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Reading, writing and erasing mRNA methylation

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Abstract | RNA methylation to form N^6-methyladenosine (m^6A) in mRNA accounts for the most abundant mRNA internal modification and has emerged as a widespread regulatory mechanism that controls gene expression in diverse physiological processes. Transcriptome-wide m^6A mapping has revealed the distribution and pattern of m^6A in cellular RNAs, referred to as the epitranscriptome. These maps have revealed the specific mRNAs that are regulated by m^6A, providing mechanistic links connecting m^6A to cellular differentiation, cancer progression and other processes. The effects of m^6A on mRNA are mediated by an expanding list of m^6A readers and m^6A writer-complex components, as well as potential erasers that currently have unclear relevance to m^6A prevalence in the transcriptome. Here we review new and emerging methods to characterize and quantify the epitranscriptome, and we discuss new concepts — in some cases, controversies — regarding our understanding of the mechanisms and functions of m^6A readers, writers and erasers.

The presence of N^6-methyladenosine (m^6A) in mRNAs was established by pioneering studies in the 1970s[1,2]. Shortly thereafter, the first function of m^6A was shown in studies linking the presence of m^6A to mRNA instability[3]. The next major breakthrough came in 1997, with the cloning of methyltransferase-like protein 3 (METTL3)[4], the enzyme that synthesizes nearly all m^6A in the mRNA transcriptome. Next, seminal studies in the 2000s revealed that the deletion of the yeast and *Arabidopsis* *METTL3* homologues results in specific developmental arrest in sporulation and seed development, respectively[5,6]. These studies documented that m^6A is a regulated modification that is required for specific developmental processes and spurred the development of mapping technologies to identify m^6A-containing transcripts in order to understand how m^6A influences cell differentiation and other essential processes.

The subsequent development of m^6A-mapping methods[7,8], and their use in innumerable studies, has revealed the previously hidden plethora of m^6A throughout the transcriptome, and started revealing its functional significance. Since the original publications of the m^6A-mapping methods in 2012 (REFS[7,8]), the concept that the epitranscriptome regulates mRNA fate and function in cells has become widely accepted.

m^6A-mapping studies have started to reveal the key principles that characterize the m^6A mark in mRNA: they have shown that m^6A is a selective modification, based on its enrichment in certain mRNAs[9,10], and that most m^6A-modified mRNAs contain only a single m^6A site, but some mRNAs contain 20 or more m^6A sites[7–9]. Most m^6A sites appear to be constitutive, as their distributions along the mRNAs are very similar in different tissues and cell lines[10], with a transcriptome-wide enrichment of m^6A in the 3′ untranslated region (UTR) and near stop codons[7,8].

The mapping studies also revealed correlation between m^6A abundance and the structure of a specific gene. The presence of a long internal exon, which is defined as an exon that is much larger than a typical ~140 bp exon, is a strong inducer of m^6A addition in the transcribed mRNA[8,11–14]. Since the genomic architecture is the same in all tissues, the m^6A sites that are induced by long internal exons would be common to all tissues. Importantly, long internal exons are not the sole determinant of m^6A addition, since many transcripts lacking this feature also contain m^6A (REFS[8,11–14]).

Finally, m^6A mapping identified specific gene categories that are associated with transcripts containing a disproportionately high level of m^6A. This provided the first mechanistic link between the differentiation phenotypes that were observed in yeast and plants in the 2000s and specific transcripts that could control those effects. Analysis of mRNAs with large numbers of mapped m^6A sites revealed an enrichment for genes that regulate development and cell fate specification[7,8,12]. In contrast, highly stable transcripts encoding 'housekeeping' genes, such as ribosomal proteins, were found to be de-enriched in m^6A (REF.[10]).

Mapping studies have also suggested that some m^6A sites might be regulated in a tissue-specific or

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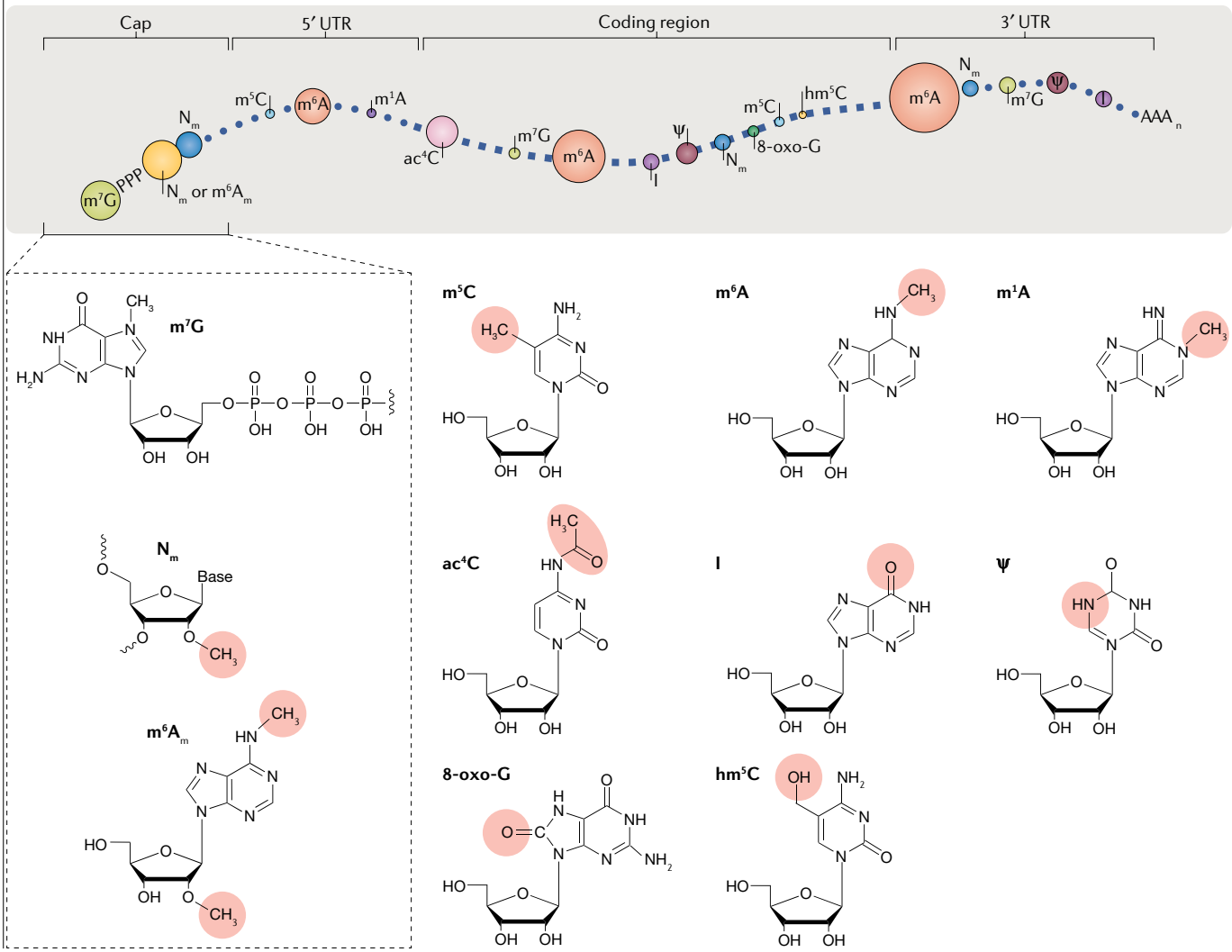
disease-specific manner, indicating regulation of m^{6}A stoichiometry — that is, the fraction of transcripts that contain the m^{6}A mark at a specific nucleotide position. However, other studies have suggested that the appearance of altered m^{6}A stoichiometry in some mapping studies may instead reflect changes in mRNA expression, which affects the efficiency of m^{6}A detection[15]. Changes in m^{6}A levels have been observed in the 5′ UTRs of various mRNAs after various cellular stresses or in different developmental states[7,8,16]. These changes might be an artefact of altered levels or detection of *N*^{6},2′-*O*-dimethyladenosine (m^{6}A$_m$)[9,17], a related nucleotide that also binds to m^{6}A antibodies[18]. Thus, the concept of a 'dynamic' epitranscriptome remains to be established.

Since the first m^{6}A-mapping studies, additional mRNA modifications have been discovered and mapped (BOX 1). However, m^{6}A remains the most abundant modification detected, as well as the best characterized at the functional level.

To date, over 500 m^{6}A-mapping studies have been deposited in gene expression databases, encompassing m^{6}A maps in diverse organisms and in response to different signalling pathways, drug treatments, gene knockdown and disease states. Overall, these studies are

Box 1 | **Overview of additional nucleotide modifications on mRNA**

N^6-methyladenosine (m^{6}A) is the most abundant modification on mRNAs, with an average of one to three m^{6}A modifications per transcript. Ten other modifications have been described on mRNAs. Their localizations in the mRNA (see the figure, upper panel) and their chemical structures (see the figure, lower panel) are shown. Modified nucleotides are presented in each transcript region according to the current understanding of where these modifications are most frequent in mRNA. The extended cap structure is enriched with modifications and comprises the m^{7}G cap, with its unusual 5′–5′-triphosphate bond, and 2′-*O*-methyl (N$_m$) modifications in the first and, sometimes, second positions in mRNA. In the case that the first nucleotide is A$_m$, the nucleotide can be additionally modified to form a doubly methylated nucleotide: *N*^{6},2′-*O*-dimethyladenosine (m^{6}A$_m$). Whereas m^{6}A$_m$ is thought to be a modification that is found exclusively in the cap, N$_m$ and m^{7}G may be also found in the transcript body. The dimensions of the circles are a schematic representation of the abundance of each modification within the transcript (for example, m^{7}G is highly abundant at the cap, m^{6}A is highly enriched near the stop codon area, and ac4C is highly abundant at the beginning of the coding sequence). The chemical structures of the base-modified nucleotides identified in eukaryotic mRNAs are presented in the lower part of the figure. Chemical groups that have been added to or isomerized in canonical nucleotides are indicated by the pink shading.



starting to reveal which transcripts are regulated by m^{6}A and how m^{6}A may regulate gene expression in diverse cellular contexts. However, the exact stoichiometry of specific m^{6}A sites and whether and how this stoichiometry changes in different conditions are not known. In the interest of space, m^{6}A mapping and measurement techniques are summarized in TABLE 1 and in Supplementary Information. These include the use of antibody-based enrichment to map m^{6}A, the development of techniques to overcome the challenges of interpreting m^{6}A maps and determining the stoichiometry of m^{6}A modifications.

