CHEMICAL REVIEWS

Review

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Nucleic Acid Oxidation in DNA Damage Repair and Epigenetics

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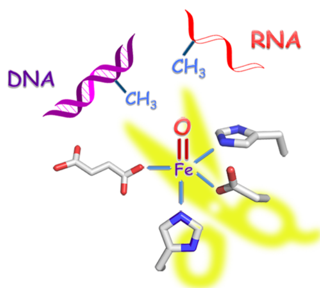

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1. INTRODUCTION

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.

DNA methylation damage caused by methylating agents can occur on different positions of bases or backbones. The location depends on the chemical reaction type (S_N1 or S_N2 nucleophilic substitution), as well as the susceptibility of the position. In general, the S_N1 type of methylating agent (e.g., *N*-methyl-*N*′-nitrosourea, MNU) methylates both nitrogen and oxygen atoms in nucleic acids, whereas the S_N2-type agents (e.g., methylmethane sulfonate, MMS) tend to attack the nucleophilic *N*-position of exposed bases (Figure 1A).[3−5] The resulting lesions exhibit different levels of cellular toxicity and mutagenic influence and can be promptly reversed by enzymes through either nucleophilic substitutions or oxidative demethylations (Figure 1B).[3,6] This review discusses the oxidative demethylation repair pathway only.

N^1-Methyladenine (m^1A) and N^3-methylcytosine (m^3C) are major lesions formed in single-stranded DNA (ssDNA) in the presence of S_N2-type methylating agents.[3,4,6] Methylations in these two positions compromise Watson−Crick base pairing during DNA replication, resulting in cytotoxcity.[7] Through an unprecedented oxidative demethylation mechanism revealed over 10 years ago, the Fe^{II}/α-ketoglutarate- (α-KG-) dependent AlkB family dioxygenases can repair these methylating DNA lesions.[8,9] 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

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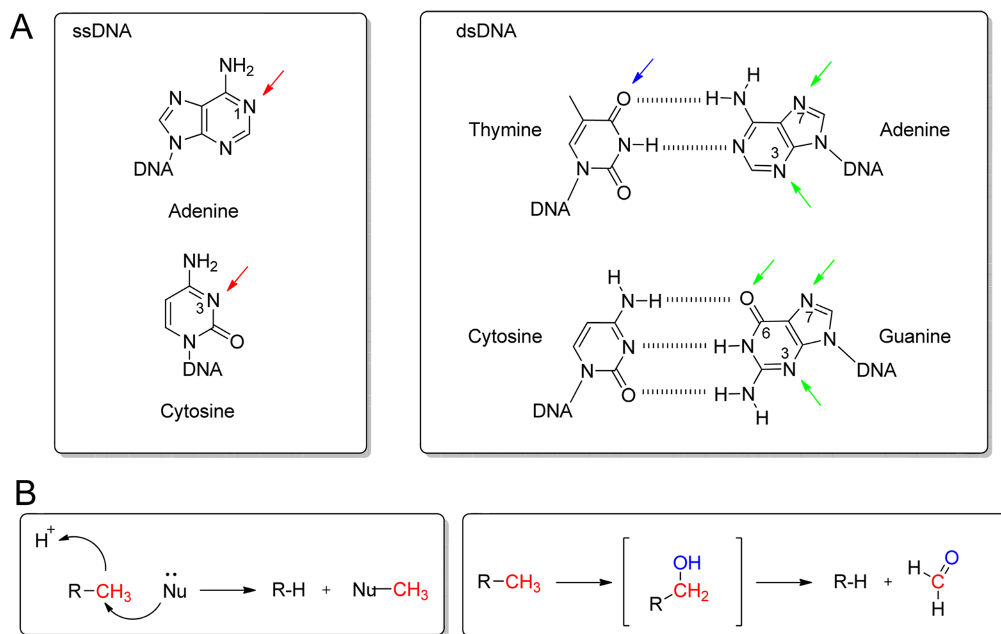

Figure 1. Nucleic acid methylations. (A) Potential methylation sites in double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) upon reaction with MNU and MMS. The blue arrow indicates the predominant methylation sites that can be introduced by MNU; red arrows, predominant sites introduced by MMS; and green arrows, sites introduced by both MNU and MMS. (B) Methylations in DNA, RNA, or proteins can be reversed by enzymes through either nucleophilic substitution or oxidative demethylation mechanism.

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 lysine,[10] arginine,[11−13] and histidine[14,15] 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.[16,17] 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 Fe^{II}/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.[18] The milestone discoveries of histone demethylation indicate that epigenetic methylation marks on other macromolecules could be reversed through the same oxidative demthylation 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.[19] A significant amount of methylation is present on the nitrogen atoms of bases, such as N^3-methylcytosine in ribosomal RNA (rRNA), N^1-methyladenosine in transfer RNA (tRNA), and N^7-methylguanosine in messenger RNA (mRNA).[20,21] 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.[22] Our recent discovery of two RNA N^6-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.[23,24]

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.[25] 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.[26] Although the enzymes that catalyze DNA methylation in mammals have been well characterized,[27−29] 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.[30−33] 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

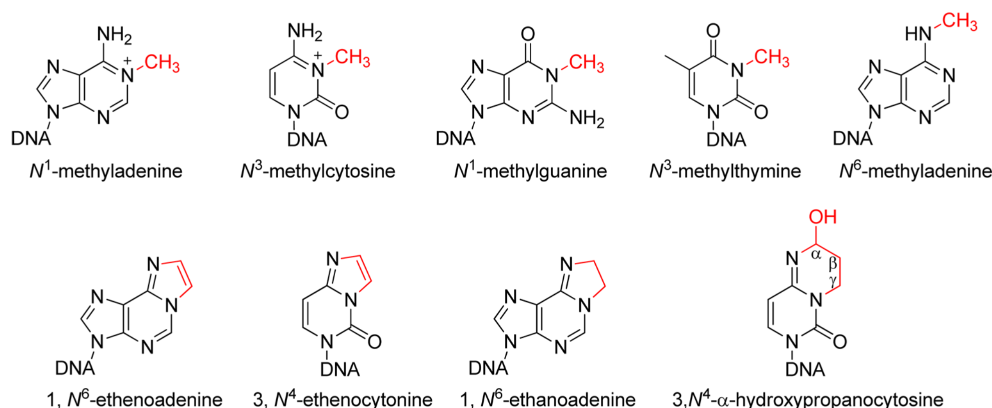

Figure 2. DNA/RNA lesions that AlkB can repair. Exogenous or endogenous methylating agents can introduce various DNA methylations as shown, which are known substrates of AlkB. Exocyclic DNA adducts of 1,N^6-ethenoadenine (εA) and 3,N^4-ethenocytosine (εC) are generated by exposure to electrophilic vinyl chloride (VC) metabolites, chloroethylene oxide (CEO), or chloroacetaldehyde (CAA) introduced exogenously or endogenously from lipid peroxidation.[54,55] 1,N^6-Ethanoadenine (EA) is produced by the reaction of adenine with the anticancer agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU).[57,58] 3,N^4-α-Hydroxypropanocytosine is produced by lipid peroxidation.[59]

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 alkylation 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 O^6-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 S_N2-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 Fe^{II}/α-KG-dependent dioxygenases, which has recently emerged as a versatile family of nonheme oxidation enzymes that perform a variety of critical functions.[35]

Early evidence suggested that AlkB is capable of protecting cells from lethal effects by repairing MMS-induced DNA damage under alkylation threats[36−38] in a process different from the DNA-repair mechanisms known at the time.[39] 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 m^1A and m^3C, which are major DNA lesions induced by MMS.[39] A bioinformatics study largely instigated this field. The study used sequence profile analysis, which predicted that AlkB is an Fe^{II}/α-KG-dependent dioxygenase.[42] These results inspired subsequent biochemical tests of the catalytic activity of the AlkB protein.

