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REVIEW

Reversible RNA Modification N^1-methyladenosine (m^1A) in mRNA and tRNA

Chi Zhang [a], **Guifang Jia** *,[b]

Synthetic and Functional Biomolecules Center, Beijing National Laboratory for Molecular Sciences, Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China

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Abstract More than 100 modifications have been found in RNA. Analogous to epigenetic DNA methylation, epitranscriptomic modifications can be written, read, and erased by a complex network of proteins. Apart from N^6-methyladenosine (m^6A), **N^1-methyladenosine** (m^1A) has been found as a reversible modification in tRNA and mRNA. m^1A occurs at positions 9, 14, and 58 of tRNA, with m^1A58 being critical for tRNA stability. Other than the hundreds of m^1A sites in mRNA and long non-coding RNA transcripts, transcriptome-wide mapping of m^1A also identifies >20 m^1A sites in mitochondrial genes. m^1A in the coding region of mitochondrial transcripts can inhibit the translation of the corresponding proteins. In this review, we summarize the current understanding of m^1A in mRNA and tRNA, covering high-throughput sequencing methods developed for m^1A methylome, m^1A-related enzymes (writers and erasers), as well as its functions in mRNA and tRNA.

The RNA modification N^1-methyladenosine

Shortly after the discovery of the base N^1-methyladenine (m^1A) in 1961 [1], Dunn et al. isolated 1-methyladenosine mononucleotide from RNA [2]. m^1A has been discovered in tRNA [3], rRNA [4–6], mRNA [7–10], and mitochondrial (mt) transcripts [9,10]. m^1A, N^3-methylcytidine (m^3C), and N^7-methylguanosine (m^7G) are the most commonly methylated nucleotides with a positive electrostatic charge under physiological conditions [11]. The electro-chemical interaction resulting from the positive charge of m^1A is critical for the function and structure of tRNA [11]. The methyl group on m^1A in mRNA blocks the Watson-Crick base pairing, affecting reverse transcription and protein translation [7–10].

m^1A was first found in yeast $tRNA^{Phe}$ [3]. Decades later, m^1A has also been found in 264 out of 564 tRNA sequences in bacteria, archaea, and eukaryota, which can occur at positions 9, 14, and 58 [12]. Among them, m^1A58 is conserved in bacteria, archaea, and eukaryota, and m^1A58 is critical for

* Corresponding author.
E-mail: guifangjia@pku.edu.cn (Jia G).
[a] OCRID: 0000-0003-1762-0639.
[b] OCRID: 0000-0002-4186-6922.

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tRNA stabilization [12,13]. A recent study has reported that m^{1}A58 can be reversed by the human AlkB homolog 1 (ALKBH1) demethylase [13].

In contrast to the high abundance of m^{1}A in tRNA, m^{1}A is present in mammalian mRNA with a low abundance (m^{1}A/A: 0.015%–0.054% in mammalian cells and up to 0.16% in mammalian tissues) [7,8]. The development of m^{1}A-sequencing methods and new single-base resolution methods [7–10] has facilitated unraveling the presence of m^{1}A in nuclear, cytosolic, and mt-encoded transcripts [9,10]. For instance, Li et al. found 473 m^{1}A sites in mRNA and long non-coding (lncRNAs) transcripts in HEK293T cells. The majority of these m^{1}A sites are located within the 5′-untranslated region (5′ UTR) [9], which is consistent with previous findings [7,8]. Interestingly, 22 m^{1}A sites have been identified in 10 of 13 mt transcripts, with 21 sites located in the coding sequence (CDS) and one in the 3′ UTR [9]. m^{1}A at CDS of mt transcripts affects mitochondrial translation, suggesting that m^{1}A might regulate the normal function of mitochondria.

m^{1}A sequencing

In order to comprehensively explore m^{1}A methylation in transcriptome, it is necessary to develop m^{1}A high-throughput sequencing. The low abundance of m^{1}A in mRNAs necessitates highly sensitive detection methods. In addition, m^{1}A can be rearranged to m^{6}A under alkaline conditions (Dimroth rearrangement) [14]. Therefore, the signature of m^{1}A in mRNA may disappear during the preparation of mRNA fragmentation or mRNA digestion, which makes it difficult to reduce the noise in m^{1}A measurement and sequencing. m^{1}A blocks the Watson-Crick interface and effectively stalls reverse transcription (RT), thus inducing truncations or mutations in RT products [15–18]. This feature can be employed to identify m^{1}A sites, and accordingly, the high-throughput m^{1}A sequencing methods were developed based on this idea.

