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ALKBHs-facilitated RNA modifications and de-modifications

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Abstract

The AlkB gene that protects *E.coli* against methylation damage to DNA was identified more than 3 decades ago. 20 years later, the AlkB protein was shown to catalyze repair of methylated DNA base lesions by oxidative demethylation. Two human AlkB homologs were characterized with similar DNA repair activities and seven additional human AlkB homologs were identified based on sequence homology. All these dioxygenases, ALKBH1–8 and FTO, contain a conserved α-ketoglutarate/iron-dependent domain for methyl modifications and de-modifications. Well-designed research over the last 10 years has identified unforeseen substrate heterogeneity for the AlkB homologs, including novel reversible methyl modifications in RNA. The discoveries of RNA demethylation catalyzed by AlkB family enzymes initiated a new realm of gene expression regulation, although the understanding of precise endogenous activities and roles of these RNA demethylases are still undeveloped. It is worth mentioning that the AlkB mechanism and use of α-ketoglutarate have also emerged to be essential for many enzymes in epigenetic reprogramming that modify and de-modify methylated bases in DNA and methylated amino acids in histones.

Keywords

AlkB homologs; DNA repair; RNA metabolisms; Oxidative demethylation; YTH domain proteins

1. Introduction

In 1983, Sekiguchi and coworkers isolated seven mutants of *E. coli* that were sensitive to methylating agent but not to UV light [1]. Most of these mutations were mapped to the region of the *E. coli* genome encoding the well known AlkA DNA glycosylase. One

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Conflict of interest

The authors declare no conflict of interest.

mutation was novel and the gene responsible was named AlkB. Despite many studies, no function was assigned to the *E. coli* AlkB gene for 2 decades. Yet, an important clue to the activity of the AlkB protein was reported in 2000; it was shown that the reactivation of methylated single-stranded DNA phages was considerably reduced in *E. coli* lacking the AlkB gene [2]. One year later, Aravind and Koonin [3] employed sequence profiling analysis to show that AlkB and other enzymes define a novel family of the 2OG-Fe(II) oxygenase superfamily. They conclude that the AlkB protein catalyzes oxidative detoxification of alkylated bases and further suggest that the more distant homologs of AlkB detected in eukaryotes and in plant RNA viruses might be involved in RNA demethylation. These bioinformatics analysis were soon to be verified experimentally for the *E. coli* AlkB and two human homolgos, ALKBH2 and ALKBH3 [4–7]. A year later, five more human homologs of the *E.coli* AlkB enzyme are identified, ALKBH4–8 [8]. They hypothesize that these homologs could be back-up enzymes for the DNA repair activities of Alkbh2 and 3 or may code for novel DNA or RNA repair activities. With the addition of the FTO (fat mass and obesity-associated) protein the mammalian family of AlkB proteins now consists of 9 members (Fig. 1). Recent research has identified remarkable diverse substrate specificities for the AlkB homologs. Yet, the activities of some homologs are still hard to pin down. In this review, we focus on the AlkB homologs acting on RNA.

2. Past and present of AlkB mediated RNA repair

Researchers have speculated on the functional roles of AlkB family proteins on RNA modifications. In 2003, reviewing the novel finding of *E. coli* and human AlkBs in mediating DNA, and possible RNA, repair by oxidative demethylation, Begley and Samson suggested that the “RNA repair” activities of AlkB and ALKBH3 might have evolved to regulate biological RNA methylation [9]. In 2010, we proposed that post-transcriptional RNA modifications can be dynamic and may play additional roles in gene expression regulation in a grand challenge commentary [10]. The AlkB-mediated demethylation was specifically proposed as a potential pathway for RNA demodification. In recent years these assumption has been confirmed in several important studies. However, the RNA repair activities of AlkB and its human homolog’s have not been con-firmed *in vivo*, although recombinant AlkB from *E. coli* and ALKBH3 from mammals efficiently demethylate 1 mA and 3mC in RNA. In an early study, these hydroxylases were shown to restore the biological function of chemically methylated mRNAs and tRNAs [11]. As the relevant modifications, 1mA and 3mC, also are naturally occurring modifications in some RNAs, introduced enzymatically, they speculated that a low level of AlkB-mediated removal of enzymatically introduced methyl modifications may be accepted, due to rapid replenishment by the methyltransferases. This raises the same question as for the 5-hydroxymethyluracil (5hmU) modification in DNA. In a recent study [12], TET is shown to directly produce 5hmU in mouse embryonic stem cell DNA by oxidizing thymine (T). Thus, 5hmU in DNA is introduced by TET enzyme(s) and by reactive oxygen species and at present there is no indications that the SMUG glycosylase that recognize and excise 5hmU has a specificity or partner proteins that can aid in distinguishing between ROS and TET induced 5hmU. Thus, the historical distinction on chemically introduced *damaged bases* in DNA and RNA as

opposed to enzymatically introduced *regulatory bases* in DNA and RNA might have to be seen in a new light.