2.1.2. Mechanism. In 2002, two independent groups made breakthroughs showing that AlkB directly reverses m^1A and m^3C to unmethylated bases in DNA through an oxidative demethylation mechanism in the presence of iron(II), α-KG, and dioxygen.[8,9] To date, the substrates of AlkB have been extended to N^1-methylguanine (m^1G), N^3-methylthymine (m^3T),[43−45] 1,N^6-ethenoadenine (εA),[46−50] 3,N^4-ethenocytosine (εC),[49,51−55] 1,N^6-ethanoadenine (EA),[56−58] 3,N^4-α-hydroxypropanocytosine,[59] and m^6A (Figure 2).[56] 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 m^1A and m^3C 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

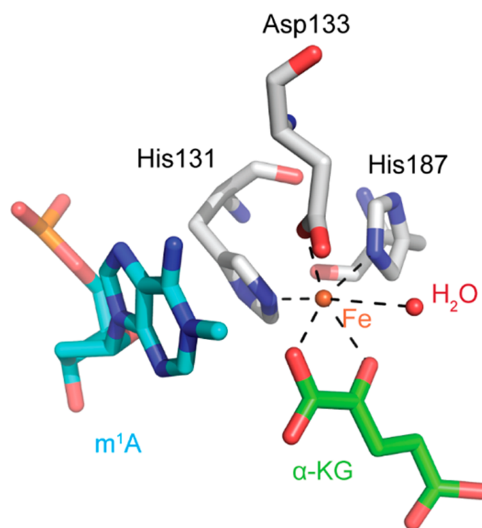

Figure 3. View of active-site stereochemistry with ligand coordination [Protein Data Bank (PDB) ID 2FD8]. His131, Asp133, and His187 are ligands to iron.

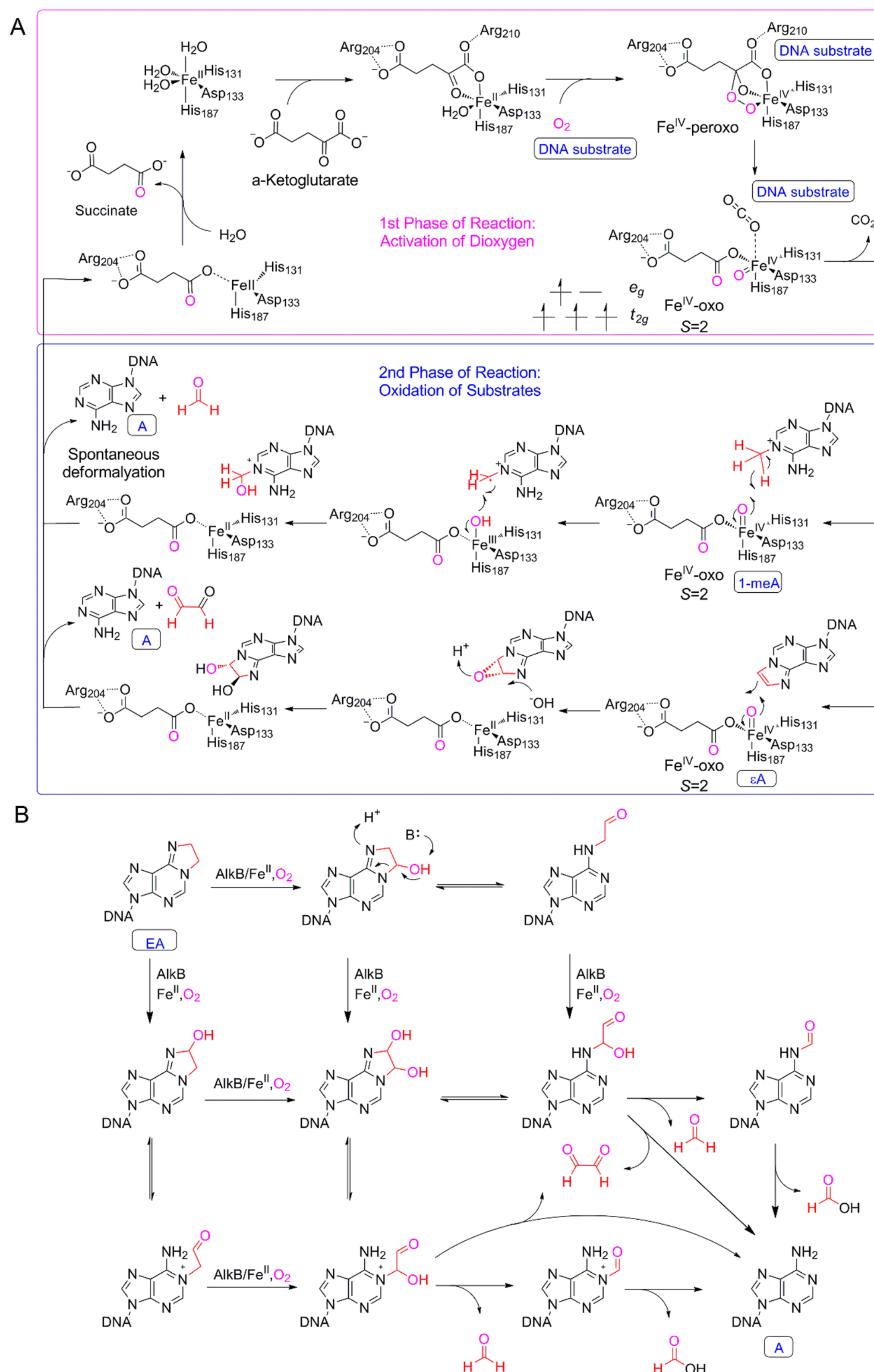

Figure 4. Proposed mechanisms of AlkB-mediated oxidative dealkyaltion toward (A) m^{1}A and 1,N^6-ethenoadenine (εA) and (B) 1,N^6-ethanoadenine (EA). The EA demethylation involves two steps of oxidation, one at N^1 and a second at N^6, as well as further hydrolysis to completely restore the lesion back to adenosine.

