N^6-methyladenosine–encoded epitranscriptomics

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N^6-methyladenosine (m^6A) is the most abundant internal modification in eukaryotic mRNA. Recent discoveries of the locations, functions and mechanisms of m^6A have shed light on a new layer of gene regulation at the RNA level, giving rise to the field of m^6A epitranscriptomics. In this Perspective, we provide an update on the various effects of mammalian m^6A modification, which affects many different stages of the RNA life cycle.

Over 100 different types of chemical modifications have been found in RNAs from all three kingdoms of life. Most modifications have been identified in abundant cellular RNAs, such as rRNAs and tRNAs, and these modifications fine-tune RNA structure and function. However, despite their biological importance, modifications of mRNA and long noncoding RNA (lncRNA) have not been intensely studied until recently. Nevertheless, several internal mRNA modifications have been identified, such as m^6A, inosine (I), 5-methylcytosine (m^5C), and pseudouridine (Ψ)[1–9]. In addition, 2′-O–methylation is known to occur within the first few nucleotides in an mRNA. First discovered in 1974, m^6A is the most abundant of these internal mRNA and lncRNA modifications in eukaryotes[1,8,9]. However, initial functional studies on m^6A were hindered by the lack of methods for its detection; such detection had been challenging because m^6A is indistinguishable from adenosine during reverse transcription, and there are no known chemical reagents that can specifically label m^6A.

Recent discoveries and technological developments have revived interest in this modification. In 2011, Jia *et al.* discovered the m^6A demethylase fat mass and obesity associated (FTO)[10], whose existence suggested that m^6A modification is reversible and dynamic. In 2012, two groups independently combined anti-m^6A immunoprecipitation and deep-sequencing methods and identified tens of thousands of m^6A sites in mammalian mRNA at a resolution of ~100–200 bases (refs. 3,4). This method, m^6A-specific methylated-RNA immunoprecipitation and next-generation sequencing (m^6A/MeRIP-seq), has identified peaks in >25% of all transcripts in human cells. These m^6A peaks are not evenly distributed along the transcript but are enriched in long exons, near stop codons and in 3′ untranslated regions (UTRs), although m^6A also occurs in the coding region and 5′ UTR. Subsequent improvements of the technology have further refined m^6A detection; for example, Schwartz *et al.* have optimized the anti-m^6A pulldown protocol and have eliminated many false positives by using methylation-deficient yeast strains[11]. In addition, UV light–induced antibody-RNA cross-linking followed by reverse transcription has enabled single-nucleotide-resolution mapping of m^6A in the human transcriptome[12–14]. These studies have confirmed the RRACH (in which R represents A or G, and H represents A, C or U) motif as the m^6A consensus motif, as characterized by early studies[15–17]. However, the majority of RRACH motifs in cellular RNA lack m^6A modification. Instead, m^6A levels in mRNA and lncRNA vary substantially[18,19], which has also been shown by site-specific cleavage and radioactive labeling followed by ligation-assisted extraction and thin-layer chromatography (SCARLET)[20]. The SCARLET method has revealed modified fractions ranging from 7% to 88% among 12 m^6A sites in human cells, thus indicating that m^6A is a dynamic modification in cells.

The study of m^6A RNA modification is a rapidly developing field. Many excellent recent reviews have provided insights into progress in determining the biological roles of m^6A (for example, refs. 21–24). Here, we focus on the recent advances in elucidating mechanisms of m^6A function, including selective m^6A methylation and the interaction of m^6A with cellular machineries, thus leading to regulation of gene expression.

m^6A 'writers' and 'erasers'

Analogously to DNA methylation and histone modifications, the m^6A RNA modification can be added by writer enzymes and removed by eraser enzymes (**Fig. 1**). The first m^6A writer, the METTL3 methyltransferase, was identified from HeLa nuclear extracts as a component of a multiprotein complex[25]. Subsequent studies identified a second methyltransferase, METTL14, and Wilms' tumor 1–associating protein (WTAP) in the mammalian RNA-methylation complex[26,27]. WTAP had previously been found to interact with the METTL3 homolog in *Arabidopsis*[28]. One additional protein, KIAA1429, also associates with this complex[29]. WTAP translocates the METTL3–METTL14 complex to mRNA targets in nuclear speckles, thus improving methylation efficiency[26,30]. Knockdown of METTL3 or METTL14 causes ~40% global m^6A loss in human mRNAs, whereas WTAP depletion leads to even larger decreases in m^6A levels[26,29]. A substantial fraction (around 30%) of target sites of m^6A writers in human cells are located in introns, thus suggesting that m^6A methylation may occur cotranscriptionally before or during splicing[26,27]. Because various precursor mRNA (pre-mRNA) splicing factors colocalize with the m^6A methyltransferases in nuclear speckles[26,31], intronic m^6A residues may regulate splicing-factor binding, thereby influencing alternative splicing.