In this Review, we discuss recent findings that have reshaped our thinking about how m^{6}A readers, writers and erasers regulate the m^{6}A epitranscriptome and how the epitranscriptome, in turn, affects the fate of mRNA in cells.

Life cycle and cellular fate of m^{6}A mRNA

The 'life cycle' of an mRNA destined for m^{6}A methylation starts during transcription. The writing and erasing of m^{6}A occurs primarily during this nuclear phase, as the m^{6}A writer complex, which comprises the core *N*^{6}-adenosine methyltransferase METTL3 and its adaptors (FIG. 1) (see below), and m^{6}A erasers are predominantly localized in the nucleus[4,19,20]. During the nuclear phase, m^{6}A can bind specific nuclear readers, which may impact mRNA splicing or other nuclear processes. Upon export to the cytoplasm, m^{6}A binds specific cytosolic reader proteins that affect the stability, translation and/or localization of mRNAs.

mRNA instability

The best-established function for m^{6}A, which was identified in 1978, is to cause mRNA instability[3]. This study used radioisotope metabolic labelling to compare the half-lives of m^{6}A-containing mRNAs and mRNAs that lack m^{6}A. This provided the first evidence of a functional role for m^{6}A. More recent studies suggest that the cytosolic m^{6}A-binding protein YTH domain-containing family protein 2 (YTHDF2, also known as DF2), contributes to the destabilizing effect[21] of m^{6}A (FIG. 1). However, DF2 depletion only slightly stabilizes m^{6}A-modified mRNAs (~30% average increase in mRNA half-lives). This contrasts with METTL3 depletion, which is associated with a more prominent increase in mRNA half-lives[14]. Thus, the effects of m^{6}A on mRNA stability are unlikely to be mediated solely by DF2.

Cytosolic mRNAs do not undergo m^{6}A methylation, because m^{6}A formation occurs co-transcriptionally in the nucleus[22–25]. Thus, unlike in the phosphorylation of proteins which can occur in a signal-dependent manner in the cytosol, signalling pathways do not induce m^{6}A in cytosolic mRNA, and m^{6}A does not induce transcriptome turnover[26] in response to a signalling pathway. Instead, m^{6}A is an imprint of nuclear events that marks transcripts for a shorter half-life when they arrive in the cytosol.

mRNA translation

Numerous studies have linked m^{6}A to translation upregulation via three distinct mechanisms. The first involves the canonical m^{6}A reader YTHDF1, also known as DF1, which is proposed to bind to the eukaryotic translation initiation factor eIF3, a multiprotein complex that recruits the small ribosome subunit to mRNAs to enhance their translation[27]. Since m^{6}A and DF1-binding sites are located mostly around the stop codon and in 3′ UTRs, this model implies that DF1 recruits eIF3 to these regions. It is not yet clear how eIF3 recruitment to stop codons would enhance translation, given that eIF3 is typically positioned upstream of the AUG start codon in order to facilitate translation initiation[28,29].

Another mechanism of m^{6}A-mediated translation enhancement involves direct binding of 5′ UTR m^{6}A to eIF3 (REF.[30]). m^{6}A modification enhances translation only if it is present in the 5′ UTR, presumably reflecting the effects of m^{6}A-dependent positioning of eIF3 upstream of start codons. Surprisingly, m^{6}A-mediated translation initiation does not require eIF4E, the 7-methylguanosine-containing mRNA cap-binding protein that recruits eIF3 (REF.[28]). Thus, the presence of m^{6}A bypasses the normal requirement for eIF4E and may be a mode of translation that is important when eIF4E function is impaired. Because only a few mRNAs contain m^{6}A in the 5′ UTR, this mechanism is limited to a small subset of the m^{6}A-containing mRNAs[30]. However, upon stress, heat shock protein-encoding mRNAs and other transcripts containing m^{6}A in their 5′ UTRs are induced, potentially enhancing their translation during stress[30,31]. Also, m^{6}A seems to be enriched in circular RNAs, and m^{6}A may enhance the translation of open-reading frames found in these transcripts through this mechanism[32].

The third mechanism of translational enhancement involves direct translation activation by METTL3 (REFS[33,34]). In this model, METTL3 methylates mRNAs in the nucleus but is suggested to remain bound to the transcript upon export to the cytoplasm. Once in the cytoplasm, METTL3 binds eIF3, which interacts with the mRNA cap-associated proteins. This could create mRNA looping between a METTL3 present at the mRNA 3′ UTRs and the 5′ mRNA cap of the same mRNA. This was proposed to enable ribosomes at stop codons to reload into the 5′ UTR of transcripts. It remains unclear how METTL3 can bind to RNA without the METTL14 adaptor, as was proposed[33,34]; how ribosome reloading could occur, since ribosomes normally disassemble at stop codons; and how this mechanism can regulate cytosolic mRNA, since METTL3 is not normally detected in the cytoplasm using standard immunofluorescence approaches[4].

A role for m^{6}A in promoting translation of the majority of m^{6}A-modified mRNA is generally not supported by ribosome profiling studies. A comparison of ribosome profiling data from control and *METTL3*-knockout cells shows negligible changes in mRNA translation, except for the small set of mRNAs with m^{6}A in their 5′ UTR[30]. These data are inconsistent with the idea that DF1 or METTL3 (both of which bind m^{6}A throughout the transcript body) enhances translation[27,34]. Other studies appear to support a role for METTL3 in translation, on the basis of polysome profiling in some cell types[33,34], but these effects are modest. More studies will be needed to determine whether m^{6}A has a general

Transcriptome turnover
Term used to indicate the rate at which mRNAs are produced and degraded in order to control the final mRNA quantity and quality in a cell.

mRNA looping
A mechanism of interaction between the 3′ UTR of an mRNA and the 5′ UTR of the same mRNA to favour ribosome reloading and a high translation rate.

Table 1 | **m^{6}A mapping and measuring techniques**

Technique	Description	Advantages	Limitations
Antibody-based detection methods			
meRIP-seq/ m^{6}A-seq[7,8]	Transcriptome-wide mapping of m^{6}A-antibody enriched regions. To identify a peak, it requires the enrichment of the m^{6}A antibody-bound regions (IP) over the RNA input	• Low amount of input material required • Easy RNA library preparation steps	• Inability to distinguish m^{6}A from m^{6}A$_m$ • RNA sequencing of the input material is required to call m^{6}A sites • Inability to call m^{6}A sites with single-nucleotide resolution • Inability to call multiple m^{6}A sites within peaks • Increased risk of false positives
m^{6}A-CLIP/IP[13]	Transcriptome-wide mapping of the position of m^{6}A antibody UV-crosslinked to mRNA at single-nucleotide resolution. To identify a peak, it requires the enrichment of the m^{6}A IP over the RNA input. Identification of the m^{6}A sites within the peaks by using a mutation and truncation signature	• Use of a mutations-and-truncations signature to identify m^{6}A sites within the peaks ensures high level of specificity • Detection of multiple m^{6}A sites per transcript	• No stoichiometric information • High quantity of mRNA material required • Laborious cloning method
miCLIP[9]	Transcriptome-wide mapping of the position of m^{6}A antibody UV-crosslinked to mRNA at single-nucleotide resolution. To identify a site, it requires the presence of a specific mutational signature at the target site.	• Use of a C-to-T mutation signature to identify m^{6}A sites ensures high level of specificity • Detection of multiple m^{6}A sites within the same peak • Ability to distinguish m^{6}A from m^{6}A$_m$	• No stoichiometric information • High quantity of mRNA material required • Complex library protocol
LAIC-seq[140]	Transcriptome-wide mapping of the m^{6}A antibody-enriched regions. To identify a peak, it requires the enrichment of the m^{6}A bound region over the input and depletion of the same region in the post-IP supernatant	Semi-stoichiometric information	• Empirical titration of the antibody concentration required • Inability to distinguish m^{6}A from m^{6}A$_m$ • Laborious method • Need for external methylated and non-methylated spike-in controls
meRIP-qPCR[142]	Quantification of the methylation levels of a specific region of interest relative to the input	• Semi-stoichiometric information at specific m^{6}A sites • Easy preparation steps • Low amount of input material required	• Inability to distinguish stoichiometry of adjacent m^{6}A sites • High level of variability between replicates • Need for external methylated and non-methylated spike-in controls
m^{6}A ELISA[153]	ELISA kit to detect the amount of m^{6}A antibody-enriched RNA	• Standardized method • Low input material • Commercially available and ready to use	Quantification of m^{6}A on mRNAs is readily contaminated by m^{6}A present on rRNA and snRNA contaminants
Digestion-based detection methods			
LC-MS[154]	Digestion to single nucleotide and UV detection of m^{6}A based on its physicochemical properties	• Quantitative method • Standardized method • Easy preparation steps	• Inability to distinguish m^{6}A on mRNA from m^{6}A on rRNA or snRNA contaminants • No sequence context and localization information
SCARLET[141]	RNase H site-specific cleavage, splinted ligation, ribonuclease digestion and thin-layer chromatography to quantify m^{6}A at one specific site	• Exact measurement of the m^{6}A stoichiometry at the specific site • High level of accuracy	• Low throughput • Ability to detect only one site per transcript at a time • Laborious method that requires large amounts of input mRNA
2D-TLC[155]	RNase T1 digestion followed by a 2D thin-layer chromatography to quantify the amount of m^{6}A a GAC context. The signal is normalized to total adenosine present in cellular mRNA	• Specific detection of m^{6}A on mRNA because unable to detect AAC sites present in rRNA • High level of accuracy	• Only detects m^{6}A preceded by guanosine, not adenosine • Laborious method which requires radioactivity • Ability to detect only one site per transcript at the time?
m^{6}A-specific deoxyribozymes[143]	RNA-cleaving deoxyribozymes optimized to cleave m^{6}A in DRACH sequence motif	Potential measurement of m^{6}A in the canonical m^{6}A sequence motif	• Method has not yet been applied genome-wide or to detect specific sites on mRNAs • Requires design of deoxyribozyme specific for each site of interest
MazF[144]	Site-specific fluorescent quantification of m^{6}A sites found in an ACA context upon MazF cleavage	• No antibody required • m^{6}A stoichiometry information at specific ACA sites	Only detects m^{6}A sites in an ACA sequence context
MAZTER-seq[117]	Transcriptome-wide mapping and quantification of m^{6}A sites in an ACA context detected upon MazF cleavage	• Global mapping method that does not require m^{6}A antibody enrichment • m^{6}A stoichiometry information at ACA sites on mRNAs • Low false-positive rate • Less laborious than any antibody-based mapping method	• Only detects m^{6}A sites in an ACA sequence context • Inability to distinguish ACA sites in close proximity