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.[66] 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 then yields a high-valent, high-spin (S = 2) iron(IV)-oxo species[67] and converts α-KG to succinate. It is proposed that the iron(IV)-oxo species then undergoes

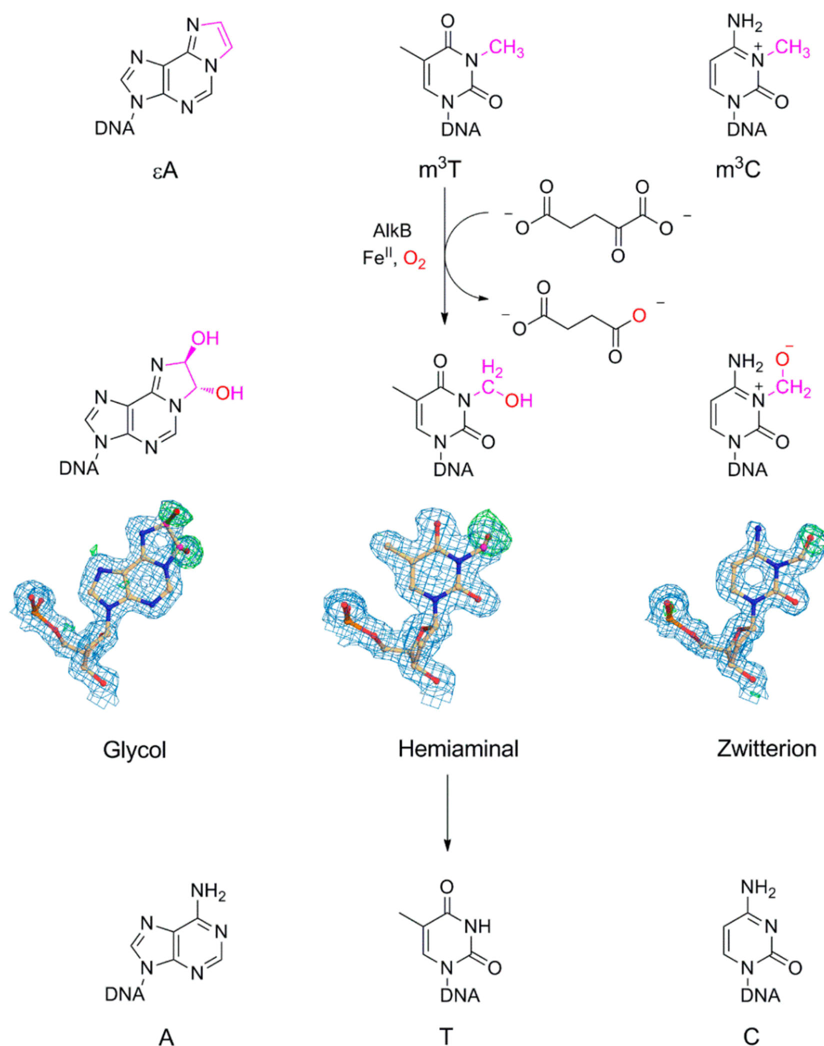

Figure 5. Oxidative demethylation mediated by AlkB. Oxidative repair of εA, m^{3}T, and m^{3}C by AlkB with intermediates glycol (PDB ID 3O1U), hemiaminal (PDB ID 3O1P), and a zwitterion (PDB ID 3O1S), respectively, captured during oxidation in crystals.

conformational reorientation upon the release of CO_2 from the active site.[64,68−70] The Fe^{IV}=O double bond is reoriented to the trans position to His187, which draws the Fe^{IV}=O oxygen closer to the target methyl carbon.[71] In the second phase of the proposed mechanism, the highly oxidative Fe^{IV}=O species could abstract an H atom from the methyl group of the aberrant methyl adduct to yield an Fe^{III}—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.[63] 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 m^{3}T), and a zwitterionic intermediate (from m^{3}C) (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 m^{3}C contains a better leaving group than the neutral hemiaminal thymine derived from m^{3}T at physiological pH; therefore, m^{3}C undergoes a decomposition more quickly than m^{3}T to liberate the intact base. This might partially explain the higher repair rate of AlkB to m^{3}C and m^{1}A than to m^{3}T and m^{1}G.[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 $\pi-\pi$ stacking in the active-site pocket. Tyr78, Lys134, Asp135, and Glu136 coordinately recognize the flipped bases through the formation of specific hydrogen bonds. Compared to other similar dioxygenases, AlkB contains 90 unique N-terminal residues to form a flexible substrate-binding lid that docks the diverse alkylated nucleotide substrates in optimal catalytic geometry (Figure 6).[48,60,71,80,81]

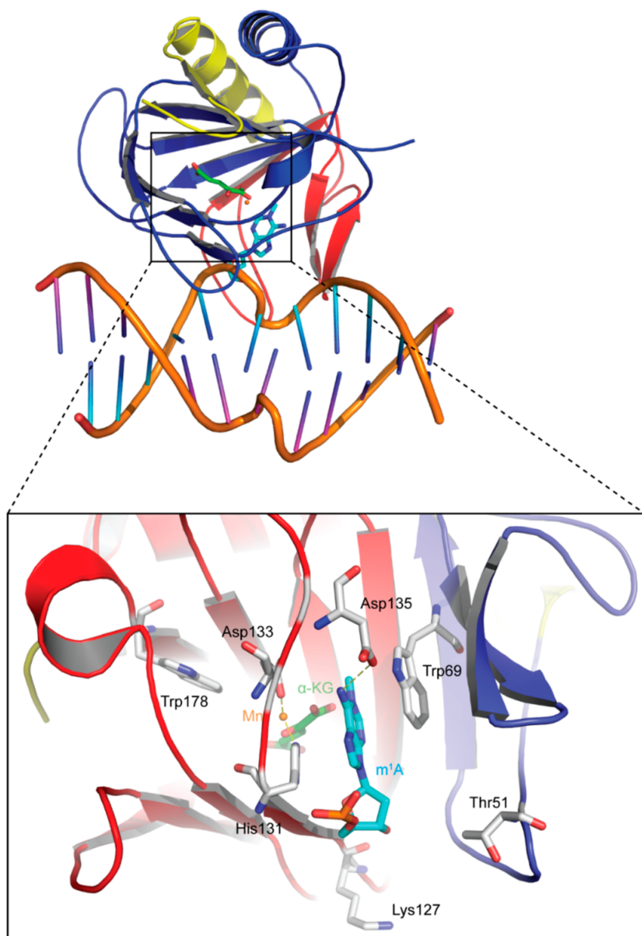

Figure 6. Crystal structure of an AlkB−dsDNA complex (PDB ID 3BIE). The protein is colored according to subdomain organization with the N-terminal extension in yellow (residues 13−44), the nucleotide-recognition lid in red (residues 45−90), and the catalytic core in blue (residues 91−214). Manganese(II) (orange) replaces iron(II) in the structure to eliminate catalytic activity. The flipped-base m^{1}A is shown in blue, α-KG in green, protein residues in white, DNA backbone in beige, bases in the m^{1}A-containing DNA strand in cyan, and bases of the complementary strand in purple.

