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RNA epigenetics

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Summary

Mammalian messenger and long non-coding RNA contain tens of thousands of post-transcriptional chemical modifications. Among these, the N^6-methyl-adenosine (m^6A) modification is the most abundant and can be removed by specific mammalian enzymes. M^6A modification is recognized by families of RNA binding proteins that affect many aspects of mRNA function. mRNA/lncRNA modification represents another layer of epigenetic regulation of gene expression, analogous to DNA methylation and histone modification.

Keywords

RNA; modification; N6-methyl; m6A; demodification

Introduction

Over 100 types of post-transcriptional modifications have been identified in cellular RNA starting in the 1950s (http://mods.rna.albany.edu/). For example, the human ribosomal RNA (rRNA) contains over 200 modifications consisting of three major types [1]: ~100 2′-*O*-methylated nucleotides (Nm), ~100 pseudouridines (Ψ), and ~10 base methylations (e.g. 5-methyl-cytosine, m^5C). Each human transfer RNA (tRNA) contains on average 14 modifications consisting of various base methylations, Ψ, Nm and chemically elaborate, modified wobble bases that require catalysis by multiple enzymes [2, 3]. rRNA modifications are generally used as quality control checkpoints in ribosome assembly [4]. tRNA modifications outside the anticodon loop are generally used to maintain tRNA stability or modulate tRNA folding, whereas modifications in the anticodon loop are generally used to tune decoding capacity and to control decoding accuracy [5].

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Up until two years ago, internal modifications in mRNA and long non-coding RNA were very much neglected. Discovered in the 1970s [6-9], the most abundant internal mRNA/lncRNA modification is made of N^6-methyl adenosine (m^6A), present on average in over 3 sites per mRNA molecule ([10-13], Fig. 1A). Other types of modifications such as m^5C or 2′*O*-methylated nucleotides have also been indicated to occur internally in mRNA ([9, 14] Fig. 1B, 1C), and many m^5C modification sites have now been identified [15, 16]. A common feature of these modifications is that their presence cannot be detected by the commonly used reverse transcriptases in cDNA synthesis. It was therefore extremely difficult to map these modifications at single nucleotide resolution. Global m^6A modification was shown to be functionally important as siRNA knockdown of a known human m^6A-methyltransferase (METTL3) led to apoptosis in cell culture[17]. Suggested functions for m^6A modification include effects on mRNA splicing, transport, stability, and immune tolerance [17, 18].

Interest in mRNA/lncRNA modification was revived in 2011 upon the discovery that m^6A modification is the cellular substrate for the human enzyme FTO [19]. FTO belongs to a family of human genes that are homologous to the *E. coli* AlkB protein which catalyzes oxidative reversal of methylated DNA and RNA bases [20, 21]. In genome-wide association studies, the human FTO gene is highly associated with diabetes and obesity in the human population [22, 23]. FTO knockout mice are much leaner than the wild-type mice, presumably due to perturbations in controlling cellular metabolism [24]. The discovery of FTO acting on m^6A in mRNA/lncRNA indicates that m^6A modification is subject to sophisticated cellular control.

The discovery of this first RNA demodification enzyme also highlights the idea that RNA modification may act as epigenetic markers and controls akin to DNA methylation and histone modification [25, 26]. Three groups of proteins are needed for epigenetic control that maintains specific modification patterns in cell type and cell state dependent manners. “Writers” catalyze chemical modification at specific sites; “erasers” remove modification at specific sites; and “readers” recognize the modified sites in DNA or histones (Fig. 2A). For m^6A in mRNA/lncRNA, members in all three groups of proteins have now been found in mammalian cells (Fig. 2B). However, the current list of these proteins likely represents just the beginning. In particular, the number of reader proteins that recognize m^6A modified mRNA/lncRNA sites will certainly expand greatly in the coming years. As of today, only the m^6A modification has been shown to exhibit all signatures of epigenetic regulation. This review therefore focuses on m^6A modifications in mRNA/lncRNA with an emphasis on its effect on human health and disease.

