textsuperscript{23} Recently, several potential mRNA substrates of Fto, including Drd3, Konj6, and Grin1 in the dopaminergic signaling pathway, have been identified by comparing mA-IP (immunoprecipitation)-enriched mRNA peaks from the wild type to peaks observed in Fto−/− mouse brain.\textsuperscript{162} The protein levels of these species decrease with little change in the mRNA level, suggesting that mA has a potential suppressing effect on mRNA translation.\textsuperscript{162} Indeed, the overall negative impact of mRNA mA methylation on gene expression was revealed recently.\textsuperscript{163} The characterization of the first reader protein of mA further revealed an mA-dependent mRNA degradation mechanism.\textsuperscript{163}

The demethylation mechanism of FTO was thought to be similar to the mechanism of the AlkB family proteins. However, later studies demonstrated that FTO not only converts mA to N6-hydroxymethyladenosine (hmA), but also converts hmA to N6-formyladenosine (fA) in sequential oxidation steps in RNA, albeit with lower efficiency (Figure 10). Unlike the direct oxidation products of mA and mC by AlkB, both hmA and fA are relatively stable and can be detected and characterized in vitro and in vivo.\textsuperscript{164} The increased stability of hmA most likely stems from the difference between hydroxymethylations on exocyclic nitrogen in hmA and on endocyclic nitrogen in other hemiaminal intermediates such as hmT. Molecular dynamics simulations suggest that hmA fits the active site of FTO in a manner similar to mA.\textsuperscript{164} Both hmA and fA hydrolytically decompose in water with a half-life of about 3 h under physiological conditions, suggesting potential roles of these oxidation products in mRNA in vivo, such as modulating RNA–protein interactions.\textsuperscript{164}

FTO is involved in human energy homeostasis in a significant way. Mutation of FTO impacts ~1 billion members of the human population.\textsuperscript{165} Small-molecule inhibitors of FTO have been developed with the aim of eventually producing therapies for obesity and diabetes.\textsuperscript{166,167}

3.2. ALKBH5

ALKBH5 is a ubiquitously expressed protein with two possible regulators: either the protein arginine methyltransferase 7 (PRMT7) upon genotoxic stresses or hypoxia-inducible factor 1α (HIF-1α) under hypoxia conditions.\textsuperscript{168,169} Located primarily in the nucleus, ALKBH5 has been identified as an α-KG-dependent dioxygenase with the ability to activate the decarboxylation of α-KG in the presence of iron(II) and ascorbic acid.\textsuperscript{168} Photocross-linking-based mRNA-bound proteomics profiles have revealed ALKBH5 as a potential mRNA-binding protein.\textsuperscript{170,171} Little was known about the biological roles of ALKBH5 until very recently, however, when our group successfully characterized this protein as a mammalian RNA demethylase capable of removing the methyl group of mA from RNA both in vitro and in vivo.\textsuperscript{24}

Recombinant ALKBH5 has been shown to efficiently demethylate mA-containing nucleic acids in vitro. Indeed, knockdown of ALKBH5 in HeLa cells for 48 h resulted in a ~9% increase of the mA level in total mRNA, whereas overexpression of ALKBH5 for 24 h led to a ~29% decrease of the mA level in total mRNA. Therefore, mA in mRNA is the primary physiologically relevant substrate for ALKBH5, although mA in other RNA species, such as rRNA and lncRNA, could also serve as potential substrates.\textsuperscript{24}

ALKBH5 exhibits higher demethylation activity toward mA-containing consensus sequences than nonconsensus sequences.\textsuperscript{24} Similarly to FTO, ALKBH5 prefers to demethylate mA in single-stranded substrates over double-stranded ones.\textsuperscript{24} Crystalllographic studies revealed a unique loop presented in ALKBH5 that confers single-stranded substrate selectivity (Figure 9B).\textsuperscript{172} In contrast to FTO, neither hmA nor fA can be detected when mA undergoes oxidative demethylation by ALKBH5, perhaps because of differences in the protein active sites (Figure 10).\textsuperscript{173} A structure of substrate-bound ALKBH5 will be valuable for further understanding both its substrate selectivity and its catalytic mechanism.

