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The dynamic epitranscriptome: *N*^{6}-methyladenosine and gene expression control

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Abstract

N^6-methyladenosine (m^{6}A) is a modified base that has long been known to be present in noncoding RNAs, ribosomal RNA, polyadenylated RNA and at least one mammalian mRNA. However, our understanding of the prevalence of this modification has been fundamentally redefined by transcriptome-wide m^{6}A mapping studies, which have shown that m^{6}A is present in a large subset of the transcriptome in specific regions of mRNA. This suggests that mRNA may undergo post-transcriptional methylation to regulate its fate and function, analogous to methyl modifications in DNA. Thus, the pattern of methylation constitutes an mRNA 'epitranscriptome'. The identification of adenosine methyltransferases ('writers'), m^{6}A demethylating enzymes ('erasers') and m^{6}A binding proteins ('readers') is helping to define cellular pathways for the post-transcriptional regulation of mRNAs.

It is well established that DNA and proteins undergo dynamic chemical modifications that influence their function. For instance, methylation of cytosine residues in DNA to form 5-methylcytosine (5mC) can have widespread effects on gene expression by recruiting specific DNA-binding proteins[1]. Similarly, phosphorylation of proteins — a reversible chemical event initially thought to be restricted to just a few targets — is involved in nearly every aspect of cellular physiology[2]. However, until recently, mRNA had not been shown to be extensively subjected to such chemical modifications that might alter its function.

Two groups showed in 2012 that a large fraction of cellular mRNA contains adenosine residues that are methylated to form *N*^{6}-methyladenosine (m^{6}A)[3, 4]. In addition, the characterization of adenosine methyltransferases and m^{6}A demethylating enzymes has provided insights into the pathways that dynamically regulate adenosine methylation in mRNA and how this process contributes to human disease[3, 5, 6]. Collectively, these studies demonstrate for the first time that mRNA is susceptible to dynamic, reversible chemical modification. Thus, analogous to methylation of DNA and phosphorylation of proteins, adenosine methylation in mRNA represents an additional layer of regulation that can potentially alter mRNA function and influence the way genes are expressed.

A flurry of recent discoveries has also pointed to important roles for m^{6}A in regulating important cellular pathways and processes, highlighting the potentially broad role for m^{6}A

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in regulating mRNA fate (**Box 1**). In this Review, we discuss the discovery of the m^{6}A modification, including early studies that first revealed the existence of m^{6}A as well as more recent studies that have profiled the features of m^{6}A on a global scale. We also analyse the strengths and limitations of current methods used to study m^{6}A and highlight improvements that are needed to move the field forward. Furthermore, we emphasize our current knowledge of adenosine methylation and demethylation pathways in mammalian systems and the insights that studies of these pathways have provided for the role of m^{6}A in human disease. Last, we discuss the questions that are driving m^{6}A research and the implications that their answers might have for our understanding of RNA biology.

Serendipitous discovery of m^{6}A

The finding that polyadenylated RNA contains m^{6}A was a serendipitous discovery made by several groups that were characterizing the mRNA 5' structure in mammalian cells in 1974[7, 8], and validated by others the following year[9-12]. The original goal of these studies was to characterize the methylation of the recently described 5' cap of mRNAs. These studies were made possible because radioactive [^{3}H]-methionine, the methyl source in most biochemical reactions, had become commercially available with high specific activity. [^{3}H]-methionine could be applied to cells and is then metabolically incorporated into S-adenosylmethionine (SAM), the enzymatic cofactor that participates in many methylation reactions in the cell.

After digestion of the poly(A) RNA, it was clear that the radioactivity was not confined to the caps. Radioactivity was also detected in the mononucleotides, with the majority found in a modified nucleotide, *N*^{6}-methyladenosine (m^{6}A)[7, 8]. In most studies, m^{6}A was the only modified base that was detected, although one study also reported the presence of 5-methyl-cytosine, although at a fourth of the abundance of m^{6}A[12, 13]. Radioactive methyl groups were also found within what seemed to constitute an extended cap structure (**Box 2**)[14]. The finding of m^{6}A in RNAs was not unprecedented, as m^{6}A was known to be in bacterial tRNA and other noncoding RNAs[15-19].

However, these initial studies provided the first evidence that m^{6}A is present in mammalian poly(A) RNA, as well as mRNA encoded by diverse viruses[20-24]. Subsequent studies showed that m^{6}A is a prevalent nucleotide in poly(A) mRNA from nearly all higher eukaryotes and plants[7, 8].

The early studies examined poly(A) RNA, not mRNA. However, since mRNA is highly enriched in poly(A) RNA preparations, it was tempting to speculate that the m^{6}A in poly(A) RNA derived from mRNA. Based on this, m^{6}A might potentially be present at a level of ~3-4 m^{6}A residues per mRNA[7-12]. This measurement relies on the assumption, which is now known to be incorrect, that m^{6}A is evenly distributed in all cellular transcripts. Nevertheless, these early investigators made the seminal proposal that post-transcriptional methylation of adenosine residues in mRNA might influence that fate of mRNA in cells, potentially analogous to the functional consequences of protein modifications.

Reluctance to accept m^{6}A

Despite the interesting possibility that mRNA is regulated by adenosine methylation, there were very few studies that further investigated the original findings on m^{6}A. The initial interest in m^{6}A was largely abandoned due to considerable reluctance to accept the idea that this modification is biologically relevant. One concern was that m^{6}A could have arisen from contamination from small amounts of known m^{6}A sources[25], such as ribosomal RNA[26] and small nucleolar RNAs[27, 28]. Another factor was that these early studies used poly(A) RNA, which is often contaminated with mitochondrial mRNA, tRNA, and certain rRNAs owing to their poly(A) tracts[29-31]. Indeed, most preparations of poly(A) mRNA invariably contain ribosomal RNA bands. In some studies, rigorous gradient purification methods were used to remove this persistent contaminant[32], which supported the idea that m^{6}A is a constituent of mRNA. However, most studies did not use these approaches, questioning the relevance of studies using poly(A) mRNA. Indeed, it is now known that up to half of the RNA pool in poly(A) RNA fractions constitutes diverse noncoding RNA species[33-36].

In addition to the concern about a contamination artifact, another concern was that mutation of specific m^{6}A sites did not result in a change in RNA fate in cells. For example, point mutations that depleted m^{6}A within the *src* transcript of Rous sarcoma virus did not affect *src* nuclear location, RNA expression levels, splicing, translation, packaging of the RNA into virions or infectivity of the mutant virus[37, 38]. These studies raised the possibility that m^{6}A has a minimal impact on mRNAs.

Another problem was that few endogenous mRNAs were shown to contain m^{6}A. If m^{6}A was indeed highly prevalent, it should be seen in numerous cellular mRNAs. Although m^{6}A was frequently detected in viral mRNAs[39], the only endogenous mammalian mRNA shown to contain m^{6}A was the *prolactin* mRNA[40, 41]. Therefore, it was plausible that m^{6}A was preferentially introduced into foreign mRNAs. The prevalence of m^{6}A in poly(A) mRNA might reflect the incorporation of m^{6}A into a small number of hypermethylated cellular mRNAs.

Addressing these issues was a formidable challenge, as no methods existed that could globally detect m^{6}A sites in the transcriptome. Identifying m^{6}A is challenging, as m^{6}A reverse transcribes to a T during reverse transcription, and because m^{6}A is not susceptible to chemical treatments that might facilitate its detection. This inability to identify endogenous m^{6}A-containing mRNAs further dampened interest in investigating the functional roles of this putative mRNA modification.