AlkB prefers to repair methylation damages in ssDNA rather than those in double-stranded DNA (dsDNA), suggesting its association with replication forks or its functional coupling with transcription.[75,82−84] Methylations at the N^1 site of adenine and the N^3 site of cytosine alter the Waston−Crick base pairing, thus disrupting DNA double helices.[85,86] These methylations could block replication and transcription, leading to recruitment of the repair enzyme. These two modifications could also occur in RNA and are exposed in ssDNA. In later research, AlkB was indeed shown to repair these adducts in RNA both in vitro and inside cells.[82,87,88] Repairing these lesions in mRNA and tRNA was suggested as part of an adaptive response to protect bacteria against chemical methylations.[87,89−91]

One advance in probing the structure and substrate preferences of AlkB involves a disulfide cross-linking approach to stabilize the labile protein−DNA complex.[92,93] AlkB interacts almost exclusively with the damage-containing strand and utilizes a unique base-flipping mechanism to access the damaged base. AlkB squeezes together the two bases that flank the flipped-out base to maintain the base stack. Therefore, attempts to access the damage in the dsDNA of a rigid duplex structure is thermodynamically less favorable than attempts to access the same damage in ssDNA. Because AlkB binds to DNAs regardless of their sequences, the complementary strand in dsDNA serves as a noncompetitive inhibitor for the repair, leading to the preference of AlkB for ssDNA over dsDNA (Figure 6).[81,94]