ALKBH5 colocalizes with nuclear speckles that are rich in various mRNA processing factors. ALKBH5 appears to play a broad role in mRNA transport and other RNA metabolism pathways in an mA-demethylation-dependent manner.\textsuperscript{24} In addition, knockout of the Alkbh5 gene in mice led to increased mA levels in mRNA isolated from mouse organs compared to those of wild-type littermates, supporting the hypothesis that
m^6A in mRNA is a physiologically relevant substrate for ALKBH5. Alkbh5-deficient mice display impaired male fertility resulting from compromised spermatogenesis, aberrant apoptosis, and altered gene expression in the testes. Considered alongside the unearthing of FTO as an RNA demethylase, this discovery points to the broad functions in mammals played by dynamic methylation/demethylation of m^6A in RNA.

4. OTHER ALKB HOMOLOGUES

In addition to the four AlkB homologues described above, other AlkB homologues have also been shown to catalyze oxidative reactions and play functional roles in biological systems. So far, only ALKBH8 has been conclusively identified as a tRNA hypermodification enzyme. ALKBH6 has no documented function. The functions of the other homologues are still unclear. Further investigation is required to unravel the enigma of these proteins.

4.1. ALKBH8

The ALKBH8 protein is the only tRNA-hypermodification enzyme characterized in the AlkB family. In addition to the AlkB domain, ALKBH8 also contains a N-terminal RNA-recognition motif (RRM) and a C-terminal Trm9-like methyltransferase domain. ALKBH8 has been thought to contribute to bladder cancer progression by increasing the production of reactive oxygen species. ALKBH8 is conserved in most multicellular eukaryotes, from plants (A. thaliana), worms (C. elegans), and insects (A. mellifera) to mammals. Knockdown of ALKBH8 leads to a fatal defect in cardiac development in D. melanogaster, whereas an internal deletion in the gene encoding ALKBH8 results in embryonic lethality or sterility in animals surviving to adulthood in C. elegans. The expression of the ALKBH8 protein is also temporally and spatially regulated. ALKBH8 is widespread in larvae, yet expressed only in a small number of neurons in adult C. elegans.

The crystal structure of the RRM and AlkB domain of ALKBH8 indicates that the binding between the RRM domain and RNA is strong but largely nonspecific, with a basic N-terminal α-helix that makes critical contributions to its binding capabilities (Figure 11). The protein loops that interact with the nucleotide substrate are completely disordered, and a disorder-to-order transition is likely responsible for this substrate specificity. The iron(II)-binding site in ALKBH8 is solvent-exposed, which might lead to uncoupled α-KG turnover. However, in the absence of nucleic acid substrate, the basal α-KG oxidation level of ALKBH8 is lower than that of AlkB. This lowered α-KG turnover of ALKBH8 has been attributed to the catalytically inactive orientation of α-KG and Arg334 in the active site, which requires a conformational change upon tRNA binding to be catalytically active.

The Trm9-like methyltransferase domain of ALKBH8 catalyzes the methylation of 5-carboxymethyluridine (cm5U) to 5-methoxycarbonylmethyluridine (mcm5U) in tRNAs with UPyN (Py = C/U) as an anticodon triplet sequence, whereas the AlkB domain catalyzes the hydroxylation of mcm5Ut to (S)-mchm5U specifically in tRNA^{Gly}_{UCC} (Figure 12). ALKBH8 is the first enzyme found in the AlkB family that mediates the process of hydroxylation instead of the process of demethylation of nucleic acids. ALKBH8 uses a mechanism similar to that of other AlkB family proteins. However, the hydroxylation product is stable because the hydroxyl group is at the 5-α position, which is connected to a stable C—C bond instead of the C—N linkage that leads to decomposition in water. The additional hydroxyl group has been thought to enhance certain codon—codon interactions and might promote its ability to decode specific codons. The decoding ability of the total...
tRNA pool can regulate the translation of individual mRNA depending on the codon bias of the specific mRNA. Therefore, the identification and characterization of genes that are translationally affected by these modifications represents an interesting future research direction to explore.

4.2. ALKBH1, -4, and -7

4.2.1. ALKBH1. The first human protein described as a functional AlkB homologue was ALKBH1,95 which shows the strongest similarity to AlkB.97 Potential demethylation activity toward m1A and m1C was not detected right away, however.109 Later, as progress was made, disputes arose among different researchers regarding the expression levels, enzymatic activities, and biological roles of ALKBH1. Overall, ALKBH1 is widely expressed in human tissues. The highest mRNA expression levels were detected using North blots in heart and skeletal muscles.184 On the basis of polymerase chain reaction (PCR) and microarray analysis, however, the spleen was reported to have the highest level of ALKBH1.185,186 In terms of activity, one study suggested that ALKBH1 might exhibit demethylation activity toward m1C in vitro.184 A different study detected lyase activity of ALKBH1 at abasic sites independent of iron(II) or α-KG.187–189 Recently, another group demonstrated that ALKBH1 could serve as a histone dioxygenase that acts specifically on histone H2A in vitro and in vivo.190 The biological significance of the oxidation functions discovered for ALKBH1 remains unclear. Alkbh1-deficient mice display sex-ratio distortion and impaired differentiation in placental trophoblast lineage and neurons.190–193