The first-generation m^{1}A sequencing methods have been reported by two independent groups [7,8] (**Figure 1**). The procedures of these two methods are largely similar. They both use the m^{1}A antibody to enrich m^{1}A-modified mRNA fragments and take advantage of the characteristics of m^{1}A to induce mismatch or truncation during the RT process. However, there are also differences between these two methods. Dominissini et al. use Dimroth rearrangement to change m^{1}A to m^{6}A and then analyze m^{1}A sites based on mismatch rates. Li et al. use *E. coli* AlkB to demethylate m^{1}A to normal A and then calculate m^{1}A sites based on truncation position. The resolution of the first generation sequencing method is around 130 nucleotides [7,8]. To identify m^{1}A sites more precisely for a more accurate picture of m^{1}A distribution, researchers have recently developed the second-generation m^{1}A sequencing method.

A highly processive reverse transcriptase thermostable group II intron reverse transcriptase (TGIRT) is used in the second-generation m^{1}A sequencing method to optimize the RT step [9,10] (Figure 1). TGIRT exhibits excellent read-through efficiency and relatively high mutation frequency at the site of m^{1}A, resulting in a higher signal-to-noise ratio for detecting the precise position of m^{1}A, and therefore achieving a single-base resolution.

Using the second-generation m^{1}A sequencing method, Li et al. [9] identified 740 m^{1}A sites in the transcriptome of 293 T cells, including 418 and 55 sites found in mRNA and lncRNA transcripts, respectively. The majority of these m^{1}A sites in mRNA are within the 5′ UTR, which agrees with the previous findings [7,8]. Interestingly, Li et al. find 24 m^{1}A sites at the first nucleotide (cap + 1) and three sites at the second nucleotide (cap + 2) of 5′ end of the transcripts. Ribosome profiling demonstrates that sites in the 5′ UTR and cap + 1 position, but not those in the CDS or 3′ UTR, are correlated with higher translation efficiency. This observation that there is a correlation between m^{1}A sites in the 5′ UTR and translation efficiency is in agreement with a previous report [7].

According to the RNA location, writer enzyme involved, and sequence-structural features, Li et al. divide the m^{1}A sites in mRNA transcripts into three categories. These include tRNA methyltransferase 6/61A (TRMT6/61A)-dependent m^{1}A sites in nuclear-encoded mRNAs (53 sites), TRMT6/61A-independent m^{1}A sites in nuclear-encoded mRNAs, and m^{1}A sites in mt-encoded mRNAs (22 sites). TRMT6/61A-dependent m^{1}A sites are barely enriched in the 5′ UTR and conform to a GUUCRA tRNA-like motif and T-loop-like structures. m^{1}A sites in mt-mRNAs are mainly located in CDS, and play a role in inhibiting mitochondrial translation. Among the ten m^{1}A-modified mt-RNAs, m^{1}A modifications on 5 mt-RNAs are installed by mt-tRNA methyltransferase TRMT61B. Would the random mismatches occurring during the RT process of TGIRT yield false positive results? Li et al. used a method called reverse calculation to test this possibility. They take the sample of demethylase (−) (*i.e.*, m^{1}A) as background and the sample of demethylase (+) (*i.e.*, A) as signal to call m^{1}A peaks. As a result, they have called 17 peaks, confirming the high accuracy of TGIRT enzyme. Using primer extension assay, they also verify several m^{1}A sites in mt mRNA, confirming again the authenticity and accuracy of their method [9].

Surprisingly, about the same time, another group adopted a similar approach but reached a very different conclusion [10]. Safra et al. identified only 15 m^{1}A sites in mRNAs and lncRNAs, with 9 in cytosolic mRNAs, 1 in cytosolic lncRNAs, and 5 in mt-mRNAs. The 10 m^{1}A sites in cytosolic transcripts are installed by TRMT6/61A, whereas one m^{1}A site in mt-mRNA *ND5* is methylated by TRMT10C. They also find that m^{1}A within 5′ UTR or CDS of cytosolic mRNAs leads to translational repression, indicating that m^{1}A might be related to ribosomal scanning or translation.