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 Fe^{II}/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 HXDX$_n$H 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 m^{1}A and m^{3}C in vitro and in vivo,[82,109,110] but reduced activity toward m^{3}T in 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.[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 m^{1}A and m^{3}C 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.

```
AlkB      RYAPGAKLSLHQDKDEPDLR--APIVSVSLGLPAIFQFGGLKRND----------------- 163
ALKBH1    YYRLDSTLGIHVD--RSELDHSKPLLSFSFGQSAIFLLGGLQRD-----------------E 188
ALKBH2    YKDGCDHIGEHRD-DERELAPGSPIASVSFGACRDFVFRH-KDSRG--------KSPSRR-V 211
ALKBH3    YRNEKDSVDWHSD-DEPSLGRCPIIASLSFGATRTFEMRK-KPPPE--------ENGDYTYV 232
ALKBH4    CPERGSAIDPHLD-DAWLWG--ERLVSLNLLSPTVLSMCR-EAPGSL----LLCSAPSAAPE 212
ALKBH5    DYQPGGCIVSHVD---PIHIFERPIVSVSFFSDSALCFGCKFQFKP-----------IRVSE 241
ALKBH6    QYLPGEGIMPHED-GPLYYP---TVSTISLGSHTVLDFYEPRRPEDD----DPTEQPRPPPR 88
ALKBH7    DLEARGYIKPHVD-SIKFCG--ATIAGLSLLSPSVMRLVH-TQE----------------PG 152
ALKBH8    QYEPGQGIPAHIDTHSAFEDEIVSLSLGSEIVMDFKHPDG---------------------- 267
FTO       FGMGKMAVSWHHDENLVDRSAVAVYSYSCEGPEEESEDDSHLEGRDPDIWHVGFKISWDIET 282

AlkB      PLKRLLLEHG-----------------DVVVWGGESRLFY-HGIQPLKAG---------- 195
ALKBH1    APPPMFMHSG-----------------DIMIMSGFSRLLN-HAVPRVLPNPEGEGLPHCL 230
ALKBH2    AVVRLPLAHG-----------------SLLMMNHPTNTHWYHSLPVR------------- 241
ALKBH3    ERVKIPLDHG-----------------TLLIMEGATQADWQHRVPKE------------- 262
ALKBH4    ALVDSVIAPSRSVLCQEVEVAIPLPARSLLVLTGAARHQWKHAIHR-------------- 258
ALKBH5    PVLSLPVRRG-----------------SVTVLSGYAADEITHCIRPQ------------- 271
ALKBH6    PTTSLLLEPR-----------------SLLVLRGPAYTRLLHGIAAARVDALDAASSPPN 131
ALKBH7    EWLELLLEPG-----------------SLYILRGSARYDFSHEILRDEESFFGER----- 190
ALKBH8    IAVPVMLPRR-----------------SLLVMTGESRYLWTHGITCRKFDTVQASESLKS 310
FTO       PGLAIPLHQG-----------------DCYFMLDDLNATHQHCV---LA----------- 311

AlkB      ------------------------FHPLTTDCRYNLTFRQAGKKE---- 216
ALKBH1    EAPLPAVLPRDSMVEPCSMEDWQVCASYLKTARVNMTVRQVLATDQNFP 279
ALKBH2    --------------------------KKVLAPRVNLTFRKILLTKK--- 261
ALKBH3    --------------------------YHSREPRVNLTFRTVYPDPRGAP 285
ALKBH4    --------------------------RHIEARRVCVTFRELSAEFGPGG 281
ALKBH5    ---------------------------DIKERRAVIILRKTRLDAPRLE 293
ALKBH6    AAACP---------------SARPGACLVRGTRVSLTIRRVPRVLRAGL 165
ALKBH7    --------------------------RIPRGRRISVICRSLPEGMGPGE 213
ALKBH8    ---------------GIITSDVGDLTLSKRGLRTSFTFRKVRQTPCNCS 344
FTO       ----------------------------GSQPRFSSTHRVAECSTGTLD 332
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Figure 7. Sequence alignment of human AlkB family proteins with AlkB. Conserved residues highlighted in red represent histidines and carboxylates as iron(II)-binding residues, as well as the characteristic RXXXXXR region, which binds the cofactor α-KG.

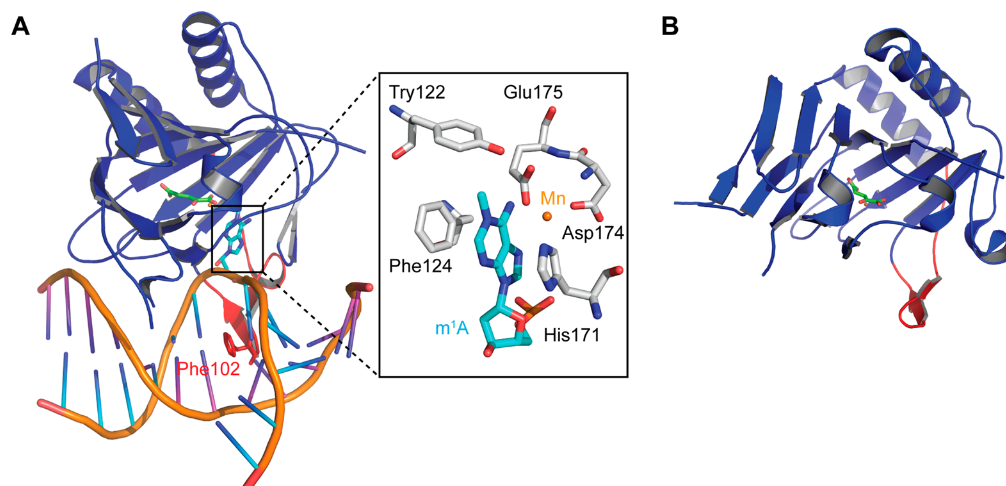

Figure 8. Crystal structures of AlkB human homologues: (A) ALKBH2–dsDNA complex (PDB ID 3BUC) and (B) ALKBH3 (PDB ID 2IUW). The protein is colored in blue, with the β-hairpin (ALKBH2, residues 89–108; ALKBH3, residues 113–129) in red. Manganese(II) is shown in orange, α-KG in green, protein residues in white, DNA backbone in beige, bases in the m^1A-containing DNA strand in cyan, and bases of the complementary strand in purple.

In the same oxidative dealkylation mechanism, ALKBH2 reverses DNA damage by using an active iron center as AlkB (Figure 3).[117,118] ALKBH2 prefers to repair damage in duplex DNA over ssDNA, however.[82,83,92] Crystallographic studies have revealed that, unlike AlkB, ALKBH2 makes extensive contact with both strands of duplex DNA.[93] Unlike certain glycosylases, ALKBH2 does not contain a damage-checking site.[119] Rather, ALKBH2 appears to detect damaged bases by probing their base-pair stability.[120] Consider m^1A 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–T base pair.[121] This reduced stability facilitates the recognition and repair of m^1A by ALKBH2. Compared to AlkB, ALKBH2 contains a unique short hydrophobic β-hairpin in proximity to the active site; this hairpin is significant to the preference of ALKBH2 for double-stranded DNA substrates (Figure 8A).[122,123] More specifically, the aromatic finger residue, Phe102, intercalates into the duplex stack to facilitate the base flipping. Phe124,

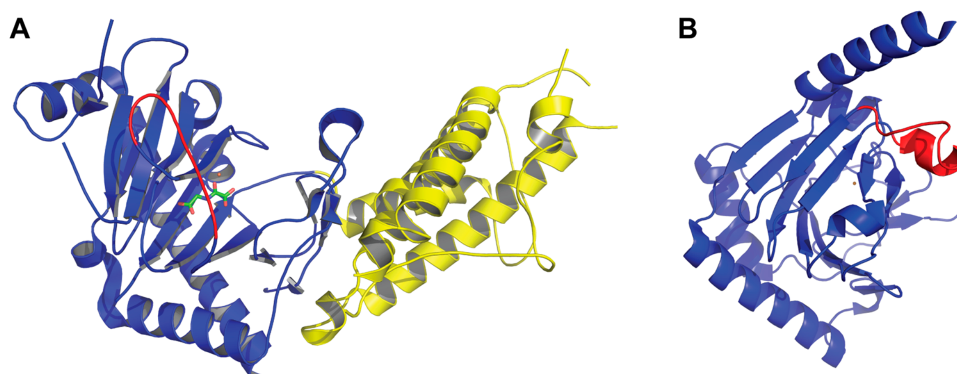

Figure 9. Crystal structures of RNA demethylases: (A) Human FTO (PDB ID 3LFM), where the N-terminal AlkB-like domain (residues 32−326) and C-terminal domain (residues 327−498) are colored in blue and yellow, respectively; the extra loop for single-stranded substrate recognition is highlighted in red; iron(II) is shown in orange; and *N*-oxalylglycine (green) was used to substitute α-KG to afford a catalytically inert FTO. (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. Mn(II) (orange) replaces iron(II) in the structure.

His171, and Glu175 coordinate with other protein residues to recognize and flip the damaged base.

ALKBH2 is frequently down-regulated in gastric cancer[124] and is also involved in the growth of brain tumor cells,[125] glioblastoma,[126] colorectal cancer,[127] and bladder cancer.[128] Knockdown of ALKBH2 increases the sensitivity of cancer therapies, such as photodynamic therapy (PDT) mediated by Photofrin and chemotherapy with cisplatin.[129,130] Based on these observations, ALKBH2, together with its repair partners,[131] 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^1A and m^3C (resides 230−243), with lowered activity toward m^1T and εA.[43,47,61,82,83,109] Although these two proteins work on similar substrates, ALKBH2 prefers double-stranded substrates, whereas ALKBH3 favors single-stranded nucleic acid substrates.[82,83,92] ALKBH3 can also demethylate m^1A and m^3C in RNA,[82,90,110] suggesting the possibility of repairing RNA lesions.[82,87,132] 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.[133] 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.[133]