4.2.2. ALKBH4. ALKBH4 has been found to activate the decarboxylation of α-KG, yet so far, it has not been observed to demethylate nucleic acid substrates.110,194 Yeast two-hybrid screens identified its potential protein partners that interact with DNA or chromatin, suggesting that ALKBH4 might play a role in gene regulation.195 Very recently, ALKBH4 was shown to mediate the demethylation of a nonmethylated site in actin (K84me1) in vivo to perhaps regulate the actin–myosin interaction as well as actomyosin-dependent processes such as cytokinesis and cell migration.196 Such an ALKBH4-mediated regulation of actomyosin dynamics is dependent on the conserved residues of the active-site pocket (Figure 7), suggesting the involvement of its catalytic activity. Further efforts are needed to biochemically verify this demethylation reaction in vitro. Whereas the overexpression of ALKBH4 only marginally alters the global gene expression pattern in the HEK293 cell line, homozygous Alkbh4 mutant mice display early embryonic lethality,195,196 which indicates that Alkbh4 plays an essential role in early developmental processes.

4.2.3. ALKBH7. ALKBH7 is a mitochondrial resident protein that does not manifest repair activity toward nucleic acid substrates.110,197 Required for alkylation- and oxidation-induced programmed necrosis, human ALKBH7 triggers the collapse of the mitochondrial membrane and initiates large-scale loss of mitochondrial function that leads to energy depletion and cellular demise.198 Deletion of Alkbh7 in mice dramatically increases body weight and body fat, an indication of its involvement in fatty acid metabolism.199

5. TET FAMILY DIOXYGENASES

5.1. Active DNA Demethylation in Mammals

DNA methylation in the form of 5mC numbers among the best-characterized epigenetic modifications and is essential for genomic imprinting, gene regulation, and development in mammals.200,201 Methylation patterns are initially established by de novo DNA methyltransferases (DNMTs), namely, DNMT3A and DNMT3B,202 and then maintained by the maintenance methyltransferase of DNMT1 during DNA replication.201,202 The proper function of DNA methylation requires the dynamic regulation of reciprocal processes. Although enzymes that catalyze DNA methylation have been well characterized, the demethylation process in mammals remained elusive for several decades before the discovery of TET enzymes and ShmC.30,203

DNA methylation could be lost at the newly synthesized DNA strand during replication in the absence of DNMT1, which has been termed passive demethylation. However, such replication-dependent passive demethylation cannot explain all cellular demethylation events. For example, immediately after fertilization the male pronucleus observably loses almost all 5mC.204 The genetic materials from the sperm and the egg have not yet fused to form one nucleus; rapid demethylation at this stage could therefore not result from replication, thus suggesting an alternative active demethylation pathway. Indeed, TET proteins have been found to catalyze the sequential oxidation of 5mC to ShmC, SfC, and ScaC.30–33 The resulting SfC and ScaC could be removed by thymine DNA glycosylase (TDG) and replaced with unmethylated cytosine through BER.

5.2. TET Proteins and Mechanism of Oxidation

As a product of the TET-mediated oxidation of 5mC, ShmC was found to be highly abundant in the genome of neuron cells and mouse embryonic stem cells (ESC).30,203 This enzymatic activity of TET resembles that of thymine hydroxylase, which can successively oxidize the methyl group on the thymine base to its alcohol, aldehyde, and carboxylic acid forms.205 Thymine hydroxylase belongs to the family of Fe(II)/α-KG-dependent dioxygenases, and computational analysis has identified homologues of thymine hydroxylases in mammals, including TET family proteins.206,207 TET proteins (TET1–TET3) were initially identified as a fusion partner of the histone H3K4 methylation transferase MLL (mixed-lineage leukemia).206,207 They contain several conserved domains, including a CXXC region that specifically recognizes clustered unmethylated CpG
dinucleotides, a cysteine-rich region, and an α-KG dioxygenase DSBH core fold (Figure 13A). Biochemical and crystallographic studies have revealed that the CXXC region binds to the unmodified cytosine; this region is thought to target unmethylated cytosine clusters. The other two domains are responsible for catalytic activity both in vitro and in vivo. The DSBH fold of TET proteins, featured in all dioxygenases, contains the signature HXDXnH motif to coordinate iron(II) and a conserved R residue for α-KG binding. A putative iron(IV)-oxo species is generated to oxidize the inert C—H bond of 5mC to form 5hmC, 5fC, and 5caC in a nonprocessive manner. Consistent with this mechanism, introducing mutations into the iron-binding sites or adding common inhibitors of α-KG-dependent dioxygenases to TET proteins would abolish their activity. The ability to initiate iterative oxidation of 5mC, 5hmC, and 5fC suggests the existence of a less selective substrate-binding site to accommodate all of these substrates. The structure of the catalytic domain of human TET2 has just been reported (Figure 13B); this research shows that the Cys-rich region folds around the DSBH domain, thus confirming that the region is essential to catalytic activity. The substrate-binding pocket in the active site contains residues that can accommodate different modifications of 5mC, as expected. It will be very interesting to further dissect the substrate recognition and oxidation mechanism with the structure now available.