These two studies share some similar findings, while disagreement arises in terms of the number of m^{1}A sites in human mRNAs. Safra et al. suggest that m^{1}A is a rare modification in human mRNAs based on their observation that only a dozen m^{1}A sites have been identified using their method [10]. In contrast, Li et al. have identified hundreds of m^{1}A sites, and thus they consider m^{1}A a prevalent modification in human mRNAs [9]. Why do the numbers of m^{1}A sites identified in these two studies differ so much? As speculated in the technology preview [19], differences in experimental procedures may confer differences in the quality of sequencing datasets. For instance, 1) compared to AlkB demethylation, the alkaline conditions used for Dimroth rearrangement might affect RNA integrity and rearrangement efficiency of m^{1}A to m^{6}A; 2) a competitive elution step at m^{1}A antibody immunoprecipitation is omitted in [10], together with the lack of optimization of the RT step

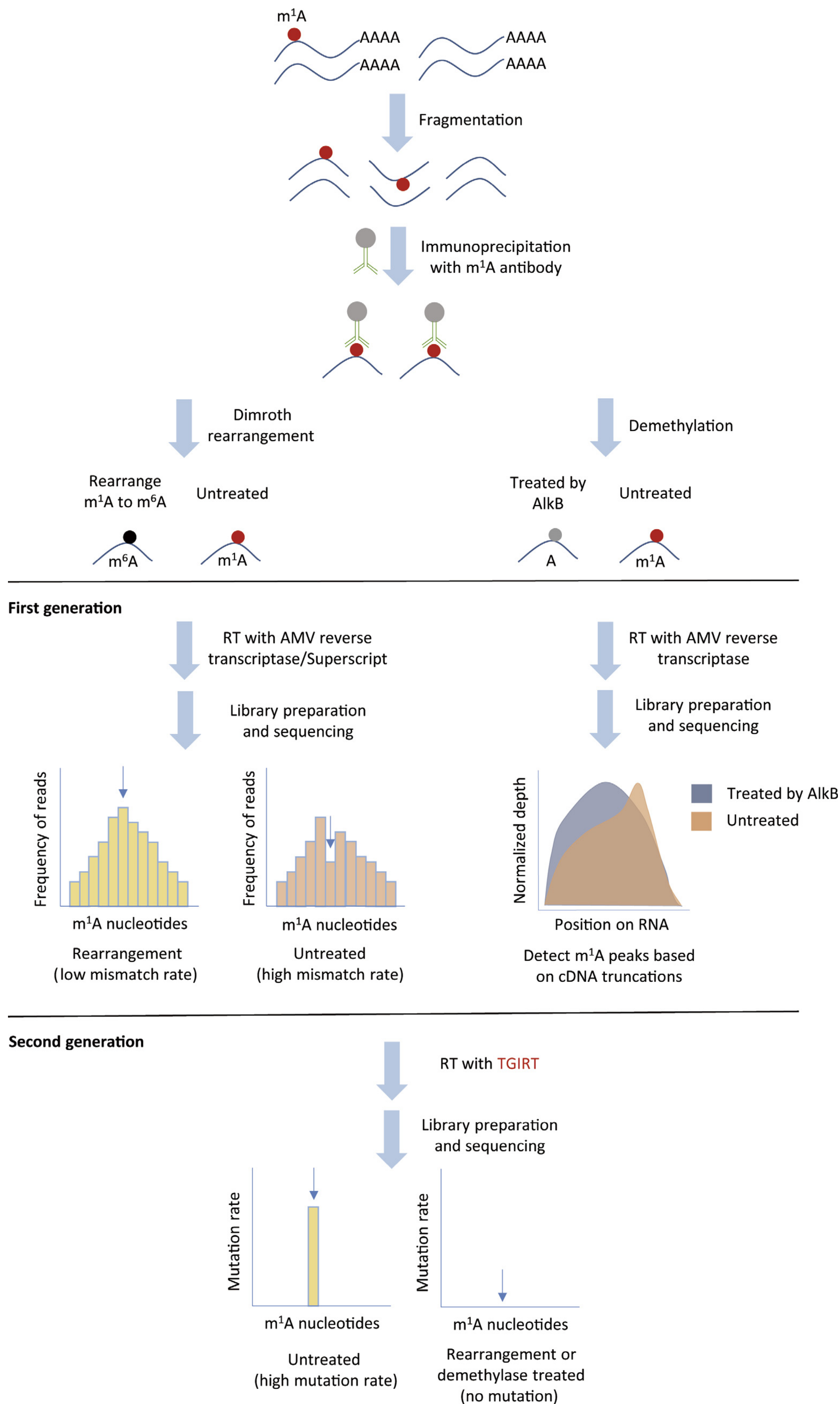

Figure 1 Schematic outline of the two generations of m^{1}A sequencing methods
RT, reverse transcription; TGIRT, thermostable group II intron reverse transcriptase.

