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REVIEW

RNA m^{6}A modification and its function in diseases

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Abstract ***N*^{6}-methyladenosine (m^{6}A) is the most common post-transcriptional RNA modification throughout the transcriptome, affecting fundamental aspects of RNA metabolism. m^{6}A modification could be installed by m^{6}A "writers" composed of core catalytic components (METTL3/METTL14/WTAP) and newly defined regulators and removed by m^{6}A "erasers" (FTO and ALKBH5). The function of m^{6}A is executed by m^{6}A "readers" that bind to m^{6}A directly (YTH domain-containing proteins, eIF3 and IGF2BPs) or indirectly (HNRNPA2B1). In the past few years, advances in m^{6}A modulators ("writers," "erasers," and "readers") have remarkably renewed our understanding of the function and regulation of m^{6}A in different cells under normal or disease conditions. However, the mechanism and the regulatory network of m^{6}A are still largely unknown. Moreover, investigations of the m^{6}A physiological roles in human diseases are limited. In this review, we summarize the recent advances in m^{6}A research and highlight the functional relevance and importance of m^{6}A modification in *in vitro* cell lines, in physiological contexts, and in cancers.**

Keywords RNA modification; m^{6}A; immunity; cancer; epigenetics

Introduction of m^{6}A

To date, more than 100 chemical modifications have been identified in RNA, but most of their functions are still unknown. RNA modifications involve adjusting the RNA structures and functions. Although some RNA modifications, instead of being static, can be dynamically modified by enzymes with opposite modifying activities, indicating that these functional dynamic RNA modifications could be a new layer of gene regulation, termed "RNA epigenetics" [1]. *N*^{6}-methyladenosine (m^{6}A) RNA modification was first discovered in 1974 in purified poly(A) RNA fraction [2]. However, the study of this marker was hampered due to lack of methods for mapping m^{6}A within mRNAs.

Using m^{6}A specific antibodies, two independent groups performed m^{6}A RNA immunoprecipitation, followed by high-throughput RNA sequencing (MeRIP-seq) to map m^{6}A throughout the transcriptome [3–5]. Their results first revealed that m^{6}A was widely distributed in mRNA. More surprisingly, the mapping approaches showed that m^{6}A modification was highly enriched near stop codons and in 3′-UTRs in certain mRNAs, and the m^{6}A levels varied in different cell contexts, indicating the critical roles of m^{6}A in cells. A few months before these two studies, He group described that m^{6}A was a major substrate of fat mass and obesity-associated protein FTO [6], indicating that m^{6}A RNA modification was dynamically regulated, and its dysregulation probably correlated with diseases (Fig.1). These results encouraged the researchers to investigate the function of m^{6}A and identify and characterize its modulators ("writers," "erasers," and "readers").

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m^{6}A writers

METTL3

The first protein responsible for m^{6}A formation (m^{6}A writer) is the 70-kDa fraction (MT-A70, now referred to METTL3) with S-adenosylmethionine (SAM)-binding activity isolated from the nuclear fractions of HeLa cells by Bokar *et al*. [7]. METTL3 is a critical subunit of (*N*^{6}-adenosine)-methyltransferase [8]. It was highly conserved throughout eukaryotes, and METTL3 knockdown in

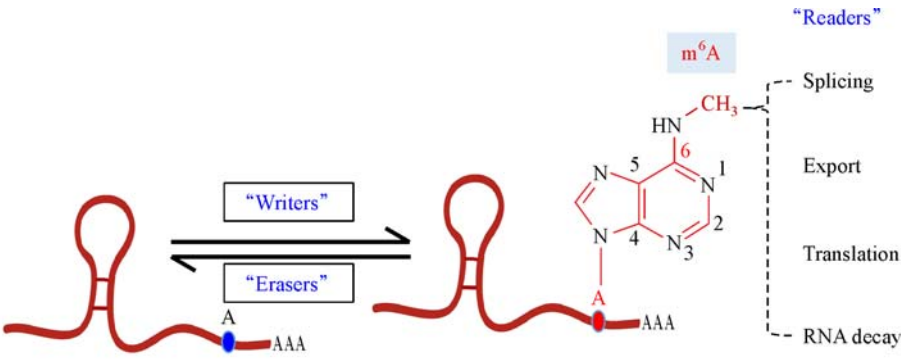

Fig. 1 Dynamic m^{6}A system. m^{6}A marker is installed at the 3′-UTR by m^{6}A "writers" co-transcriptionally and could be reversed by m^{6}A "erasers." The marker is recognized in nucleus and cytoplasm by different m^{6}A "readers" and executes different functions involved in RNA metabolism, including splicing, export, translation, and RNA decay.