5.3. TET-Mediated DNA Demethylation

In contrast to methylation on nitrogen, methylation on the carbon atom is much more challenging to reverse because of the inert nature of the C—C bond under physiological conditions. The substrate-binding pocket in the active site contains residues that can accommodate different modifications of 5mC, as expected. It will be very interesting to further dissect the substrate recognition and oxidation mechanism with the structure now available.

Although the TET1–TET3 proteins all have the ability to oxidize 5mC, their functions and expression levels vary among cell types and tissues. Mouse Tet1 and Tet2 are highly expressed in ESC cells. They have been proposed to regulate pluripotency and lineage differentiation. On the other hand, mouse Tet3 protein is specifically enriched in the paternal pronucleus at the zygotic stage. This enrichment concurs with the loss of 5mC and the appearance of 5hmC in the paternal genome, thereby suggesting that mouse Tet3 plays a critical role in zygotic epigenetic reprogramming.

Figure 13. Domain architecture of TET proteins. (A) TET proteins contain a DNA-binding CXXC region in the N-terminus and a catalytic core in the C-terminus. The catalytic core is composed of a Cys-rich region and a DSBH fold. The number of amino acids for each protein is indicated. Sequence alignment of the catalytic motif is shown. Sequences used in the alignment include AlkB, Trypanosoma brucei JBP1 (tbJBP1), tbJBP2, human TET1–TET3, and mouse Tet1–Tet3. Conserved iron(II)- and α-KG-binding sites are highlighted in red columns. (B) Crystal structure of the human TET2 bound to a 5mC-containing dsDNA (PDB ID 4NM6). The Cys-rich region (residues 1129–1312) and DSBH core (residues 1313–1936) are colored in yellow and red, respectively. The active-site iron is shown in orange, the α-KG analogue of NOG in green, structural zinc ions in gray, protein residues in white, flipped 5mC in blue, DNA backbone in beige, bases in the 5mC-containing DNA strand in cyan, and bases in the complementary strand in purple.
conditions. Therefore, all of the oxidative derivatives, namely, 5hmC, 5fC, and 5caC, are quite stable under cellular conditions. Although oxidation of 5mC could be functionally regarded as “demethylation” given that the oxidized derivatives are more hydrophilic and would recognize binding proteins different from 5mC, the complete reversion of methylation nevertheless requires additional processes for conversion.

In addition to the passive demethylation as the oxidized cytosine derivatives are diluted during replication, three replication-independent demethylation mechanisms have been proposed following TET-catalyzed 5mC oxidation (Figure 14).

Figure 14. Dynamic regulation of cytosine methylation/demethylation in mammalian genomic DNA. The DNA 5mC pattern is established and maintained by DNMTs, but can undergo either passive dilution during replication or active TET-mediated demethylation.

Biochemically confirmed, the first mechanism recruits TDG to excise 5fC or 5caC to generate an abasic site that is then replaced by cytosine through the BER pathway.\(^{31,218,219}\) The second mechanism proposes the deamination of 5hmC to form 5-hydroxycytosine (5-hmC) by AID (activation-induced cytidine deaminase) and APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) family proteins. 5hmC is then removed by TDG or SMUG1 (single-stranded-selective monofunctional uracil DNA glycosylase 1) and finally repaired by BER.\(^{220}\) This mechanism is still under debate because purified AID/APOBEC deaminases disfavor modified cytosines in vitro.\(^{221}\) Although not yet revealed, the third mechanism proposes a putative decarboxylase to directly convert 5caC to cytosine.\(^{222}\)