Techniques used to study m^6A in mRNA/lncRNA

A pre-requisite for mRNA/lncRNA transcriptome studies is the copying of RNA into cDNA by reverse transcriptase (RT). M^6A modification does not affect Watson-Crick base pairing, and it behaves like an unmodified adenosine for the commonly used RTs. A widely applied method for m^6A study is to use immunoprecipitation (IP) with a commercial m^6A-antibody followed by high throughput sequencing (m^6A-seq or MeRIP-seq, [27, 28]). The mRNA/lncRNA mixture is first chemically fragmented to produce suitably sized RNA segments for deep sequencing and to increase the resolution of m^6A detection. The fragmented RNA is

split in two: one is used for m^{6}A-antibody IP to enrich RNA segments that contain m^{6}A, and the other is used as the reference. The location of m^{6}A modification is obtained by comparing the sequencing read profiles of both samples. This method could readily identify tens of thousands of candidate m^{6}A modification sites in mammalian mRNA/lncRNA at an average resolution of ∼100 nucleotides [27, 28]. Studies prior to the advent of high throughput sequencing have determined a consensus sequence for mammalian m^{6}A modification consisting of RRACH (R=A,G, H=A,C,U, m^{6}A site underlined, [13]). Indeed, this consensus sequence is present in a majority of m^{6}A/MeRIP-seq peaks. Peaks without this consensus sequence are likely m^{6}A-antibody binding artifacts as demonstrated in a yeast m^{6}A study [29].

To map transcriptome-wide m^{6}A sites at or near single nucleotide resolution, a combination of high coverage sequencing and bioinformatics was used in the yeast m^{6}A study for ∼1,300 m^{6}A sites [29]. This approach may not be readily applicable to mammalian RNA where the number of m^{6}A sites is at least one order of magnitude greater and the context of m^{6}A modification is much more diverse. It was shown recently that the HIV RT is sensitive to the presence of m^{6}A in RNA using the single molecule real time sequencing method by Pacific Biosciences [30]. The *Thermus thermophilus* DNA polymerase I can work as a reverse transcriptase in the presence of Mn^{2+}; this RT activity is sensitive to the presence of m^{6}A modification in the RNA template [31]. It remains to be seen whether these particular RT activities will be further developed for high resolution, transcriptome-wide identification of m^{6}A sites.

Liu *et al* developed a low throughput method that can directly determine the presence and the modification fraction of candidate m^{6}A site at single nucleotide resolution (termed SCARLET, [32]). The SCARLET method starts with total polyA$^+$ RNA. Hybridization of a specific 2′-*O*-Me-2′-deoxy oligonucleotide enables a single, site-specific cut by RNase H at the 5′ of the candidate site which is first identified from the m^{6}A/MeRIP-seq data. The cut site is radio-labeled with ^{32}P, followed by targeted ligation with a long, single-stranded DNA oligo. The sample is then digested with ribonucleases to completion; the only remaining nucleic acid is the ^{32}P-labeled candidate adenosine nucleotide linked to the DNA oligo. This ^{32}P-labeled product is purified on denaturing gels, and digested with another nuclease to obtain two ^{32}P-labeled products, 5′p-A and 5′p-m^{6}A which are separated by thin-layer chromatography and visualized by phosphorimaging. SCARLET not only can detect the presence of m^{6}A, it also determines the modification fraction of that site which has not been possible using m^{6}A-antibody based techniques.

M^{6}A writers

The first m^{6}A-methyltransferase identified is the protein encoded by the METTL3 gene [33]. This gene is conserved from mammals to yeast. Knockdown or deletion of METTL3 led to a wide range of phenotypes such as apoptosis in human cell lines, viability in plants and drosophila, or sporulation defects in yeast [17, 34-36].