Table 1 (cont.) | **m^{6}A mapping and measuring techniques**

Technique	Description	Advantages	Limitations
m^{6}A sensing RT-based detection methods			
4SedTTP-RT[147]	Detection of m^{6}A sites using the RT-dependent truncation signature when the 4Sed-dTTP nucleotide is used instead of a dTTP nucleotide during cDNA synthesis	• No input required • Potential to detect every m^{6}A site with single-nucleotide resolution independently of the sequence motif	• Method never yet applied genome-wide • Background truncation signature causes high false-positive rate • High false-negative rate. When calling a site, the set-up requires a parallel condition of eraser depletion as a control condition
Tth polymerase[145]	m^{6}A site detection by using the *Tth* DNA polymerase in a primer extension assay; the *Tth* polymerase preferentially incorporates dTTP opposite A rather than opposite m^{6}A	• Low number of laborious steps • Method already used to validate a few m^{6}A mRNA sites	• Incorporation of dTTP opposite from a m^{6}A is dependent on reaction time and RNA concentration, which can vary between samples • Low throughput • Ability to detect only one site per transcript
RT-KTQ polymerase[146]	m^{6}A site detection by measuring the increased mis-incorporation error rate at m^{6}A sites induced by the RT-KTQ enzyme, a KlenTaq DNA polymerase variant with reverse-transcriptase activity	• Potential to detect every m^{6}A site with single-nucleotide resolution independently of the sequence motif • Potential to provide stoichiometric information • Low number of laborious steps	• Ineffective detection of m^{6}A at the 5′ end of the fragment • High false-positive rate • Method never applied genome-wide or to detect specific m^{6}A sites on mRNAs
Ligation-based detection methods			
T3/T4 DNA ligase-qPCR[149]	qPCR measurement of the proportional decrease in ligation efficiency of two probes close to the m^{6}A site of interest when m^{6}A is present	• Potential measurement of m^{6}A stoichiometry • Method already validated at specific sites on mRNA • Easy preparation steps	• Dependence on efficiency of the ligation reaction • Low throughput
SELECT[148]	qPCR measurement of the proportional decrease in ligation efficiency at the nick left by the *Bst* DNA polymerase during elongation if m^{6}A is present	• Measurement of m^{6}A stoichiometry • Method already validated at specific sites on mRNA • Easy preparation steps	• Dependence on two selective steps: efficiency of the *Bst* polymerase and efficiency of the ligation reaction • High false-positive rate • Low throughput
Hybridization-based detection methods			
m^{6}A melting-qPCR[151]	DNA oligo-hybridization at the m^{6}A site of interest on the mRNA and measurement of the difference in the melting properties of the created RNA–DNA duplex when m^{6}A is present compared with A	• Relative information provided about m^{6}A stoichiometry at specific sites • Method validated at specific sites on rRNA and snRNAs	Low level of sensitivity
Direct RNA-based detection methods			
Nanopore[150]	Detection of changes of current caused by m^{6}A compared with A while the mRNA travels through the pores	• No need for complex library preparation steps • No PCR biases • Direct investigation of m^{6}A relation to isoform and transcript features • Potential measurement of m^{6}A stoichiometry • Direct measurement of the number of m^{6}A sites per isoform	• Low level of change in current caused by m^{6}A • High general base error rate • Method never applied to cellular mRNAs
SMRT[152]	Detection of changes in the binding of the labelled nucleotides during the cDNA synthesis	• Direct investigation of m^{6}A relation to isoform and transcript features • Direct measurement of the number of m^{6}A sites per isoform	• High base error rate • Low sensitivity levels

CLIP, UV crosslinking immunoprecipitation; IP, immunoprecipitation.

role in enhancing mRNA translation and the precise mechanism involved.

mRNA splicing

Some of the strongest evidence of a role for m^{6}A in regulating splicing comes from studies in *Drosophila melanogaster*, where intronic m^{6}A affects the splicing of *Sex lethal*, a gene central to sex determination[35–37]. In mammals, the link between m^{6}A and mRNA splicing is less clear. To establish whether m^{6}A regulates splicing in mammals, a major approach has been to determine whether m^{6}A is located near exonic or intronic splice junctions, where it could directly influence splicing. However, mapping m^{6}A in intronic regions is challenging, because the amount of intronic RNA in cells is very low.

Some research groups have shown that m^{6}A is located near the exonic 5′ splice site[38]. However, other researchers who have performed high-resolution m^{6}A mapping have found no enrichment of m^{6}A near exonic 5′ splice sites and a lack of m^{6}A in introns[14]. Yet others have

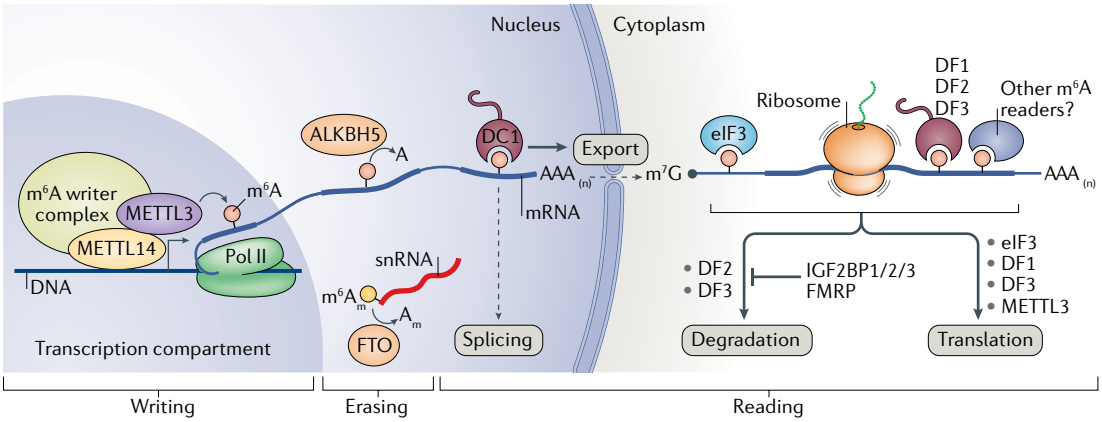


Fig. 1 | **The m⁶A mRNA life cycle.** The 'life cycle' of an mRNA destined for N^6-methyladenosine (m⁶A) methylation starts in the nucleus during transcription. The m⁶A writer complex (BOX 1), which comprises the core methyltransferase-like protein 3 (METTL3) and its adaptors, is located in the nucleus, where it adds m⁶A co-transcriptionally. The m⁶A erasers are largely localized in the nucleus as well. The main m⁶A eraser acting on m⁶A in mRNAs is ALKBH5. Fat mass and obesity-associated protein (FTO) has recently been found to preferentially target m^6A_m, not m⁶A, with its major target being m^6A_m in small nuclear RNAs (snRNAs). While in the nucleus, m⁶A can bind specific nuclear reader proteins, mainly YTHDC1 (DC1), which may affect splicing or other nuclear processes such as mRNA export. Upon mRNA export to the cytoplasm, m⁶A binds to specific reader proteins that affect the stability, translation and/or localization of the mRNA. In the cytoplasm, the m⁶A readers YTHDF1 (DF1), YTHDF3 (DF3), the eukaryotic translation initiation factor eIF3, and METTL3 all favour the translation of m⁶A mRNAs. YTHDF2 (DF2) and DF3 mediate the degradation of m⁶A mRNAs, while the insulin-like growth factor 2 mRNA-binding proteins (IGF2BP1/2/3) and the synaptic regulator FMRP (a polyribosome-associated RNA-binding protein known to have a central role in neuronal development and synaptic plasticity) enhance m⁶A mRNA stability. A_m, methylated A; m^6A_m, N^6,2′-*O*-dimethyladenosine.

found m⁶A near splice sites in both exonic and intronic regions[39]. In all cases, nascent RNAs were examined and showed different m⁶A patterns. The accuracy of the mapping approaches or the methods used to prepare the nascent RNA may explain these discrepancies.

Regardless of whether m⁶A is found in the proximity of splice junctions, most studies have shown that the number of METTL3-dependent splicing events is small[12,14]. In embryonic stem (ES) cells, only a small fraction of known alternatively spliced exons showed altered splicing compared with *METTL3*-knockout ES cells. This observation was made in two independent studies, which used different algorithms for detecting alternative splicing. In one study, only 360 of 241,964 exons were alternatively spliced in *METTL3*-knockout ES cells[14]. In another study, 1,269 exons out of ~240,000 showed alternative splicing in *METTL3*-knockout ES cells[12]. Only 34 of these exons contained m⁶A. In another study, only 360 differential alternative splicing events were observed in *METTL3*-knockout ES cells, of which less than a third contained m⁶A sites[14]. Overall, these studies suggest that m⁶A has limited roles in directly controlling mRNA splicing. Nevertheless, even though m⁶A may only affect splicing in a small number of genes, these m⁶A-dependent splicing events might be functionally important[8,12].

If m⁶A does affect splicing, YTH domain-containing protein 1 (YTHDC1, also referred to as DC1), may be involved, as it interacts with splicing regulators[40] including SAM68 (REF.[41]), SC35 (REF.[42]), SRSF1 (REF.[42]) and SRSF3 (REFS[38,43]) (see the section on readers below), thus suggesting a link between DC1 and splicing.

A further complication is that the splicing alterations seen upon m⁶A depletion might be an indirect effect. For example, some of the transcripts that have the most annotated m⁶A sites are prominent splicing regulators, such as *SON*[44], *HNRNPC*[38] and *HNRNPF*[45]. Thus, depletion of m⁶A might affect the expression of splicing-regulatory proteins, making it difficult to differentiate direct effects of m⁶A on mRNA splicing from indirect effects mediated by altered levels of splicing-regulatory proteins.

m⁶A-mediated phase separation

A major mechanism by which m⁶A-modified mRNAs are targeted for regulation is through a process called liquid–liquid phase separation. The link between m⁶A and liquid–liquid phase separation was revealed by examining the amino acid sequences of YTH domain-containing family (DF) proteins, which each contain a large glutamine/proline/glycine-rich ~30 kDa low-complexity domain. Some low-complexity domains have the ability to interact with each other and to 'phase separate' into gels, polymers or liquid droplets within the cytosol. DF proteins were shown to phase separate into liquid droplets when incubated with RNAs containing multiple m⁶A residues[46]. These RNAs recruit and juxtapose DF proteins, causing them to undergo phase separation and to form RNA–protein droplets. These m⁶A mRNA-rich droplets then partition into endogenous phase-separated liquid droplets such as stress granules, P-bodies or neuronal RNA granules[46] (FIG. 2). Thus, m⁶A makes an mRNA more likely to partition into these membraneless compartments, where mRNAs may be stored, degraded or used to transport mRNAs to dendritic arbors in neurons.