The discovery of m^6A erasers in 2011 and 2012 has indicated that the m^6A modification is reversible[10,32] (**Fig. 1**). The first m^6A eraser to be discovered, FTO, is linked to human body-weight regulation[33], although the phenotypic mutations in the locus encoding the FTO gene are now known to influence the transcription of

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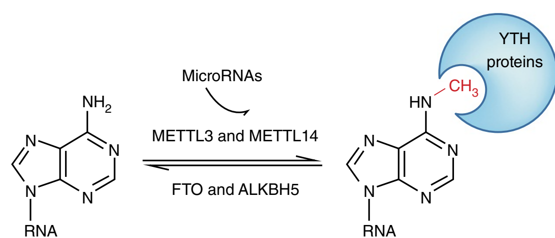

Figure 1 m^{6}A epitranscriptomics: writers, erasers and readers. Dynamic m^{6}A modification is regulated by writers METTL3 and METTL14, and erasers FTO and ALKBH5. miRNAs guide writers to specific target sites. m^{6}A directly recruits readers, e.g., YTH-domain proteins containing a hydrophobic pocket to directly accommodate the *N*^{6}-methyl group of m^{6}A.

adjacent genes rather than FTO itself[34]. The second m^{6}A eraser to be discovered, ALKBH5, is associated with fertility in mice[32]. Both FTO and ALKBH5 belong to the nonheme Fe(II)- and α-ketoglutarate (KG)-dependent dioxygenase AlkB family of proteins. FTO oxidizes m^{6}A, generating *N*^{6}-hydroxymethyladenosine as an intermediate modification and *N*^{6}-formyladenosine as a further-oxidized product[35]. These demethylation intermediates, which are present in isolated mRNAs from human cells as well as mouse tissues, may also possess regulatory functions similar to those of the oxidized derivatives of m^{5}C in DNA. In addition to these known m^{6}A writers and erasers, there may be other proteins yet to be discovered that are responsible for regulating the m^{6}A patterns in the cell.

One essential question regarding m^{6}A is how the methylation machinery selectively targets a subset of consensus motifs in the transcriptome. It remains unclear why RRACH motifs adjacent to stop codons are more likely to be methylated. *Cis* elements such as the neighboring RNA sequences or secondary structures are likely to have roles in methylation specificity. Schwartz *et al.* have shown that m^{6}A sites have a static topology across four dynamic cellular systems when sequencing is performed at very high coverage, but the m^{6}A/MeRIP-seq method is insensitive to quantitative differences in methylation levels at individual sites between samples[36]. Several other studies have indicated that the m^{6}A content in total mRNA can vary substantially among different cell types or tissues[3,4,37]. The cell type–specific m^{6}A patterns suggest the presence of *trans*-regulatory factors that function in a cell type–dependent manner. One crucial way to better understand selective m^{6}A deposition is to identify additional factors interacting with the writer and eraser machineries.

Recently, Chen *et al.* have suggested that microRNAs (miRNAs) can act as *trans*-regulatory factors in conferring methylation specificity[37] (**Fig. 1**). The authors have shown that miRNAs modulate METTL3 binding to mRNAs via a sequence-dependent pairing mechanism, thus directing m^{6}A modification. Although Argonaute (AGO) proteins are known to mediate the binding of miRNAs to their target mRNAs, AGOs do not seem to participate in the regulation of m^{6}A modification. Thus, it is likely that miRNAs associate with other yet-unknown RNA-binding proteins in regulating m^{6}A deposition. These results reveal a function of miRNAs in guiding m^{6}A writers to specific RNA sites for methylation and partially explain the mechanism of methylation specificity.