Recent studies discovered another human methyltransferase-like 14 (METTL14) protein that can also catalyze m^{6}A RNA methylation, and the METTL14 protein forms a stable heterodimer complex with METTL3 [37]. Both METTL3 and METTL14 belong to the same

methyltransferase superfamily; they are 43% identical in their primary sequences. Knockdown of either METTL3 or METTL14 leads to a marked decrease of m^{6}A content in mRNA and causes mouse embryonic stem cells (mESCs) to lose their self-renewal capability [38]. Both METTL3 and METTL14 are catalytically active *in vitro* in the methylation of single stranded RNA oligo substrates. These results indicate that both proteins are catalytic subunits of the complex. Each enzyme may methylate a distinct and overlapping set of m^{6}A sites.

The METTL3-METTL14 core complex has been found to interact with WTAP [37, 39]. WTAP is a protein known to be involved in mRNA splicing [40]. siRNA knockdown of WTAP also leads to a significant decrease of m^{6}A content, but WTAP protein itself does not show any methyltransferase activity *in vitro* [37]. These results indicate that WTAP acts as an accessory protein that may be needed to enhance the methyltransferase selectivity or for sub-nuclear localization of the methyltransferases [37, 39].

M^{6}A erasers

The first m^{6}A eraser identified is the protein encoded by the FTO gene [19]. FTO belongs to the family of Fe^{2+}-α-ketoglutarate dependent dioxygenases and removes the methyl-group of m^{6}A through successive oxidation ([41], Fig. 3A). FTO overexpression led to a ∼15-20% reduction of m^{6}A content, whereas siRNA knockdown of FTO lead to ∼20% increase of m^{6}A content in human cell lines. FTO reaction generates two intermediate products, N^6-hydroxymethyladenosine and N^6-formyladenosine that are stable for several hours in the mammalian cell [41]. These intermediates may be used to recruit specific proteins that recognize this particular chemical feature.

Another m^{6}A eraser known to date is the protein encoded by the ALKBH5 gene [42]. ALKBH5 belongs to the same protein family as FTO. Other members in this family that have known cellular substrates include ALKBH2 for DNA methylation repair, ALKBH3 for RNA methylation repair, and ALKBH8 for hydroxylation of a specific human $tRNA^{Gly}$ [43, 44]. ALKBH5 directly removes the methyl-group of m^{6}A without the accumulation of any detectable intermediate product ([45], Fig. 3B).

M^{6}A readers

Numerous mammalian proteins were found to preferentially bind a synthetic RNA oligo derived from Rous Sarcoma Virus genomic RNA with or without a specific m^{6}A residue as bait [27]. The three proteins detected with highest confidence include ELAVL1, YTHDF2 and YTHDF3. ELAVL1 or HuR (human antigen R) belongs to the ELAVL family of RNA binding proteins that contain several RNA-binding domains, and selectively bind *cis*-acting AU-rich elements (AREs) located in the 3′ UTR regions of mRNA [46]. ELAVL1 is known to stabilize ARE-containing mRNAs [47, 48], and RNA-ELAVL1 interactions have been shown to regulate the stability of many mRNAs in embryonic stem cells in a m^{6}A-dependent way [38]. Both the YTHDF2 and YTHDF3 proteins belong to a superfamily of RNA binding proteins containing the YTH domain [49]. The YTH domain is conserved in eukaryotes and is particularly abundant in plant genomes. Except for YTHDF2, further studies are needed to validate whether these proteins are authentic m^{6}A readers *in vivo*.

A more recent study shows that the YTHDF2 protein directly recognizes m^{6}A-modified RNA *in vitro* and *in vivo* [50]. YTHDF2 protein binds a single-stranded RNA oligo containing a single m^{6}A with ∼15-fold higher affinity for the modified RNA *in vitro*. YTHDF3 and another member of this family, YTHDF1 are also shown to prefer this m^{6}A-containing RNA oligo by 5-20 fold *in vitro*. In human cell lines, YTHDF2 binds over 3,000 cellular RNA targets, most are located in mRNAs. YTHDF2 directly competes with ribosomes for translatable mRNA molecules in the cytoplasm. YTHDF2 binding results in mRNA localization to mRNA decay sites such as processing bodies. YTHDF2 is basically a triage factor for m^{6}A-containing mRNA. When sufficient amounts of ribosomes are available, these mRNAs are bound by the ribosome for active translation, whereas decreasing amount of available ribosome enables their binding to YTHDF2 protein for targeted re-localization and degradation.