different cells remarkably reduced m^{6}A in mRNAs [5,9–11]. Genetic deletion of METTL3 [10,12,13] led to the near-complete loss of m^{6}A, suggesting METTL3 as the major catalytic component of m^{6}A methyltransferase multiprotein complex. The presence of METTL3 in both cytoplasm and nucleus [11,14] indicated that m^{6}A modification might occur in nucleus and cytoplasm. However, by chromatin fraction coupled with HITS-CLIP [15], followed by RNA sequencing, Ke *et al*. [16] showed that m^{6}A was formed on nascent RNAs usually before splicing. Minimal differences were noted on the content and the location of m^{6}A in cytoplasmic mRNA and chromatin-associated nascent pre-mRNA or nucleoplasmic mRNA at steady-state [16], suggesting that m^{6}A methylation occurred co-transcriptionally. This idea was supported by recent studies that METTL3 interacted with chromatin and the transcription machinery [17–19].

METTL14

METTL14, a homolog of METTL3, was initially discovered as another putative m^{6}A methyltransferase by different approaches from independent groups [10,20,21]. METTL3 interacts with METTL14, and knockdown of METTL14 reduces the m^{6}A content *in vitro* and *in vivo*. In addition, purified METTL14 manifested methyltransferase activities [10,20], suggesting that METTL14 is a distinct m^{6}A writer. However, recent independent crystallization studies [22–24] demonstrated that METTL3, not METT14, bound to SAM and catalyzed m^{6}A formation. Based on crystal structure analysis of METTL3–METTL14 complex, METTL14 possessed a degenerated SAM-binding domain, which was not functional. As an alternative, METTL14 was critical for substrate RNA binding and the methyl group positioning to facilitate the catalytic activities of METTL3. Thus, METTL14 was an essential adaptor for METTL3 activities. METTL3 and METTL14 constitute the core m^{6}A methylation complex. However, METTL3 or METTL14 functions or activities, other than m^{6}A methylation, need further investigation.

Wilms tumor suppressor-1-associated protein (WTAP)

WTAP was found to interact with METTL3 in 2008 by Zhong *et al*. [12] via two-hybrid screening. In the interaction between mum2 and Ime4 [25], the WTAP and METTL3 homologs , were required to form m^{6}A in yeast. Furthermore, the WTAP–METTL3 interaction was observed both *in vitro* and *in vivo* [20,21,26]. Although WTAP alone did not exhibit any methyltransferase activity, WTAP knockdown led to METTL3 and METTL14 degradation and reduced the m^{6}A levels remarkably [20]. Detailed characterization of WTAP revealed the importance of WTAP that guided and localized the METTL3–METTL14 complex into nuclear speckles to efficiently methylate target RNAs [26]. Furthermore, Schwartz *et al*. [21] revealed the WTAP-dependent and WTAP-independent m^{6}A modification sites upon WTAP depletion. WTAP-dependent sites were located at internal positions and were topologically static, whereas the WTAP-independent sites were found in the cap structure of the transcripts [21], indicating the complexity of co-transcriptional regulation.

KIAA1429

In a proteomics screening performed by Schwartz *et al*. to isolate METTL3-associated proteins, KIAA1429 was among the top candidates [21]. KIAA1429 knockdown by siRNA led to a considerably more striking reduction of m^{6}A peaks in mRNA than knockdown of either METTL3 or METTL14, suggesting that KIAA1429 was required for the intact catalytic activity of the methyltransferase complex. Meanwhile, virilizer interacted with Fl(2)d

[27,28], the *Drosophila* homologs of KIAA1429 and WTAP, respectively, which were involved in controlling the sex determination in females by alternative splicing of *Sxl* transcripts.