of TGIRT; 3) a random 10-nt barcode (unique molecular identifier, UMI) in the DNA 3′-adaptor is used in [9] to remove repetition caused by PCR and improve the accuracy of detection, while lack of UMIs could lead to trouble in data analysis and mutation rate calculation [10]. In addition, the lower number of raw sequencing reads in the study [10] could also explain the fewer m^{1}A sites identified.

m^{1}A writers: m^{1}A methyltransferases

tRNA m^{1}A methyltransferase (MTase) has been extensively studied, since m^{1}A was first found in tRNA. Given m^{1}A58 is dominant and conserved across the three domains of life, studies of tRNA m^{1}A MTases started from looking for m^{1}A58 MTase.

tRNA m^{1}A58 MTase was initially characterized using partially purified protein fractions from bovine liver, which exhibits m^{1}A MTase activity *in vitro* toward *E. coli* tRNA$_2^{Glu}$ [20]. The genes encoding m^{1}A58 MTase were firstly identified in *Saccharomyces cerevisiae* [21]. The *S. cerevisiae* m^{1}A58 MTase comprises two subunits, tRNA adenine-*N*^{1}-methyltransferase non-catalytic subunit and tRNA adenine-*N*^{1}-methyltransferase catalytic subunit, encoded by two essential genes *Trm6* and *Trm61*, respectively [21]. Trm61 is responsible for AdoMet-binding and catalytic function as an enzyme, while Trm6 is critical for tRNA binding [22]. The purification and characterization of the m^{1}A58 MTase from *S. cerevisiae* [21,22] has facilitated the identification of other m^{1}A58 MTases from humans and other organisms. In eukaryota, m^{1}A58 MTase consists of Trm6 and Trm61 orthologs, whereas prokaryotic m^{1}A58 is installed by a single protein encoded by orthologs of *Trm61* [23]. TRMT61A and TRMT6 are the human othologs of yeast Trm61 and Trm6, respectively, which are responsible for m^{1}A58 modification of cytoplasmic tRNAs [24] (**Figure 2**).

Human mt-tRNAs are known to contain m^{1}A at positions 9 and 58 [24], which are catalyzed by TRMT10C and TRMT61B, respectively [25,26] (Figure 2). In addition, m^{1}A is also found at position 1322 of 28S rRNA, which is catalyzed by the human nucleolar protein nucleomethylin (NML; also known as RRP8) [27].

m^{1}A is present in human nuclear-encoded mRNA and mt-mRNA [9]. tRNA m^{1}A MTases are found to be able to write m^{1}A in some mRNA transcripts as well. For instance, TRMT6/61A can install m^{1}A sites within a GUUCRA tRNA-like motif with T-loop-like structure in some nuclear mRNAs, whereas TRMT61B methylates half of the identified m^{1}A sites in mt-mRNA transcripts [9] (Figure 2). In addition, TRMT10C can add m^{1}A at the 1374 position of mt-mRNA *ND5* [10] (Figure 2). Apparently, more m^{1}A mRNA MTases may be identified in future.

m^{1}A erasers: m^{1}A demethylases

m^{1}A is discovered to be the second reversible RNA modification. The first reversible RNA modification, m^{6}A, is demethylated by two AlkB family proteins, *i.e.*, fat mass and obesity-associated (FTO) and ALKBH5 [28,29]. Similarly, two AlkB family proteins ALKBH3 and ALKBH1 have been found to demethylate m^{1}A [8,13] (Figure 2).

E. coli AlkB is an Fe^{II}/α-ketoglutarate (α-KG)-dependent dioxygenase that repairs *N*-alkylated DNA lesions [30,31]. In mammals, nine homologs of AlkB, *i.e.*, ALKBH1-ALKBH8 and FTO, have been identified [32,33]. ALKBH3 and ALKBH2 are known DNA-repair proteins that protect the genomic integrity of mammalian cells [34,35]. Unlike ALKBH2 that repairs DNA alkylation lesions in double-stranded DNA (dsDNA), ALKBH3 repairs *N*-methylated bases, for instance, m^{1}A, m^{3}C, *N*^{3}-methylthymine (m^{3}T), and *N*^{1}-methylguanine (m^{1}G), in single-stranded DNA (ssDNA)