Improved detection of m^{6}A

The major breakthrough that resulted in the acceptance of m^{6}A as a highly prevalent mRNA modification was the development of novel tools and methods for detecting m^{6}A in mRNAs (**Box 3**). For example, the m^{6}A immunoblotting technique showed that m^{6}A-containing poly(A) RNAs span a broad range of sizes, ranging from less than 500 bases to over 7 kb[3]. This resembles the distribution expected for mRNAs, suggesting that mRNAs are the m^{6}A-containing species in these blots.

m^{6}A immunoblots also allow changes in m^{6}A levels to be measured. For example, the intensity of the m^{6}A smear in the immunoblot differs markedly when comparing RNA derived from different tissues and cancer cell lines, with the highest m^{6}A levels in the brain, heart and kidney[3]. m^{6}A-containing mRNAs are low in the fetal brain, but increase during development, achieving a maximal level in the adult brain[3]. These data argued against initial theories that m^{6}A might be a fixed feature of mRNA, like the 5' cap modification, and suggested that it acts as a tissue-specific regulator of mRNA fate.

Finally, pulldown of the *low density lipoprotein receptor 1*, *metabotropic glutamate receptor 1*, and *Dopamine receptor D1A* transcripts followed by m^{6}A immunoblotting analysis revealed that each of these mRNAs is endogenously methylated in cells[3].

The methylated transcriptome

The methylated transcriptome was first defined using the MeRIP-Seq technique and an essentially equivalent approach, termed m^{6}A-Seq (**Box 4**)[3, 4]. These are next-generation sequencing techniques that involve selective immunoprecipitation and sequencing of m^{6}A-containing RNA fragments[3]. MeRIP-Seq led to the identification of 13,471 m^{6}A peaks derived from 4,654 genes in the mouse brain. Analysis of HEK293T cell RNA led to the identification of 18,756 m^{6}A peaks from 5,768 genes. Highly similar numbers were obtained in HepG2 cells, which showed 12,769 m^{6}A peaks among 6,990 mRNAs[4]. These results provided the first unequivocal demonstration that m^{6}A is a widespread mRNA modification, and thus laid to rest many of the previous doubts whether m^{6}A was indeed a prevalent modification in mRNA.

However, m^{6}A was not found in all mRNAs or just a few highly methylated mRNAs, but in ~25% of all transcripts. Thus, these studies ruled out earlier ideas that m^{6}A might be an obligate feature of all mRNAs as a result of biogenesis or processing. Importantly, the distribution of m^{6}A along the length of transcripts was nonrandom, suggesting that m^{6}A has functional roles in mRNA[3, 4]. Furthermore, the sites of methylation in mRNAs were typically found in portions that showed higher evolutionary conservation than other regions[3], further suggesting that m^{6}A has conserved regulatory roles.

Importantly, although many transcripts were methylated in both brain and HEK293 cells, others were methylated in only one tissue[3]. This indicates that methylation is not exclusively regulated by *cis*-acting sequences in the mRNA and that *trans*-acting tissue-specific factors probably contribute to the specificity of methylation in different cell types. However, when a transcript was methylated in both tissues, the m^{6}A peaks were typically localized to the same region of the transcript.

Interestingly, not all m^{6}A peaks detected by MeRIP-Seq were found in mRNAs. In the brain for example, although 94.5% of m^{6}A peaks were found within mRNAs, the rest mapped to several classes of (long) noncoding RNAs[3]. These data indicate that noncoding RNAs are also important targets of m^{6}A.

MeRIP-Seq also demonstrated that different m^{6}A peaks exhibit markedly distinct m^{6}A stoichiometries. It is important not to view transcripts as either "methylated" or

"unmethylated." As with any modification in cells, some copies of a transcript will be methylated and some will be unmethylated. An indirect measure of stoichiometry can be obtained by normalizing the number of MeRIP-Seq reads within each peak to the transcript abundance[3, 42]. A rank of m^{6}A peaks based on this method shows that m^{6}A stoichiometry varies considerably, with the majority of m^{6}A peaks likely exhibiting low stoichiometry[3]. This approach can be used to determine mRNAs with high stoichiometry m^{6}A, which are therefore most likely to be influenced by methylation pathways in any given cell type.

Asymmetric m^{6}A distribution in mRNAs

A striking result from the MeRIP-Seq analysis was the finding that m^{6}A residues are enriched in specific regions of the mRNA[3, 4] (Figure 1). The majority of m^{6}A peaks are located in the vicinity of the stop codon, with a roughly even distribution both upstream and downstream of this site[3]. Additionally, a subset of mRNAs contains m^{6}A residues in their 5' untranslated regions (UTRs)[3, 4]. Typically, the 5'UTR of a transcript is relatively short; thus, the concentration of m^{6}A residues within the 5'UTR is strikingly high compared with other regions of the transcript[4]. Interestingly, the fraction of cellular mRNAs that have m^{6}A in the 5'UTR seems to exhibit tissue-specific differences[4, 43], indicating that regulation by 5'UTR m^{6}A residues may be more frequently utilized in certain tissues than others. Liver and liver-derived cells (HepG2) appear to have a higher proportion of 5'UTR m^{6}A than do other tissues. Thus far, no tissues have been identified which completely lack m6A, although the overall abundance of m^{6}A within RNA appears to vary across different tissue types[3].

m^{6}A residues are also found in the 3'UTR outside of the region adjacent to the stop codon[3]. However, these m^{6}A residues were less abundant than the m^{6}A residues near the stop codon. Furthermore, m^{6}A residues were found within the coding sequence, but again, the relative abundance per unit length of the sequence was proportionately smaller than in other regions of the transcript. Finally, m^{6}A was not found in poly(A) tails[3], which is consistent with early metabolic labelling studies[25].

The enrichment of m^{6}A residues near the stop codon and the 5'UTR is markedly distinct from the binding properties of other known mRNA regulatory elements. For example, microRNA-binding sites are enriched at the 5' and 3' ends of the 3'UTR[44]. Many other mRNA-binding proteins bind to the 3'UTR of mRNAs[45], which is also the most structured portion of the molecule[46]. Moreover, few mRNA-binding proteins show preferential binding to the 5'UTR[45]. Thus, the enrichment of m^{6}A residues in the 5'UTR and around the stop codon is unusual compared with other mRNA regulatory elements, and suggests unique regulatory functions for this modification.

How is methylation targeted?—The asymmetric distribution of m^{6}A residues in mRNA immediately raises the question of how this specificity is achieved. Analysis of the MeRIP-Seq-generated peaks using the motif-discovery algorithm FIRE[47] identified enrichment of G-A-C and A-A-C consensus motifs within these peaks, with the former being the most prominent. Variants of this motif with preferences for residues 5' and 3' to the core motif were also found, including G-(G/A)-A*-C-U (* indicates putative m^{6}A)[3].

These motifs were found in nearly 90% of all peaks[3], and importantly, other motifs may exist for a smaller subset of m^{6}A residues[3, 4].

It is noteworthy that the motifs identified by bioinformatic analysis of MeRIP-Seq data were remarkably similar to the motifs predicted using biochemical approaches in the 1970s[20, 24, 48-50]. These experiments relied on the digestion of mRNAs using ribonucleases that selectively cleave RNA after specific nucleotides, and showed that the central adenosines in the GAC and in the less common AAC motifs are the methylation sites. Follow-up studies using in vitro methylation assays with purified nuclear extracts and synthetic RNAs showed that methylation only occurred on RNAs containing a GAC sequence, and not variants, such as GAU or UAC, or GACG[51]. Furthermore, mutation of GAC sequences to GAU was sufficient to block adenosine methylation[38]. The sequences discovered by FIRE analysis of the MeRIP-Seq dataset show that these m^{6}A motifs apply to the majority of cellular m^{6}A-containing mRNAs.