Notably, the effects of m⁶A on mRNA degradation are most prominent when multiple m⁶A residues are

Liquid–liquid phase separation
(LLPS). Condensation into a dense phase of RNA and proteins that often resembles a liquid droplet and is strictly related to microenvironment conditions, such as pH, temperature and salt concentration.

Low-complexity domain
Regions of a protein in which specific amino acids are over-represented compared with their amino acid proportions found in the proteome.

Stress granules
Membraneless cytoplasmic liquid–liquid phase compartments where RNA and RNA-bound proteins are found under conditions of cellular stress.

P-bodies
Membraneless liquid–liquid phase compartments where RNA and RNA-bound proteins are assembled in order to store and/or degrade the RNA.

RNA granules
RNA- and protein-containing liquid–liquid phase compartments assembled in order to transport RNA to dendrites in neurons.

Allosteric adaptor
A protein that indirectly influences the effects of a second protein on the bound target. In this case, the second protein may directly influence the function of the target.

present in clusters[14]. The reason for the prominent effect of m^{6}A clusters may be that these clusters are particularly efficient at inducing phase separation of DF proteins, thereby making mRNA more efficiently targeted to phase-separated compartments involved in mRNA degradation.

Nuclear export of mRNA

m^{6}A may enhance mRNA export from the nucleus by binding DC1. Interactions between DC1 and SRSF3 may promote mRNA export since SRSF3 functions as a key adaptor for the NXF1-dependent mRNA export pathway[47]. Consistent with this idea, cells deficient in the RNA demethylase ALKBH5, which show increased m^{6}A, also show accelerated mRNA export[19]. However, many mRNAs lack m^{6}A and are still exported from the nucleus, making it unclear why m^{6}A would be needed for export. Additionally, there is little evidence of global variations in mRNA export rates in the transcriptome[48]. Nevertheless, these studies point to the possibility of a role for m^{6}A in affecting mRNA export pathways.

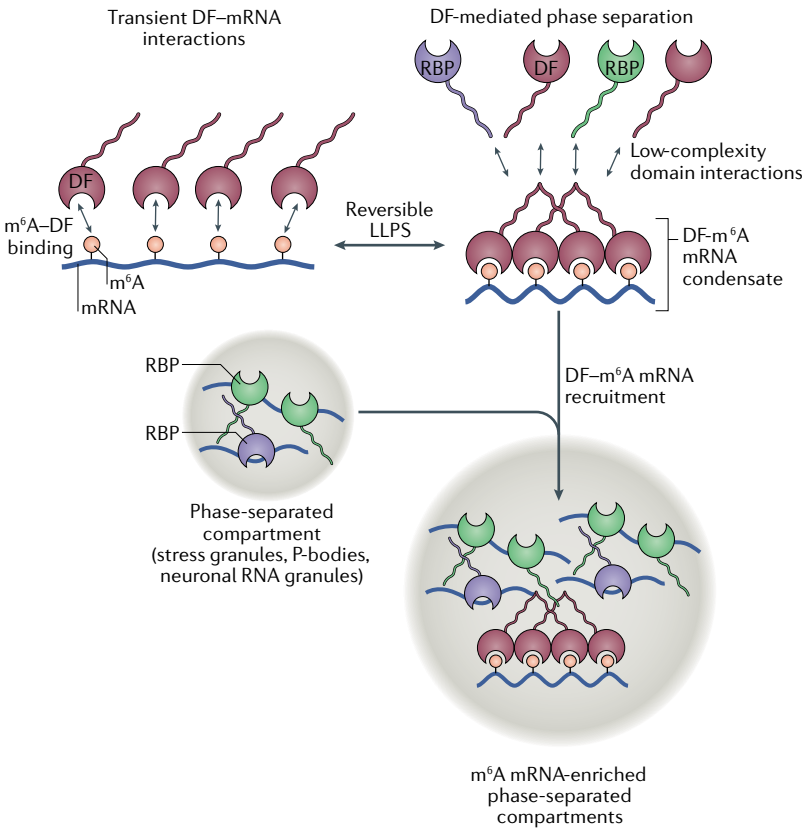


Fig. 2 | **m^{6}A recruits YTHDF proteins, leading to their phase separation.** YTHDF (DF) proteins were shown to undergo liquid–liquid phase separation (LLPS) in the presence of mRNAs containing multiple *N*^{6}-methyladenosine (m^{6}A) residues. The low-complexity domain of each DF has the ability to mediate protein–protein interactions that induce the formation of phase-separated 'condensates'. These DF–m^{6}A mRNA condensates are then recruited to pre-existing membraneless compartments formed by phase separation, such as stress granules, P-bodies and neuronal RNA granules. Understanding how the phase separation of DF proteins is regulated, which thus controls the recruitment or release of mRNA and its associated proteins from these membraneless compartments, will be instrumental in clarifying how DF proteins are regulated in controlling m^{6}A fate in the cytoplasm. Of note, the nuclear m^{6}A reader YTHDC1 may undergo a similar phase separation process. YTHDC1 has a low-complexity domain that may recruit m^{6}A-modified mRNA in specific nuclear structures to favour splicing or other nuclear processes. RBP, RNA-binding protein.

m^{6}A writers

m^{6}A is added to mRNA by a multi-subunit writer complex (BOX 2) in a highly specific manner — only certain mRNAs contain m^{6}A, and among these, only a small fraction of the potential m^{6}A consensus sites are methylated. The basis for this transcript specificity and site specificity remains poorly understood.

Although recent attention has focused on m^{6}A in mRNA, the vast majority of m^{6}A in total cellular RNA is actually located in the much more abundant ribosome RNAs. Four methyltransferases are encoded in the mammalian genome to generate m^{6}A in distinct RNAs. m^{6}A in mRNAs and other RNA polymerase II-derived transcripts are primarily formed by the METTL3–METTL14 heterodimer, of which METTL3 is the enzymatic component and METTL14 is an allosteric activator that also binds to the target RNA[49–51]. The single m^{6}A in the 28S ribosomal RNA (rRNA) is formed by the rRNA *N*^{6}-adenosine-methyltransferase ZCCHC4 (REFS[52,53]), whereas the single m^{6}A in the rRNA of the 18S subunit is formed by the METTL5–TRMT112 complex, of which METTL5 is the catalytic subunit and TRMT112 is an allosteric adaptor[53]. Lastly, the single m^{6}A in the U6 small nuclear RNA (snRNA), an snRNA involved in splicing, is catalysed by METTL16 (REF.[54]). METTL16 also catalyses the formation of m^{6}A in U6-like sequences in the *MAT2A* mRNA, which encodes the enzyme responsible for *S*-adenosylmethionine (SAM) biosynthesis[54,55]. Additionally, METTL16 catalyses the formation of m^{6}A in a small number of other mRNAs and noncoding RNAs[56].

The METTL3–METTL14 heterodimeric complex is responsible for the vast majority of m^{6}A sites in mRNA. Genomic deletion of METTL3 or CRISPR-mediated inactivation of METTL14 in mouse ES cells results in the loss of more than 99% of total m^{6}A in poly(A) RNA[12]. Thus, very few m^{6}A residues in poly(A) RNA are catalysed by METTL16 or other potential methyltransferases. For this reason, deletion of METTL3 or METTL14 has been used to document numerous m^{6}A-dependent functions[11,12]. Here, we focus on how the METTL3–METTL14 complex is regulated and how it determines the distribution of m^{6}A in the transcriptome.

METTL3: the catalytic subunit

The connection between m^{6}A and cellular physiology was revealed by the discovery and cloning of METTL3 as the m^{6}A-forming enzyme in mRNA. The original purification studies revealed that the methyltransferase activity was mediated by a >1 MDa methyltransferase complex that rapidly dissociated and became inactive during chromatography[57]. However, mixing two fractions comprising a ~200 kDa complex and a ~875 kDa complex reconstituted the methyltransferase activity[4]. It is now known that the smaller complex, designated methyltransferase A (MT-A), contains METTL3 and METTL14, while the other larger complex, designated methyltransferase B (MT-B), probably contains

Box 2 | **The m^{6}A writer-complex components**

The m^{6}A writer complex contains diverse proteins. Why m^{6}A formation is mediated by such a large complex is not known, but the individual proteins probably have specific functions or may integrate different cellular signals in order to regulate methylation. The proteins comprising the writer complex are shown in the figure.

WTAP: the key METTL3 adaptor

Wilms' tumour-associated protein (WTAP) was discovered in a screen for interactors with METTL3 in *Arabidopsis thaliana*[6]. This work and subsequent studies in yeast showed that WTAP is essential for m^{6}A formation[129]. The yeast homologues of METTL3 and WTAP (Ime4 and Mum2, respectively) interact and are required for m^{6}A formation in mRNA[129]. The binding of WTAP to METTL3 that is required for m^{6}A formation in yeast was subsequently shown to be important in mammalian cells[58,130,131]. Depletion of WTAP causes a loss of nuclear speckle localization for METTL3 and METTL14 (REF.[130]), showing that WTAP anchors the writer complex to chromatin[132].

VIRMA: a WTAP interactor important for methylation

Proteomic analyses of WTAP revealed VIRMA (originally known as KIAA1429) as one of its top interactors[133]. Depletion of VIRMA leads to a substantial loss of m^{6}A in mammalian cells[131] and in *D. melanogaster*[37]. VIRMA recruits specific cleavage and polyadenylation specificity factors (CPSF5, CPSF6), resulting in longer 3′ UTR selection[134].