Deeply involved in these demethylation processes, TDG is a member of the uracil DNA glycosylase (UDG) superfamily that flips and excises modified bases in dsDNA and initiates BER.\(^{223,224}\) TDG was first shown to remove mismatched pyrimidines from G–U and G–T pairs in dsDNA. TDG was found only recently to recognize TET-oxidized derivatives of 5caC and 5fC and participate in TET-mediated active demethylation in mammals.\(^{31,218}\) In fact, TDG exhibits a slightly higher binding affinity toward G–5fC and G–5caC base pairs than toward G–U and G–T mismatches.\(^{219}\) The crystal structure of the TDG catalytic domain in complex with 5caC-containing dsDNA reveals its preference for 5caC over other bases.\(^{219}\) These lines of evidence suggest that, instead of DNA repair, DNA demethylation might be the primary function of TDG, supporting its critical role in transcriptional regulation and mouse embryonic development.\(^{225,226}\)

5.4. 5mC Oxidation Derivatives 5hmC, 5fC, and 5caC

Nucleic acids can be oxidized to yield various oxidative lesions.\(^{3}\) The presence of 5hmC in the mammalian genome as a potential nonenzymatic oxidation product has been proposed for decades.\(^{227}\) Now recognized as products of TET-mediated 5mC oxidation, 5hmC, 5fC, and 5caC are chemically stable under physiological conditions, which might enable them to serve as potential epigenetic markers with biological functions besides their role as demethylation intermediates. To effectively explore these roles, massive sequencing methods have been developed to profile them genome-wide and with base resolution and to gain precise distribution information.\(^{5}\) The sequencing data together with other evidence suggest that 5hmC could be a regulatory marker in addition to its role as a transient oxidative intermediate.\(^{225,226}\) 5hmC is not as evenly distributed as 5mC. 5hmC is most abundant in ESCs and brain tissues (≈1% of total cytosines) with distinct patterns.\(^{32}\) This modification is enriched at distal regulatory elements in ESCs, whereas it is enriched at 5mC-depleted gene bodies of neuronal function-related genes.\(^{231,232}\) Specific 5hmC-binding proteins have been identified, some of which can result in altered chromatin structures and gene expression.\(^{233}\)

In contrast to 5hmC, 5fC and 5caC are much less abundant, and their levels are consistently lower among all cells and tissues examined so far,\(^{32}\) suggesting that 5fC and 5caC are more likely committed as transient demethylation intermediates. In mouse ESCs, the distribution of 5fC and 5caC represents the portion of 5hmC undergoing demethylation, with a preference for distal regulatory elements.\(^{233,234}\) However, further investigation is required to fully depict these intermediates.

6. CONCLUSIONS AND PERSPECTIVE

In this review, we have discussed the versatile oxidations of methyl groups in nucleic acids mediated by Fe\(^{III}/α-KG\)-dependent dioxygenases, from DNA repair to RNA/DNA demethylation. The discovery of oxidative demethylation mediated by AlkB in DNA repair opened up this new paradigm. Nine human homologues of AlkB proteins have vividly illustrated the diverse manner in which such a mechanism can affect cellular functions and regulations. Whereas some of the homologues have been well studied to debunk the myths of biological pathways, functions of other homologues are still unclear and call for further efforts. The discoveries of oxidative demethylation of epigenetic RNA and DNA methylations have added additional layers of complexity to gene expression regulation. Identification of specific binding proteins for all of these novel modifications represents a future research direction that is required to reveal their biological functions.\(^{163,235}\) Interest in RNA methylation has been revived owing to the recent identification of RNA m\(^{5}\)A methylases, yet a full characterization of the RNA m\(^{5}\)A methyltransferase remains important.\(^{161}\) High-throughput sequencing methods with base-level resolution are particularly urgent to precisely define RNA methylomes. Reversible DNA methylation will continue to attract extensive attention from researchers in broad areas of biology and medicine. The enigma of
demethylation events and the ways in which demethylation contributes to differentiation and development have yet to be fully resolved. Our knowledge of these oxidation reactions will persist in driving present and future efforts to further uncover the biological significance of these processes and to develop potential therapies that will take advantage of the critical functions of these proteins.

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Notes
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ABBREVIATIONS

ScaC S-carboxylycytosine
Sfc S-formylcytosine
ShmC S-hydroxymethylcytosine
S-hmU S-hydroxymuracil
SmC S-methylcytosine
AID activation-induced cytidine deaminase
ALKBH5 AlkB homologue 5
APOBEC apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
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