One of the immediate implications of finding very few methylation site motifs is that there are possibly only a few pathways that control and recognize methylation. A diversity of motifs would have suggested that there might be various methylating enzymes and m^{6}A-binding proteins, each linked to a specific motif consensus site. This would be analogous to the known diversity of kinases, which generally are linked to specific phosphorylation consensus sites in the proteome. Thus, the relative consistency of m^{6}A sites throughout the transcriptome suggests that there is a limited repertoire of readers, writers and erasers for this modification.

The presence of such a short methylation motif raises the question why so few are methylated in mRNAs? The most prominent m^{6}A consensus motif, GAC, is found roughly every 64 nts in RNA, which suggests that an average 2000-nt long mRNA would have ~30 m^{6}As distributed evenly along the length of the transcript. However, most transcripts lack an m^{6}A altogether[3], and in m^{6}A-modified transcripts m^{6}A is usually found the near the stop codon. Thus, the enrichment of m^{6}A at specific GAC residues, especially residues near stop codons, suggests that additional factors determine whether a GAC is targeted for methylation. Structural features adjacent to the GAC sequence could be a possible determining factor. However, m^{6}A peaks are typically found within unstructured regions of RNA[4], rather than in loops or bulges of stem— loops. Thus, although the structure may contribute in some instances to specific methylation events, it does not seem to account for the overall distribution of m^{6}A seen in the metagene analysis.

One mechanism that might allow region-specific methylation within mRNAs could be methyltransferase tethering. For example, tethering of a methyltransferase to 5’UTR elements or near the stop codon could account for the distribution of methylated GAC motifs in these regions. This model is not fully consistent with our understanding of the subcellular localization of the adenosine methyltransferase enzymes (see below) and does not explain m^{6}As within the open reading frame, but could account for some of the defining features of m^{6}A distribution in transcripts. Indeed, in agreement with a tethering model, MeRIP-Seq data indicate that most m^{6}A peaks reflect a cluster of m^{6}A residues[3]. Additionally, previous studies showed that m^{6}A residues were clustered in the Rous

sarcoma virus transcript[39]. These data are consistent with the idea that 'hotspots' of methylation occur in certain regions of mRNA, possibly as a result of a tethered methyltransferase. A global method for profiling m^{6}A at single-nucleotide resolution will be important to determine whether the distribution of m^{6}A in mammalian mRNAs is compatible with a clustering model.

Enzymes that mediate m6Amethylation

Identifying the enzymes that catalyse adenosine methylation will be an important advance for delineating the regulation of mRNA methylation in cells. Several enzymes have been identified that seem to mediate physiological adenosine methylation in mRNA:

•The METTL3–METTL14 complex

Early studies identified a large (> 1 MDa) multiprotein complex that mediates adenosine methylation[52]. Nuclear preparations were capable of methylating RNA in a SAM-dependent manner in vitro, and the substrate specificity matched the m^{6}A consensus motif established by biochemical methods[51]. Thus, mRNAs containing GAC were more efficiently methylated than mRNAs containing AAC, whereas other sequences, such as GAU, were typically unmethylated (Figure 2a). Importantly, this complex did not induce the formation of *N*,*N*-dimethyladenosine[51], unlike various Lys methyltransferases, which can produce 'higher order' methylated forms of Lys, such as di- and tri-methylated Lys[53]. Subsequent efforts focused on purifying the catalytic subunit of the methyltransferase complex[54]. The catalytic component was detected by its ability to crosslink to [^{3}H]-SAM, and was designated METTL3 (methyltransferase-like 3; also known as MT-A70 Purification of the enzyme showed that it exhibited the predicted specificity towards GAC and AAC sequences in single-stranded RNA[55, 56]. Cloning of this enzyme revealed that it has a classic SAM-binding methyltransferase domain, consistent with its function[54]. Homologues in plants (MTA)[57], *S. cerevisiae* (IME4)[58] and Drosophila (IME4)[59] have also been identified.

Recent studies have begun to clarify other components of the METTL3 multiprotein complex. A proteome-wide analysis of protein complexes identified a single interactor of METTL3, METTL14[60]. METTL14 is highly similar to METTL3 and is a predicted methyltransferase[61-63]. Subsequent studies confirmed that both METTL3 and METTL14 form complexes in cells and that purified METTL14 also selectively methylates GAC sequences in vitro[61-63]. Furthermore, the methyltransferase activity of both METTL3 and METTL14 are synergistically enhanced when they are mixed[62, 63]. It is not clear why two methyltransferases are present in the methyltransferase complex, but it is possible that these highly similar enzymes can function independently or each have unique forms of regulation.

Importantly, knockdown of either *METTL3* or *METTL14* results in reduced m^{6}A peaks in more than half of all m^{6}A-containing mRNAs in mouse embryonic stem cells[63], confirming that METTL3 and METTL14 physiologically target mRNAs for methylation.

In addition to METTL14, Wilms tumor 1 (WT1)-associated protein (WTAP) was found to associate with METTL3/14. The importance of this interaction was first established in yeast and Arabidopsis, in which the WTAP homologs Mum2 and AtFIP37, respectively, were

shown to associate with METTL3 and were required for efficient methylation of mRNA[57, 64]. Analysis of mammalian WTAP showed that it too is required in mammalian cells for cellular mRNA methylation[61, 62]. Although WTAP binds to WT1, this tumor suppressor-oncogene is not involved in mRNA methylation as knockdown of *WT1* does not affect cellular m^{6}A levels in mRNA[61]. WTAP seems to interact with numerous cellular proteins, thus, despite its name, many of its functions are unrelated to WT1[65]. WTAP, which lacks methyltransferase domains or activity, was shown to bind to the METTL3—METTL14 complex[61, 62], and induce its localization to nuclear speckles[61]. Thus, WTAP may facilitate mRNA methylation by translocating METTL3—METTL14 to nuclear speckles.

Where in the cell does methylation occur? Because of the readily detectable levels of METTL3, METTL14 and WTAP in nuclear speckles[54, 61], this is likely to be an important site for methylation. A nuclear site for methylation is also supported by early studies that showed that nuclear extracts contain methyltransferase activity[51, 66]. Additionally, *WTAP* knockdown prevents METTL3 localization to speckles and reduces cellular m^{6}A levels[61]. The localization of METTL3 in speckles suggests that this protein may methylate speckle-associated RNAs, such as snRNAs and pre-mRNA, and function during splicing reactions (see below). Additional support for nuclear mRNA methylation comes from PAR-CLIP analysis of METTL3-binding sites in the transcriptome, which revealed an exceptionally high number of binding sites in intronic mRNA sequences[61].

However, early studies also detected methyltransferase activity in cytosolic extracts, which supports the idea that at least some methylation could occurs in the cytoplasm[51]. Cytoplasmic methylation would imply a role for m^{6}A in the control of mRNAs fates that do not affect splicing or nuclear export. Furthermore, the localization of METTL3 seen using different antibodies showed a granular cytoplasmic staining or perinuclear staining[67], and immunostaining for the Drosophila homologue, IME4, indicated some immunoreactivity in the cytoplasm[59]. Notably, METTL3 is expressed as both long and short alternatively spliced isoforms, which remain to be characterized[68, 69]. Conceivably, some of the variability in the staining and localization may reflect these isoforms.