Another open question pertaining to methylation control is how the m^{6}A writers cooperate with erasers or other protein factors in regulating m^{6}A dynamics in response to various stresses or other stimuli. Zhou *et al.* have found that, in response to heat shock, certain adenosine residues in the 5′ UTRs of newly synthesized mRNAs are preferentially m^{6}A methylated[38]. They have shown that this stress-increased 5′-UTR methylation is the result of competitive binding between the m^{6}A eraser FTO and YTHDF2, a well-characterized m^{6}A-'reader' protein. Upon heat shock, YTHDF2 translocates from the cytoplasm to the nucleus and preserves the 5′-UTR methylation of stress-induced transcripts by preventing the m^{6}A eraser FTO from demethylating the residues. Furthermore, this stress-responsive 5′-UTR m^{6}A methylation promotes cap-independent translational initiation, which will be discussed in detail below. Perturbation of m^{6}A writers and erasers changes the global m^{6}A levels in the cell and leads to a variety of severe phenotypes (**Table 1**). How m^{6}A achieves such wide-ranging physiological effects is only beginning to be understood.

Mechanisms underlying m^{6}A function

m^{6}A can exert its function through the recruitment of specific binding proteins, so-called m^{6}A readers, but the modification also affects RNA structure and has its own consequences on RNA-mediated processes. In this section, we discuss the known m^{6}A readers and their biological mechanisms as well as other functional consequences of m^{6}A.

Direct readers: YTH-domain proteins. m^{6}A can be directly recognized by YTH-domain proteins (**Fig. 1**). In 2012, Dominissini *et al.* used m^{6}A methylated RNA as bait to pull down binding proteins from human cell lysates and identified YTH-domain proteins as selective binders of m^{6}A methylated RNA[3]. Mammalian YTH-domain proteins have five members: YTHDF1–3, which are primarily localized in the cytoplasm, and YTHDC1 and YTHDC2, which are localized in the nucleus[22,39–41]. A cocrystal structure shows a hydrophobic pocket within the YTH domain directly accommodating the methyl group of m^{6}A[41–44]. The functions of YTHDF1 and YTHDF2 have been characterized in detail[39,40]. YTHDF2 recognizes many m^{6}A-containing mRNAs that are not being actively translated and recruits them to cytoplasmic processing (P) bodies for degradation. YTHDF1, in contrast, selectively binds m^{6}A-modified mRNAs and promotes their protein synthesis by interacting with the translation machinery. Wang *et al.* have shown that YTHDF1 promotes ribosome loading of m^{6}A-modified mRNAs and interacts with translation initiation factors, thus facilitating translation initiation[39]. This effect is prominently observed during cellular stress responses. Wang *et al.* have suggested that, in response to arsenite treatment, YTHDF1 is driven into stress granules and stabilizes the formation of stalled translation-initiation complexes within the stress granules. Once stress is relieved, YTHDF1-bound mRNAs can rapidly resume translation from their stalled position. Interestingly, YTHDF1 and YTHDF2 share a large set of common target mRNAs. Wang *et al.* have suggested that YTHDF1 binds these shared targets earlier and promotes their translation before they are recognized by YTHDF2, which facilitates their decay[39]. Thus, YTHDF1 and YTHDF2 cooperate to improve the translation efficiency of transcripts that have short lifetimes.

Interestingly, all m^{6}A-reader proteins identified to date contain low-complexity (LC) domains characterized by a biased amino acid composition. In fact, many RNA-binding proteins contain LC domains, which can promote the formation of RNA granules[45,46]. RNA granules, including stress granules and P bodies in mammalian cells, are membrane-free cellular structures composed of RNA and RNA-binding proteins. Although RNA granules were first observed more than 100 years ago, the mechanism underlying their formation and function has been elusive. McKnight and colleagues have recently shown that RNA granules consist mainly of RNA-binding proteins containing LC domains and that the LC domains are indispensable for RNA-granule formation both *in vitro* and *in vivo*[45,47]. For example, the YTHDF2 protein contains a Pro/Gln/Asn (P/Q/N)-rich domain, which has an essential role in recruiting the YTHDF2–RNA complex to P bodies for RNA decay[40]. The METTL14 methyltransferase has a glycine-rich domain that may regulate the enzyme's subnuclear localization[22]. It remains unclear precisely

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Table 1 Phenotypes of m^{6}A perturbation in cells and organisms

