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Structural determinants of nucleobase modification recognition in the AlkB family of dioxygenases

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Abstract
Iron-dependent dioxygenases of the AlkB protein family found in most organisms throughout the tree of life play a major role in oxidative dealkylation processes. Many of these enzymes have attracted the attention of researchers across different fields and have been subjected to thorough biochemical characterization because of their link to human health and disease. For example, several mammalian AlkB homologues are involved in the direct reversal of alkylation damage in DNA, while others have been shown to play a regulatory role in epigenetic or epitranscriptomic nucleic acid methylation or in post-translational modifications such as acetylation of actin filaments. These studies show that that divergence in amino acid sequence and structure leads to different characteristics and substrate specificities. In this review, we aim to summarize current insights in the structural features involved in the substrate selection of AlkB homologues, with focus on nucleic acid interactions.

Keywords
AlkB homologues, substrate specificity, structural features, nucleic acid interactions

1. Introduction

The AlkB family of dioxygenases has been studied extensively over the recent decades. As the name suggests, all of the members of this family share homology with the *E. coli* DNA repair protein AlkB (EcAlkB). Being part of the adaptive (Ada) response in *E. coli*, EcAlkB expression is strongly upregulated by DNA damage caused by alkylation. Upon sensing of alkylated adducts, a cysteine residue in the alkyltransferase Ada is irreversibly methylated. The methyl transfer to the protein reduces the negative charge of the zinc–thiolate center, thereby lowering its repulsive interaction with DNA. Consequently, Ada is turned into a strong transcription activator of the Ada operon, consisting of four DNA repair proteins (AlkA, Ada, AlkB and AidB) (Teo et al., 1984). Similar adaptive response mechanisms have been found in many bacterial species, although the response genes are sometimes grouped into different operons (Mielecki and Grzesiuk, 2014). Also in higher eukaryotes including yeast, plants and mammals, members of the AlkB family play pivotal roles (Aas et al., 2003; Korvald et al., 2012; Kurowski et al., 2003; Meza et al., 2012). In humans, this family draws particular interest because of its involvement in DNA repair and progression of cancer, as they are frequently overexpressed in tumors and counteract the effects of alkylating drugs such as temozolamide used in chemotherapy (Johannessen et al., 2013; Kaina and Christmann, 2019). Therefore, homologs such as hALKBH8 and hALKBH3 are targeted in anti-cancer therapies (Shimada et al., 2009; Ueda et al., 2017). Besides, the AlkB homologue FTO is related to the onset of obesity (Dina et al., 2007) whereas ALKBH5 activity is regulating fertility in mice (Zheng et al., 2013).

Like the other family members, the prototypical EcAlkB is an iron- and α-ketoglutarate-dependent dioxygenase (Aravind & Koonin, 2001) catalyzing the direct reversal of a wide variety of modified nucleobases. Oxygen is used to convert adducts into an unstable aldehyde, which is spontaneously released from the base, causing complete reversal of the alkylation. During the repair reaction, the required co-factor α-ketoglutarate is converted into succinate and CO_2 (Falnes et al., 2002; Trewick et al., 2002). Through several biophysical experiments, Ergel et al. (2014) showed sequential binding of all (co-)substrates in order to avoid premature turnover of the alpha-ketoglutarate. Furthermore, Bleijlevens et al. (2008) showed that release of unmethylated DNA is facilitated by increased flexibility of the protein upon formation of succinate.

All of the investigated family members contain a 200 to 300 amino acid-sized AlkB domain adopting a so-called "jelly-roll" topology. This fold, also known as a double-stranded β-helix (DSBH), typically consists of a core of eight arranged β-strands (Figure 1A and B) (Aravind and Koonin, 2001; Elkins et al., 2002; Hausinger, 2004). Strongly conserved in this family is a histidine-x-aspartate (HXD) motif located at the 'open end' of the DSBH core, followed by a second histidine further downstream. These three residues are involved in coordinating the catalytic Fe atom. Further complementation of the octahedral coordination of iron is provided by two oxygen atoms of the α-ketoglutarate cofactor. Often, binding of α-ketoglutarate is further stabilized by two additional, conserved arginine residues (Yu et al., 2006; Yang et al., 2008). A feature shared amongst the nucleobase-binding members of the AlkB family is the occurrence of residues that aromatically stack against the damaged bases, although no strict conservation of aromatic residue type is seen between different family members (Holland and Hollis, 2010). A third conserved property of this family is the presence of a so-called nucleotide recognition lid consisting of a small β-sheet, contributing to substrate specificity (Yu et al., 2006).

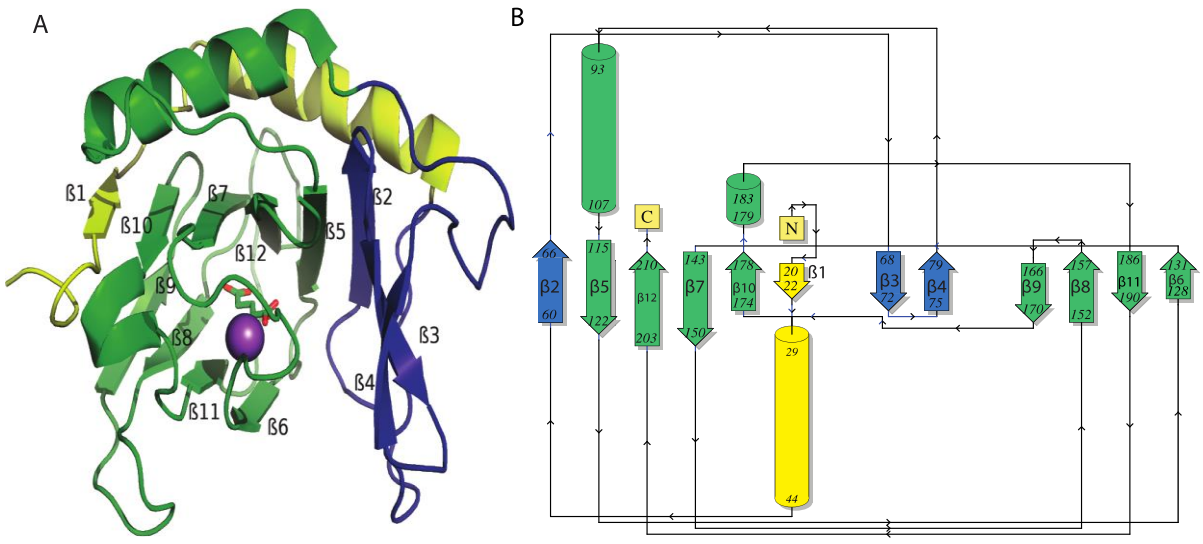

Figure 1. Overall structural features of the AlkB family of dioxygenases. (A) 3D structure of the prototypical EcAlkB (PDB ID: 3BIE, figure rendered with Pymol). Green: catalytic "jelly-roll" core. Blue: nucleotide-recognition lid. Yellow: N-terminal domain. (B) Topology diagram of EcAlkB (figure made with the PDBsum webserver (Laskowski et al., 1997)).