3. Regulatory DNA modifications

Known regulatory modifications in mammalian genomic DNA have for long been restricted to the methylation of the 5-position of cytosine (5mC). In recent years, the further hydroxylation of 5mC to 5-hydroxymethylCytosine (5hmC), 5-formylCytosine (5fC) and 5-carboxylCytosine (5caC) by the AlkB like TET enzymes have added complexity to the understanding of modified cytosines in DNA. It is very interesting to notice that the final removal of these cyto-sine modifications is carried out by DNA glycosylases [13]. Thus, the processing of nearby cytosine modifications in opposing DNA strands, like clustered oxidative DNA damage, by the base excision repair (BER) enzymes must be coordinated to avoid producing double-strand DNA breaks (DSBs) [14]. In a recent study [15] it is shown that TET1-TDG-BER dependent active DNA demethylation is highly coordinated to avoid double strand breaks in DNA. Very recently, the repertoire of regulatory methylated bases in mammalian genomic DNA was expanded to also include methylation of the 6-position of adenine (6mA) and the possible dynamic nature of this modifications could well indicate a role for an AlkB-like enzyme for its reversal [16–18]. For this review, it is worth mentioning that histone demethylation by hydroxylation also share homology to AlkB demethylation. The similarity of removing a methyl group from Adenine (1mA) and methylated-lysine led Shi and co-workers to discover the first histone demethylase [19], and Zhang and co-workers to look for a similar mechanism for histone lysine demethylation and they identified JHDM1 (JmjC domain-containing histone demethylase 1) following purification through six chromatography columns [20].

While the number of regulatory bases in DNA is relatively limited, there are a wide variety of regulatory bases in RNA that are often quite abundant. The known biochemical processes involved in generating and removing methylated bases from DNA and RNA are remarkably similar and here we will focus on the recent identification of the reversible status of RNA modifications and the role of the mammalian AlkB enzymes.

4. ALKBH8 mediated tRNA modifications

Amongst the nine AlkB homologs, ALKBH8 is unique by containing one additional annotated protein domain. The activity of this C-terminal methyltransferase, methylation of certain wobble uridines in some tRNAs, was elucidated due to its homology to the *S. cerevisiae* tRNA methyltransferase, Trm9 [21,22]. The AlkB domain of ALKBH8 was later shown to catalyze the hydroxylation of 5-methoxycarbonylmethyluridine (mcm^5U) at the wobble position of certain tRNAs [23,24]. This generate, unlike the other known AlkB enzymes that produces an unstable hydroxymethylated base that is spontaneously demethylated, a new stable hydroxylated base comparable to the hydroxylation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in genomic DNA [23,24].

5. Reversible methylations of mRNA

The research of RNA modifications entered a new era in 2011 with the identification of the reversible nature of the N^6-methyladenine modifications by the Fe(II)- and α-ketoglutarate-dependent fat mass and obesity-associated (FTO) protein [25] (Fig. 2). Soon after that, a second Fe(II)- and α-ketoglutarate-dependent hydroxylase, ALKBH5, was also shown to demethylate m^6A in RNA [26]. m^6A is a very common internal modification in mRNA and non-coding RNAs. It was also shown that two additional modifications, 6-hydroxymethyladenosine (hm^6A) and 6-formyladenosine (f^6A), exist in mammalian messenger RNA and that the FTO protein can further oxidize m^6A to make hm^6A and f^6A [27]. Following the discovery of FTO, two laboratories developed sequencing methods to profile m^6A in the mammalian transcriptomes [28,29]. In three recent studies [30–32] m^6A-CLIP (cross-linking and immunoprecipitation) followed by reverse transcription was used to map m^6A at high-resolution throughout the transcriptome. The mutational signature induced by the reverse transcriptase allows for potential single-base resolution mapping of m^6A throughout the transcriptome. Although these methods are not suitable for use in low input materials, they allowed further insight on, for example, the location of m6A and its possible role in regulating alternative polyA choice.