RBM15/15B: mediators of methylation specificity

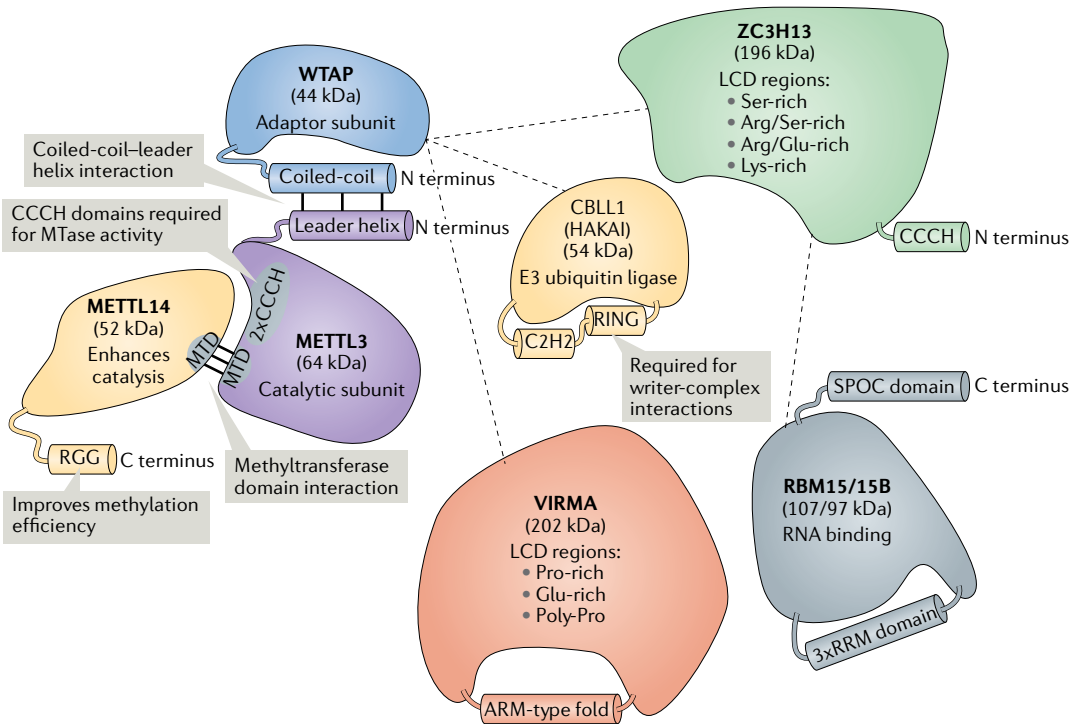
Proteomic studies of WTAP initially identified RBM15 and RBM15B, two paralogous RNA-binding proteins, as probable WTAP interactors[133]. This was confirmed in a study that showed that RBM15 and RBM15B interact with METTL3 in a WTAP-dependent manner[69], and knockdown of RBM15 and RBM15B reduces m^{6}A in mRNA. The *D. melanogaster* homologue of RBM15, Spenito (Nito), is required for m^{6}A formation in flies[37]. RBM15/15B contains RNA-binding domains, and thus may facilitate the recruitment of the writer complex to specific sites in mRNA.

ZC3H13–WTAP and ZC3H13–RBM15/15B

ZC3H13 is a WTAP interactor[133], and its depletion in *D. melanogaster* causes loss of m^{6}A[135,136]. ZC3H13 is thought to bind RBM15/15B and to link it to WTAP[135]. Similarly to WTAP, ZC3H13 is important for nuclear localization of the writer complex[137]. Deletion of ZC3H13 results in the loss of ~80% of cellular m^{6}A in mammalian cells[135], suggesting that some m^{6}A sites are formed independently of ZC3H13.

CBLL1/HAKAI

CBLL1, also known as HAKAI, was first identified as an E3 ubiquitin ligase that interacts with the E-cadherin complex[138]. As with the other writer-complex proteins identified so far, CBLL1/HAKAI was first identified in the WTAP interaction proteome[133]. CRISPR-mediated deletion of CBLL1/HAKAI results in a partial reduction in global m^{6}A levels[139].



Confirmed interactions are shown as solid lines; dash–dotted lines indicate interactions that have not yet been characterized. ARM-type fold, armadillo-type fold; C2H2, C2H2 zinc finger domain; CCCH, CCCH zinc finger domain; LCD, low-complexity domain; METTL3, methyltransferase-like protein 3; MTD, methyltransferase domain; RING, RING zinc finger domain; RRM, RNA recognition motif; SPOC, spen paralogue and orthologue C-terminal.

additional adaptor proteins that facilitate the recruitment of METTL3 with RNA.

The methyltransferase protein within the megadalton complex was determined by crosslinking ^{3}H-SAM to a ~70 kDa protein within the MT-A complex. The cloned enzyme[4], initially termed MT-A70 (MT-A subunit, 70 kDa), and now referred to as METTL3, enabled antibody development and METTL3 deletion studies in yeast, cancer cells and plant development, which precipitated the development of m^{6}A-mapping methods.

Although little is known about how the writer complex is regulated, its rapid dissociation may be physiologically relevant; the regulated assembly of MT-A and MT-B-like components in vivo may control its activity.

METTL14: an allosteric adaptor of METTL3

More recent studies have proposed that some m^{6}A sites are formed by a second putative methyltransferase, METTL14. METTL14 was purified and shown to have in vitro m^{6}A-synthesizing ability independent of METTL3 (REF.[58]). This study proposed that METTL3 and METTL14 assemble into a complex, with each protein methylating distinct target sites[58]. A separate study came to a different conclusion, proposing that METTL14 stabilizes METTL3 and that the proteins act together to mediate m^{6}A formation[59].

The first study that attempted to resolve this discrepancy used a bioinformatic analysis of putative adenosine methyltransferases in various genomes[60]. This study showed that the METTL14 sequence has a disrupted SAM-binding motif, suggesting that it is catalytically inactive. The authors thus proposed that METTL14 is an inactive partner in the METTL3–METTL14 complex. This hypothesis was proved shortly thereafter when three groups described the crystal structure of the METTL3–METTL14 complex[49–51], demonstrating that METTL14 is inactive, since it lacks a SAM-binding site. Overall, the crystal structures showed that METTL3 and METTL14 form a single methyltransferase in which METTL14 likely contains the RNA-binding site and is an allosteric activator of the enzymatic activity of METTL3 (REFS[49–51]).

So why did early findings report that METTL14 had robust methyltransferase activity? Subsequent work showed that human METTL14 expressed and purified in insect cells forms a heterodimer with the insect homologue of METTL3 (REF.[50]), suggesting that the enzymatic activity attributed to METTL14 in earlier studies was derived from co-purified, contaminating insect METTL3. Together, these studies overturned the concept that METTL14 is a second, independent methyltransferase responsible for m^{6}A formation in mRNA.

Notwithstanding the evidence, the idea that METTL14 is a second methyltransferase remains widespread in the literature, partly owing to experiments in which m^{6}A levels persisted after CRISPR-mediated *METTL3* inactivation[11,61]. This contrasts with other studies in which genomic deletion of *METTL3* resulted in the complete loss of m^{6}A (REF.[12]). The residual m^{6}A seen in some studies is likely due to incomplete CRISPR-mediated deletion of *METTL3* or *METTL14*, resulting in enzyme hypomorphs. Indeed, exon skipping over CRISPR-induced mutations is well documented[62]. METTL3 and METTL14 seem to be very prone to forming hypomorphs when CRISPR technologies are used, making it crucial to validate that METTL3 or METTL14 protein has been deleted and that any residual truncated protein lacks enzymatic activity.

Site- and transcript-specific selectivity

As the m^{6}A consensus sequence, DRACH (D = A, G, or U; R = G or A; H = A, C or U), appears once every ~57 nucleotides in mRNA, every transcript is predicted to have many potential methylation sites. However, very few of these DRACH sequences are methylated. Furthermore, despite the wide prevalence of DRACH sequences, only specific transcripts acquire m^{6}A. The basis for this site-specific and transcript-specific methylation is poorly understood.

One mechanism to select transcripts for methylation may be the recruitment of the writer complex to specific promoters by transcription factors (FIG. 3). For example, in human embryonic stem cells, the SMAD2/3 transcription factors bind METTL3, METTL14 and the METTL3 adaptor Wilms' tumour-associated protein (WTAP), thus promoting the methylation of SMAD2/3-induced transcripts in response to TGFβ signalling[23]. Approximately 150 genes were shown to be regulated through this mechanism[23]. In acute myeloid leukaemia (AML) cells, the CAATT-box binding protein CEBPZ binds and recruits METTL3 to ~70 different gene promoters to increase the methylation of the transcripts generated from these genes[24]. Importantly, these mechanisms appear to mediate only a very small fraction of the total number of m^{6}A-containing transcripts in the cell, indicating that other, currently unknown mechanisms must account for the majority of m^{6}A in the transcriptome. Other promoter features, such as the number of CpG islands and the promoter structure[63], have been found to be correlated with m^{6}A levels in mRNA and may recruit factors that account for the specificity in methylation.

Transcript specificity and the position of m^{6}A may also depend on histone modifications. Mapping studies have shown that m^{6}A is preferentially found in long internal exons[8,63]. Exons have unique features, such as substantially higher nucleosome density than introns[64–66], and high levels of certain histone modifications, such as trimethylation of histone H3 lysine 36 (H3K36me3)[67] and H4K20me1 (REF.[66]). Thus, chromatin marks may guide mRNA methylation. Recent studies have reported that H3K36me3 is associated with m^{6}A formation in mRNA[22]. Interactions between METTL14 and H3K36me3 were proposed to guide the writer complex to nascent RNA and to specific regions of the transcript body (FIG. 3). Knockdown of Setd2, the methyltransferase responsible for H3K36me3 formation, led to an ~40% drop in m^{6}A levels in mRNA, thus linking this pathway to a substantial proportion of methylation events[22]. However, the specific transcripts or specific sites that require H3K36me3 for methylation are not fully understood.

What determines which DRACH site is targeted for methylation? Besides long internal exons, m^{6}A is

Exon skipping
A specific mechanism of mRNA splicing used to 'skip over' an exon normally included in the processed mRNA. Thus, in this case, the final processed mRNA will not include this exon.