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Nucleic acid-interacting AlkB homologues need to be able to discern damaged bases from canonical bases. For AlkB and homologues repairing 1-methyl adenine (1mA), the mechanism of damage detection is thought to rely on the adoption of a Hoogsteen base pair between 1mA and thymine in duplex DNA. Other lesions, such as 3-methyl cytosine (3mC) show other features in duplex DNA, yet they all have in common that the thermal stability of the base-pairing is weakened (Lu et al., 2010). The process of searching and interrogating base pair stability is thought to occur through multiple mechanisms such as sliding, hopping or a combination of both (Yi et al., 2012). To our knowledge, no kinetic studies on the AlkB family have been done so far to elucidate their way of scanning. For hALKBH2 and hALKBH3, associated proteins PCNA and ASCC3, respectively, have been identified to assist in sliding over the DNA (Dango et al., 2011; Fu et al., 2015). EcAlkB on the other hand has been reported to interact with single-strand binding protein (Ssb) and RecA, which is possibly promoting the oxidative repair of modified ssDNA (Mohan et al., 2018; Nigam and Anindya, 2018; Shivange et al., 2016). Although the sliding/hopping mechanism of EcAlkB is largely unknown, the low affinity for canonical bases and the dynamic state of EcAlkB when bound to the end product succinate is thought to result in a more transient binding. This transiency then promotes one-dimensional scanning for nucleobase adducts (Bleijlevens et al., 2008).

As noted by Huffman et al. (2005), upon detection of modified bases, most DNA repair enzymes flip out the damaged base by intercalating in the double helix. hALKBH2 extensively contacts the complementary strand, thereby forcing out the damaged base and filling the vacant space in the double helix with the aromatic ring of a phenylalanine residue (F102) from a DNA-interacting loop. Conversely, EcAlkB forces the nucleosides flanking the methylated base to stack on one another, thereby squeezing out the modified nucleobase. Stacking of these nucleobases requires one of the two sugars to be inverted, by forcing the sugar moiety to rotate 180°. This inversion then further requires stabilisation by EcAlkB through a network of hydrogen bonds.

Through bioinformatical sequence analysis of bacterial AlkB homologues, Van den Born et al. (2009) proposed four different subfamilies of AlkB homologues, termed 1A, 1B, 2A and 2B, each characterized by the presence of specific amino-acid residues in the nucleotide recognition domain. Human homologues were classified in the same subfamilies. For example, hALKBH2 and hALKBH3 were assigned to subfamily 1B. Together with subfamily 1A and 2A, these are thought to be involved in DNA repair. On the other hand, the AlkB domain of hALKBH8, belonging to subfamily 2A, is involved in removal of tRNA modifications. However, the amino-acid composition of the nucleotide recognition lid, and thus indirectly the classification into subfamilies, is insufficient to predict substrate specificity.

Many aspects of the AlkB family have been reviewed over the past two decades, such as RNA repair (Falnes and Rognes, 2003; Falnes et al., 2007; Alemu et al., 2016), DNA damage repair (Drabløs et al., 2004; Sedgwick, 2004; Nieminuszczy and Grzesiuk, 2007), base-flipping mechanisms (Sundheim et al., 2008), reaction mechanism (Mishina and He, 2006), cellular functions (Ougland et al., 2015) and substrate promiscuity (Fedeles et al., 2015). Lastly, Lu et al. (2014) and Shen et al. (2014) compare the TET family of dioxygenases with the AlkB family.

In this review, we aim to give a concise, state-of-the-art view on the structural features contributing to the substrate specificity towards different nucleic acid modifications.

2. Structural features of the promiscuous substrate specificity of the *E. coli* DNA repair protein AlkB

EcAlkB has been shown to have a remarkably substrate promiscuity, acting on both RNA and DNA (Aas et al., 2003) and a plethora of nucleobase lesions (Delaney et al., 2005; Delaney and Essigmann, 2004; Frick et al., 2007; Li et al., 2013, 2012, 2010; Maciejewska et al., 2010; Mishina et al., 2005; Singh et al., 2014). However, 1mA and 3mC in ssDNA are assumed to be its preferred substrates *in vivo* (Delaney and Essigmann, 2004). To shed light on the broad substrate specificity, different crystal structures of DNA-AlkB complexes have been solved (Yang et al., 2008; Yu et al., 2006; Zhu and Yi, 2014) (see Table 1). The accommodation of this wide range of alkylated bases is attributed to a flexible nucleotide recognition lid capable of dynamically adapting to the lesion to be repaired (Yu et al., 2006). However, the amino acid composition and flexibility of the nucleotide recognition lid are not the sole determinants of substrate specificity. EcAlkB is also able to repair many different residues through so-called K_m/k_{cat} compensation (Yu and Hunt, 2009). For example, 3mC has a smaller volume than 1mA, and thus is not as well accommodated in the active site as 1mA as evidenced by a higher K_m value. However, it seems that the active site stereochemistry of AlkB has evolved in such a way to have a higher turnover-rate (k_{cat}) for 3mC compared to 1mA.

Table 1. Published (co-)crystal structures of AlkB homologues.

Source	Protein	Substrate	Modification	PDB ID	Reference
E. coli	AlkB	dsDNA	1mA	3BI3	Yang et al., 2008
E. coli	AlkB	ssDNA	3mC	3I3M	Yu & Hunt , 2009
E. coli	AlkB	ssDNA	1mG	3KHC	Holland & Hollis, 2010
E. coli	AlkB	dsDNA	6mA	4NID	Zhu & Yi, 2014
Human	AlkBH1	dsDNA	“bulged”	6KSF	Zhang et al., 2020
Human	AlkBH2	dsDNA	1mA	3BTY	Yang et al., 2008
Human	AlkBH3	—	—	2IUW	Sundheim et al., 2006
Human	AlkBH5	—	—	4NJ4	Aik et al., 2014
Human	AlkBH7	—	—	4QKD	Wang et al., 2014
Human	AlkBH8	—	—	3THP	Pastore et al., 2012
Human	FTO	nucleotide	3mT	3LFM	Han et al., 2010
Human	FTO	ssDNA	6mA	5ZMD	Zhang et al., 2019

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The substrate specificity of EcAlkB has been the subject of many different studies. Early on, it was already noted that AlkB has a preference for 3mC and 1mA but poorly repairs 3mT modifications (Koivisto et al., 2004 and Yi et al., 2010). Structural and mutational analysis by Holland and Hollis (2010) suggested that the carboxylate side chain of residue D135 interacts with the exocyclic amine of methylated adenine and cytosine to provide selective binding. This interaction was further investigated by Maciejewska et al. (2012) who observed a higher catalytic activity on other AlkB substrates at a pH corresponding to their cationic form. Their

findings confirmed the preference of EcAlkB for the protonated form of the methylated bases, further explaining the importance of the negatively charged D135. Other bases such guanine and thymine would encounter potential electrostatic repulsions from the negative charge. On top of this, the relatively larger size of this aspartate residue as compared to alanine also limits the affinity for larger adducts such as 1,N^6-ethenoadenine (εA) (Holland and Hollis, 2010). Lastly, D135 also has a role in the stabilization of the transition state of the substrates. In many homologues displaying affinity towards 1mA and 3mC, the negatively charged residue is conserved (E175 in hALKBH2; E195 in hALKBH3), highlighting its importance in the recognition of 1mA and 3mC (Sundheim et al., 2006; Yang et al., 2008). Only recently, it was also established that EcAlkB, hALKBH2 and hALKBH3 are able to revert the epigenetic modifications 5-methylcytosine (5mC) *in vitro* (Bian et al., 2019). In this case, two acidic residues (D174/E175 in hALKBH2, and D135/E136 in EcAlkB) are important, since MD simulations pointed out that both contact the methylated base through water-mediated hydrogen-bonding interactions.