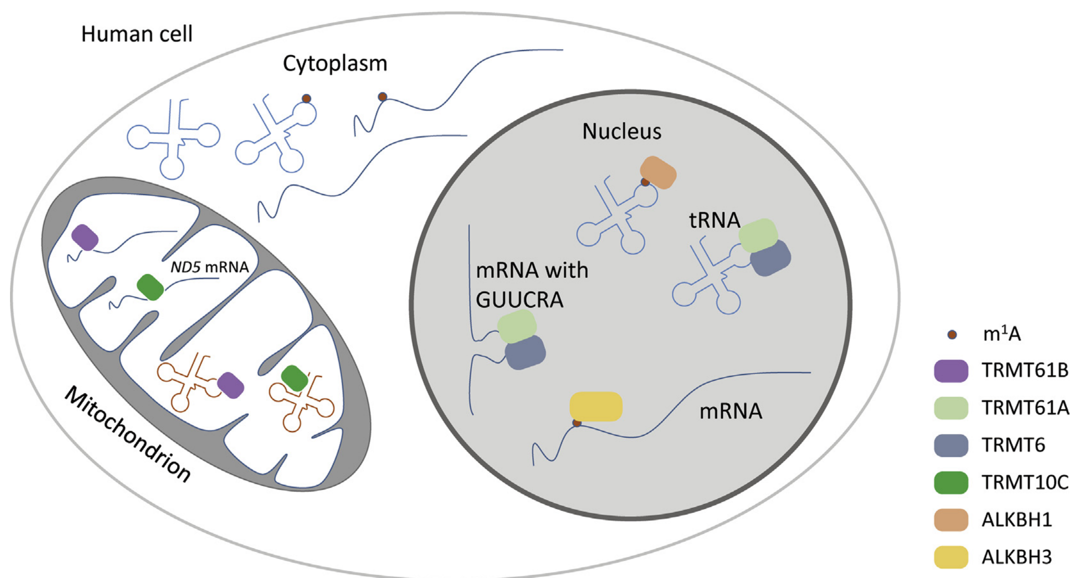

Figure 2 The methylation and demethylation of m^{1}A in tRNA and mRNA
In nucleus, m^{1}A modifications in pre-tRNA and specific pre-mRNA are installed by tRNA m^{1}A methyltransferase complex TRMT6/61A and erased by AlkB homolog proteins ALKBH1 and ALKBH3, respectively. In mitochondrion, mt-tRNA and a subset of mt-mRNA are methylated by mt-tRNA m^{1}A methyltransferase TRMT61B and TRMT10C. mt, mitochondrial.

or ssRNA [35]. ALKBH3 is coupled with the helicase activating signal cointegrator 1 complex subunit 3 (ASCC3) to unwind dsDNA for dealkylation [36]. ALKBH3, also known as prostate cancer antigen-1 (PCA-1), is highly expressed in a few human cancers and promotes apoptotic resistance and angiogenesis in prostate cancer and pancreatic cancer [37,38]. Due to its demethylation activity of m^1A *in vitro*, expectedly, ALKBH3 is confirmed to function as an m^1A mRNA demethylase *in vivo* [8]. Notably, a recent study reports that ALKBH3 can function as a tRNA demethylase to promote protein synthesis in cancer cells [39]. ALKBH3 displays demethylation activities toward m^1A, m^3C, and m^6A in tRNA to enhance protein translation efficiency *in vitro*, whereas knockdown of *ALKBH3* increases m^1A levels in tRNA and decreases protein synthesis in cancer cells [39].

ALKBH1 shows the highest sequence homology with *E. coli* AlkB and exhibits enzymatic activities toward a wide range of substrates. For instance, ALKBH1 has weak demethylation activity toward m^3C in ssDNA [40]. ALKBH1 also acts as a histone dioxygenase during neural development, which specifically removes the dimethylation of K118 or K119 on histone H2A [41]. However, Wu et al. did not observe the demethylation activity on histone H2A in $ALKBH1^{-/-}$ embryonic stem cell lines. Rather, they found ALKBH1 is a DNA N^6-methyladenine (m^6dA) demethylase that regulates epigenetic gene silencing [42]. ALKBH1 also exhibits apurinic/apyrimidinic (AP) lyase activity, cleaving DNA at abasic sites via a β-elimination mechanism [43]. Interestingly, ALKBH1 has been recently identified as an m^1A demethylase in cellular tRNAs [13]. Knockdown of *ALKBH1* increases the m^1A methylation level and the cellular level of the targeted $tRNA_i^{Met}$. These data indicate that the function of m^1A58 is to stabilize $tRNA_i^{Met}$, leading to the attenuated initiation of protein translation. Demethylation of other targeted tRNAs by ALKBH1 affects translation elongation by decreasing the usage of tRNAs in protein synthesis [13]. Furthermore, ALKBH1 can hydroxylate 5-methylcytosine (m^5C) to 5-formylcytosine (f^5C) in cytoplasmic $tRNA^{Leu}$ and mt-$tRNA_i^{Met}$, resulting in an increase in mitochondrial protein translation [44,45]. The diverse roles and enzymatic activities of ALKBH1 are in agreement with its multiple cellular localizations in the nucleus, cytoplasm, and mitochondria.