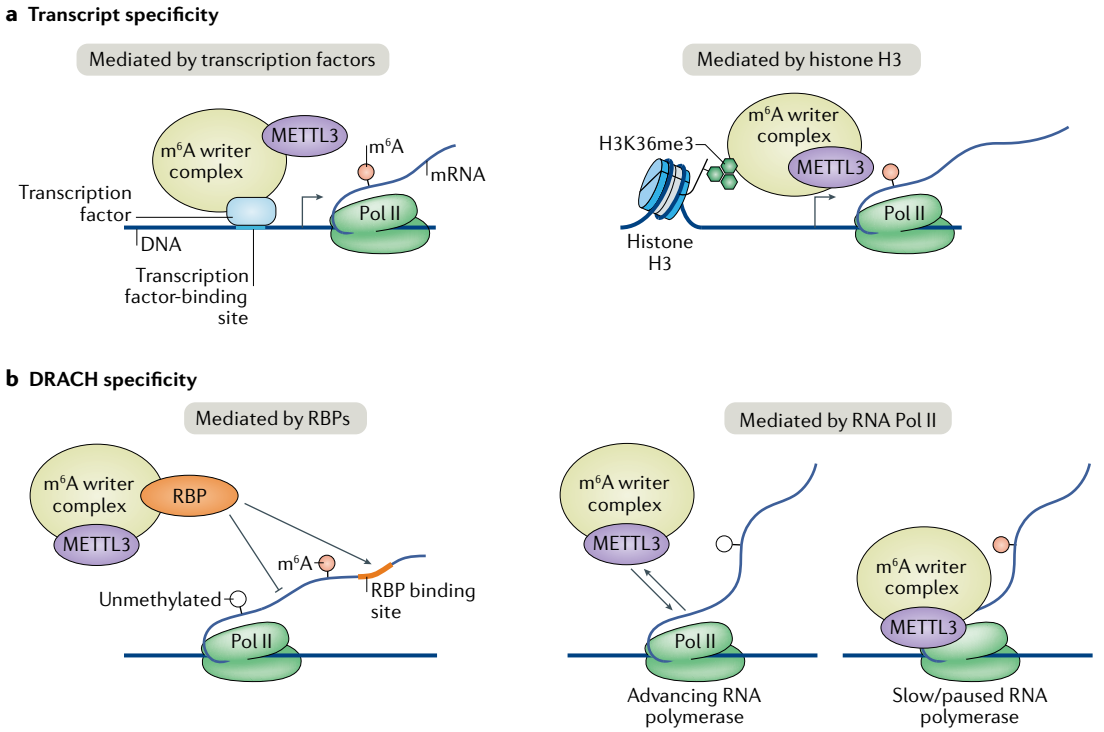


Fig. 3 | **Mechanisms for transcript and site specificity in m^{6}A writing. a** | Recruitment models for the N^6-methyladenosine (m^{6}A) writer complex to specific transcripts. Two models are shown: in one model, the recruitment of the m^{6}A writer complex to DNA motifs is mediated by transcription factors; in the other model, recruitment of the m^{6}A writer complex to specific DNA locations is guided by histone modifications, such as histone H3 lysine 36 trimethylation (H3K36me3). **b** | Recruitment of the m^{6}A writer complex to specific mRNA regions may favour the methylation of proximal DRACH consensus sequences within the transcript. Two models are shown. In the RNA-binding protein (RBP)-mediated recruitment model, the m^{6}A writer complex is brought into the proximity of DRACH sites during transcription by RBPs that bind to specific sites in the mRNA, so that nearby DRACH sites can be methylated. An example of such an RBP is RBM15/15B, which is part of the writer complex and may facilitate m^{6}A writer-complex binding to RNA. In the RNA polymerase II (Pol II)-mediated recruitment model, the slowing down of Pol II, or its pausing, favours recruitment of the m^{6}A writer complex. Pol II pausing may occur in specific regions of the transcript, leading to the accumulation of m^{6}A at these sites. METTL3, methyltransferase-like protein 3.

also found near stop codons. It is unlikely that the stop codon itself directs methylation, since methylation occurs in the nucleus and the stop codon is recognized in the cytosol by ribosomes. Instead, the terminal exon–exon junction in mRNA (which is typically near the stop codon)[68] is likely the key structural feature that accounts for enrichment of m^{6}A near stop codons[11,13]. The enrichment of m^{6}A in this region is seen in nascent, chromatin-bound mRNA[14], further indicating that this stop codon-proximal enrichment of m^{6}A is dictated by m^{6}A writing events early in the mRNA life cycle and is not a consequence of selective cytosolic degradation of mRNAs that contain m^{6}A outside of these regions.

As methylation occurs co-transcriptionally[25], events during transcription probably dictate which DRACH sites are methylated. Localization of the m^{6}A writer complex to H3K36me3 marks could confer site specificity in the nascent transcript. Alternatively, METTL3 has been detected bound to RNA polymerase II[63], suggesting that the m^{6}A writer complex could be recruited to RNA polymerase II to induce methylation (FIG. 3). Slowing RNA polymerase II, by using chemical inhibitors or RNA polymerase II mutants, increased both the recruitment of METTL3 and m^{6}A levels in mRNA[63]. However, it should be noted that it is not clear whether RNA polymerase II normally slows near the stop codon region or in large internal exons, which would explain the enrichment of m^{6}A at these sites.

Lastly, RNA-binding components of the writer complex may target the writer complex to mRNA and facilitate the methylation of adjacent sites (BOX 2; FIG. 3). One component of the methylation complex, RBM15/15B, contains RNA-binding domains, and RBM15/15B-binding sites are near m^{6}A sites in mRNA[69]. It is not clear what fraction of m^{6}A sites are the result of writer-complex recruitment to specific transcript regions by RBM15/15B.

Although m^{6}A formation is thought to be primarily nuclear, m^{6}A writing could occur in the cytoplasm in some circumstances. For example, RNA viruses acquire m^{6}A in their genome even though their genome is transcribed in the cytoplasm[70,71]. The ability of these transcripts to acquire m^{6}A suggests that some METTL3 is present in the cytoplasm[71]. Other studies have also detected METTL3 in the cytoplasm[72,73]. It is not clear whether the METTL3-containing writer complex that assembles in the cytoplasm is similar to the canonical nuclear writer complex.

Nuclear speckles
Membraneless compartments enriched in pre-mRNA splicing factors, located in the interchromatin regions of the nucleoplasm and implicated in different aspects of RNA metabolism.

m^{6}A readers

A major mechanism by which m^{6}A affects the fate of mRNAs is by recruiting m^{6}A-binding proteins. The YTH domain-containing proteins were the first m^{6}A-binding proteins to be discovered[8] and provided a mechanistic basis for understanding the effects of m^{6}A in mRNA (TABLE 2). m^{6}A can also influence mRNA by destabilizing the mRNA structure, which can affect the binding of diverse RNA-binding proteins. Here we summarize some of the major discoveries about the m^{6}A readers and provide insights on how bona fide m^{6}A-binding proteins can be identified.

Direct readers

The YTH domain as a m^{6}A-binding module. The discovery that YTH domain-containing proteins bind m^{6}A came from an in vitro m^{6}A-RNA pull-down experiment that recovered YTHDF2 and YTHDF3 (REF.[8]). The YTH domain was previously suggested to be an RNA-binding domain, based on its homology with other RNA-binding domains[74]. Subsequent studies formally demonstrated that the ~150 amino acid YTH domain binds RNA in an m^{6}A-dependent manner[75,76].

There are five YTH domain-containing proteins in the mammalian genome, which fall into three classes: YTHDC1 (also called DC1), YTHDC2 (also called DC2) and the family of YTHDF (called DF) proteins. DC1 is predominantly nuclear[41], the DF proteins are cytosolic[21,77–80] and DC2 can be both nuclear and cytosolic[81].

Structural studies[82–85] have demonstrated that the selectivity of YTH domain-containing proteins for binding the methyl moiety of m^{6}A is achieved mainly through a 'tryptophan cage', in which two or three tryptophans encase the methyl group.

DC1 as a nuclear m^{6}A reader. DC1 has been linked to mRNA splicing[43], epigenetic silencing mediated by the noncoding RNA *XIST*[69] and the nuclear export of mRNA[47]. DC1 was found to be localized in dynamic and cell cycle-regulated dot-like structures in the nucleus, which were termed 'YT bodies' on the basis of the original name for DC1, YT521-B[86]. YT bodies are now thought to be nuclear speckles, on the basis of the colocalization of DC1 and SRSF proteins[43], as well as proteomic studies of nuclear bodies[87]. Notably, like the DF proteins, DC1 contains a large low-complexity domain[69]. Phase separation may thus be essential for DC1 function.

Since nuclear speckles are associated with sites of active transcription, DC1 may bind mRNAs shortly after they are transcribed and methylated. Upon binding, DC1 may influence splicing. DC1 binds SRSF3, which is thought to enable DC1 to promote exon inclusion by

Table 2 | **Three main categories of m^{6}A readers**

Mechanism of m^{6}A recognition	Protein name	Cellular localization	Effect on m^{6}A RNA upon binding
Direct: direct binding to m^{6}A	YTHDC1	Nucleus	Preferentially binds to m^{6}A sites in noncoding RNA (ncRNA); may bind mRNAs and affect their splicing and export
	YTHDC2	Nucleus and cytosol	Weakly binds m^{6}A; highly expressed in testes; has been implicated in mRNA degradation and translation initiation regulation
	YTHDF1	Cytosol	Preferentially binds m^{6}A sites in cytosolic mRNAs; promotes translation of a subset of m^{6}A-containing mRNAs
	YTHDF2	Cytosol	Preferentially binds to m^{6}A in cytosolic mRNAs; promotes degradation of m^{6}A mRNAs by targeting them to P-bodies
	YTHDF3	Cytosol	Preferentially binds m^{6}A in cytosolic mRNAs; can recognize circular RNA; promotes the translation and degradation of m^{6}A-containing mRNAs
	eIF3	Cytosol	Binds to m^{6}A sites in the 5′ UTRs of mRNAs; promotes their cap-dependent and YTHDF1-independent translation
	METTL3	Nucleus and cytosol	Binds a small fraction of m^{6}A mRNAs in the cytoplasm; promotes their translation
	Ribosome	Cytosol	Recognizes m^{6}A during translation; ribosome stalling may occur at m^{6}A sites
m^{6}A switch: binding regulated by m^{6}A-induced structural changes	HNRNPC and HNRNPG	Nucleus	Preferentially bind noncoding RNAs; may bind m^{6}A on mRNAs and affect their splicing
	HNRNPA2B1	Nucleus	Preferentially binds noncoding RNAs; mediates m^{6}A-dependent microRNA processing events; may affect splicing
	IGF2BP1, IGF2BP2, IGF2BP3	Nucleus and cytosol	Bind m^{6}A with weak binding affinity; promote the stability of m^{6}A mRNAs
Indirect: binding to bona fide m^{6}A-binding proteins	FMRP	Nucleus and cytosol	Recognizes m^{6}A in the coding sequence with weak binding affinity; directly binds YTHDF2 and so indirectly maintains the stability of m^{6}A-containing mRNAs

FMRP, fragile X retardation protein; HNRNPA2B1, heterogeneous nuclear ribonucleoprotein A2B1; IGF2BP1, insulin-like growth factor 2 mRNA binding protein 1; METTL3, methyltransferase-like protein 3; YTHDC1, YTH domain-containing protein 1; YTHDF1, YTH domain-containing family protein 1.

displacing SRSF10 (REF.[43]). These effects are consistent with a potential role of m^{6}A in shaping RNA splicing kinetics though the recruitment of specific splicing factors. However, it is currently unclear whether splicing regulation is a major function of m^{6}A and whether m^{6}A-mediated regulation of splicing is mediated by DC1 or other m^{6}A-dependent mechanisms (see the section above on mRNA splicing).