The pivotal role of the acidic residue D135 in discrimination of charged adducts has been strengthened by other studies. For example, in an attempt to alter the substrate specificity of EcAlkB, Zhu and Yi (2014) mutated D135 to isoleucine. The affinity of EcAlkB towards the uncharged epigenetic 6mA increased significantly while the activity towards 1mA remained rather constant, inferring differential effects of mutations on the accommodation of modified bases such as 1mA and 6mA in the active site. On the other hand, mutations such as D135L showed a higher activity towards 6mA. Additionally, the authors stated that the loop region surrounding D135 (K134-L139) is important in modulating substrate specificity, since it is part of a divergent active site region when comparing homologues with different specificities.

Other efforts also indicated specificity-relevant positions. In an attempt to facilitate tRNA sequencing, demethylation of m1G and N2,N2-dimethylguanosine by EcAlkB was mutationally improved (Dai et al., 2017; Zheng et al., 2015), as these modifications block reverse transcription during template preparation. Again, position 135 appeared important in substrate recognition, since D135S mutants catalyzed demethylation of m1G much more efficiently. By altering large residues within the Van Der Waals radius of N2,N2-dimethylguanosine, based on the modelled structure of the binary complex, a L118V mutant was found to also accommodate and efficiently demethylate the bulky adduct. Therefore, it can be concluded that steric effects in the active site contribute to specificity towards well defined modifications.

Fang et al. (2013) used energy decomposition analysis to investigate the catalytic role of different residues in EcAlkB. Nine residues were proposed to be of major importance in the catalytic cycle: T51, R73, Y76, K127, Q132, Q155, R161, F185 and R210 (figure 2). Three of these residues (T51,Y76 and R161) were also investigated by Holland and Hollis (2010). T51 and Y76 are both thought to be involved in the stabilization of the transition state (Fang et al., 2013). Moreover, crystal structures of holoenzyme and substrate bound enzyme yielded more detailed information on the function of Y67 (Holland and Hollis, 2010). When EcAlkB is scanning DNA for lesions, Y76 points away from the active site. Upon recognition of substrate, such as 1mA or 3mC, residue Y76 rotates and provides hydrophobic stacking to accommodate the modified base. R161, on the other hand, is involved in the correct discrimination of the damaged base. R210 stabilizes the binding of the catalytic co-substrate α-ketoglutarate.

Besides being a co-substrate, it also serves a structural function, holding together the jelly-roll fold of EcAlkB (Bleijlevens et al., 2008). Interestingly, many of the above-mentioned residues are structurally conserved in human AlkB homologues (Duncan et al., 2002).

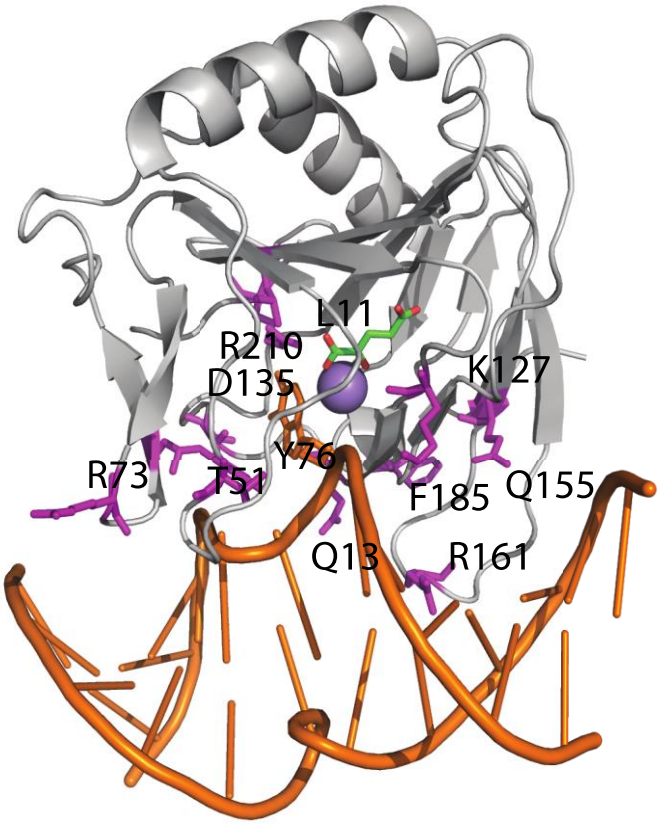

Figure 2. EcAlkB residues involved in DNA interaction. Interacting amino acids are in magenta; DNA and flipped base are in orange. Purple: catalytic iron; green: α-ketoglutarate co-substrate (PDB ID: 3BIE, figure rendered with Pymol).

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3. Interactions of AlkB homologues with the modified nucleobase

An intriguing AlkB homologue having a different specificity towards methylated DNA is human AlkBH1. Although it has the highest sequence similarity to *E. coli* AlkB (52% similar and 23% identical) (Ougland et al., 2012; Wei et al., 1996), it behaves quite differently. hALKBH1 is reported to have insignificant activity towards 1mA in DNA, but efficiently repairs 3mC (Westbye et al., 2008). On top of this, it is also reported to have lyase activity, cutting the DNA sugar-phosphate backbone at apurinic or apyrimidinic sites. However, this activity is independent of the iron and alpha-ketoglutarate-dependent oxidation reaction (Müller et al., 2010). In addition, it also demethylates histones (Ougland et al., 2012), 6mA in DNA (Wu et al., 2016) 5mC in mt-tRNA (Haag et al., 2016), 1mA in tRNAs (Liu et al., 2016) and other modifications (Kawarada et al., 2017). Recently, Zhang et al. (2020) elucidated the crystal structure for hALKBH1 and its preference for 6mA. The substrate binding pocket appears more similar to that of EcAlkB compared to other homologs, although it is more exposed to the

solvent. The modified base is sandwiched between two residues Y177 and H231, as compared to W69 and H131 of EcAlkB. More difference is seen at the equivalent position of D135 in EcAlkB, where hALKBH1 contains a serine (S235). S235 leaves more space in the active site to accommodate the 6mA modification. Replacement of S235 by a larger residue such as aspartate or glutamate showed significant decreases in catalytic activity, further stressing the importance of the size of the active site of hALKBH1.