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 (Figure 8B).[122,134,135] 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;[135] 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.[136] ALKBH3 is overexpressed in various cancer cells[133] and exhibits a potential role in brain tumors,[125] lung cancer,[137] rectal carcinoma,[138] papillary thyroid cancer,[139] colorectal cancer,[127] prostate cancer,[140] pancreatic cancer,[141] and urothelial carcinoma.[142] 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.[22] 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.[24,143−145] The demethylation function of m^6A in mRNA and other RNAs by these two enzymes revealed a previously uncharacterized, reversible regulatory mechanism present in mammals.[23,24] 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.[146] Several genome-wide association studies (GWASs) then found FTO to be associated with human fat mass and obesity in 2007.[143−145] The *Fto* knockout mice showed multiple phenotypes, including the increased possibility of postnatal lethality, postnatal growth retardation, and reduced fat mass.[147] FTO has the highest expression in brain tissues.[106] FTO has also been linked to food intake,[148] development,[149] cancer,[150] and other emerging functions.[151] Studies indicated that FTO is homologous to the AlkB family dioxygenases[105,106] and exhibits weak demethylation activity toward m^3T in ssDNA[106] and N^3-methyluracil (m^3U) in ssRNA.[152] 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.[149] The crystal structure of FTO supports the preference of FTO for ssRNA; in this crystal structure, an extra loop collides with the

complementary strand of a potential duplex substrate (Figure 9A).[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.[153] In 2011, our group discovered FTO as the first RNA demethylase that reverses m^{6}A methylation in mammalian mRNA and other nuclear RNAs.[23]

The m^{6}A level is quite low in the genomic DNA of higher eukaryotes;[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.[19] m^{6}A is also present in tRNA, rRNA, small nuclear RNA (snRNA), and long noncoding RNA (lncRNA).[155−157] This methylation in mRNA is installed by mRNA m^{6}A methyltransferases at a consensus sequence of Pu[G > A]m^{6}AC[A/C/U].[157−161] Transcriptome-wide profiling of m^{6}A reveals that this modification is present in all regions of mRNA, but is highly enriched around the stop codon region of the mRNA.[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.[23] Recently, several potential mRNA substrates of Fto, including *Drd3*, *Kcnj6*, and *Grin1* in the dopaminergic signaling pathway, have been identified by comparing m^{6}A-IP (immunoprecipitation)-enriched mRNA peaks from the wild type to peaks observed in *Fto*$^{-/-}$ mouse brain.[162] The protein levels of these species decrease with little change in the mRNA level, suggesting that m^{6}A has a potential suppressing effect on mRNA translation.[162] Indeed, the overall negative impact of mRNA m^{6}A methylation on gene expression was revealed recently.[163] The characterization of the first reader protein of m^{6}A further revealed an m^{6}A-dependent mRNA degradation mechanism.[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 m^{6}A to N^6-hydroxymethyladenosine (hm^{6}A), but also converts hm^{6}A to N^6-formyladenosine (f^{6}A) in sequential oxidation steps in RNA, albeit with lower efficiency (Figure 10).[164] Unlike the direct oxidation products of m^{1}A and m^{3}C by AlkB, both hm^{6}A and f^{6}A are relatively stable and can be detected and characterized in vitro and in vivo.[164] The increased stability of hm^{6}A most likely stems from the difference between hydroxymethylations on exocyclic nitrogen in hm^{6}A and on endocyclic nitrogen in other hemiaminal intermediates such as hm^{3}T. Molecular dynamics simulations suggest that hm^{6}A fits the active site of FTO in a manner similar to m^{6}A.[164] Both hm^{6}A and f^{6}A 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.[164]

FTO is involved in human energy homeostasis in a significant way. Mutation of FTO impacts ∼1 billion members of the human population.[165] Small-molecule inhibitors of FTO have been developed with the aim of eventually producing therapies for obesity and diabetes.[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.[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.[168] Photocross-linking-based mRNA-bound proteomics profilings have revealed ALKBH5 as a potential mRNA-binding protein.[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 m^{6}A from RNA both in vitro and in vivo.[24]

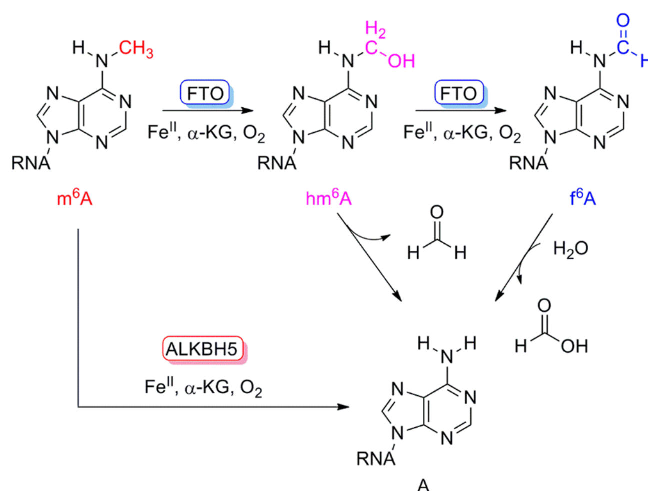

Figure 10. Oxidative demethylation of m^{6}A in RNA mediated by FTO and ALKBH5. Note that ALKBH5-mediated oxidation of m^{6}A should also generate hm^{6}A as an intermediate, which can decompose in the active site of the protein. In the case of FTO, both hm^{6}A and f^{6}A are observed during the oxidation reaction.

Recombinant ALKBH5 has been shown to efficiently demethylate m^{6}A-containing nucleic acids in vitro. Indeed, knockdown of ALKBH5 in HeLa cells for 48 h resulted in a ∼9% increase of the m^{6}A level in total mRNA, whereas overexpression of ALKBH5 for 24 h led to a ∼29% decrease of the m^{6}A level in total mRNA. Therefore, m^{6}A in mRNA is the primary physiologically relevant substrate for ALKBH5, although m^{6}A in other RNA species, such as rRNA and lncRNA, could also serve as potential substrates.[24]