The distribution of m^{6}A within transcripts also supports the idea that mRNA methylation might occur in the cytosol. MeRIP-Seq analysis of m^{6}A localization in the transcriptome shows that m^{6}A residues are highly enriched around stop codons. Cellular recognition of stop codons is mediated solely by ribosomes bound to release factors[70]. Thus, stop codon-bound ribosomes could in principle tether a methyltransferase, resulting in stop codon-adjacent adenosine methylation. Importantly, ribosomes bound to stop codons have key regulatory roles in controlling nonsense-mediated decay and potentially other processes[71]. As ribosome function is predominantly cytosolic[72], a ribosome-mediated methylation pathway would indicate a cytosolic location for methylation, at least for stop-codon proximal m^{6}A residues.

Could other methyltransferases drive m^{6}A formation?

The possibility that additional methyltransferases have a role in adenosine methylation is supported by the fact that a small number of m^{6}A peaks do not seem to contain the AAC or

GAC consensus site[3, 4]. Furthermore, m^{6}A can be found at the first position in many mRNAs after the m^{7}G cap, resulting in a m^{7}Gpppm^{6}Am structure (Figure 2a, see **Box 2** for other methylated forms of the 5' cap). This adenosine contains two methyl modifications: one at the 2' position of the ribose ring, and the other at the N^6 position of adenosine (see **Supplementary Table 1**). Importantly, the residues following the methylated adenosine can be any base[50]. Because of the lack of a cytosine following and a guanosine immediately preceding the adenosine residue, these m^{6}A residues differ from the GAC and AAC consensus motifs. Thus, methylation of these noncanonical m^{6}A motifs might be mediated by other methyltransferases.

There are numerous candidate methyltransferases that could mediate the methylation of these residues. METTL3 is part of a superfamily of at least three different types of mammalian methyltransferases of unclear function[73]. The adenosine methyltransferase activity that targets the U6 small nuclear RNA, which is different from METTL3[28], could potentially target certain m^{6}A residues in mRNA. Thus, these or other enzymes could contribute to mRNA methylation. Interestingly, analysis of cellular extracts from mammalian cells produced two distinct adenosine methyltransferase activities, each of which could be partially purified into distinct fractions[74]. One fraction, similarly to METTL3, methylated diverse RNA templates; however, a separate activity selectively labelled RNA containing a 5' cap[74]. As most studies of adenosine methylation used noncapped RNAs as substrates, the activity of the cap-dependent adenosine methyltransferase might have been missed and additional methyltransferasesmay also contribute to m^{6}A formation in cells.

m^{6}A demethylation pathways

An important recent discovery was the finding that endogenous enzymes can demethylate m^{6}A. The presence of m^{6}A erasers suggests that the effects of m^{6}A can potentially be reversed, and that cellular demethylation pathways might be a mechanism of dynamic regulation of m^{6}A within mRNAs. Notably, these erasers are not expressed in yeast, pointing to the potential role of passive RNA degradation as a mechanism to remove m^{6}A in certain cell types[75]. Whether m^{6}A demethylases preferentially target mRNA or whether they target rRNA and snRNA has not yet been established; however, early evidence points to a role for these enzymes in mRNA demethylation.

FTO

The first enzyme identified as an m^{6}A demethylase was FTO (fat mass and obesity-associated protein)[5]. FTO has a controversial history as numerous enzymatic activities have been ascribed to the enzyme. Bioinformatic analysis showed that FTO is a member of the superfamily of Fe(II)/2-oxoglutarate (2-OG)-dependent oxygenases, which typically mediate oxygen transfer to specific target molecules[76, 77]. FTO has highest homology to the AlkB subfamily of Fe(II)/2-OG-dependent oxygenases, which comprises eight family members in humans and has known functions in nucleotide base demethylation reactions. These reactions occur by hydroxylation of methyl substituents on bases, forming a hydroxymethyl substituent[78, 79]. Hydroxymethyl substituents are highly unstable when connected to nitrogen atoms in a nucleotide base, and spontaneously decompose to formaldehyde,

resulting in demethylation (**Figure 2b**) Thus, based on homology, FTO was thought to be potentially involved in similar hydroxylation—demethylation reactions.

Initial studies first demonstrating that FTO could mediate oxidative demethylation of nucleotide bases showed demethylation of 3-methyl thymidine (3mT) in the context of single-stranded DNA[77]. A subsequent study demonstrated that FTO could also demethylate 3-methyl uracil (m3U) in single-stranded RNA[80]. Interestingly, m3U is a minor constituent of ribosomal RNA[81], potentially linking FTO to ribosome function.

Furthermore, m^6A in RNA has been shown to be an additional substrate for FTO. One study showed that FTO demethylated m^6A with a catalytic efficiency that was substantially higher than the activity of FTO towards 3mU[5]. Overexpression of FTO in HeLa cells reduced the level of m^6A in purified poly(A) RNA by ~18%, whereas *FTO* knockdown increased m^6A levels by 23% in poly(A) mRNA. Consistent with this, another study showed that FTO overexpression greatly reduced the levels of m^6A in cellular RNA[3], and the size of bands produced were consistent with mRNA being a target of this enzyme. Thus, m^6A is a physiologic target of FTO.

Definitive evidence for the specificity of FTO towards mRNA came from MeRIP-Seq analysis of *FTO*-knockout brain tissue. Surprisingly, MeRIP-Seq data showed that m^6A levels are generally unaffected in most mRNAs in *FTO* knockout brain[42]. However, for a small subset of mRNAs, m^6A peaks are markedly higher in the *FTO*-knockout tissue than in wild-type[42]. Thus, these data demonstrate that FTO demethylates mRNAs, although only a few m^6A-containing mRNAs are targeted. At present it is unknown why certain mRNAs become targeted by FTO. Conceivably, sequence or structural context might account for this selectivity, but further studies are needed to determine the role of such *cis*-acting elements in targeting FTO to specific m^6A residues.

FTO was originally proposed to target m^6A in nuclear RNAs[5]. Nuclear m^6A-containing RNAs include certain noncoding RNAs, U6 snRNA, rRNA, and pre-mRNAs. The proposed selectivity towards nuclear RNA was based on immunostaining results showing that FTO is in nuclear speckles[5]. However, more recent results show that FTO is also found in the cytoplasm in various cell types, including dopaminergic neurons, hypothalamic neurons and mouse embryonic fibroblasts[42, 82, 83]. Thus, FTO may also target cytosolic mRNAs, which would allow FTO to have roles in regulating cytosolic mRNA processing events.

The activity of FTO may involve the formation of oxidized m^6A intermediates. Demethylation of m^6A involves a hydroxylation reaction to form N^6-hydroxymethyl-adenosine (hm^6A) (see **Supplementary Table 1**). This modification is highly labile and spontaneously decomposes to adenosine within a few hours. In vitro oxidative demethylation reactions using FTO also produces other oxidized species, such as N^6-formyl-adenosine (f^6A), in analogy to the diverse oxidized forms of cytosine generated by the FTO-homolog Tet proteins[84]. It is not clear yet whether these labile oxidized intermediates of m^6A have specific functions.

Studies of FTO target specificity and function will be facilitated by small-molecule inhibitors of FTO that will enable temporal regulation of the specific m^6A residues. Rhein, a

bioactive component of rhubarb, is a moderately selective FTO inhibitor[85]. The development of potent and selective inhibitors may be useful for treatment of type 2 diabetes, obesity, and other physiological processes linked to FTO.

ALKBH5

Another recently described m^{6}A demethylase is ALKBH5, an FTO homologue of the AlkB family[6]. A systematic test of each of the nine mammalian AlkB homologues showed that ALKBH5 catalyses the demethylation of an m^{6}A-containing RNA with nearly as high a rate as FTO, and showed specificity for demethylation of m^{6}A over various other methylated nucleotides in single-stranded RNA[6].