The AlkB domain of hALKBH8 is reported to be specific towards the methylated uridines in the anticodon of specific tRNAs such as $tRNA_{UCG}^{Arg}$, and $tRNA_{UCC}^{Gly}$. The AlkB domain differs in that the nucleotide recognition lid of hALKBH8 is very disordered and only becomes structured upon binding of its natural substrate. The transition is held responsible for this specificity (Pastore et al., 2012). In contrast to other homologues, hAlkBH8 rather acts as a hydroxylating enzyme (much like the Tet family of proteins) than as a demethylatase (van den Born et al., 2011).

FTO is the ninth human AlkB homologue, also referred to as hALKBH9. FTO preferentially demethylates 6mA (Jia et al., 2011) and cap-localised N6,2'-O-dimethyladenosine (m6Am) (Mauer et al., 2017) both *in vitro* and *in vivo*, thereby regulating the functioning of modified mRNAs including their stability, translation, splicing and nuclear export (Alarcón et al., 2015; Liu et al., 2015; Tan et al., 2018), and of snRNAs (Mauer and Jaffrey, 2018). The dynamic demethylase activity of FTO affects many biological processes, including cell differentiation and development (Geula et al., 2015), circadian rhythm (Fustin et al., 2013), viral infection (Lichinchi et al., 2016) and cancer progression (Li et al., 2017). Throughout the years, additional FTO substrates have been reported, such as m3T, m3U and 1mA, although considered physiologically less relevant (Gerken et al., 2007; Jia et al., 2008; Wei et al., 2018). So far, no crystal structure of FTO in complex with 6mA-containing RNA has been published yet. Nonetheless, many FTO structures in complex with nucleobases and inhibitors are available. The first FTO structure, in complex with m3T and m3U nucleotides, provided the primary insights in the FTO structural features and catalytic mechanism (Han et al., 2010; PDB ID: 3LFM). Later, FTO-6mA models were published, either using molecular dynamics (Y. Fu et al., 2013) or using a structural overlap with AlkB-6mA crystal complex (Zou et al., 2016; PDB ID: 4NID). Only recently, Zhang and colleagues successfully crystallized FTO in complex with a 6mA-containing ssDNA oligonucleotide, providing novel details on FTO substrate specificity and nucleoside discrimination (Zhang et al., 2019; PDB ID: 5ZMD).

FTO consists of two large domains: an N-terminal domain (residue 1-326) which possesses the active site embedded in the highly conserved DSBH fold. The C-terminal domain (residues 327-505) shares no homology with AlkB. Although the C-terminal domain has no catalytic activity, it appears essential for folding, catalysis and binding of substrates (Han et al., 2010; Waheed et al., 2019). The deoxyribose and nucleobase rings are accommodated in a hydrophobic pocket, interacting with residues I85, Y108, L109, V228, S229, W230 and H231. The nucleobase is stacked between two aromatic residues, Y108 and H231, analogous to other AlkB homologues. The N6-methyl group is specifically bound in another hydrophobic pocket, stabilised by a robust H-bonding network between R96, Y106 and Y108, L203 and R322. (Figure 3)

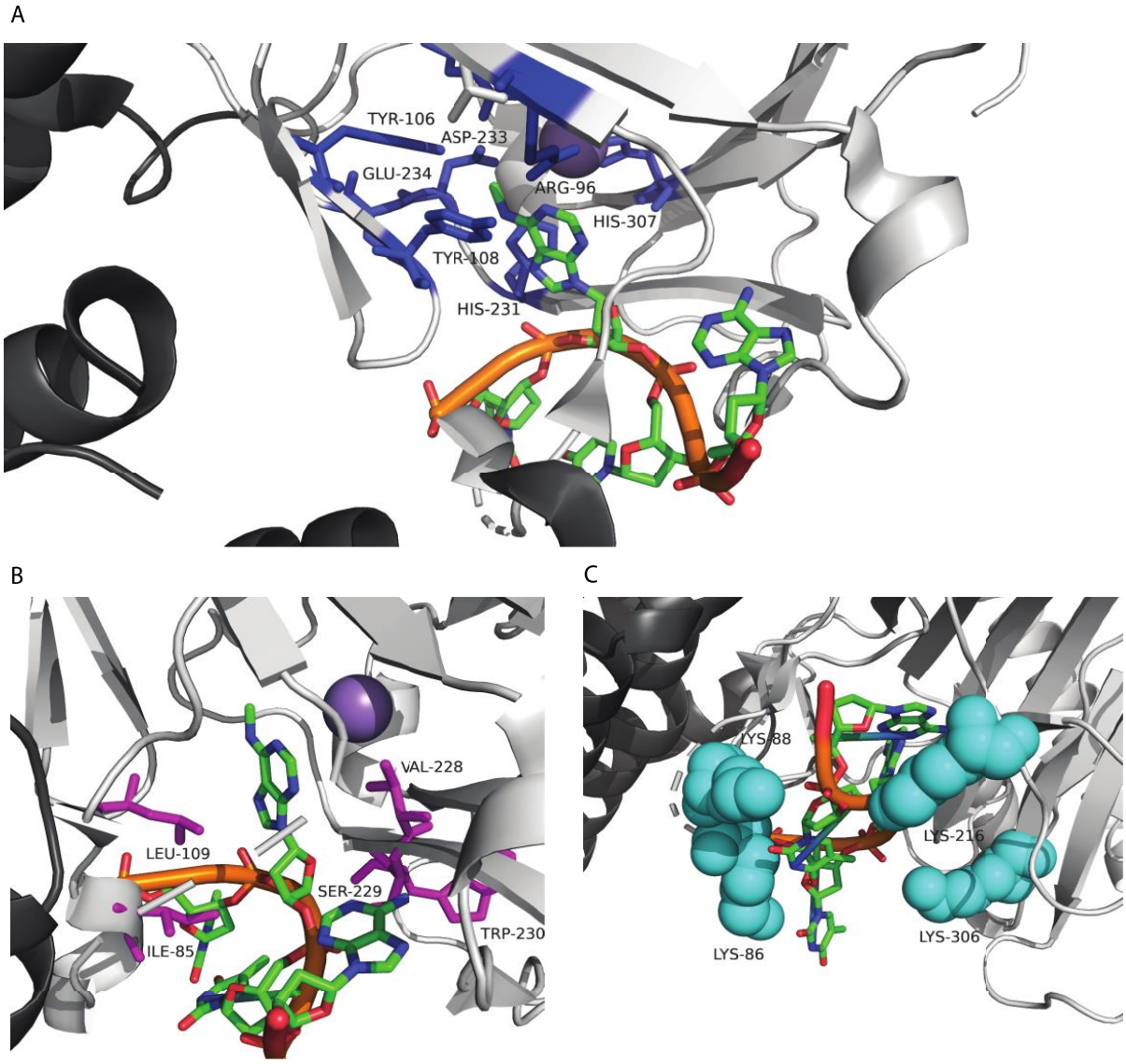

Figure 3. Structural features of FTO-6mA interaction. (A) Detailed representation of flipped 6mA in the FTO catalytic site (blue). (B) Hydrophobic pocket (magenta) stabilizing N6-methyl group. (C) DNA bending by two lysine pincers (cyan) (PDB ID: 5ZMD, figures rendered with Pymol). Orange: DNA phosphate backbone.