RBM15/15B and METTL16

m^{6}A is highly enriched in certain mRNA species [4] and specific regions of a transcript [5]. However, the mechanism of this selection is still poorly understood. RBM15 and RBM15B, two other WTAP interactors identified by coimmunoprecipitation, bound to U-rich regions in transcripts that were closely adjacent to DRACH, the consensus sequence of m^{6}A [29], suggesting that RMB15 and RMB15B could recruit methyltransferase complex to DRACH consensus sequence sites for m^{6}A methylation. Furthermore, RBM15/15B targets lncRNA *XIST* [29], which was involved in mediating X-chromosome inactivation and gene silencing during development, for methylation. Therefore, the formation of specific m^{6}A sites in specific mRNA might require distinct components of methyltransferase complex. This idea was further supported by the findings of METTL16 [30], which was shown to specifically form m^{6}A in U6 snRNA and U6-like hairpins of *MAT2A* mRNA in a C-m^{6}A-G context.

m^{6}A erasers and the dynamics of m^{6}A modification

FTO

FTO belongs to the non-heme Fe(II)- and α-KG-dependent dioxygenase AlkB family of proteins and demethylated m3T and m3U in single-stranded DNAs and single-stranded RNAs [31]. The demethylase activity of FTO against m^{6}A [6] was further characterized, and for the first time, m^{6}A residues in RNAs were found to be real substrates of FTO *in vitro*. This study was first to indicate that the ubiquitously distributed m^{6}A modifications on mRNA might be reversely regulated by methyltransferases and demethylases, thereby serving as a previously unappreciated layer of regulation on mRNAs. Furthermore, FTO-mediated m^{6}A demethylation was involved in adipogenesis [32–34], acute myeloid leukemia (AML) cell transformation, leukemogenesis [35,36], and ultraviolet-induced DNA damage response [19], emphasizing the importance of m^{6}A modification in different cell contexts. Although FTO catalyzed the demethylation of m^{6}A residues both *in vitro* and *in vivo* [6,37], FTO was instead the genuine demethylase of m^{6}Am, which was highly similar to m^{6}A and enriched in the 5′-UTR adjacent to the N^7-methylguanosine (m^{7}G) cap [38]. In this study, FTO manifested a catalytic activity to m^{6}Am nearly 10 times greater than m^{6}A, despite the cap m^{6}Am levels were considerably lower (less than 1/20) than those of m^{6}A [39].

ALKBH5

As a member of the ALKB family, ALKBH5 is another m^{6}A demethylase. ALKHB5 depletion was related to the increase of m^{6}A, whereas its overexpression reduced m^{6}A in mRNAs of human cell lines [40]. ncRNAs, which are small nucleolar RNAs, and snRNAs could also be the targets of ALKBH5 [41]. In addition, a specific m^{6}A site in *FOXMI* mRNA was demethylated by ALKBH5 in an RNA–RNA interaction-dependent manner [42]. ALKBH5 knockout mice bore defects in spermatogenesis because of elevated apoptosis of meiotic metaphase-stage spermatocytes [40], emphasizing the fundamental significance of m^{6}A in mammalian cells.

Dynamics of m^{6}A modification

The broad distribution of m^{6}A throughout the transcriptome and the discovery of FTO as m^{6}A "eraser" spurred the hypothesis that in parallel to reversible functional modifications of DNA and histones, reversible m^{6}A RNA modifications might represent an unappreciated layer of regulation, affecting fundamental aspects of mRNA metabolism. This hypothesis became noteworthy in the RNA modification field.

However, FTO showed greater enzyme activity against m^{6}Am in 5′-UTR than against m^{6}A in the internal of mRNA [43]. In addition, in HeLa cells, m^{6}A formation primarily occurred co-transcriptionally and maintained in nucleoplasmic and cytoplasmic mRNAs, whereas no mRNA methylated and demethylated in the cytoplasm [16]. Thus, m^{6}A might not be subjected to reversible "epigenetic" changes.

Nevertheless, m^{6}A methylation and demethylation were proven by human AML studies [35], showing that FTO knockdown remarkably increased m^{6}A abundance of m^{6}A peaks containing transcripts. Specifically, FTO targeted the internal m^{6}A sites of the *ASB2* and *RARA* mRNAs to regulate the stability and expression of these two transcripts [35]. Importantly, FTO was present and functional both in the nucleus and cytoplasm in this study. FTO also targeted the m^{6}A of several oncogenes (*ADAM19*, *EPHA3*, and *KLF4*) to promote the growth of glioblastoma stem cell (GSC) and tumor progression; ALKBH5 demethylated 3′-UTR m^{6}A sites in *NANOG* and *FOXM1* mRNAs, thereby increasing their expression in breast cancer stem cells and GSC, respectively. In addition, m^{6}A was also demethylated during stress responses. Zhou *et al*. [44] demonstrated that the nuclear localization of YTHDF2 induced by heat shock stress could prevent the m^{6}A in the 5′-UTR of stress-induced transcripts from demethylation by FTO. Moreover, Xiang *et al*. showed that in response to ultraviolet-induced DNA damage,