ALKBH5 seems to affect mRNA export pathways. In *ALKBH5* knockdown cells, increased levels of poly(A) mRNA were detected in the nucleus[6], suggesting that ALKBH5 influences the expression of protein regulators of mRNA export. A more speculative hypothesis is that the mRNAs retained in the nucleus could be those that are hypermethylated in *ALKBH5* knockdown cells. Although this idea is intriguing, it will require the demonstration that the retained, hypermethylated mRNAs in the nucleus are direct ALKBH5 targets.

ALKBH5-knockout mice grow to adulthood, but with a marked increase in the levels of apoptotic cells in the testes[6], indicating a defect in spermatogenesis. The restriction of the knockout phenotype to testes is consistent with the distribution of the enzyme, which is primarily expressed in testes, with lower expression levels in the spleen and lung. It is intriguing that phenotypes associated with m^{6}A-deficiency in lower organisms relate to gametogenesis and meiosis[57, 59, 64, 75], suggesting a possibly evolutionarily conserved function for m^{6}A in these processes.

It will be important to determine if ALKBH5 demethylates mRNA, noncoding RNAs, or both. *ALKBH5* knockdown increased m^{6}A levels in poly(A) mRNA by ~9%, whereas ~50X overexpression of ALKBH5 reduced m^{6}A levels by ~29%[6]. The subtle changes in m^{6}A levels in cellular poly(A) mRNA suggests that this enzyme, similarly to FTO[42], only demethylates certain m^{6}A residues in mRNAs. Selected mRNAs were tested for their susceptibility to ALKBH5-mediated demethylation, which showed that in some cases, the m^{6}A level increased in *ALKBH5* knockdown cells[6]. The identification of the specific ALKBH5 targets using MeRIP-Seq will be useful for characterizing the specificity of this enzyme.

What is the difference between these two FTO and ALKBH5? Currently, the pathways that activate or inhibit these enzymes are unknown. However, ALKBH5 appears to be markedly enriched in the nucleus[6], unlike FTO, which is readily detected in the cytosol[42, 82, 83]. Thus, ALKBH5 may target nuclear RNAs, whereas FTO may be capable of targeting mature mRNAs. The tissue distribution of these enzymes is another major difference. Whereas FTO is highly brain enriched[77], ALKBH5 is predominantly expressed in testis, with substantially lower levels in other tissues[6]. Thus, ALKBH5 may confer demethylase activity in tissues lacking FTO, and vice versa. MeRIP-Seq analysis of the specific targets of ALKBH5 and FTO will address whether these enzymes have redundant targets or if they each target different types of mRNAs.

Mechanisms of m^{6}A action

Based on the localization of methyltransferase and demethylase enzymes, m^{6}A may be introduced and removed in either the nucleus or the cytosol. Similarly, m^{6}A may have a different effect on an mRNA, depending on whether it is detected in the nucleus or the cytosol. Currently, several functions have been ascribed to m^{6}A that begin to tease apart these compartment-specific m^{6}A roles.

Protein recruitment

m^{6}A is likely to have a role in facilitating RNA—protein interactions. In principle, methylation of adenosine could either block or induce RNA—protein interactions (Figure 3a). To date, several m^{6}A-binding proteins have been identified from mammalian cellular extracts using RNA pulldown approaches followed by mass spectrometry[4, 75, 86]. These include the mammalian proteins YTHDF1, YTHDF2 and YTHDF3, each of which contain a YTH RNA-binding domain[87]. A YTH-domain containing protein, MRB1, was also found to be a m^{6}A-binding protein in yeast[75]. The RNA-binding protein HuR (also known as ELAVL1) was also detected in m^{6}A-RNA pulldowns from mammalian lysates[4]. Several other proteins were shown to bind m^{6}A using yeast extracts [75]. However, since these studies used a pulldown approach, it was not clear which of these proteins directly bind to m^{6}A or are instead part of a m^{6}A-binding ribonucleoprotein complex. Also, it is not clear if these proteins bind m^{6}A in living cells.

YTHDF1, YTHDF2 and YTHDF3 binding to m^{6}A has been validated in vitro[86, 88]. The binding affinity was relatively weak, ranging from 400 nM t~1.2 μM[86, 88]. Although this binding is relatively weak compared with established sequence-specific RNA-protein interactions[89], it is possible that these proteins function by binding transiently to m^{6}A.

Do these proteins bind m^{6}A in living cells? Transcriptome-wide methods for monitoring binding sites of RNA-binding proteins in living cells, such as HITS-CLIP, iCLIP, and PAR-CLIP[90-92] will be essential for assessing the physiologic targets of these RNA-binding proteins. These approaches involve crosslinking of mRNA with target proteins in live cells, followed by the recovery of the target proteins with bound RNA fragments. Analysis of the bound RNA enables the identification of the binding sites within the mRNA at near single nucleotide resolution[90]. The major criteria for establishing that a putative m^{6}A-binding protein is a bona fide interactor is that the binding sites of the protein need to precisely match m^{6}A residues in the transcriptome. An m^{6}A-binding protein might physiologically function to bind to m^{6}A in rRNA or in ncRNAs (such as U6), and not mRNA; therefore overexpression of an m^{6}A-binding protein can lead to nonphysiological binding to mRNA. For this reason, putative m^{6}A-binding proteins and their binding partners should be assessed by analysis of the endogenous protein.

Analysis of the binding sites of the endogenous protein will indicate its physiological binding partners. Comparing the binding sites to m^{6}A peaks obtained by MeRIP-Seq is problematic, as these peaks are ~200 nt-wide regions. Thus, alignments using MeRIP-Seq data will not show if the RNA-binding protein directly binds to the m^{6}A in the mRNA. Single-nucleotide resolution m^{6}A mapping techniques will therefore be needed to

confidently identify m^{6}A-binding proteins. Additionally, it is important to demonstrate that any putative m^{6}A-binding protein shows decreased binding to mRNA in METTL3/14-deficient cells.

What kind of binding selectivity can be achieved with such a small modification like a methyl group? Based on our understanding of protein binding to methylated amino acids, it seems that considerable specificity can be achieved. For example, 53BP1, which binds methyl-Arg, exhibits a >20-fold increase in affinity between the nonmethylated and methylated form of a peptide ligand, 52.9 μM and >1,000 μm, respectively[93]. Similarly, L3MBT1, which binds methyl-Lys in histone proteins, exhibits a ~80-fold increase in affinity between the nonmethylated and methylated forms (6 μM and >500 μM respectively)[94]. Thus, the methyl group on adenosine is may introduce >20-fold binding selectivity.

Conformational changes

m^{6}A can also affect RNA by altering RNA structure or folding. The two hydrogen bonds that constitute the A•U basepair still form if the adenosine is methylated, but they are slightly destabilized[95] (Figure 3b). Although the overall effect of methylation on the stability of an RNA duplex is subtle, it could influence certain types of interactions in which duplex stability is particularly important, such as microRNA—mRNA interactions[96]. Thus, subtle alterations in binding and basepair stability could contribute to some of the effects of m^{6}A in cells.

A more pronounced effect of adenosine methylation would occur on A-A•U or U-A•U base triples. This interaction involves a Hoogsteen basepair between an adenosine or uracil on the adenosine base in a A•U Watson-Crick basepair (Figure 3b). This interaction will not form if the N^6 position is methylated as both hydrogens on this nitrogen are H-bond donors in the base triple. Thus, the formation of A-A•U base triples is blocked by methylation. A-U•A base triples have been seen in several mammalian noncoding RNAs, including MENβ and MALAT1[97]. Interestingly, MALAT1 also contains m^{6}A on the basis of MeRIP-Seq analysis[3]. Thus, methylation could act as a trigger to disrupt RNA structures that depend on hydrogen bonding of both N^6 hydrogens on adenosine.

m^{6}A effects on mRNA fate and function

The mechanisms through which m^{6}A regulates cellular mRNAs are still in the process of being uncovered. However, studies to date have provided important insights into our understanding of how m^{6}A contributes to the processing, localization, and functional roles of mRNAs in cells.