High-resolution mapping allowed further insight on the location of m^6A and suggest a role for m^6A in regulating alternative polyA choice [31]. Similarly, 5-hydroxymethylcytosine (5hmC), a modifications that recently have attracted much attention due to its presence in genomic DNA, also occurs in RNA and was recently shown to be introduced by the TET enzyme and mark polyadenylated RNAs in Drosophila [33]. Transcriptome mapping of cells and organisms lacking ALKBH5 and/or FTO has not yet been completed. Thus, no information on the specificity of the methyltransferases METTL3 and METTL14 [34] or the demethylases ALKBH5 and FTO exist and data published on the sequence specificity of m^6A in the mammalian genome so far thus only portray the combined effect of m^6A deposition by methyltransferases and its removal by demethylases. The selectivity of the methylation deposition and removal remain unanswered questions.

The discovery and characterization of reader proteins that recognize m^6A have significantly advanced functional understanding of this modification in gene expression regulation. The YTH family proteins selectively recognize methylated RNA and affect metabolism and processing of the target mRNAs [28,35,36]. The methylation can promote translation, mediate mRNA decay, and affects localization and splicing. Upon heat shock, YTHDF2 can preserve m^6A methylation of stress-induced transcripts [37]. The m^6A methylation also occurs on lncRNA and pri-miRNA. It serves as a structural switch to tune RNA secondary structure and affects binding by RNA-binding proteins [38]. The methylation on pri-miRNA can be recognized by HNRNPA2B1 to accelerate miRNA maturation [39].

More recently, two papers portray 1-methylAdenine (m^1A) in eukaryotic mRNA [40,41], and describe a potential role for ALKBH3 for its reversible and dynamic nature. m^1A modifications in mRNA are less frequent than m^6A, 10-fold less in cell lines but 3-fold less in tissues, and can occur on a few thousands of mRNA transcripts in humans. Unlike m^6A, m^1A appears around start codon and strongly correlates with protein production. The unique

features of m^1A indicate a role in translation of methylated mRNA. The methyltransferase introducing the m^1A modification is not yet identified.

6. The biology of reversible modifications in RNA

The reversible nature of some RNA modifications is a very recent discovery and the biological consequences of aberrant dynamics of m^6A (and m^1A) have therefore just now become a potential topic of study. Based on mutant analysis in model organisms, it seems clear that m^6A modifications in mRNA have unique roles in meiosis [26,42–44] (Fig. 2). The precise role of individual mRNAs may now be studied due to the ability to map m^6A sites that are dynamically regulated during meiosis. The m^6A modification in mRNA also has crucial roles for embryonic stem cells and depletion of the METTL3 m^6A methyltransferase results in impaired differentiation and restricted lineage priming [45,46]. It is also shown that m^6A in mRNA is associated with the timing of circadian periods [47].

Initially, the extraordinary interest in FTO was sparked by its association with obesity. However, the relevant polymorphic variants in the introns of the *FTO* gene does not seem to affect FTO expression or activity and it is more likely that these polymorphisms have an effect, as a long-range promoter, for the downstream *IRX3* gene [48]. Less attention has been credited to the study that revealed that an inactivating *FTO* mutation is responsible for an autosomal-recessive lethal syndrome which is suggestive of a crucial role of FTO in global gene expression regulation [49]. The *in vivo* relevant RNAs serving as substrate for FTO is still unknown and mutant cells and organisms should be used for its identification.

7. Future studies

The dynamically regulated m^6A and m^1A bases in RNA are chemically stable and recent data indicate that they carry out their function by interacting with reader proteins. The distinct and highly conserved positioning of these modifications further argues for such a role. Yet, m^6A and m^1A could also affect hydrogen binding in certain RNA structures since the positions methylated are involved in “Watson-crick” base pairing. In order to advance our understanding of RNA methabolisms by reversible base modifications, focus could be on:

- **i.** The development of quantitative sequencing methods for m^6A with less input materials (current methods require millions of cells). Highly accurate sequencing results could allow site-directed mutagenesis studies to get insight on individual mRNA transcripts that are dynamically regulated by environmental stress or through periodic regulations like the cell cycle and the circadian rhythm.
- **ii.** Biochemical and cellular characterization of the writer complex, additional readers, and existing and potentially new erasers, including structural characterizations.
- **iii.** The extensive post-transcriptional gene expression regulation through RNA methylation in various biological processes, as well as their impacts on various human diseases.

iv. Posttranslational events that impact RNA methylation writers, readers and erasers to reveal how selective writing, reading and erasing are achieved.

v. More than 100 post-transcriptional base modifications have been identified in various RNA-species. Thus, it is very likely that many of these are dynamic or even reversible. The impacts of dynamic and potentially reversible modifications on other RNA species such as tRNA and rRNA.

vi. The activities of ALKBH6 and ALKBH7 remains a mystery. ALKBH7 is the only AlkB homolog containing a conserved mitochondrial signal and is involved in programmed necrosis and fat metabolism [50,51]. The exact role and underlying mechanism of ALKBH4 still need further studies [52].