METTL3/METTL14 was recruited to damaged sites to install m^{6}A on RNAs that can be removed by FTO in nucleus [19]. Hence, m^{6}A demethylation could occur both in the nucleus and cytoplasm with substantial functional outcomes.

m^{6}A readers

YTH domain-containing proteins

The manner on how m^{6}A executes its effects remains to be unknown. Biochemical approaches have been used to screen the m^{6}A-binding proteins ("readers"). Primarily, YTHDF2 and YTHDF3 bound to m^{6}A in an RNA pulldown experiment performed by Dominissini *et al*. [5]. This observation was further confirmed by using gel shift assays and crystallography [45–47]. The YTH domain-containing proteins have five members in mammalian genomes—YTHDC1, YTHDC2, YTHDF1, YTHDF2, and YTHDF3. By using the individual-nucleotide-resolution UV crosslinking and immunoprecipitation (iCLIP) method [29,48], the binding sites of the individual proteins in an endogenous context were mapped and revealed that all the YTH domains containing proteins bound to m^{6}A in specific contexts, except YTHDC2, which showed primarily noncoding RNA-binding activities. Although YTHDF proteins closely resembled each other and were predominantly cytoplasmic, their function could be diverse. YTHDF2 mediated the decay of target mRNAs, evidenced by the interactions between YTHDF2 and P-bodies [47]. In contrast, the half-lives of mRNAs were not affected by YTHDF1 depletion, whereas its interaction with eIF3 and other translation initiation factors suggested that it might affect translation [49].

Interestingly, tethering experiment performed by Kennedy *et al*. showed that all YTHDF proteins promoted translation [50], whereas those performed by Du *et al*. [51] showed that all three YTHDF proteins triggered mRNA degradation and deadenylation. Consistently, studies from viruses showed that all YTHDF proteins promoted degradation of virus-encoded m^{6}A transcripts [52–54].

YTHDC1 was initially critical for alternative splicing events when its overexpression or depletion caused splicing abnormalities. Studies of YTHDC2 function were limited. YTHDC2 bound to noncoding RNAs and promoted translation of HIF1a via its helicase function [29,55].

eIF3

Eukaryotic initiation factor 3 (eIF3), a component of the translation pre-initiation complex, directly bound to m^{6}A in 5′-UTR and mediated an eIF4E-independent translation initiation [43]. Importantly, in another study, eIF3 was recruited to 5′-UTR by YTHDF1, which bound to m^{6}A near stop codon [49].

HNRNPA2B1

HNRNPA2B1 was an m^{6}A reader that interacted with the microRNA microprocessor complex protein DGCR8 and promoted miRNA biogenesis. Loss of HNRNPA2B1 showed similar processing and alternative splicing defects as the deletion of METTL3. However, the poor overlap of HNRNPA2B1 binding sites with m^{6}A suggested that HNRNPA2B1 might not directly bind to m^{6}A [56]. Indeed, m^{6}A could affect the structure of a region in the RNAs to make it more single-stranded or accessible, which in turn, facilitated the binding of heterogeneous nuclear ribonucleoprotein [57].

IGF2BPs

Insulin-like growth factor 2 mRNA binding proteins (IGF2BPs, IGF2BP1/2/3) were previously known as highly conserved oncofetal RNA-binding proteins that contained two RNA recognition motif (RRM) domains and four K homology (KH) domains [58]. IGF2BP expression was often correlated with malignant transformation and poor prognosis of various cancer types. IGF2BP affected multiple aspects of target RNAs, including localization, translation, and stability [58]. However, the mechanism of how IGF2BPs function was obscure. Huang *et al*. [59] re-identified IGF2BPs as a new class of m^{6}A readers by m^{6}A RNA bait pulldown experiment combined with a computational screening of m^{6}A-binding proteins. In their study, IGF2BPs recognized the consensus GG(m^{6}A)C sequence of mRNA targets through their K homology domains. Interestingly, in contrast to YTHDF2, which promoted the decay of target mRNAs, IGF2BPs promoted the translation of target mRNAs (*MYC*, for example) by increasing their stability and storage [59]. The findings of m^{6}A readers with distinct regulatory functions suggested the diversity of m^{6}A functions. Interestingly, m^{6}A readers may function in context-specific manners.