The YTHDF2 protein is made of two functional domains: one directly binds the m^{6}A-modified RNA, and the other is required for mRNA localization within the cytoplasm [50]. Among YTHDF1-3, the RNA binding domain is highly conserved, but the conservation of the other domain is far less pronounced. Therefore, these YTHDF proteins may affect mRNA metabolism in different ways, even though they can all interact with m^{6}A-containing RNAs in a similar manner.

Since tens of thousands of m^{6}A-sites have already been found, other m^{6}A-reader proteins are certain to exist. Other m^{6}A binding proteins may directly interact with the modified adenosine like YTHDF2, or they may indirectly sense m^{6}A-modified RNA through changes in the RNA structure [51]. The biological function of m^{6}A modification is executed through their reader proteins. As more m^{6}A-readers are identified, we anticipate a rapid growth in our understanding of how m^{6}A affects all aspects of mRNA and lncRNA function.

M^{6}A modification in human health and disease

The current knowledge on the writer, eraser and readers of m^{6}A modification is summarized in Table 1. The m^{6}A modification regulates a variety of biological processes and has been linked to numerous human diseases. Both m^{6}A methyltransferases (METTL3 and METTL14) are crucial for cell development, and their depletion causes cell death. Diseases associated with METTL3 include prostatitis and aicardi syndrome, while diseases associated with METTL14 include alcohol dependence and alcoholism. The WTAP protein, known to affect mRNA splicing, is associated with Wilms tumor, kindney cancer, and other ailments. Variants within introns of m^{6}A demethylase gene *FTO* have been indicated to increase risk for obesity and diabetes, although recent studies suggest that these variants within *FTO* introns form long-range functional connections with the homeobox gene *IRX3* at the DNA level [52]. Another identified m^{6}A demethylases, ALKBH5, has been reported to affect Smith magenis syndrome, hypoxia and others. Except the still unclear biological role of YTHDF3, the three other identified m^{6}A readers (YTHDF1, YTHDF2, ELAVL1) are related to mRNA stability. Diseases associated with the m^{6}A readers include cancer, leukemia, hepatitis, Alzheimer's Disease, arthritis, prostatitis, hypoxia, pancreatitis and others.

RNA epigenetics beyond m^{6}A in mRNA/lncRNA

Most RNA methylations can be in principle reversed by another enzyme. In practice, the most likely candidates for reversal include m^{5}C in mRNA and in tRNA [16], N^1-methyl-A (m^{1}A) in tRNA, and N^1-methyl-G (m^{1}G) in tRNA (Fig. 4A). m^{5}C is the epigenetic marker of DNA and it can be reversed by the Tet1/Tet2 enzymes and the DNA repair enzyme thymine-DNA glycosylase (TDG; Fig. 4B, [53]). A similar oxidative reversal pathway may also work for m^{5}C modification in RNA, although the actual enzymes catalyzing such reactions in RNA have not yet been identified. m^{1}A is present in almost every tRNA species in eukaryotes; it is required for the stability of some tRNAs [54, 55]. The *E. coli* AlkB enzyme can reverse m^{1}A in RNA when m^{1}A modification was introduced by chemical methylation agents [56]. An AlkB-like enzyme in a human cell may therefore potentially reverse endogenous m^{1}A in some tRNAs to control their stability. m^{1}G is present in about half of tRNA species in eukaryotes; it is needed for accurate decoding or for tRNA stability [57, 58]. No natural enzyme is yet known that reverses m^{1}G with high efficiency; however, m^{1}G should be readily reversible based on its chemical feature.

Finding reader proteins for these other methylatons, however, may be far more challenging. To our knowledge, RNA binding protein that directly recognizes m^{5}C-modified mRNA has not yet been identified. For tRNAs, these modifications are generally used to control decoding accuracy and efficiency and to confer stability. It is unclear whether any reader protein is needed for their direct recognition. For now, m^{6}A modification is clearly the prominent marker of RNA epigenetics.