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FTO substrate specificity for the 6mA nucleobase is mainly achieved by residues E234 and R96. E234 specifically forms H bonds with N6 and N7 of the 6mA purine ring (Figure 4), adopting a unique stabilizing conformation which can only be observed in presence of 6mA substrates (Zhang et al., 2019). In presence of m3T, however, the sidechain of E234 is pushed out of the catalytic pocket, resulting in a much weaker interaction between its amide nitrogen and O4 of 3mT (Han et al., 2010). In the FTO-1mA complex, R96 and E234 form steric clashes with the methyl group of 1mA, resulting in reduced binding affinity (Zhang et al., 2019). In general, the aforementioned residues cannot explain discrimination between 6mA and m6Am substrates

since they carry the same nucleobase but differ in ribose moiety. Hence, no difference in substrate binding and catalysis between 6mA and m6Am was could be detected by Zhang and colleagues, despite the 2'OH of 6mA showing a favorable weak interaction with S229. Substrate specificity of FTO is believed to primarily result from the interaction of residues in the catalytic pocket with the nucleobase, rather than the ribose ring. Interactions with adjacent nucleobases and RNA structures distant from the catalytic pocket may form an additional layer for discriminating between 6mA substrates, although the precise mechanism is still unknown.

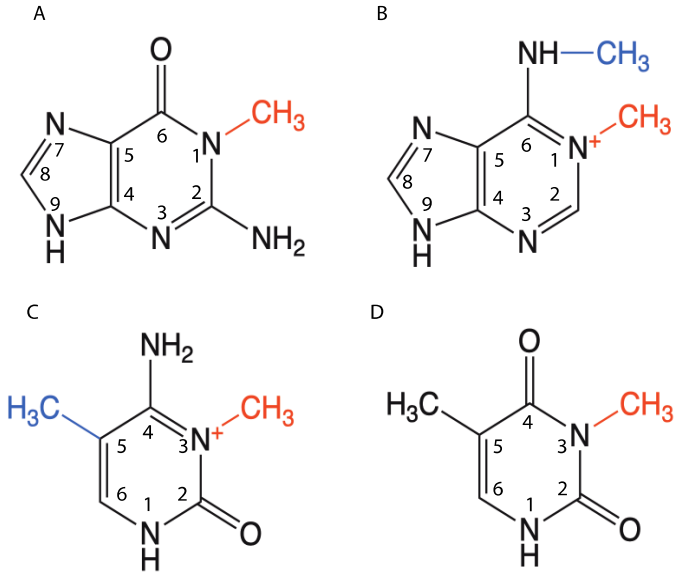

Figure 4. Nucleobase modifications oxidised by the AlkB family of homologues. (A) 1mG (B) 1mA (red), 6mA (blue) (C) 3mC (red), 5mC (blue). (D) 3mT.

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hALKBH5 is another homologue preferentially demethylating 6mA. Localizing in the nucleus, hALKBH5 is thought to be involved in mRNA processing critical for sperm development (Thalhammer et al., 2011; Zheng et al., 2013) and can be associated to certain types of cancer (Li et al., 2017). Currently, no crystal structures in complex with modified nucleobases are available. Nevertheless, several crystal structures of hALKBH5 in complex with its cofactors have been published (Aik et al., 2014; Feng et al., 2014; Xu et al., 2014) as well as NMR structures to explain conformational protein dynamics in the active site (Purslow et al., 2018). hALKBH5 adopts the typical DSBH fold containing the catalytic pocket, flanked by functionally important N-terminal and C-terminal regions. The N-terminal region (residues 1-66) is largely α-helical, resembling the hALKBH8 RNA recognition motif (RRM) believed to be involved in tRNA interactions. The C-terminal region (residues 293–394), on the other hand, is largely disordered due to a substantial number of proline, arginine and serine residues. Potentially, phosphorylation events (S361, S371, S374, S384) in this region may serve as an additional mechanism for regulating RNA interactions. Within its active site, hALKBH5 shows a hydrophobic 6mA-binding pocket similar to that of FTO. Also, hALKBH5 contains aromatic

residues which are likely involved in stacking of the methylated bases (Aik et al., 2014; Feng et al., 2014). However, some subtle differences in the nature of these residues might contribute to a more specific binding of 6mA. For example, Y139 is thought to reinforce the binding of hALKBH5 to 6mA (Feng et al., 2014). Also, the active site of ALKBH5 appears more open than that of FTO, meaning hALKBH5 may accept bulkier RNA secondary structures. Another interesting observation is that hALKBH5 lacks the two polar residues immediately downstream of the HXD motif, unlike the other AlkB homologues (Han et al., 2010; Sundheim et al., 2006; C.-G. Yang et al., 2008). The ALKBH5 loop following the $H_{204}XD_{206}$ motif contains more hydrophobic residues, proline 207 and isoleucine 208, respectively (Figure 5). This finding is in accordance with the mutational studies by Zhu and Yi (2014), who observed increased affinity of AlkB for 6mA by mutating the D135 residue to isoleucine.

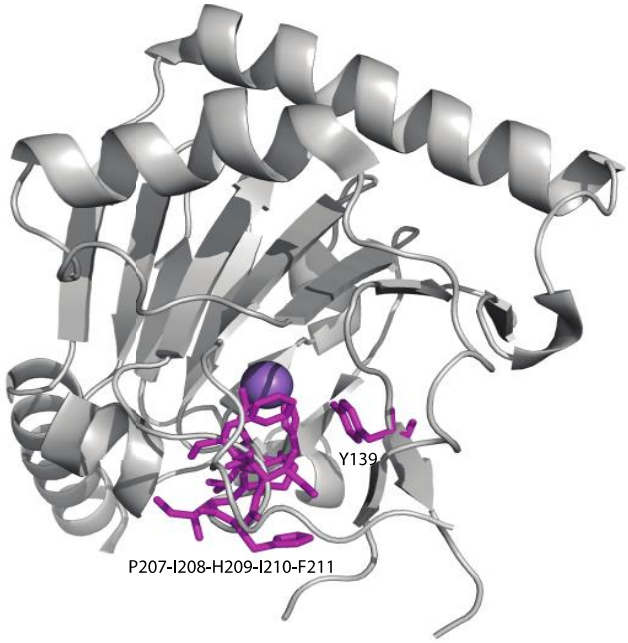

Figure 5. Structure of AlkBH5 in the absence of substrate. Magenta: hydrophobic residues lining the active site. (PDB ID: 4NJ4, figure rendered in Pymol)

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4. Double-strand selectivity