m^{6}A and mRNA metabolism

The function of m^{6}A in RNAs was first described in 1978, showing that m^{6}A in mRNAs were remarkably reduced when RNA synthesis was blocked by either actinomycin D or 5,6-dichloro-I-β-d-ribofuranosyl-benzimidazole [60]. Thus, m^{6}A possibly promoted RNA decay. Correspondingly, m^{6}A-containing mRNAs had reduced half-lives, compared with mRNAs lacking m^{6}A in ES cell [9,13]. In addition, by conditional deletion of METTL3 in T cells [61,62], we could investigate the *in vivo* physiological functions of m^{6}A in adult mammalian cells. We found that

METTL3 depletion resulted in disrupted naïve T cell homeostasis [61]. Specifically, overexpression of SOCS family proteins prevented the activation of IL-7-STAT5 signaling, which was critical for naïve T cell homeostasis, due to the increased half-lives of m^{6}A-containing mRNAs of SOCS family genes [61]. Similarly, *Socs* mRNA levels were also elevated in METTL3 depleting Tregs [62]. Furthermore, m^{6}A was recognized by YTHDF2, which delivered m^{6}A-target mRNAs to P-bodies for degradation. The half-lives of the YTHDF2-target mRNAs increased upon its depletion [47].

The enrichment of m^{6}A in long exons and transcripts [5] with alternative splicing variants clearly indicated that m^{6}A might regulate the process of splicing. m^{6}A facilitated the binding of a splicing regulator, HNRNPC, to its targets by m^{6}A-induced structural switch [63]. Similarly, the m^{6}A, specifically catalyzed by METTL16 in the hairpins of MAT2A pre-mRNAs, was required for the splicing of this intron [30]. Furthermore, depletion of m^{6}A reader protein YTHDC1 caused splicing abnormalities, which could only be resolved by YTH domain-containing YTHDC1 proteins [64]. In flies, the splicing of *Sxl* transcripts was critical in sex determination in females [27,28]. This process depended on m^{6}A formation on *Sxl* transcripts formed by m^{6}A methyltransferase complexes, namely, virilizer, Fl (2) d, and IME4 [65–67]. m^{6}A also affected mRNA translation by the interaction of m^{6}A readers and translation machinery proteins. In one study, YTHDF1 bound to m^{6}A near the stop codon to facilitate the binding of eIF3 to the 5′-UTR for translation initiation [49]. eIF3 was also recruited and bound to m^{6}A in 5′-UTR to promote translation [43].

m^{6}A in cancers

m^{6}A modification affects fundamental aspects of mRNA metabolism. Given that deregulated m^{6}A modulators result in dysregulation of gene expression related to cell differentiation, homeostasis, and response to stresses, m^{6}A is indeed involved in cancer.

In a recent study, METTL3 was overexpressed in AML cells [68]. METTL3 depletion induced differentiation and apoptosis of AML cell lines and delayed leukemia progression [68]. m^{6}A promoted the translation of *c-MYC*, *BCL2*, and *PTEN* mRNAs in the human AML MOLM-13 cell line [68]. In another study, METTL3 promoted the maintenance of a leukemic state by binding to the promoter of active genes, installing m^{6}A modification within the target mRNA transcripts and resulting in enhanced translation of those transcripts by relieving ribosome stalling [69]. METTL3 overexpression was also observed in human hepatocellular carcinoma (HCC) and associated with poor prognosis of patients with HCC [70]. Experimentally, the proliferation, migration, and colony formation of HCC cell were inhibited by METTL3 knockdown *in vitro*. Knockout of METTL3 *in vivo* suppressed HCC tumorigenicity and lung metastasis [70].