Concluding remarks

Although mRNA modification has been known since the 1970s, its functional importance in mRNA metabolism and its effect on human biology have not been extensively studied in the past. The discovery of the m^{6}A eraser protein FTO in 2011 directly links m^{6}A modification in mRNA/lncRNA to human health and disease. Subsequent studies show that the m^{6}A modification is connected to many aspects of human biology. The field of RNA epigenetics is still in its infancy. We look forward to many exciting discoveries in the coming years.

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Abbreviations

m^{6}A	N^6-methyl adenosine
m^{5}C	5-methyl cytosine
Nm	2′-*O*-methyl nucleotides
rRNA	ribosomal RNA
tRNA	transfer RNA
mRNA	messenger RNA
lncRNA	long non-coding RNA

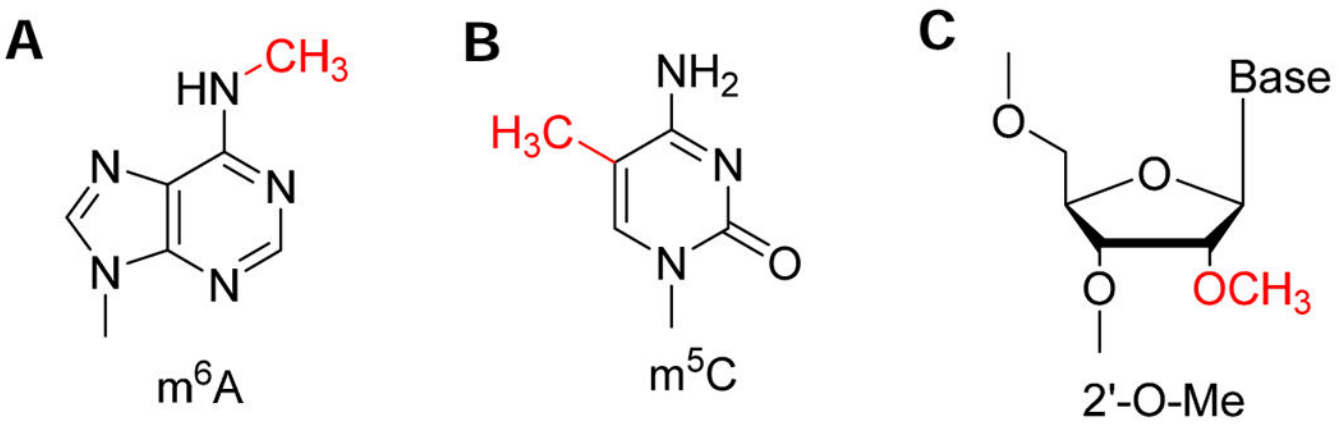

Fig. 1. Chemical structure of internal mRNA/lncRNA modifications

(A) m^6A; (B) m^5C; (C) Nm.