Species / cell lines	m^{6}A perturbation	Phenotype	References
Human and mouse / ESC	METTL3 KO	Impaired ESC exit from self-renewal toward differentiation	63,64
Human / HEK293T	METTL3, METTL14 KD	Decreased cell proliferation	52
Human / U2OS; mouse / embryonic fibroblasts	METTL3 KD	Circadian-period elongation	65
Human / HeLa	METTL3 KD	Cell death	1
Human and mouse	FTO KO	Obesity	66,67
Mouse / ESC	METTL3, METTL14 KD	Loss of self-renewal capability	27
Mouse	ALKBH5 KO	Spermatogenesis	32
Drosophila	IME4 KO	Viability	68
Budding yeast	IME4 KO	Halted sporulation	69
Arabidopsis thaliana	MTA KD, KO	Embryonic development, plant growth patterning	28,70

ESC, embryonic stem cells; KD, knockdown; KO, knockout; IME4 and MTA, homologs of METTL3.

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how LC domains contribute to granule formation, but phosphorylation of LC-domain residues has been shown to inhibit or reverse granule formation[47], thus adding a new layer of complexity in the regulation of granule formation. The prevalence of LC domains in proteins engaged in m^{6}A biology suggests that m^{6}A readers and the mRNAs bound to them can localize at specific cellular sites, where they can interact with other RNA-binding proteins within the same granules. Therefore, the identification of specific protein and RNA molecules in granules containing YTH-domain proteins would be of great importance to elucidate the biological pathways regulated by m^{6}A modification.

m^{6}A affects RNA structure. m^{6}A can also alter RNA structure. Biophysical studies using UV melting and NMR have measured the effect of m^{6}A modification on base-pairing thermodynamics and structural perturbation of RNA duplexes[48,49]. Kierzek and Kierzek have found that one single m^{6}A residue can destabilize an RNA duplex by up to 1.4 kcal/mol, and the actual degree of destabilization depends on the location of the m^{6}A relative to the end of the duplex[48]. By NMR, Roost *et al.* have shown that the m^{6}A-U pair in a stable RNA duplex is paired via canonical Watson-Crick geometry, which rotates the N^6-methyl group into the high-energy *anti* conformation[49]. Both studies have also reported that m^{6}A in unpaired positions, such as in a hairpin loop, stack better than the unmodified base and can contribute to the stabilization of the RNA secondary structure. This is because m^{6}A in unpaired RNA regions adopts the relaxed (*syn*) conformation[50], thus placing the methyl group in contact with an adjacent base pair and probably adding a favorable hydrophobic stabilization. Therefore, the effect of m^{6}A on RNA structure is highly context dependent.

Consistently with the results of these thermodynamic studies, transcriptome-wide RNA structural mapping analyses *in vitro* and *in vivo* have revealed that RNA structures adjacent to the m^{6}A sites are more single-stranded than the nonmethylated RNA region[49,51]. Thus, it appears that m^{6}A can function as an RNA-structure remodeler within mRNA and lncRNAs. This modulation of RNA structure driven by m^{6}A can serve as another layer of control affecting RNA-protein, RNA-RNA and RNA-DNA interactions transcriptome wide. Cotranscriptional m^{6}A methylation could potentially affect the folding of newly synthesized transcripts. RNA transcripts with different structures can interact with different binding partners, thus resulting in their distinct participation in different biological pathways, as illustrated by the m^{6}A-switch studies described below.

Indirect m^{6}A readers: m^{6}A switches. The concept that m^{6}A modulates RNA-protein interactions by affecting RNA structure has been exemplified by a recent report demonstrating that m^{6}A influences the accessibility of RNA-binding motifs to their RNA-binding proteins[52] (**Fig. 2**). This mechanism regulating RNA-protein interactions on the basis of m^{6}A-dependent RNA structural remodeling is termed the 'm^{6}A switch'[52,53]. Liu *et al.* have found that m^{6}A can alter local RNA structure and hence increase the accessibility of RNA-binding motifs to HNRNPC, a protein that belongs to the large family of heterogeneous nuclear ribonucleoproteins. HNRNPC is an abundant nucleus-localized protein that binds nascent RNA transcripts and controls their processing[54–57]. It preferentially binds to U-tract motifs in pre-mRNA. However, U-tract motifs are frequently buried within local RNA structures, and hence their interactions with HNRNPC are inhibited. Liu *et al.* have found that the U-tract motifs located in the stems of RNA stem-loop structures often base-pair with the m^{6}A consensus motif (RRACH). Methylation of the adenosine residue within RRACH leads to destabilization of the stem structure, causing the U-tract motif to be single-stranded and more readily accessible for HNRNPC binding. In this case, the m^{6}A reader HNRNPC does not directly recognize the methyl group; instead, it recognizes the structural change induced by m^{6}A modification.