Methylated bases occur frequently in a variety of RNA species and the specificity of demethylases is not well characterized regarding the possibility that other factors restrict or initiate substrate recognition. Also, individual enzymes might have several relevant substrates, both regarding the modification itself and the type of RNA molecule.

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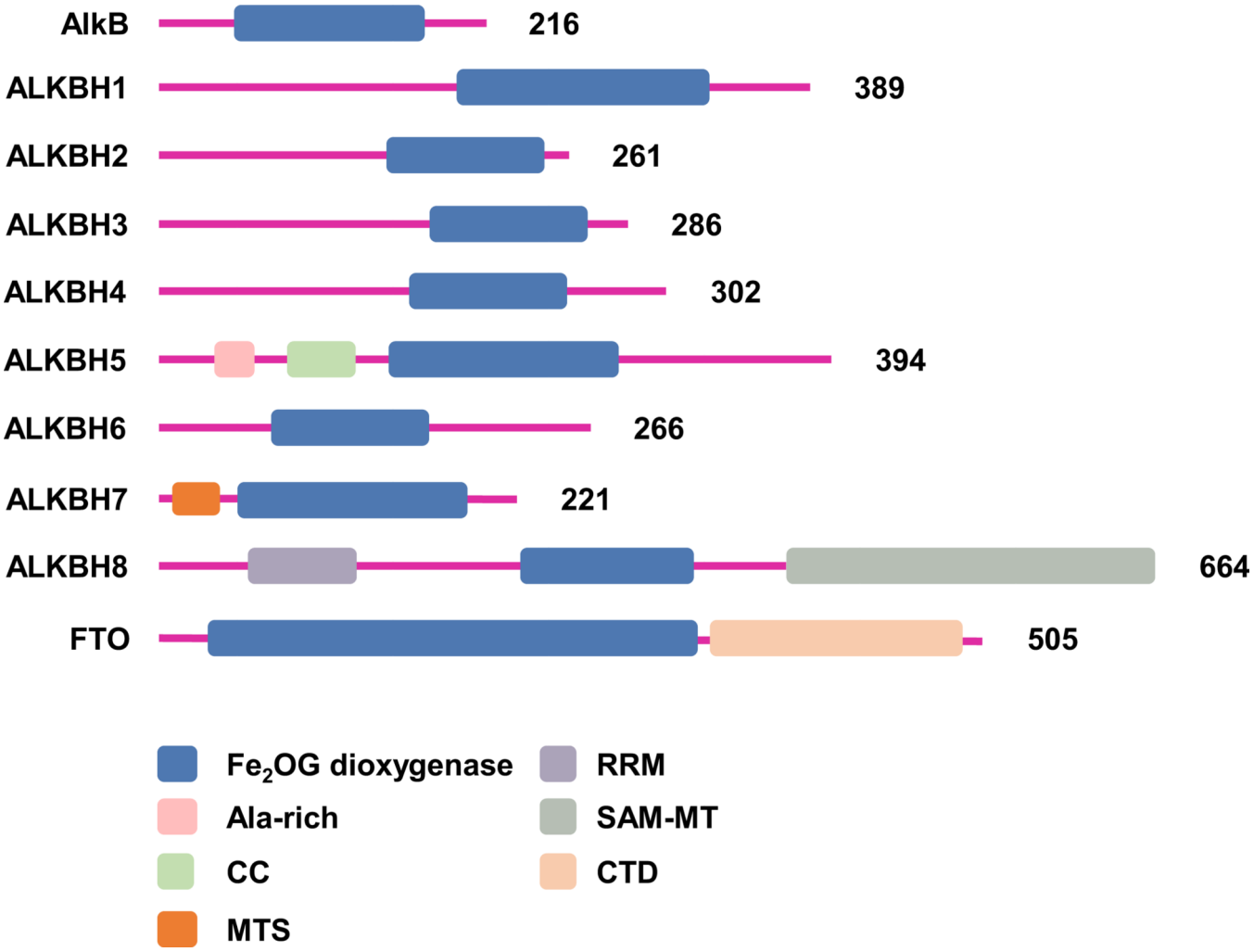

Fig. 1.