One difference in substrate specificity seen in the AlkB family is selectivity for either single-stranded (ss) or double-stranded (ds) DNA. Whereas EcAlkB and hALKBH3 have a strong preference for ssDNA (Aas et al., 2003), hALKBH2 is reported to be equally active on dsDNA and ssDNA, but only marginally on RNA (Falnes, 2004; Ougland et al., 2004). Although highly similar in structure and fold, both EcAlkB and hALKBH3, as compared to hALKBH2, contain divergent β-hairpins interacting with the damaged strand (Monsen et al., 2010). Chen et al. (2010) describe part of this β-hairpin as a “strand determination hand motif” (figure 6A). While in hALKBH2 this hand is in an open conformation (figure 7B), it is in a closed “fist” conformation in hALKBH3 (figure 6C). In EcAlkB, this loop is much shorter, folding in a rather closed conformation (figure 6D). The open hand in hALKBH2 fits well with the major binding groove of dsDNA. Another crucial aspect in the dsDNA substrate selectivity is the F102 finger

present in the hALKBH2 hand domain (figure 6B). It intercalates in the free space generated by the flipped-out base, thereby stabilizing the DNA duplex by means of π-π interactions. On the contrary, the fist conformation of hALKBH3 is stabilized by a bulky salt bridge formed between three ionized amino acids (R122, E123, and D124; RED). Replacing these with corresponding residues found in the hALKBH2 hand (V101-F102-G103; VFG) renders hALKBH3 much more active towards dsDNA. Conversely, when the VFG hand in hALKBH2 was replaced by the RED fist, all activity towards methylated double-stranded substrates was abolished, whereas the activity on ssDNA was largely retained (Monsen et al., 2010). Comparative molecular dynamics simulations by Pang et al. (2013) additionally suggest that the DNA duplex and the DNA-protein interactions are much more stable in hALKBH2 than in EcAlkB. Intriguingly, Chen et al. (2016) demonstrated, using LC-MS, the preferential repair of minor substrates such as m1G and m3T in double-stranded DNA by EcAlkB, as opposed to its presumed preference for single-stranded DNA (Dinglay et al., 2000). To our knowledge, no explanation for this preference has been given so far.

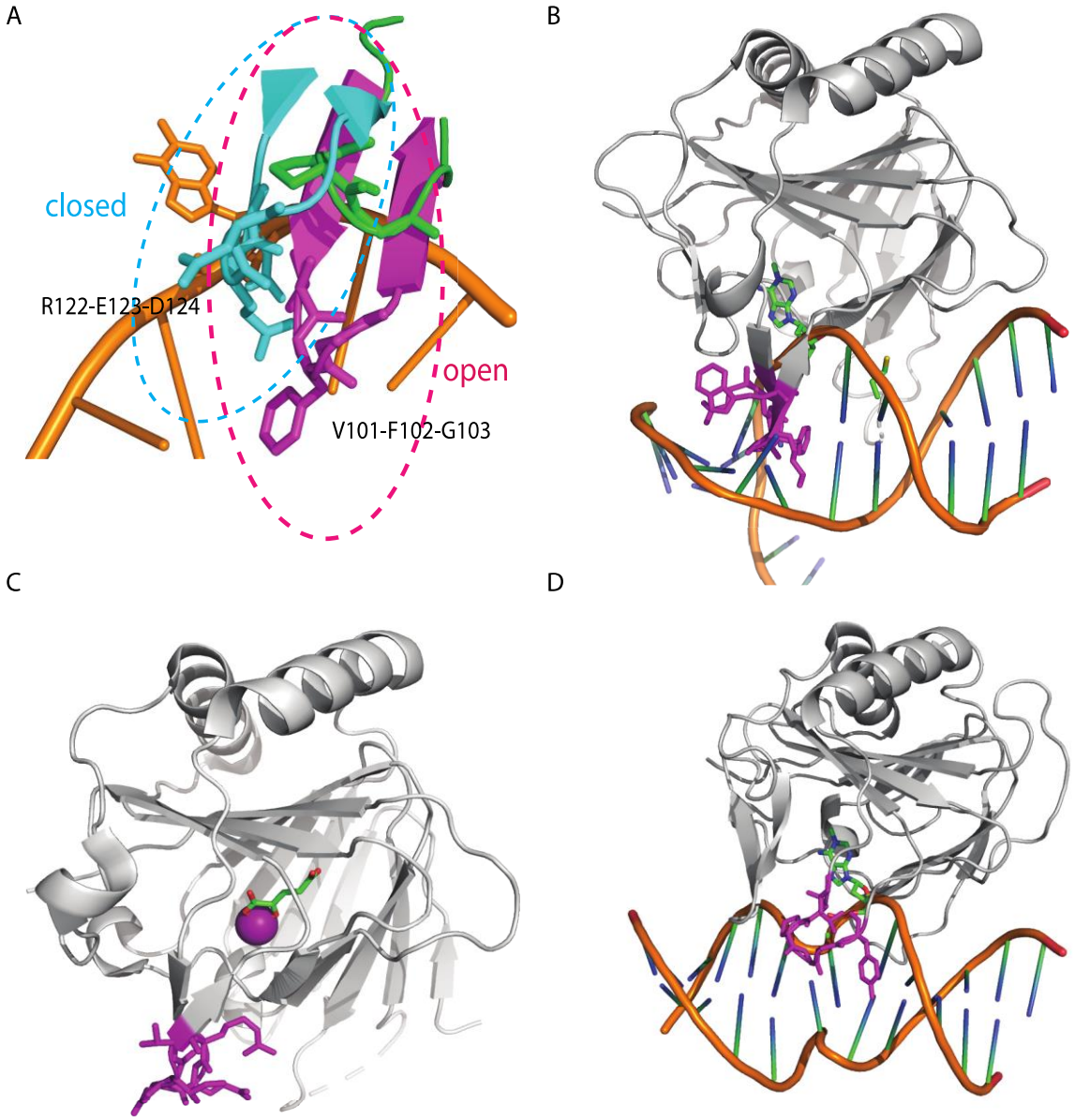

Figure 6: Divergent ß hairpins operating at the DNA-protein interaction interface. (A) Loop of EcAlkB (T51-P52-G53) (green; PDB ID: 3BIE) overlayed with the homologous hairpins in hAlkBH3 in closed conformation (cyan; PDB ID: 2IUW), and hAlkBH2 in open "hand" conformation (magenta; PDB ID: 3BTY); orange: 1mA lesion in DNA. (B) hAlkBH2 loop (magenta) interacting with 1mA in dsDNA. (C) hAlkBH3 (PDB ID: 2IUW). (D) EcAlkB interacting with 1mA methylated dsDNA (PDB ID: 3BIE).

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5. Single-strand selectivity

A large difference in substrate selectivity can also be found between the different AlkB homologues in their ability to repair single-stranded nucleic acids. Besides a domain homologous to AlkB, human ALKBH8 contains an RRM-containing domain and a methyl transferase domain (Pastore et al., 2012). The RNA-binding domain partially explains the selectivity towards RNA, but in addition, a basic N-terminal helix and certain basic patches of the AlkB domain (figure 7) are thought to be involved in RNA interaction. Strong binding of RNA molecules was, however, only observed when the AlkB domain of hALKBH8 is expressed together with the RMM domain.