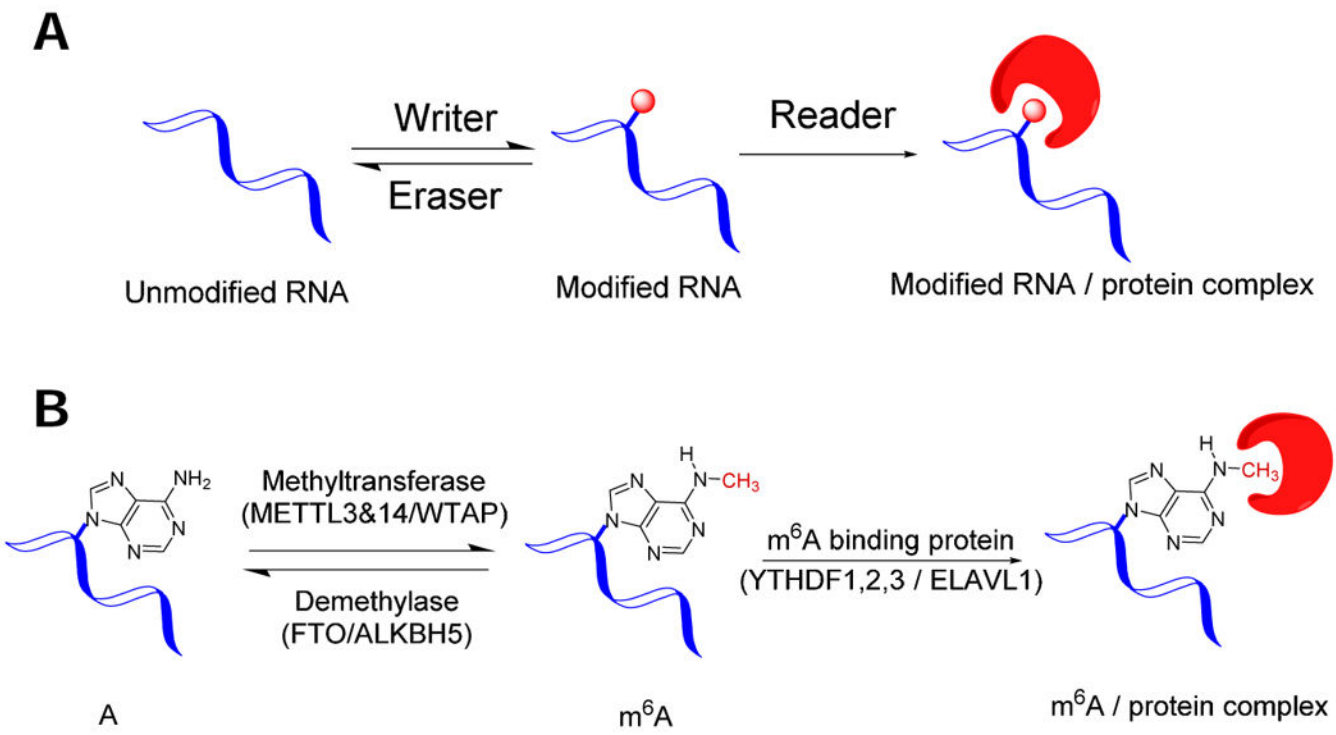

Fig. 2. RNA epigenetic marking and control requires three groups of proteins

(A) Schematic plot for general RNA modification. Writer = modification enzymes; eraser = demodification enzymes; reader = RNA binding proteins that recognize specific sites in the modified mRNA/lncRNA. (B) Schematic plot for the m^6A modification.