m^{6}A demethylases also play essential roles in cancer. Both FTO and ALKBH5 could promote tumorigenesis of GSC [42,71]. Under hypoxia conditions, ALKBH5 mediated m^{6}A demethylation of pluripotency factors, such as *NANOG* and *KLF4*, thereby promoting the breast cancer stem cell (BCSC) phenotype [42,60]. FTO was also overexpressed in certain AML cells [35,36]. Similar to the effects of ALKBH5 in GSC tumorigenesis, FTO enhanced oncogene-mediated cell transformation and leukemogenesis of AML cells by reducing m^{6}A levels in *ASB2* and *RARA* mRNA transcripts, resulting in enhanced leukemic activity of AML cells [35]. R-2-hydroxyglutarate (R-2HG) inhibited the demethylation activity of FTO, thereby stabilizing the *MYC/CEBPA* transcripts to exert a broad anti-leukemic activity [36].

Conclusions

In 1974, m^{6}A modification was discovered initially in the poly(A) RNA fractions and was predicted to be functional in mRNA processing [2]. The discovery of the m^{6}A demethylase FTO [6,31] suggested that dynamic regulation of m^{6}A modification on target RNAs might represent a previously undefined layer of reversible regulation in parallel to reversible DNA and histone modifications. Its importance has already been appreciated for decades. This idea sequentially triggered the “golden rush”on the screening and characterization of possible m^{6}A “writers,” “erasers,” and “readers.”

Given that m^{6}A affects multiple aspects of the mRNA metabolism, which in turn, regulates the expression of genes, dysregulation of key modulators of m^{6}A will be certainly implicated in cancer. The overexpression levels of m^{6}A modulators, such as METTL3 [68–70], FTO [35,36], and ALKBH5 [72], in different cases of cancers emphasize the potential therapeutic importance of targeting m^{6}A modulators. To date, the *in vivo* functions of m^{6}A system in different cell types and in different microenvironments are gradually known and will give us accumulating evidence to further define the concepts of RNA epigenetics. Further investigations are needed to broaden our views regarding the principles of the m^{6}A biological function and its disease relevance.

Over 100 different modifications have been identified on histones; how those different modifications coordinate to regulate the transcription prompted the well-known concepts of histone code hypothesis. Similarly, over 100 different RNA modifications are known; how those different RNA modifications regulate RNA metabolism may constitute the “RNA code” (Fig.2). Acquiring experimental evidence on the communication between

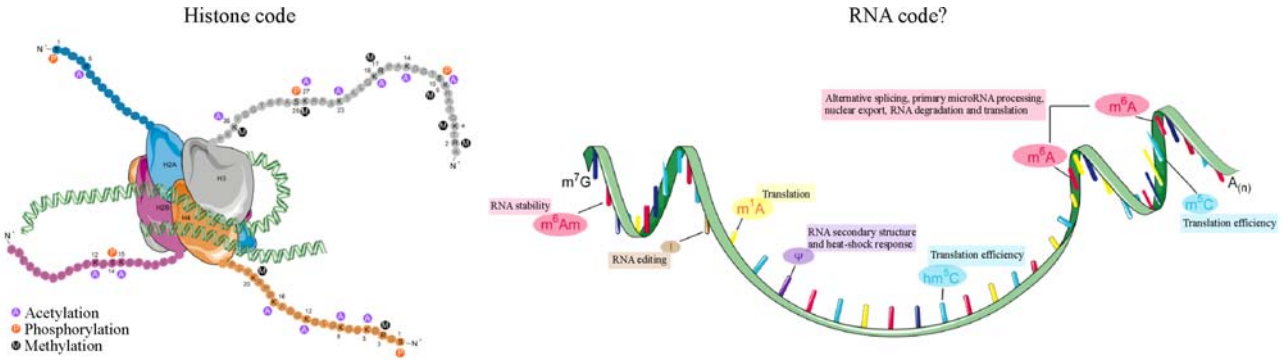

Fig. 2 Does "RNA code" exist? How over 100 histone modifications coordinate to regulate the transcription prompted the well-known concepts of histone code hypothesis. Similarly, over 100 different RNA modifications are known, and how those different RNA modifications may regulate the RNA metabolism may constitute the "RNA code."

different RNA modifications in the same RNA transcripts of the same cells in response to various stimulations is an exciting research goal.

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Compliance with ethics guidelines

Jiyu Tong, Richard A. Flavell, and Hua-Bing Li declare that they have no conflict of interest. This manuscript is a review article and does not require an approved research protocol by the relevant institutional review board of ethics committee.

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