Effects on mRNA splicing

One of the earliest proposed roles for m^{6}A was as a regulator of splicing (Figure 3c). This was initially based on studies that characterized the m^{6}A content of the pre-mRNA in the nucleus, compared to the m^{6}A content in mature cytoplasmic mRNA. In these studies, pre-mRNA was found to be methylated at ~4 m^{6}A residues per mRNA, whereas the mature mRNA was methylated at ~2 m^{6}A residues per mRNA[32]. These studies documented that

methylation occurs in the nucleus, and suggested that the removal of introns resulted in the loss of total m^{6}A content per mRNA.

More recent studies support this idea. PAR-CLIP analysis shows that the vast majority of METTL3-binding sites in transcripts occurs in introns[61]. Thus, intronic sequences may be a major target of nuclear methylation. The localization of METTL3 and METTL14 to nuclear speckles also supported the idea that m^{6}A is linked to splicing, and these sites may enable intronic methylation.

MeRIP-Seq does not fully support the concept that methylation is highly targeted to introns. MeRIP-Seq studies showed that m^{6}A was preferentially found in exons, and only ~5-7% of m^{6}A peaks in introns. However, MeRIP-Seq studies to date have been performed on steady state cellular RNA, which contains a low concentration of the highly labile pre-mRNA and introns. Thus, m^{6}A may be introduced into introns at a relatively high level, which is then followed by rapid intron excision and degradation. Although the prevalence and distribution of m^{6}A within intronic sequences needs to be more thoroughly examined by performing MeRIP-Seq on premRNA, one possibility is that m^{6}A is targeted to specific intronic regions in order to influence splicing efficiency.

The function of m^{6}A in splicing seems to be linked to regulating isoform diversity by alternative splicing. mRNAs that exhibit multiple isoforms due to alternative splicing are significantly more likely to contain m^{6}A and exhibit METTL3 binding than mRNAs that are expressed as only one spliced isoform[4, 61]. Additionally, m^{6}A is more likely to be found in introns and exons that undergo alternative splicing[4]. Thus, m^{6}A appears to contribute to alternative splicing of mRNA. However, a mechanistic relationship between the presence of m^{6}A and splicing events is not yet established.

Regulation of mRNA translation

There are conflicting results as to whether m^{6}A affects protein translation *in vitro*. Early studies using a rabbit reticulocyte lysate system showed that translation of m^{6}A-containing *DHFR* mRNA was increased by about 50% compared with mRNA[98]. However, more recent studies using both *in vitro* translation as well as transfection of reporter mRNAs into cells have shown that adenosine methylation leads to reduced translation compared with unmethylated transcripts[99] (Figure 3d). Thus, the effects of m^{6}A on protein production do not seem to be uniform among different mRNAs. One possibility is that this effect is determined in part by other *cis*-acting factors within the transcript, or perhaps that the location of m^{6}A within an mRNA influences its ability to interact with specific *trans*-acting factors which mediate its effects on translation.

Effects on mRNA expression and degradation

Analysis of differential mRNA expression levels in control and *METTL3* knockdown cells suggested that m^{6}A stabilizes mRNA[4] (Figure 3e). Loss of methylation correlated with reduced expression levels of transcripts that contain m^{6}A[4]. This effect was most prominent for mRNAs that contain m^{6}A in introns. These studies support the general idea that m^{6}A is needed for normal mRNA expression levels. A possible explanation for these data is that

m^{6}A is needed for proper splicing of mRNA, which when disrupted results in impaired splicing and subsequent mRNA degradation.

Studies using *FTO*-knockout mice provide an opportunity to determine how altered m^{6}A levels influence mRNA abundance and protein production. In these mice, five mRNAs that have been tested had increased m^{6}A peaks (that is, that are hypermethylated) and also exhibited slightly increased mRNA levels, but there was no consistent effect on the level of the encoded proteins: in some cases, protein levels were decreased, whereas for other transcripts the protein levels were slightly increased or were unchanged[42].

Furthermore, other reports suggest that m^{6}A promotes mRNA degradation[63, 86]. One study focused on YTHDF2, a mammalian protein that has been identified to bind to m^{6}A using pulldown approaches[86]. YTHDF2 was shown to promote mRNA degradation of thousands of cellular transcripts[86]. Consistent with this idea, YTHDF2 is found in a subset of cellular P-bodies. Additionally, PAR-CLIP studies probing the binding sites of overexpressed epitope-tagged YTHDF2 identified target mRNAs that are also known to contain m^{6}A. In some transcripts, the PAR-CLIP site seems to overlap with the m^{6}A peak[86]. Further analysis will be required to define the specific identity and number of m^{6}A-containing mRNAs that are subjected to YTHDF2 regulation in cells.

Another study used *METTL3* and *METTL14* knockdown to identify mRNAs that were methylated by these methyltransferases, and monitored the stability of these mRNAs in mouse embryonic stem cells[63]. In knockdown cells, many METTL3- and METTL14-target mRNAs showed a modest increase in stability, suggesting that m^{6}A functioned to induce mRNA stability. The authors proposed that m^{6}A displaces HuR, a known mRNA stabilizer. Indeed, *in vitro* HuR binding to its typical U-rich binding site was impaired by adjacent m^{6}A residues[63]. Previous studies had suggested that HuR might be an m^{6}A-binding protein[4]; however, this more recent analysis argued that m^{6}A reduces HuR binding, thereby facilitating mRNA degradation.

In both of these studies, the effect of m^{6}A on mRNA stability was measured by monitoring total mRNA levels. Since every mRNA exists as a pool of both methylated and nonmethylated forms, it will be important to directly follow the fate of only the mRNA molecules that contain m^{6}A. This is important, as the stoichiometry of m^{6}A in the vast majority of m^{6}A-containing mRNAs is likely to be very low. These experiments will be useful to distinguish between the functional roles of YTHDF2 and HuR in mRNA degradation.

Relationship between m^{6}A and microRNAs

Analysis of the MeRIP-Seq dataset revealed a strong correlation between the presence of m^{6}A and microRNA-binding sites[3]. 67% of 3'UTRs that contain m^{6}A peaks also contain at least one TargetScan-predicted microRNA-binding site. Because ~30% of genes have microRNA-binding sites in their 3'UTRs[100], this is a significantly enhanced association. Importantly, the m^{6}A peak and microRNA site did not overlap. In general, the m^{6}A peaks are most abundant near the stop codon and generally decrease in frequency along the 3'UTR length, whereas the microRNA target sites are enriched at the 5' and 3' end of 3'UTRs[44].

The strong association between m^{6}A in 3'UTRs and the presence of microRNA-binding sites suggests an interplay between mRNA methylation and microRNAs. It is possible that the presence of a microRNA-binding site induces pathways that lead to mRNA methylation. Indeed, in the brain, mRNAs containing brain-enriched microRNA-binding sites were more likely to have an m^{6}A than mRNAs that contain sites for microRNAs that are not abundant in brain[3]. Further studies will be necessary to determine whether microRNAs contribute to the induction of methylation at upstream regions of the transcript. Additionally, it will be important to determine whether adenosine methylation contributes to the actions of microRNA-induced mRNA silencing.

Future directions

The results of transcriptome-wide m^{6}A mapping techniques have resolved many of the longstanding concerns regarding the physiological relevance of m^{6}A and have renewed interest in this mRNA modification. Currently, an understanding of the function of m^{6}A and how mRNA methylation is regulated remain poorly understood, despite many important recent advances. Resolving these issues will require efforts on several different fronts.