The HNRNPC-binding motifs in the human transcriptome vastly outnumber the copies of HNRNPC proteins per cell. Via the m^{6}A-switch mechanism, m^{6}A methylation directs HNRNPC to specific binding sites near m^{6}A modifications. HNRNPC has been associated with RNA transcription, stability and regulation of alternative splicing[54,56]. Hence, changes in HNRNPC binding patterns directly affect the downstream function of HNRNPC at the target substrates. Up to ~8% of all mapped HNRNPC sites, representing ~40,000 m^{6}A switches, have been found to regulate HNRNPC binding and influence mRNA abundance and alternative splicing in human cells. However, HNRNPC mediates only ~16% of m^{6}A-dependent pre-mRNA–processing changes. Therefore, it seems likely that other direct or indirect m^{6}A readers exist that mediate m^{6}A-dependent pre-mRNA–processing events.

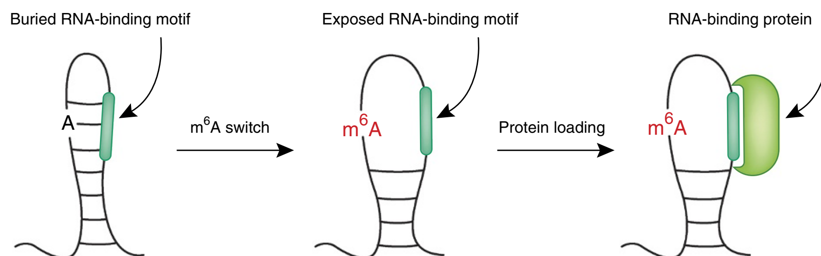

Figure 2 m^{6}A switches. m^{6}A alters local RNA structure, thereby exposing RNA binding motifs and facilitating protein binding (m^{6}A switch). Proteins that are regulated by m^{6}A switches, such as HNRNPC, are referred to as indirect readers of m^{6}A.

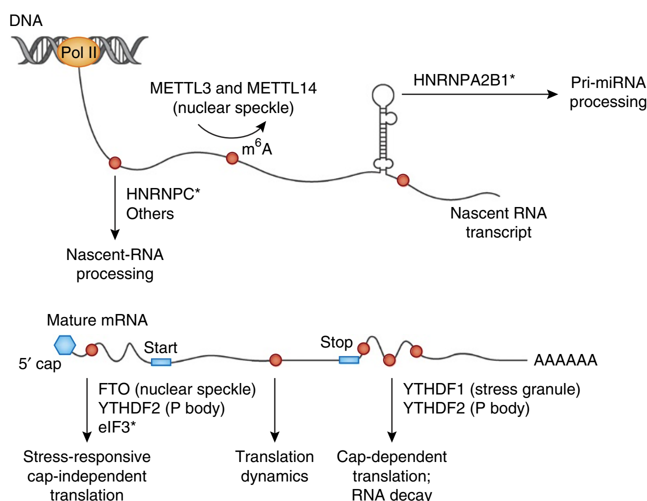

Figure 3 m^{6}A-regulated RNA metabolism in mammalian cells. m^{6}A-modified nascent RNA transcripts are selectively recognized by specific RNA-binding proteins for processing into mature mRNAs or miRNAs. 5′-UTR m^{6}A dynamics, regulated by FTO and YTHDF2 in response to stress, alters eIF3 recruitment and influences cap-independent translation. m^{6}A methylation in the coding region of mRNAs affects translation dynamics through interfering with tRNA selection. m^{6}A in the 3′ UTR can be recognized by YTHDF1, thus promoting cap-dependent translation, or can be recognized by YTHDF2, thus facilitating mRNA decay. All m^{6}A-reader proteins associate with specific RNA-protein granules in cells. Asterisks indicate proteins previously found to associate with RNA granules[45], but the precise granule identity remains unknown.