ALKBH5 exhibits higher demethylation activity toward m^{6}A-containing consensus sequences than nonconsensus sequences.[24] Similarly to FTO, ALKBH5 prefers to demethylate m^{6}A in single-stranded substrates over double-stranded ones.[24] Crystallographic studies revealed a unique loop presented in ALKBH5 that confers single-stranded substrate selectivity (Figure 9B).[172] In contrast to FTO, neither hm^{6}A nor f^{6}A can be detected when m^{6}A undergoes oxidative demethylation by ALKBH5, perhaps because of differences in the protein active sites (Figure 10).[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 m^{6}A-demethylation-dependent manner.[24] In addition, knockout of the *Alkbh5* gene in mice led to increased m^{6}A levels in mRNA isolated from mouse organs compared to those of wild-type littermates, supporting the hypothesis that

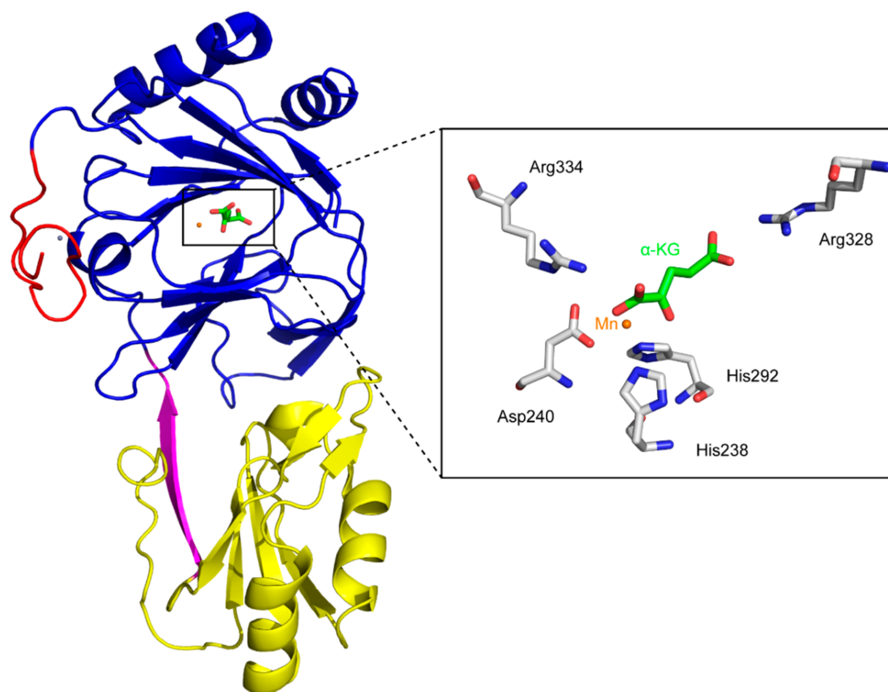

Figure 11. Structure of ALKBH8 (PDB ID 3THT). The N-terminal RRM domain (residues 25−122) and the AlkB domain (131−339) are colored in yellow and blue, respectively. The loop connecting these two domains is colored in magenta. The C-terminal structural zinc(II)-binding site is shown in red. The manganese(II) ion in the active site is shown in orange, whereas the zinc(II) ion in the structural zinc(II)-binding site is shown in gray. α-KG is labeled in green; protein residues are labeled in white.

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.[24] 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.[174−177] ALKBH8 has been thought to contribute to bladder cancer progression by increasing the production of reactive oxygen species.[178] ALKBH8 is conserved in most multicellular eukaryotes, from plants (*A. thaliana*), worms (*C. elegans*), and insects (*A. mellifera*) to mammals.[177] Knockdown of ALKBH8 leads to a fatal defect in cardiac development in *D. melanogaster*,[179] whereas an internal deletion in the gene encoding ALKBH8 results in embryonic lethality or sterility in animals surviving to adulthood in *C. elegans*.[180] 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*.[181]

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).[181] 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.[181]

The Trm9-like methyltransferase domain of ALKBH8 catalyzes the methylation of 5-carboxymethyluridine (cm^5U) to 5-methoxycarbonylmethyluridine (mcm^5U) in tRNAs with UPyN (Py = C/U) as an anticodon triplet sequence, whereas the AlkB domain catalyzes the hydroxylation of mcm^5U to *S*-5-(methoxycarbonylhydroxymethyl)uridine [(*S*)-$mchm^5U$] specifically in $tRNA^{Gly}_{(UCC)}$ (Figure 12).[174,176,182] 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.[175,183] 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.[183] The decoding ability of the total

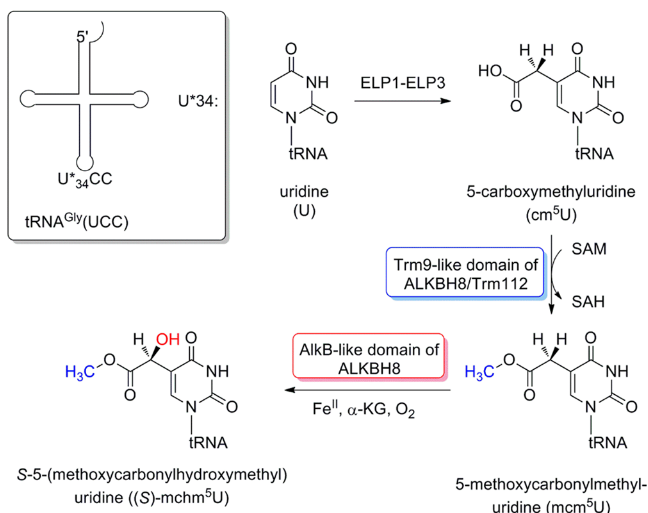

Figure 12. ALKBH8 catalyzes the hypermodification of tRNA wobble uridine. The Trm9-like methyltransferase domain of ALKBH8 catalyzes the methylation of cm^5U to mcm^5U in several tRNAs, and the AlkB-like domain of ALKBH8 catalyzes the hydroxylation of mcm^5U to (S)-$mchm^5U$ in $tRNA^{Gly}$(UCC).

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 m^1A and m^3C was not detected right away, however.[82,109] 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 m^3C 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 monomethylated 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.[197]

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.[29,199] Methylation patterns are initially established by de novo DNA methyltransferases (DNMTs), namely, DNMT3A and DNMT3B,[28,200] 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 5hmC.[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 5hmC, 5fC, and 5caC.[30−33] The resulting 5fC and 5caC 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, 5hmC 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 methyltransferase MLL (mixed-lineage leukemia).[208,209] They contain several conserved domains, including a CXXC region that specifically recognizes clustered unmethylated CpG

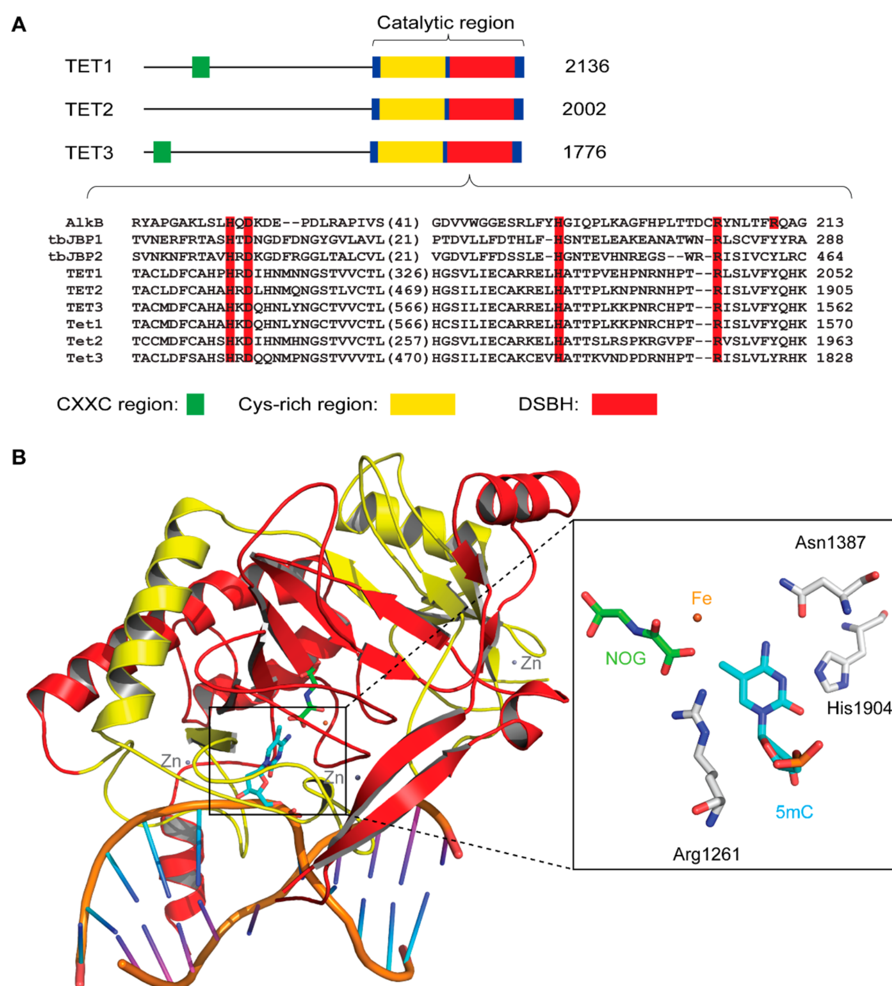

Figure 13. Domain architecture of TET proteins. (A) TET proteins contain a DNA-binding CXXC region in the N-terminus and a catalytic core in the C-terminus. The catalytic core is composed of a Cys-rich region and a DSBH fold. The number of amino acids for each protein is indicated. Sequence alignment 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.

dinucleotides, a cysteine-rich region, and an α-KG dioxygenase DSBH core fold (Figure 13A).[29,210] Biochemical and crystallographic studies have revealed that the CXXC region binds to the unmodified cytosine; this region is thought to target unmethylated cytosine clusters.[211] The other two domains are responsible for catalytic activity both in vitro and in vivo.[212] The DSBH fold of TET proteins, featured in all dioxygenases, contains the signature $HXDX_nH$ 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.[30,212,213] 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.[214] The substrate-binding pocket in the active site contains residues that can accommodate different modifications of 5mC, as expected.[214] 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.[210] 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,[215−217] thereby suggesting that mouse Tet3 plays a critical role in zygotic epigenetic reprogramming.

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. 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).

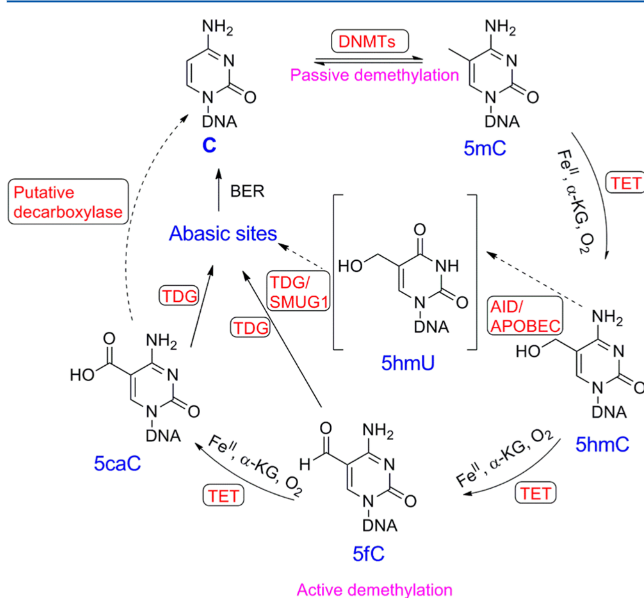