DC1 also appears to mediate the functions of m^{6}A in noncoding RNA. Transcriptome-wide binding studies of YTH proteins using UV crosslinking immunoprecipitation (CLIP) methods demonstrated that DC1 preferentially binds m^{6}A sites in noncoding RNAs, such as *XIST*, *NEAT1* and *MALAT1*, whereas the DF family members preferentially bind m^{6}A sites on mRNA[69].

Although m^{6}A is in diverse noncoding RNAs such as *NEAT1* and *MALAT1*, its function has only been probed in the case of *XIST*, the noncoding RNA that contributes to X chromosome inactivation and the silencing of genes on the X chromosome[69]. Depletion of m^{6}A enhances *XIST*-mediated silencing by recruiting DC1, which might facilitate the recruitment of other epigenetic silencing proteins[69]. Whether DC1 contributes to the functions of other noncoding RNAs is unclear.

DF proteins and cytosolic mRNAs. The YTHDF family comprises three highly similar paralogues: YTHDF1, YTHDF2 and YTHDF3 (also called DF1, DF2 and DF3, respectively)[88]. DF proteins share very high amino acid identity over their entire length. Besides the nearly identical YTH domains in DF1–DF3, the remainder of the sequence (~400 amino acids) is a low-complexity region that lacks any recognizable modular protein domain and contains several prion-like P/Q/N-rich domains[88]. As described above, these domains cause DF proteins to undergo phase separation, typically along with bound m^{6}A mRNA. This phase separation property targets m^{6}A mRNA for processing by P-bodies, stress granules and other RNA–protein assemblies[46].

Although all three DF proteins have the same ability to enhance m^{6}A mRNA phase separation[46], there is conflicting evidence about whether they each have specialized effects on m^{6}A mRNAs. Earlier studies reported that each of the three DF proteins has a different effect on m^{6}A mRNAs: DF1 enhances the translation of m^{6}A-modified mRNAs, DF2 promotes their degradation, and DF3 has both functions[21,77,80]. However, other assays showed that DF1–DF3 all had similar roles in mRNA degradation using reporter RNA degradation[89] or mRNA deadenylation assays[90]. Also, all three DF proteins seem to recruit the main cellular mRNA deadenylation complex, called CCR4–NOT, on m^{6}A mRNAs[90]. DF2 was previously shown to undergo heat shock-induced relocalization to the nucleus to regulate stress induced-m^{6}A writing pathways[79]; however, a subsequent study showed no stress-induced DF2 nuclear relocalization, which indicates that DF2 does not have a selective role in regulating m^{6}A writing pathways[46]. Additionally, DF2 may promote endonucleolytic cleavage of its bound mRNAs[91]. Given the high degree of sequence similarity between DF proteins, it is unclear how DFs would mediate different functions; more studies will be needed to resolve these divergent models for DF function on cytosolic mRNAs.

Another question is whether DF proteins bind different or the same m^{6}A sites in mRNAs. Some CLIP-based studies have suggested that most m^{6}A residues only bind one of the three DF paralogues[80], whereas others have suggested that all m^{6}A sites bind all DF paralogues in largely equivalent manners[69]. It will be important to resolve this discrepancy and to determine whether the DF proteins bind the same or different m^{6}A sites.

DC2 is an m^{6}A reader that functions primarily in testes. Unlike the other YTH proteins, which are ubiquitously expressed, DC2 is enriched in the testes[81,92–94]. *YTHDC2* knockout mice show defects in spermatogenesis without other obvious developmental defects[81,92–94]. DC2-deficient germ cells enter meiosis but undergo premature and aberrant metaphase and apoptosis without acquiring normal meiotic gene expression programmes[81,92–94].

The binding properties of DC2 are unusual. Unlike the other four YTH domain-containing proteins, which bind m^{6}A with RNA-binding affinities ranging from 200 nM to 1 μM[84,85], the DC2 YTH domain binds more weakly to m^{6}A RNAs (~5 μM)[81,84]. The YTH DC2 domain retains the tryptophan cage and thus seems to bind methylated adenine. However, the DC2 YTH domain shows sequence divergence in the region that is predicted to bind m^{6}A-adjacent residues[88]. CLIP-based studies of DC2-binding sites in the transcriptome have shown low overlap with m^{6}A sites, unlike the other YTH domain proteins[69]. Thus, the DC2 YTH domain may bind select m^{6}A sites or use a different binding mode to affect m^{6}A mRNA.

DC2 has an RNA helicase domain that shows similarities to RNA helicases that regulate translation, such as DHX29 (REF.[95]), suggesting that it might promote mRNA translation. This hypothesis was supported by the observation that translation was increased when DC2 was artificially tethered to a reporter RNA[93]. Other studies suggest that DC2 mediates mRNA degradation through recruitment of the 5′–3′ exoribonuclease Xrn1 (REFS[81,96]). In mouse testes, DC2 depletion led to a subtle increase in the expression of mRNAs with high levels of m^{6}A (REF.[81]). Overall, the reported effects are very small, raising the possibility that the function of DC2 has not been fully elucidated.

Indirect readers

In addition, m^{6}A may indirectly recruit RNA-binding proteins. Although m^{6}A can form a base pair with U, m^{6}A•U base pairs are weaker than A•U base pairs, and the presence of a single m^{6}A in a short RNA helix can destabilize it, lowering the melting temperature by 5 °C or more[97]. Therefore, m^{6}A reduces the ability of RNA to form structures, favouring the linear, unfolded form of RNA. This reduction in structure gives RNA-binding proteins that bind a single-stranded motif greater access to RNA. This concept was first demonstrated with the heterogeneous nuclear ribonucleoprotein C (HNRNPC), an abundant nuclear RNA-binding protein responsible for pre-mRNA processing including

UV crosslinking immunoprecipitation (CLIP). An antibody-based method used to identify the RNA sites directly bound by RNA-binding proteins.

splicing[38]. The presence of m^{6}A provided HNRNPC with greater access to several of its binding sites that were near m^{6}A sites[38]. The propensity of m^{6}A to be in unstructured regions of the transcriptome has been supported by transcriptome-wide assays of RNA structure[98]. This concept of m^{6}A-induced RNA unfolding is referred to as an 'm^{6}A structural switch'[38].

Importantly, an m^{6}A-induced structural switch would promote binding of essentially any RNA-binding protein that has a binding site near or overlapping with the m^{6}A site. Indeed, binding studies have shown that other RNA-binding proteins that bind near some m^{6}A sites have reduced binding efficiency when m^{6}A is absent. These proteins include heterogeneous nuclear ribonucleoprotein G (HNRNPG)[99], an RNA-binding protein involved in splicing, and A2B1 (HNRNPA2B1)[100], an RNA-binding protein involved in primary microRNA processing. Any protein that binds near an m^{6}A residue will exhibit altered binding efficiency when m^{6}A is present.

It is difficult to determine whether an RNA-binding protein binds to m^{6}A directly or whether it binds to an m^{6}A-containing RNA due to m^{6}A-induced RNA unfolding and a subsequent increase in accessibility to nearby binding sites. For example, although early gel-shift assays suggested that HNRNPA2B1 binds m^{6}A (REF.[101]), more recent studies have suggested that it was m^{6}A-induced RNA unfolding that made the labelled RNA probe more accessible to HNRNPA2B1 (REF.[100]). Thus, comparing non-methylated and methylated RNAs is complicated, since the RNAs differ by methylation status as well as structure. It is therefore critical to use RNAs that are not capable of forming structure when asking whether an m^{6}A directly binds an RNA-binding protein.

Also, m^{6}A can repel certain RNA-binding proteins. These proteins, known as 'anti-readers', are displaced by m^{6}A if m^{6}A occurs in their binding site and if the methyl group interferes with binding. An analysis of proteins that bind the methylated and non-methylated versions of the same RNA showed that G3BP1, an RNA-binding protein involved in stress granule formation, is specifically repelled by the presence of m^{6}A (REF.[102]). LIN28A, a core pluripotency regulator involved in microRNA processing, and EWSR1, a major transcriptional repressor, have also been described as anti-m^{6}A readers on the basis of structural and binding analysis[102]. It remains unclear if m^{6}A-mediated anti-reading mediates any aspect of m^{6}A biology.

Other potential readers

Ribosomes may function as m^{6}A readers. Studies using single-molecule ribosome translocation assays showed that bacterial ribosomes stall on mRNA at m^{6}A-containing codons[103]. Although bacterial ribosomes are different from mammalian ribosomes, ribosome profiling data suggest that mammalian ribosomes also have a tendency to stall at m^{6}A sites[103]. The extent to which this mechanism affects m^{6}A mRNA stability and/or translation remains unclear.

Additional m^{6}A-binding proteins have been identified from pull-down experiments using m^{6}A RNA probes and mass spectrometry. These proteins include FMRP, the fragile X mental retardation protein, and the IGF2BP proteins (IGF2BP1, IGF2BP2 and IGF2BP3). Each of these RNA-binding proteins seems to enhance m^{6}A mRNA stability[102,104]. However, it remains unclear whether these proteins bind m^{6}A directly. CLIP studies have provided evidence both in support of and against the idea that these are bona fide m^{6}A-binding proteins. On the basis of motif analysis of peaks identified in some CLIP studies, FMRP and IGF2BP proteins bind a sequence motif that resembles the m^{6}A DRACH site[102,104]; however, other CLIP studies have suggested that IGF2BP proteins bind to a different consensus sequence[105]. Another reason why these proteins do not appear to be bona fide readers is that their transcriptome-wide binding patterns, as determined by the distribution of their CLIP reads, does not match the striking stop codon-enriched m^{6}A distribution[102,104]. Furthermore, in contrast to YTH domains, FMRP and IGF2BP proteins show weak binding affinities for m^{6}A-containing RNA and poor capacity to discriminate between these RNAs and non-methylated RNA[102,104]. Thus, these proteins do not show the type of m^{6}A-binding behaviour expected from a direct m^{6}A reader.