An important next step will be to develop approaches for higher resolution mapping of m^{6}A in the mammalian transcriptome. This will allow researchers to selectively mutate specific adenosine residues and determine how methylation regulates the fate of an mRNA. Furthermore, precise m^{6}A mapping is needed to define the specific m^{6}A residues that are bound to putative m^{6}A-binding proteins. MeRIP-Seq involves immunoprecipitation of ~100-nt long fragments, which therefore results in peaks that are approximately 200 nt in resolution. The use of smaller RNA fragments has recently been described, which provides greater resolution[75]. This approach was used to map m^{6}A sites in yeast, and its applicability to more complex transcriptomes has not yet been established. Other approaches to achieve finer resolution can be envisaged, for example by crosslinking cellular RNA to m^{6}A antibodies and analysing the crosslinking site using methods that have been highly optimized for HITS-CLIP and related techniques[90-92]. Last, the inclusion of control RNAs with known m^{6}A stoichiometry could be used as standards to obtain absolute measures of m^{6}A stoichiometry. These strategies will be essential to more precisely define the specific modified m^{6}A residues in the transcriptome and the degree of methylation at specific adenosines within a transcript.

A major goal will be to determine how m^{6}A affects the fate of mRNA. In addition, there is a need to understand whether all m^{6}As have the same function, or whether their distribution within a transcript dictates their role in mRNA processing. A crucial step in this process will be the identification of high-affinity m^{6}A-binding proteins, and the demonstration that endogenous protein binds mRNAs at m^{6}A residues. This requires, in part, mapping of m^{6}A at higher resolution than is currently available. The demonstration that m^{6}A-binding proteins are indeed binding m^{6}A should in turn enable the characterization of the mechanisms through which m^{6}A regulates mRNAs.

One of the exciting aspects of m^{6}A is its potential to be reversed by FTO or ALKBH5. As mutations in *FTO* are linked to various diseases and altered neuronal function [42, 101, 102], the

identification of physiological signaling pathways that trigger m^6A demethylation are particularly important. However, evidence for dynamic reversibility of m^6A in mRNA has not been established. The recent identification of mRNAs that are targeted by FTO[42] is a first step in deciphering these demethylation pathways.

Similarly, it is not clear why certain mRNAs are targeted for methylation. This is an outstanding puzzle, as all mRNAs contain the G-A*-C consensus motif, yet only a fraction of cellular mRNAs seem to be targeted for methylation. Thus, the factors that determine whether an mRNA will be methylated are unknown. Identifying *cis*-acting elements in mRNAs that render an mRNA susceptible to methylation will be important for ultimately deciphering the methylation specificity in cells.

As mentioned above, m^6A stoichiometry varies greatly among different mRNAs. The factor that makes an mRNA a target for high stoichiometry methylation remains unclear, but is likely to be the trigger that shunts an mRNA into an m^6A-regulated processing pathway. Insights are likely to come from a more comprehensive assessment of the components of the methyltransferase complexes. Although some components have been identified, such as WTAP-1, the identification of the remaining components will likely provide insight into the basis for methylation specificity.

It remains unclear whether specific signalling pathways use mRNA methylation as an effector mechanism. Conceivably, signalling mechanisms could recruit methyltransferases or demethylases to alter m^6A levels and thereby influence mRNA fate. mRNA methylation is already known to have highly specific effects on cellular processes. For example, increased adenosine methyltransferase activity is correlated with cellular transformation[103]. Furthermore, in yeast, induction of meiosis coincides with increased expression of the METTL3 homologue IME4 and concomitant m^6A accumulation[58, 104]. IME4 also affects the developmental response to nutrient starvation in yeast[64]. These studies show that regulated adenosine methylation influences cellular processes. Ultimately, by identifying the signalling pathways that rely on mRNA methylation and demethylation, we will gain a better appreciation for the breadth of physiological roles that involve m^6A.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Biography

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Glossary

rRNA	RNAs that are a highly abundant species of cellular RNA that function in complex with ribosomes to mediate mRNA translation.
snRNA (Small nuclear RNA)	RNAs that associate with specific proteins and are frequently involved in pre-mRNA processing events such as splicing.
non-coding RNAs (ncRNAs)	RNAs that are not translated into proteins. ncRNAs include functional RNAs such as transfer RNAs, microRNAs and long ncRNAs.
Nuclear speckles	Small, dynamic, subnuclear structures that are enriched in pre-mRNA splicing factors.
Fe(II)/2-oxoglutaratedependent oxygenases	A family of proteins that catalyzes cellular oxidative reactions, most notably hydroxylation. Several members of this family are involved in nucleic acid demethylation.
Zero-mode waveguides	Nanostructures with highly confined optical observation volumes.

REFERENCES

1. Lewis JD, et al. Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. Cell. 1992; 69:905–14. [PubMed: 1606614]
2. Pawson T, Scott JD. Protein phosphorylation in signaling--50 years and counting. Trends Biochem Sci. 2005; 30:286–90. [PubMed: 15950870]
3. Meyer KD, et al. Comprehensive analysis of mRNA methylation reveals enrichment in 3' UTRs and near stop codons. Cell. 2012; 149:1635–46. [PubMed: 22608085] [Provides the first demonstration that m6A is a widespread modification in mammalian mRNAs and reveals that m6A is highly enriched surrounding stop codons and in UTRs. Also identifies many methylated noncoding RNAs which were not previously known to contain m6A.]
4. Dominissini D, et al. Topology of the human and mouse m6A RNA methylomes revealed by m^6A-seq. Nature. 2012; 485:201–6. [PubMed: 22575960] [Demonstrates, together with reference 3, that m6A is a pervasive feature of the transcriptome which exhibits a unique distribution within mRNAs. Identifies YTHDF2-3 and HuR as potential m6A binding proteins.]
5. Jia G, et al. N^6-methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. Nat Chem Biol. 2011; 7:885–7. [PubMed: 22002720] [Reveals that the obesity-associated protein, FTO, is capable of demethylating m6A residues in mRNA and points to the reversibility of this modification.]