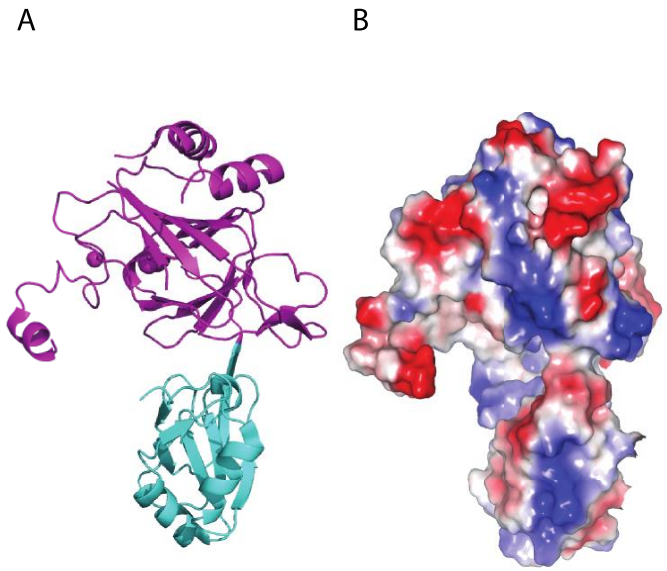

Figure 7: Structural features of hAlkBH8. (A) domain structure. Magenta: AlkB domain; Cyan: RNA-binding domain. B. Surface charge. The central basic patches (blue) in both domains are presumed to take part in RNA interaction. (PDB ID: 3THT, figures rendered in Pymol).

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Alternatively, selectivity towards RNA may be governed by a strict selectivity towards single-strands. In FTO, for example, the preference for single-stranded substrates is determined by a 13 amino acid loop insertion between β5 and β6 (figure 8). Superposition of the structures

of FTO and the hALKBH2-dsDNA complex shows steric clashes of this FTO loop with the unmethylated complementary strand (Han et al., 2010). This supports the idea that regions of AlkB homologues other than the F102 finger of hALKBH2 also contribute to substrate selection. In RNA-active hALKBH5, the loop between β8 and β9 is thought to obstruct the binding of double-stranded substrates. This loop adopts an altered sturdier conformation compared to other homologues such as hALKBH2, and is anchored by a disulfide bridge between residues C230 and C267, as confirmed by mutational studies (Aik et al., 2014; Xu et al., 2014).

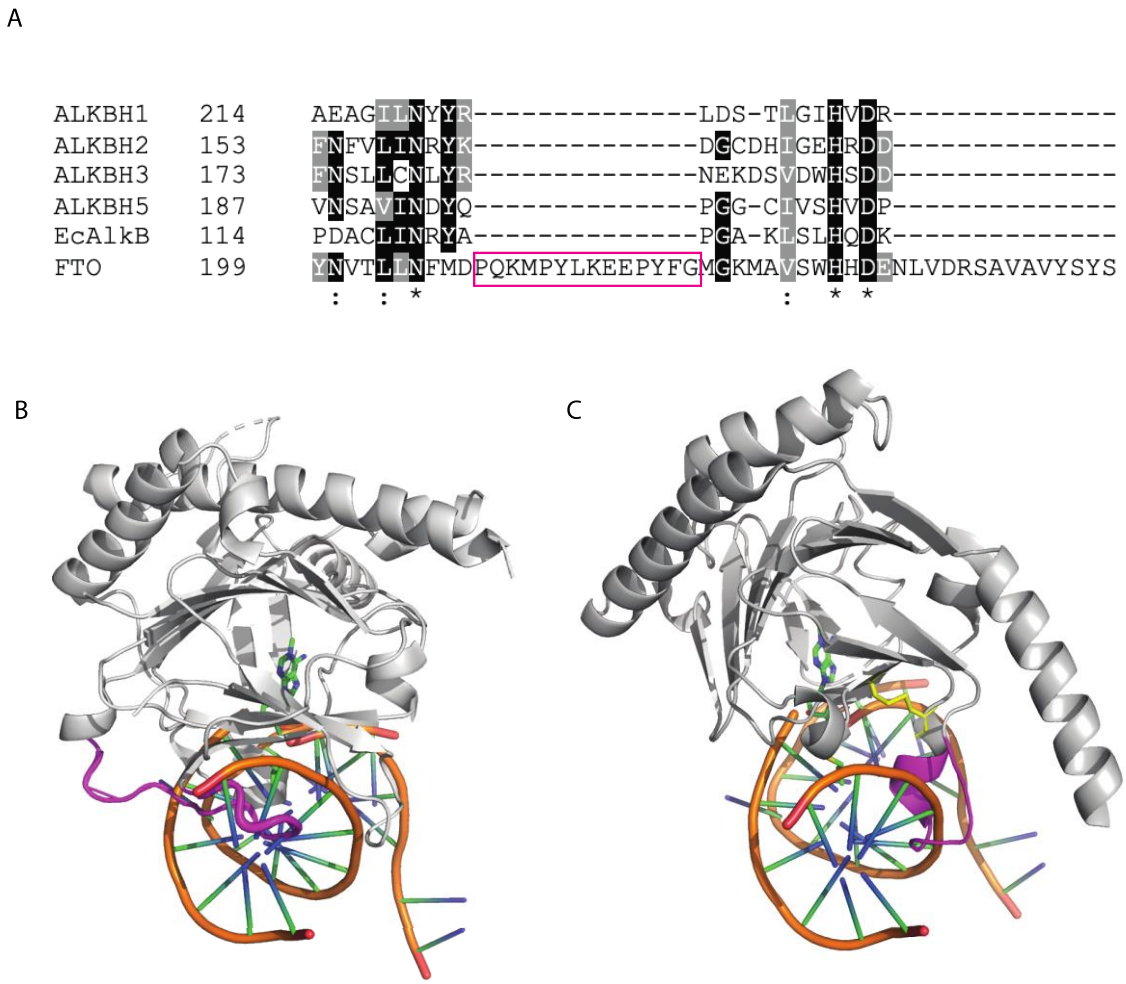

Figure 8. Steric hindrance of unmethylated complementary strand-interacting loops in ss-specific AlkB homologues. (A) Sequence alignment of homologues. (B) Overlay of the AlkB domain (PDB ID: 3LFM) with dsDNA from the ALKBH2-dsDNA complex (PDB ID: 3BTY). Loop causing steric hindrance indicated in magenta. (C) AlkBH5 (PDB ID: 4NJ4) overlayed dsDNA from the ALKBH2-dsDNA complex (PDB ID: 3BTY, ALKBH2 not shown). Loop in magenta. Rigidifying disulfide bridge in yellow.