Roles of m^1A in mRNA and tRNA

m^1dA in DNA is considered as a form of DNA damage modification, which leads to false base pairing and genomic mutations, and thus it has to be repaired [46]. On the other hand, endogenous enzymes are present to install the modification of m^1A in RNA and play important roles in tRNAs and mRNAs.

The idea that m^1A58 might be vital to tRNA structure was first proposed in studies on the 3-dimensional structure of *S. cerevisiae* initiator $tRNA_i^{Met}$ [47]. Hydrogen bonds between adenosines A20, A54, and A60 play important roles in stabilizing yeast $tRNA_i^{Met}$. In this substructure, m^1A58 is linked to A54 and A60 via hydrogen bonds, which is also structurally critical for stabilization [47]. The presence of m^1A58 in all eukaryotic initiator tRNAs implies that m^1A58 is indispensable for tRNA structure and stability [12], which has been experimentally validated in human cells [13]. Knockdown of *ALKBH1*, the m^1A58 eraser, increases the cellular level of $tRNA_i^{Met}$ [13], supporting the function of m^1A58 to maintain a stable structure of $tRNA_i^{Met}$. Furthermore, m^1A58 in human $tRNA_3^{Lys}$ is important for HIV replication. An 18-nucleotide sequence in the HIV-1 genome (GenBank accession No.: NC 001802) is complementary to the last 18 nucleotides of human $tRNA_3^{Lys}$. This sequence can be used as the primer binding site (PBS) for a virus to form a hybrid with $tRNA_3^{Lys}$ and synthesize its minus strand cDNA in the presence of reverse transcriptase. To precisely reproduce the 18 nucleotides of $tRNA_3^{Lys}$ in mature cDNA, m^1A58 $tRNA_3^{Lys}$ is required to terminate the reverse transcription process when the last 18 nucleotides of $tRNA_3^{Lys}$ have been copied [48–50].

As m^1A is a newly-discovered modification in mRNA and lncRNA, its function has not been extensively explored yet. Up till now, only the function of m^1A at CDS in mt-mRNA has been confirmed in preventing the effective translation of modified codons due to the Watson-Crick disruptive nature of m^1A [9,10]. Ribosome profiling data analysis suggest that m^1A at 5′ cap and 5′ UTR in nuclear mRNA might play a role in promoting translation [9].

Conclusion and perspective

The reversible m^1A modification in tRNA and mRNA uncovers new mechanisms underlying the epitranscriptomic regulation of gene expression. The presence and functions of m^1A in nuclear transcripts had been a subject of debate lately. Hopefully, the discovery of new m^1A writers, erasers, and readers in the future will provide convincing evidence to help resolving such argument. Although tRNA MTases have been found to modify a subset of m^1A sites in mRNA, the writer responsible for the majority of m^1A sites in mRNA is still unknown. Since ALKBH3 is a demethylase of mRNA m^1A, it is interesting to investigate whether its function in cancers is related to its demethylation activity toward mRNA m^1A, and whether other erasers for mRNA m^1A exist. Analogous to the positive charged cap m^7G modification bound by a specific RNA-binding protein, mRNA m^1A could be recognized by reader proteins as well [51]. Compared to the extensively-studied m^6A modification, our knowledge about m^1A in mRNA is limited. Functions of m^1A in mRNA and the underlying mechanisms need to be further investigated. It is anticipated that m^1A in mRNA and lncRNA might regulate RNA processing or protein translation in various manners: 1) m^1A is specifically bound by a reader; 2) m^1A controls RNA structure to affect protein-RNA interaction; 3) m^1A at CDS affects the base-pairing between codon and anticodon. It might be worth exploring whether m^1A occurs and plays important regulatory roles in other types of RNAs as well.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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