m^{6}A switches may also control the accessibility of the RNA-binding motifs of other RNA-binding proteins, thus influencing a wide range of biological activities. Further investigation of m^{6}A-altered RNA structure and functions requires highly sensitive high-throughput methods to analyze m^{6}A and its effect on RNA structures *in vivo*. Recently, Spitale *et al.* have developed an *in vivo* click-selective 2-OH acylation and profiling method (icSHAPE) and have used it to obtain a global view of *in vivo* RNA-structure profiles at base resolution[51]. The authors have observed that RNA structures at and adjacent to the m^{6}A residue tend to be more single-stranded *in vivo*. Global m^{6}A depletion by knocking out METTL3 decreases this tendency around the methylated bases, thus validating the role of m^{6}A in destabilizing RNA structures transcriptome wide.

m^{6}A-dependent miRNA maturation. m^{6}A has also been shown to promote the initiation of miRNA biogenesis[58,59]. Specifically, m^{6}A methylation in primary miRNAs (pri-miRNAs) is required for their recognition and processing by DGCR8, an RNA-binding protein of the microprocessor complex. Global m^{6}A reduction by depletion of METTL3 decreased the interactions between DGCR8 and pri-miRNAs, thus causing a global decrease in mature miRNAs. HNRNPA2B1, another member of the nuclear HNRNP protein family, has been found to be a mediator of this effect[59]. HNRNPA2B1 recognizes m^{6}A marks in pri-miRNAs and recruits DGCR8 protein complexes, thus facilitating miRNA maturation. HNRNPA2B1 also has effects on m^{6}A-dependent alternative splicing. Although the RNA-binding site of HNRNPA2B1 contains the m^{6}A consensus motif, it is still unclear whether HNRNPA2B1 recognizes m^{6}A residues directly, like the YTH-domain proteins, or indirectly, like the HNRNPC protein. Collectively, these studies reveal yet another mechanism by which m^{6}A influences RNA metabolism.

Effects of m^{6}A on translation. Whereas m^{6}A in the 3′ UTRs of mRNAs can recruit YTHDF1 and promote cap-dependent translation[39], two recent studies have indicated that m^{6}A in the 5′ UTR facilitates cap-independent translation[38,60]. A single m^{6}A in the 5′ UTR is sufficient to boost cap-independent translation through interaction with the eukaryotic translation initiation factor eIF3 and subsequent ribosome recruitment. Whether eIF3, a large complex composed of more than ten proteins[61], is a direct reader of m^{6}A or whether the m^{6}A-mediated interaction with eIF3 occurs through an m^{6}A-switch like mechanism remains unclear. As mentioned earlier, the effect of m^{6}A methylation on cap-independent translation is particularly apparent during the heat-shock stress response, when the interplay between YTHDF2 and FTO enhances the m^{6}A levels in the 5′ UTRs of newly synthesized mRNAs[38].

Finally, a recent single-molecule study has demonstrated that m^{6}A methylation within the mRNA coding region affects translation dynamics[62]. X-ray crystallography data of a ribosome–mRNA–tRNA complex have shown that whereas m^{6}A within codons pairs with U in the anticodons of tRNA, the m^{6}A-induced steric constraints lead to destabilization of m^{6}A-U pairing, specifically in the first codon position. Thus, m^{6}A alters both the initial selection and subsequent proofreading of tRNA selection, and moreover m^{6}A affects translation dynamics in a codon position–dependent manner.

CONCLUSIONS

The field of m^{6}A epitranscriptomics is growing rapidly. Recent years have witnessed major advances in m^{6}A profiling techniques and substantial gains in understanding of m^{6}A functions and mechanisms. The identification of m^{6}A-responsive RNA-binding proteins has revealed that m^{6}A regulates cognate RNAs from nascence to decay (**Fig. 3**). In addition, m^{6}A modulates RNA structure, miRNA biology and protein localization. It remains unclear how these various functions are coordinated within the cell, how they are coupled with m^{6}A biogenesis and removal, and how they are regulated in a cell type– and cell state–dependent manner.

Future studies will be necessary to improve the resolution and sensitivity of m^{6}A profiling methods, explore additional m^{6}A regulatory mechanisms and further investigate the cellular pathways involving m^{6}A and their reader proteins. Notably, all m^{6}A-related proteins discovered to date associate with RNA granules (**Fig. 3**), and the mechanisms and functions of the m^{6}A-associated RNA granules require further exploration. These studies will be of great importance to advance understanding of the complex regulatory networks underlying m^{6}A-related RNA biology and its relation to human health and disease.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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