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6. Specificity towards nucleic acid conformations

The selectivity of nucleic acid-interacting proteins is often guided by nucleic acid conformations. As mentioned above, certain modifications such as 1mA impair the standard Watson-Crick base pairing in dsDNA and induce the modified bases to adopt Hoogsteen base pairs. This altered conformation is recognized by repair proteins such as EcAlkB (H. Yang et al., 2008). hALKBH1, on the other hand, is more selective towards bulged nucleic acids that share a local unpairing feature with flanking duplexes (Zhang et al., 2020b). Structurally, hALKBH1 copes with these bulges through a stretched out, positively charged Flip1 (F1) motif and an internal stabilizing helix. The extended F1 loop strongly impairs autonomous base flipping, pointing towards the preference for bulged nucleic acids. For eraser proteins hALKBH5 and FTO, Zhang et al. (2019) demonstrated that, besides the sequence context, RNA structure is crucial in regulating substrate interaction. FTO contains two positively charged loops, each containing two pincers (residues K88 and K216; K86 and K306, respectively) (figure 3C). These pincers interact with the phosphate backbone and neighboring nucleosides, bending the RNA and positioning the methylated base in the catalytic pocket. Notably, there is large vacancy next to pincer 2 which allows tertiary RNA structures or cap structures to accommodate. These may form additional interactions with the FTO loop residues, potentially enhancing the binding affinity. Hence, the presence of favorable cap-protein interactions might explain the higher FTO activity for cap-localized m6Am compared to internal 6mA. Vice versa, m6Am is not demethylated in absence of a preceding cap structure (Mauer et al., 2017), and 6mA is more efficiently demethylated in presence of RNA loop structures compared to linear RNA (Zou et al., 2016).

7. Nucleic acid sequence specificity

Most of the DNA repair proteins of the AlkB family (e.g EcAlkB, hALKBH2 and hALKBH3) do not posses selectivity towards defined DNA sequences (Falnes, 2004). In contrast, so-called erasers such as FTO and hALKBH5 do contain sequence preferences. Transcriptome-wide 6mA mapping revealed that N6-adenosine methylation is preferentially localized on the DR(6mA)CH sequence motif (with D = not C; R = A or G; H = not U) (Dominissini et al., 2012; Meyer et al., 2012), providing an indirect preference for sequence context. However, FTO and ALKBH5 were also shown to recognize 6mA modifications at non-consensus sites, albeit with lower affinities (Zou et al., 2016). Interestingly, 6mA itself was shown to induce different RNA conformational changes dependent of the sequence context, thereby affecting its interactions with several 6mA readers (Zou et al., 2016).

8. Atypical dioxygenase specificity of hALKBH7

A puzzling member of the human AlkB family is hALKBH7. Although it contains many conserved active site residues characteristic of the family, it lacks the recognition lid typical of AlkB proteins, making the active site solvent exposed. Moreover, it lacks the arginine needed for binding the alpha-ketoglutarate co-factor (Wang et al., 2014). In spite of being studied for several years, no specific substrate has been identified. Having no nucleotide recognition lid, it most probably lacks the capability to bind and stabilize incoming nucleobases, for the recognition lid is seen as a major determinant of substrate specificity (Yu et al., 2006). Its rather negatively charged surface near the active site suggests that hALKBH7 is less likely to be a nucleic acid demethylase (Wang et al., 2014). Through structural alignment, it was predicted to function as a protein hydroxylase. Deletion of the gene pointed at a role in the

regulation and triggering of cell death caused by oxidative or alkylation-induced necrosis, underlining the hypothesis of a protein substrate (Fu et al., 2013).

9. Conclusions and outstanding questions

In this review, we compiled the current knowledge on how substrate specificity for modified nucleic acids is structurally realized by different AlkB homologues. Through comparison of the available structures, we aimed to bring together insights in the determinants of substrate specificity. It is clear that the loops and β-harpins of the AlkB domains interacting with the backbone and adjacent nucleobases of the nucleic acid substrate are major actors in substrate discrimination. Selectivity for single-stranded RNA is, for example, generated by steric exclusion of a complementary strand by a larger loop as in the case of FTO. In contrast, hALKBH2 more efficiently repairs double-stranded substrates through hydrophobic interactions by a β-hairpin stabilizing the base-stacking in the void left by the flipped-out base.

More in-depth insights in how AlkB homologues discriminate the chemistry of the modified nucleobase have been revealed by mutational and structural studies. The charges found directly downstream of the HxD motif determine the nature of the preferred modified nucleobases. For example, an acidic residue at these positions better accommodates positively charged lesions such as 1mA. On the contrary, homologues undoing adducts such as 6mA are expected to have more hydrophobic residues in this region. Secondly, the dimensions of the preferred bases are also probed by the equivalent residues at the EcAlkB position D135 as in the case of hALKBH1. Other key residues are found lining the active site, where they provide the right steric environment for each substrate as bulky residues control the size of possible accommodated nucleobases.

A third essential feature of this family of enzymes is the flexible nature of the proteins which plays at different levels. For several variants, active site flexibility provided by the nucleotide lid generates sufficient room for promiscuity. On the other hand, rigidity of certain loops is used to discriminate against substrates. Furthermore, flexibility plays a major in the sequential binding of (co-)substrate, “active site” breathing and subsequent release of unmethylated DNA.

Some outstanding questions still remain, however. The binary structures of some human AlkB homologs and their preferred physiological substrates is yet to be determined, as in the case of ALKBH3, ALKBH5 and ALKBH7. Additionally, recent work has focused on human and *E. coli* AlkB, but also homologues from other taxa might be interesting, as well as more remotely related protein families such as the Ten-eleven translocases (TET). Furthermore, co-crystals with RNA have remained elusive for members of this family, but might provide novel insights in substrate binding. Another question is to determine whether the *in vitro* promiscuity observed in some family members such as EcAlkB holds physiological relevance *in vivo*. Besides, the higher affinity of EcAlkB for dsDNA containing modifications 1mG and 3mT remains unexplained. Additionally, the dynamics of nucleic acid binding, and search and recognition of modified nucleobases are still a large caveat in current knowledge.

To answer these questions, additional structural information is required. Co-crystallisation of ALKBHs and their respective substrates could yield important clues to understand substrate recognition by each homologue. Other methods such as NMR or molecular dynamics have

already been used to investigate certain members of the AlkB family and can provide an additional strategy when co-crystallisation proves to be difficult. Additional mutational studies will provide more insight in the residues responsible for the recognition and discrimination of the different substrates and for performing the catalytic function.

To investigate the underlying molecular mechanisms of substrate binding and recognition, several methods are available. For example, the conformational dynamics of binding of AlkB have already been extensively studied by NMR, fluorescence anisotropy and catalytic assays (Bleijlevens et al., 2008; Ergel et al., 2014). Similar investigations are invaluable for the other homologues. Additionally, by expanding the number of sequences in the phylogenetic analyses of the AlkB family, one might be able to better understand the evolutionary relationships between the different variants. For example, an interesting analysis would entail the origins of the different functions and how they diverged.

Lastly, as already described by Dai et al. (2017) and Zheng et al. (2015), EcAlkb has been proven to be an interesting starting point for the development of molecular tools for RNA sequencing or methylation detection using mutagenesis of key residues. This leads to the possibility for molecular evolution of substrate specificity of other homologues. Initially, molecular evolution of AlkB homologues is strongly dependent on structural information, however the outcome of such experiments itself can provide additional information on substrate recognition. Hence, such experiments can be advantageous in further understanding of the structure-function relationships of the AlkB family.

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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