More recent studies have provided alternative explanations for the initial observations that FMRP and the IGF2BPs directly bind m^{6}A. FMRP was found to directly bind DF2 (REF.[106]), consistent with other studies that found FMRP in the 'interactome' of DF proteins[107]. Thus, FMRP may indirectly associate with m^{6}A RNA through its interaction with DF proteins. Similarly, IGF2BP proteins interact with DF proteins in pull-down studies[107], and thus probably interact with m^{6}A RNA indirectly. Another study suggested that IGF2BPs bind m^{6}A-modified mRNA due to an m^{6}A structure switch[108]. Thus, IGF2BPs might bind an RNA that is unfolded due to m^{6}A. Nevertheless, FMRP and IGF2BPs may have important roles in m^{6}A signalling, but it will be important to understand whether they function as binding partners of DF proteins or as direct readers of m^{6}A.

m^{6}A erasers

The m^{6}A 'erasers', which are demethylases that convert m^{6}A into A[109], may also shape the m^{6}A epitranscriptome. Although early reviews of m^{6}A suggested that m^{6}A erasers should be critical for m^{6}A function, by catalysing its removal in a dynamic, rapid, signal-dependent manner[110], m^{6}A erasers now appear to have a limited role under normal physiological conditions. Indeed, biochemical tracing and metabolic labelling studies show that m^{6}A levels are stable in mRNA during its life cycle in HeLa cells[3,26]. Instead, m^{6}A erasing appears to be limited to specific tissues, such as the testes, or in specific stress- and disease-relevant conditions.

Which nucleotide is demethylated by FTO?

Analysis of the fat mass and obesity-associated protein (FTO) sequence revealed distant homology to the ALKB family of dioxygenases[111], which demethylate DNA and RNA nucleotides that are alkylated by exogenous agents[112,113]. Based on this, FTO was screened for demethylase activity on methylated DNA nucleotides,

Ribosome profiling
A method used to determine the ribosome footprints on mRNAs and thus to identify the translating mRNAs.

which showed weak activity towards these substrates[111]. However, a subsequent study proposed that FTO was a demethylase for 3-methyluridine (m^3U) in RNA based on its higher activity towards this ribonucleotide compared to methylated deoxyribonucleotides[114]. A subsequent study showed even higher demethylase activity towards m^6A in mRNA, leading to the conclusion that m^6A was the bona fide substrate of FTO[109].

However, several studies have hinted that m^6A may not be a physiologically relevant target of FTO. For example, m^6A-mapping studies of the mouse *FTO*-knockout brain transcriptome did not show a clear and robust increase in m^6A sites[115], and m^6A levels were not increased in mRNA derived from *FTO* knockout mouse embryos and cells[116]. More recent transcriptome-wide mapping of m^6A stoichiometry using Mazter-Seq also demonstrated that m^6A stoichiometries throughout the transcriptome are not affected by FTO depletion[117] (see the Supplementary Information for more information on Mazter-Seq). In vitro enzymatic analysis showed that FTO demethylates m^6A at an unusually low reaction rate compared with related enzymes towards their substrates[109]. Lastly, FTO did not demethylate RNAs in an m^6A-containing sequence-specific manner[109,118,119], which is a hallmark of nonspecific enzymatic activity. Overall, these results indicate a nonspecific reaction with m^6A.

A major advance in understanding the function of FTO was the discovery that FTO has substantially higher catalytic activity for demethylating m^6A_m than for m^6A (REF.[116]). This discovery was the result of a detailed re-analysis of m^6A peak intensities of miCLIP performed in wild-type and *FTO*-knockout mice, which revealed a subtle increase in m^6A levels in FTO-deficient cells when m^6A is located in the 5′ UTR[116]. Importantly, peaks in the 5′ UTR often reflect m^6A_m, since m^6A_m is exclusively found adjacent to the m^7G cap in mRNA[120]. When directly tested in biochemical assays, m^6A_m was demethylated at a rate that was 100 times higher than that of m^6A (REF.[116]). This activity was highly sensitive to sequence context, since the m^7G was needed for demethylation, suggesting that the physiologic substrate of FTO is m^6A_m.

Although FTO can demethylate m^6A_m in mRNA, the increase in cellular levels of m^6A_m in poly(A) mRNA in *FTO*-knockout cells was fairly subtle[116]. This prompted the analysis of other RNAs in *FTO*-knockout cells using miCLIP analysis of the total cellular RNA[121] (see the Supplementary Information for miCLIP). Here, quantitative measurement of m^6A_m stoichiometry (compared with the demethylated nucleotide form, 2′-*O*-methyladenosine) showed a ~1,500% increase in m^6A_m levels in *FTO*-depleted cells in the first-transcribed nucleotide position in specific snRNA transcripts[121]. In wild-type cells, <5% of snRNA has m^6A_m in most cell types. However, *FTO*-knockout cells showed >50% m^6A_m stoichiometries, demonstrating that FTO normally demethylates m^6A_m so that most mature snRNAs contain an A_m at the first encoded nucleotide[121]. Notably, *FTO*-knockout cells display splicing defects[122]. Because snRNAs mediate splicing, it is possible that the m^6A_m form of snRNAs could have altered roles in splicing.

Overall, these robust alterations in the methylation state of m^6A_m in snRNAs confirm that snRNAs are FTO targets and suggest that FTO functions to control snRNA rather than mRNA methylation. The precise mechanism by which the FTO-regulated methyl modification affects snRNAs are unknown.

Although FTO has clear and robust effects on m^6A_m in cells, it has been proposed that FTO can act on m^6A despite its low reaction rate on this modification. *FTO* depletion in specific leukaemia subtypes leads to an ~20% increase in m^6A levels[123]. It has been proposed that FTO has an anomalous cytoplasmic localization in these cells that enables m^6A demethylation[123]. However, no specific mRNA has directly been tested for m^6A levels using the SCARLET method (see the Supplementary Information for SCARLET), to determine whether it is indeed regulated by FTO. It will be important to identify an m^6A site that shows a clear and robust change in m^6A stoichiometry in order to definitively identify FTO as a m^6A demethylase in mRNA.

ALKBH5 as a testes-enriched m⁶A eraser

The second RNA demethylase to be identified, ALKBH5, was discovered in a biochemical screen for demethylases that act on m^6A (REF.[19]). ALKBH5 is an endogenous m^6A demethylase, as *ALKBH5* knockdown and overexpression lead to increased and reduced m^6A in cells, respectively. Unlike FTO, ALKBH5 has no activity towards m^6A_m[116]. Since ALKBH5 is nuclear and appears to be localized to nuclear speckles[19], ALKBH5 likely demethylates m^6A in nuclear RNA or in mRNA during its biogenesis in the nucleus.

ALKBH5 does not seem to have roles in mouse development or physiology, as *ALKBH5*-knockout mice appear normal except for defects in spermatogenesis[19]. Notably, ALKBH5 is enriched in testis and in female reproductive tissues, suggesting that it mediates an m^6A demethylation event that is crucial for germ cell development, as was shown for spermatogenesis[19].

In contrast to normal cells, in certain cancer cells ALKBH5 appears to be upregulated, such as in glioblastoma stem cells[124,125]. ALKBH5 may also be upregulated in hypoxia, on the basis of findings that show that the hypoxia-sensing transcription factor HIF-1α binds to the *ALKBH5* promoter using chromatin immunoprecipitation (ChIP) analysis[126]. Consistent with this, ALKBH5 is induced by the hypoxic environment to which breast cancer stem cells are exposed[127]. ALKBH5 induction has also been reported following viral infection[128]. Thus, ALKBH5 may be upregulated in diverse disease contexts in order to modify the epitranscriptome.

Specific transcripts and m^6A sites can mediate the oncogenic effects of increased ALKBH5 expression. For example, the mRNA of *NANOG*, a key pluripotency gene, shows decreased m^6A levels in breast cancer cells that exhibit elevated ALKBH5 levels[127]. Another potential ALKBH5-regulated mRNA is *FOXM1*, which encodes forkhead box protein M1 (a transcription factor that regulates cell proliferation). *FOXM1* forms a duplex with an antisense RNA that enables its m^6A to be demethylated by ALKBH5, raising the possibility that ALKBH5 specificity may require unique RNA

structures[124]. Overall, these data suggest that ALKBH5-mediated m^{6}A demethylation is crucial for sperm development and may have roles in certain disease contexts in which ALKBH5 is upregulated.

In short, it is now clear that FTO and ALKBH5 demethylate m^{6}A$_m$ and m^{6}A, respectively, and that their target RNA may not be mRNA, but snRNA, at least in the case of FTO[121]. Future research should focus on identifying the major targets of these enzymes and the mechanisms that regulate these enzymes in order to induce RNA demethylation.

Conclusions

Many questions remain about how m^{6}A is added on or removed from mRNAs and how m^{6}A regulates gene expression. Additionally, although m^{6}A mapping was a pivotal advance, it does not provide information on stoichiometry. Newer methods such as Mazter-Seq[117] (see the Supplementary Information) will enable precise determination of the stoichiometries of m^{6}A at specific sites in specific mRNAs, which will answer the question of whether the epitranscriptome is indeed dynamic and variable between different cells and disease conditions. The use of Mazter-Seq and the development of new methods to enable m^{6}A stoichiometry will be crucial for revealing 'regulated' m^{6}A sites, as opposed to the 'constitutive' m^{6}A sites. Ultimately these studies will reveal the degree to which the epitranscriptome can be dynamically controlled in order to affect gene expression. It is likely that an understanding of the different m^{6}A writer components and their regulation will be key to understanding the nature and function of regulated m^{6}A sites.

In addition to understanding m^{6}A writing, it will be important to determine whether DF proteins can be activated to induce m^{6}A-containing mRNA degradation, translation and/or localization. It will also be important to understand whether the numerous newly identified m^{6}A readers are indeed recognizing m^{6}A or whether, instead, they are binders, and potentially modulators, of DF proteins. Furthermore, whether DF proteins mediate the majority of the effects of m^{6}A, or whether other pathways, possibly the m^{6}A structural switch mechanism, contribute to the actions of m^{6}A in physiological processes, remains to be elucidated.

Lastly, it will be important to precisely determine which adenosine residues are under the control of m^{6}A erasers. New data have pointed to FTO's dramatic regulation of m^{6}A$_m$ in snRNA, rather than of m^{6}A or m^{6}A$_m$ in mRNA[121]. The ability to quantitatively measure m^{6}A levels at specific sites in mRNAs will provide insights into which sites are regulated by FTO or by ALKBH5.

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Competing interests
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