Figure 14. Dynamic regulation of cytosine methylation/demethylation in mammalian genomic DNA. The DNA 5mC pattern is established and maintained by DNMTs, but can undergo either passive dilution during replication or active TET-mediated demethylation.

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-hydroxyuracil (5-hmU) 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.[228] 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.[229,230] 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 the 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.[232]

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^{II}/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^6A demethylases, yet a full characterization of the RNA m^6A 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

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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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The authors declare no competing financial interest.

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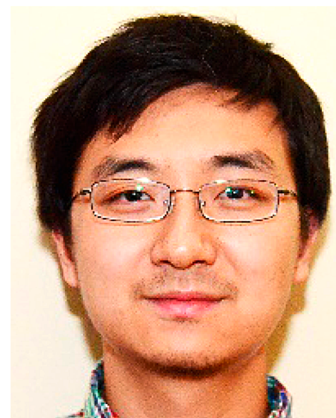
Chuan He is a Professor in the Department of Chemistry and Director of the Institute for Biophysical Dynamics at the University of Chicago. He is also a joint Professor in the Department of Chemical Biology and Director of the Synthetic and Functional Biomolecules Center at Peking University. He was born in P. R. China in 1972 and received his B.S. (1994) from the University of Science and Technology of China. He received his Ph.D. degree in Chemistry from Massachusetts Institute of Technology in 2000. After being trained as a Damon Runyon postdoctoral fellow at Harvard University from 2000 to 2002, he joined the University of Chicago as an assistant professor and was promoted to associate professor in 2008 and full professor in 2010. He is also a member of the Cancer Research Center at the University of Chicago. His research spans a broad range of chemical biology, bioinorganic chemistry, molecular biology, biochemistry, cell biology, structural biology, and genomics. He was recently selected as an Investigator of the Howard Hughes Medical Institute in 2013.

Guanqun Zheng received her B.S. degree from Nanjing University in Biochemistry (2007) and her Ph.D. degree from the University of Chicago in Chemistry (2013). She is interested in chemical modifications in biology, including the investigation of RNA methylations, characterization of modification enzymes, exploration of modification-mediated epigenetic processes, and development of sequencing methods. She is currently a postdoctoral fellow in Dr. Tao Pan's laboratory at the University of Chicago, where she is focusing on tRNA modifications and functional RNA genomics. In addition to science, she is also fond of making friends, volunteering at social events, and watching movies.



Ye Fu was born in Jiangxi, P. R. China, in 1986 and received his B.S. (2007) in Chemistry from Peking University. He then moved to the United States and began to work on the chemical biology of nucleic acid modifications in Dr. Chuan He's laboratory at the University of Chicago. Since then, he has focused on the dynamic regulation of RNA modifications by ALKBH8 and FTO proteins, as well as the dynamic regulation of other DNA modifications. He received his Ph.D. degree in 2012 from the University of Chicago. Upon graduation, he continued as a postdoctoral researcher in Dr. He's laboratory working on the genomics of nucleic acids modifications. He will be a postdoctoral fellow in Dr. Xiaowei Zhuang's laboratory at Harvard University starting in 2014. He has been a recipient of a Weil Endowed Graduate Fellowship (2011), Chinese Government Award for Outstanding Students Abroad (2011), and Yang Cao-Lan-Xian Best Thesis Award in Organic/Inorganic Chemistry (2013). In addition to science, he also enjoys outdoor activities such as hiking, rock climbing, and skiing.

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ABBREVIATIONS

5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5-hmU	5-hydroxyuracil
5mC	5-methylcytosine
AID	activation-induced cytidine deaminase
ALKBH5	AlkB homologue 5
APOBEC	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like

ASCC3	Activating signal cointegrator 1 complex subunit 3
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BER	base-excision repair
CAA	chloroacetaldehyde
CEO	chloroethylene oxide
cm^{5}U	5-carboxymethyluridine
DME	Demeter
DNMT	de novo DNA methyltransferase
DSBH	double-stranded β-helix
dsDNA	double-stranded DNA
EA	1,N^6-ethanoadenine
ESC	embryonic stem cells
f^{6}A	N^6-formyladenosine
Ft	fused toe
FTO	fat mass and obesity-associated
GWAS	genome-wide association study
HIF-1α	hypoxia-inducible factor 1α
hm^{6}A	N^6-hydroxymethyladenosine
IP	immunoprecipitation
JBP	J-binding protein
JHDM	Jumonji C domain-containing histone demethylase
JmjC	Jumonji C
lncRNA	long noncoding RNA
m^{1}A	N^1-methyladenine
m^{1}G	N^1-methylguanine
m^{3}C	N^3-methylcytosine
m^{3}T	N^3-methylthymine
m^{3}U	N^3-methyluracil
m^{6}A	N^6-methyladenosine
mcm^{5}U	5-methoxycarbonylmethyluridine
MLL	mixed-lineage leukemia
MMS	methylmethane sulfonate
MNU	N-methyl-N'-nitrosourea
mRNA	messenger RNA
PDB	Protein Data Bank
PDT	photodynamic therapy
PRMT7	protein arginine methyltransferase 7
ROS	repressor of silencing
RRM	RNA-recognition motif
rRNA	ribosomal RNA
SAM	*S*-adenosylmethionine
(*S*)-mchm^{5}U	*S*-5-(methoxycarbonylhydroxymethyl)uridine
SMUG1	single-stranded-selective monofunctional uracil DNA glycosylase 1
snRNA	small nuclear RNA
ssDNA	single-stranded DNA
TDG	thymine DNA glycosylase
TET	ten-eleven translocation
tRNA	transfer RNA
UDG	uracil DNA glycosylase
VC	vinyl chloride
α-KG	α-ketoglutarate
εA	1,N^6-ethenoadenine
εC	3,N^4-ethenocytosine

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