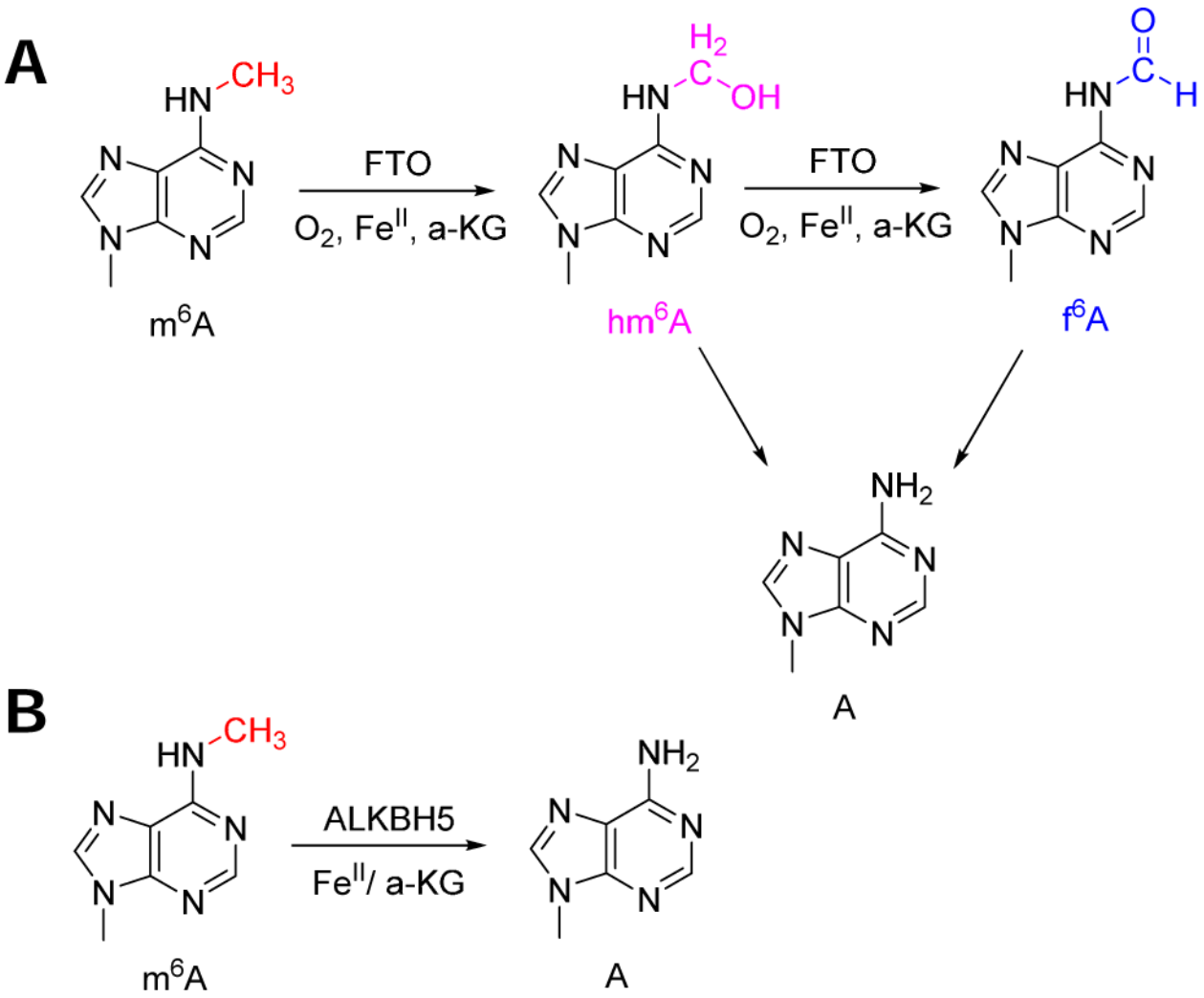

Fig. 3. Reaction mechanism of m6A erasers

(A) FTO reaction generates two intermediates that are stable for several hours before its decomposition and reversal to adenosine. (B) ALKBH5 reaction directly removes the methyl-group from m^6A.