Domain architecture of the *Escherichia coli* (*E. coli*) AlkB and the nine mammalian AlkB homologs. UniProtKB accession number for *E. coli* AlkB (P05050); and human ALKBH1(Q5XKL0), ALKBH2(Q6NS38), ALKBH3(Q96Q83), ALKBH4(Q9NXW9), ALKBH5(Q6P6C2), ALKBH6(Q3KRA9), ALKBH7(Q9BT30), ALKBH8(Q96BT7) and FTO(Q9C0B1). Fe_2OG dioxygenase (Fe(II) and α-ketoglutarate dependent dioxygenase domain); CC (Coiled Coil), MTS (Mitochondrial Targeting Signal), Ala-rich (Region with Ala stretch); RRM (RNA Recognition Motif), SAM-MT (S-adenosylmethionine dependent Methyltransferase domain), and CTD (C-Termina Domain).

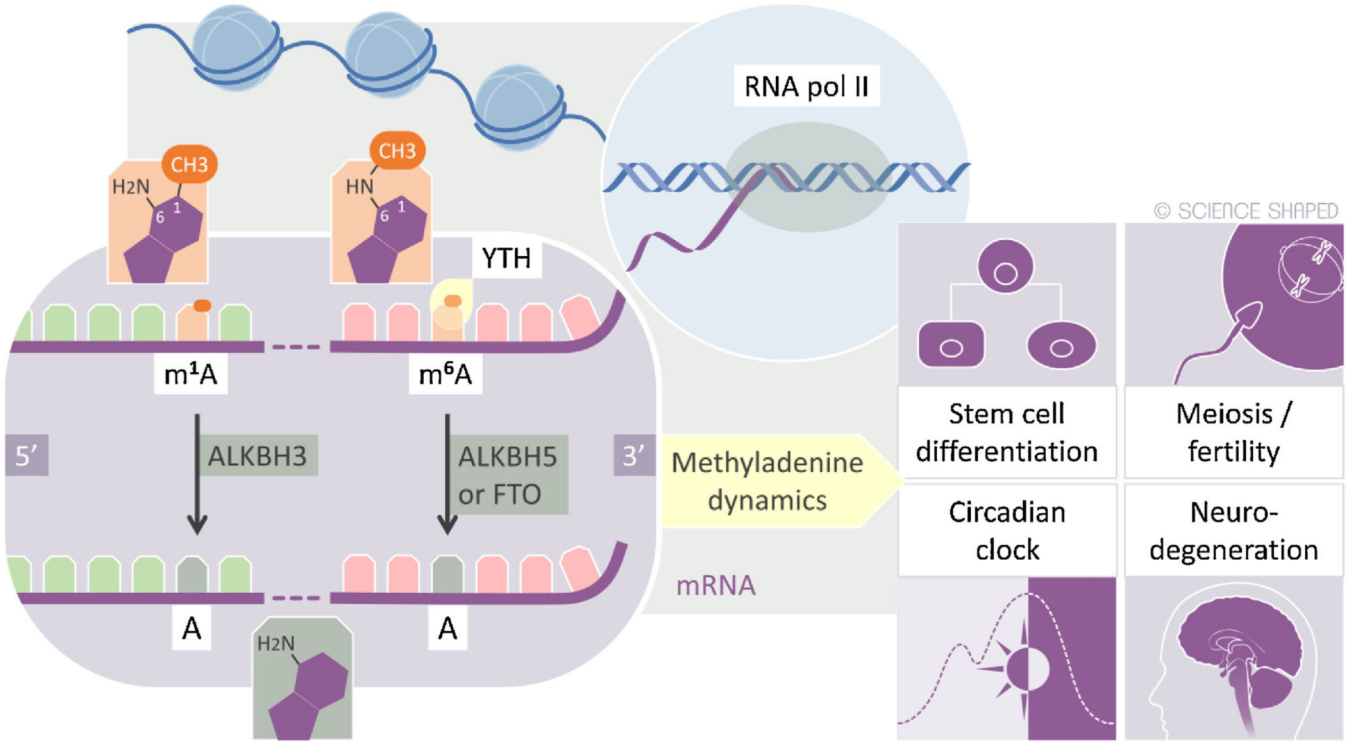

Fig. 2.

Overview of the distinct pattern of 1-methyladenine (m^1A) and 6-methyladenine (m^6A) dynamics in mammalian mRNA. Transcriptome mapping of m^1A in mRNA was done very recently so the consequences of methylation dynamics indicated is for m^6A. All other details in the main text.