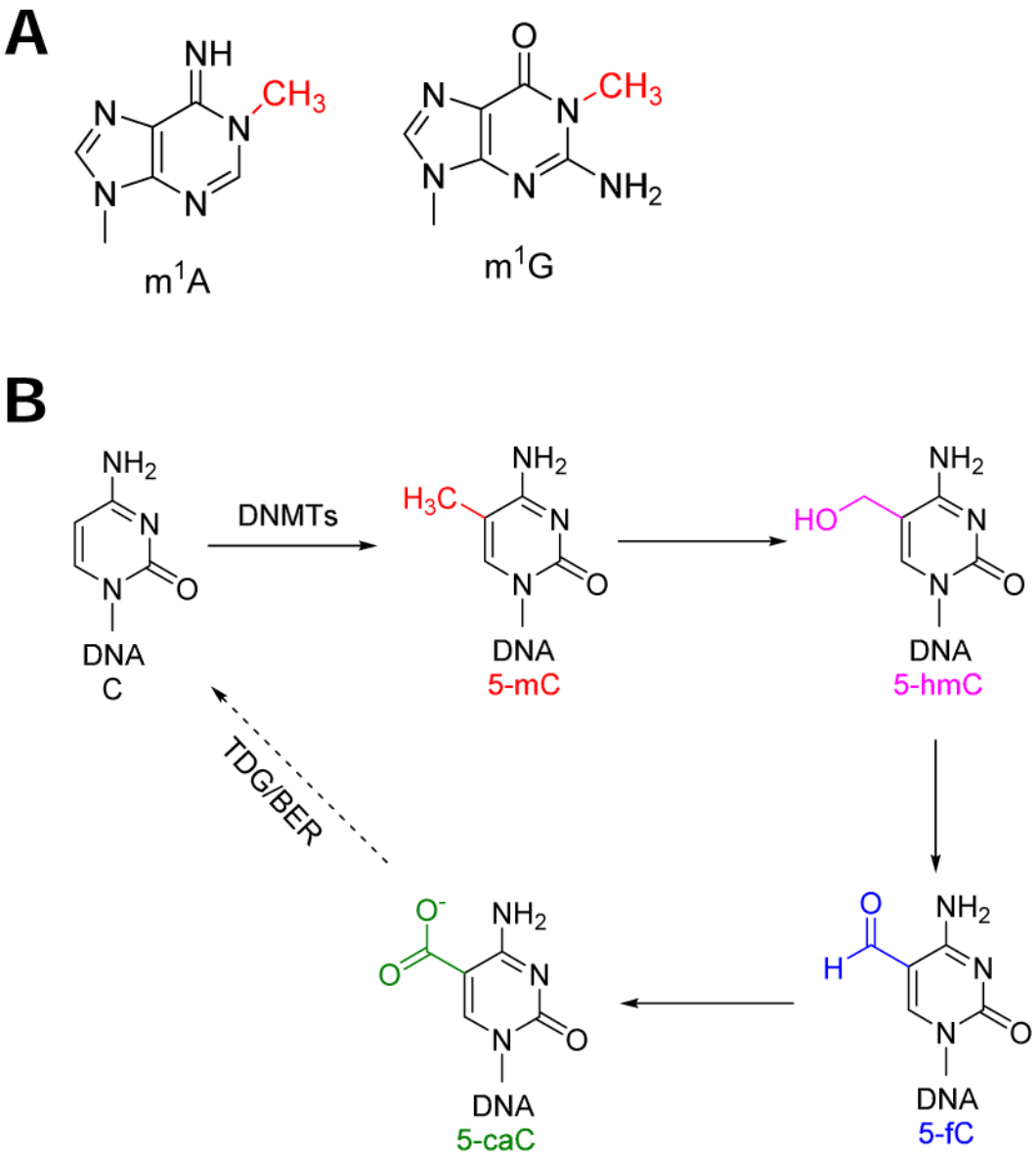

Fig. 4. Other potentially reversible RNA methylations

(A) m^1A and m^1G. (B) DNA m^5C oxidative reversal pathway.

Table 1

Human diseases associated with genes involved in m^{6}A modification (Disease information from http://www.malacards.org/)

Gene	Function	Phenotype	Associated Diseases	References
METTL3	Methyltransferase	Apoptosis, development	Prostatitis, Aicardi Syndrome	38, 59, 60
METTL14	Methyltransferase	Apoptosis, development	Alcohol dependence, Alcoholism	38, 60
WTAP	Scaffolding or localization	mRNA splicing	Wilms Tumor, Hypospadias, Sarcoma, Malignant Mesothelioma, Synovial Sarcoma	34, 60-64
FTO	Demethylase	Cellular energy homeostasis	Obesity, diabetes, Polycystic Ovary Syndrome, Heart attack, Cancer, Alcoholism, Mental Disorders, Cataract, Hepatitis	19, 65-71
ALKBH5	Demethylase	Male fertility	Hypoxia, Smith Magenis Syndrome	45, 72
YTHDF1	m^{6}A binding	mRNA stability	Pancreatic Cancer, Pancreatitis, Dermatomyositis	50
YTHDF2	m^{6}A binding	mRNA stability	Leukemia, Renal Cell Carcinoma, Breast Cancer	27, 50, 73, 74
YTHDF3	m^{6}A binding	N/A	N/A	27, 50
ELAVL1	m^{6}A binding	Apoptosis, mRNA stability	Cancer, Leukemia, Hepatitis, Anoxia, Alzheimer's Disease, Arthritis, Endotheliitis, Prostatitis, Hypoxia, Laryngitis, Keratoconus, Pancreatitis	27, 38, 75-81

N/A: no available information.