6. Zheng G, et al. ALKBH5 Is a Mammalian RNA Demethylase that Impacts RNA Metabolism and Mouse Fertility. Mol Cell. 2013; 49:18–29. [PubMed: 23177736]
7. Perry RP, Kelley DE. Existence of methylated messenger RNA in mouse L cells. Cell. 1974; 1:37–42.
8. Desrosiers R, Friderici K, Rottman F. Identification of methylated nucleosides in messenger RNA from Novikoff hepatoma cells. Proc Natl Acad Sci U S A. 1974; 71:3971–5. [PubMed: 4372599]
9. Lavi S, Shatkin AJ. Methylated simian virus 40-specific RNA from nuclei and cytoplasm of infected BSC-1 cells. Proc Natl Acad Sci U S A. 1975; 72:2012–6. [PubMed: 166375]
10. Wei CM, Moss B. Methylated nucleotides block 5'-terminus of vaccinia virus messenger RNA. Proc Natl Acad Sci U S A. 1975; 72:318–22. [PubMed: 164018]
11. Furuichi Y, et al. Methylated, blocked 5 termini in HeLa cell mRNA. Proc Natl Acad Sci U S A. 1975; 72:1904–8. [PubMed: 1057180]
12. Adams JM, Cory S. Modified nucleosides and bizarre 5'-termini in mouse myeloma mRNA. Nature. 1975; 255:28–33. [PubMed: 1128665]
13. Dubin DT, Taylor RH. The methylation state of poly A-containing messenger RNA from cultured hamster cells. Nucleic Acids Res. 1975; 2:1653–68. [PubMed: 1187339]
14. Wei C, Gershowitz A, Moss B. N6, O2'-dimethyladenosine a novel methylated ribonucleoside next to the 5' terminal of animal cell and virus mRNAs. Nature. 1975; 257:251–3. [PubMed: 1161029]
15. Schmidt W, Arnold HH, Kersten H. Biosynthetic pathway of ribothymidine in B. subtilis and M. lysodeikticus involving different coenzymes for transfer RNA and ribosomal RNA. Nucleic Acids Res. 1975; 2:1043–51. [PubMed: 807911]
16. Tanaka T, Weisblum B. Systematic difference in the methylation of ribosomal ribonucleic acid from gram-positive and gram-negative bacteria. J Bacteriol. 1975; 123:771–4. [PubMed: 807565]
17. Munns TW, Sims HF, Liszewski MK. Immunospecific retention of oligonucleotides possessing N6-methyladenosine and 7-methylguanosine. J Biol Chem. 1977; 252:3102–4. [PubMed: 323262]
18. Epstein P, Reddy R, Henning D, Busch H. The nucleotide sequence of nuclear U6 (4.7 S) RNA. J Biol Chem. 1980; 255:8901–6. [PubMed: 6773955]
19. Harada F, Kato N, Nishimura S. The nucleotide sequence of nuclear 4.8S RNA of mouse cells. Biochem Biophys Res Commun. 1980; 95:1332–40. [PubMed: 6251836]
20. Dimock K, Stoltzfus CM. Sequence specificity of internal methylation in B77 avian sarcoma virus RNA subunits. Biochemistry. 1977; 16:471–8. [PubMed: 189800]
21. Beemon K, Keith J. Localization of N6-methyladenosine in the Rous sarcoma virus genome. J Mol Biol. 1977; 113:165–79. [PubMed: 196091]
22. Furuichi Y, Shatkin AJ, Stavnezer E, Bishop JM. Blocked, methylated 5'-terminal sequence in avian sarcoma virus RNA. Nature. 1975; 257:618–20. [PubMed: 170541]
23. Sommer S, et al. The methylation of adenovirus-specific nuclear and cytoplasmic RNA. Nucleic Acids Res. 1976; 3:749–65. [PubMed: 1272797]
24. Canaani D, Kahana C, Lavi S, Groner Y. Identification and mapping of N6-methyladenosine containing sequences in simian virus 40 RNA. Nucleic Acids Res. 1979; 6:2879–99. [PubMed: 223130]
25. Perry RP, Kelley DE, Friderici K, Rottman F. The methylated constituents of L cell messenger RNA: evidence for an unusual cluster at the 5' terminus. Cell. 1975; 4:387–94. [PubMed: 1168101]
26. Choi YC, Busch H. Modified nucleotides in T1 RNase oligonucleotides of 18S ribosomal RNA of the Novikoff hepatoma. Biochemistry. 1978; 17:2551–60. [PubMed: 209819]
27. Bringmann P, Luhrmann R. Antibodies specific for N6-methyladenosine react with intact snRNPs U2 and U4/U6. FEBS Lett. 1987; 213:309–15. [PubMed: 2951275]
28. Shimba S, Bokar JA, Rottman F, Reddy R. Accurate and efficient N-6-adenosine methylation in spliceosomal U6 small nuclear RNA by HeLa cell extract in vitro. Nucleic Acids Res. 1995; 23:2421–6. [PubMed: 7630720]
29. Perlman S, Abelson HT, Penman S. Mitochondrial protein synthesis: RNA with the properties of Eukaryotic messenger RNA. Proc Natl Acad Sci U S A. 1973; 70:350–3. [PubMed: 4510280]

30. Nagaike T, Suzuki T, Ueda T. Polyadenylation in mammalian mitochondria: insights from recent studies. Biochim Biophys Acta. 2008; 1779:266–9. [PubMed: 18312863]
31. Slomovic S, Laufer D, Geiger D, Schuster G. Polyadenylation of ribosomal RNA in human cells. Nucleic Acids Res. 2006; 34:2966–75. [PubMed: 16738135]
32. Salditt-Georgieff M, et al. Methyl labeling of HeLa cell hnRNA: a comparison with mRNA. Cell. 1976; 7:227–37. [PubMed: 954080]
33. Okazaki Y, et al. Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature. 2002; 420:563–73. [PubMed: 12466851]
34. Numata K, et al. Identification of putative noncoding RNAs among the RIKEN mouse full-length cDNA collection. Genome Res. 2003; 13:1301–6. [PubMed: 12819127]
35. Ravasi T, et al. Experimental validation of the regulated expression of large numbers of non-coding RNAs from the mouse genome. Genome Res. 2006; 16:11–9. [PubMed: 16344565]
36. Jacquier A. The complex eukaryotic transcriptome: unexpected pervasive transcription and novel small RNAs. Nat Rev Genet. 2009; 10:833–44. [PubMed: 19920851]
37. Csepany T, Lin A, Baldick CJ Jr. Beemon K. Sequence specificity of mRNA N6-adenosine methyltransferase. J Biol Chem. 1990; 265:20117–22. [PubMed: 2173695]
38. Kane SE, Beemon K. Inhibition of methylation at two internal N6-methyladenosine sites caused by GAC to GAU mutations. J Biol Chem. 1987; 262:3422–7. [PubMed: 3029112]
39. Kane SE, Beemon K. Precise localization of m6A in Rous sarcoma virus RNA reveals clustering of methylation sites: implications for RNA processing. Mol Cell Biol. 1985; 5:2298–306. [PubMed: 3016525]
40. Carroll SM, Narayan P, Rottman FM. N6-methyladenosine residues in an intron-specific region of prolactin pre-mRNA. Mol Cell Biol. 1990; 10:4456–65. [PubMed: 2388614]
41. Horowitz S, Horowitz A, Nilsen TW, Munns TW, Rottman FM. Mapping of N6-methyladenosine residues in bovine prolactin mRNA. Proc Natl Acad Sci U S A. 1984; 81:5667–71. [PubMed: 6592581]
42. Hess ME, et al. The fat mass and obesity associated gene (Fto) regulates activity of the dopaminergic midbrain circuitry. Nat Neurosci. 2013; 16:1042–8. [PubMed: 23817550]
43. Saletore Y, et al. The birth of the Epitranscriptome: deciphering the function of RNA modifications. Genome Biol. 2012; 13:175. [PubMed: 23113984]
44. Grimson A, et al. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell. 2007; 27:91–105. [PubMed: 17612493]
45. Baltz AG, et al. The mRNA-bound proteome and its global occupancy profile on protein-coding transcripts. Mol Cell. 2012; 46:674–90. [PubMed: 22681889]
46. Li F, et al. Global analysis of RNA secondary structure in two metazoans. Cell Rep. 2012; 1:69–82. [PubMed: 22832108]
47. Elemento O, Slonim N, Tavazoie S. A universal framework for regulatory element discovery across all genomes and data types. Mol Cell. 2007; 28:337–50. [PubMed: 17964271]
48. Wei CM, Gershowitz A, Moss B. 5'-Terminal and internal methylated nucleotide sequences in HeLa cell mRNA. Biochemistry. 1976; 15:397–401. [PubMed: 174715]
49. Wei CM, Moss B. Nucleotide sequences at the N6-methyladenosine sites of HeLa cell messenger ribonucleic acid. Biochemistry. 1977; 16:1672–6. [PubMed: 856255]
50. Schibler U, Kelley DE, Perry RP. Comparison of methylated sequences in messenger RNA and heterogeneous nuclear RNA from mouse L cells. J Mol Biol. 1977; 115:695–714. [PubMed: 592376]
51. Harper JE, Miceli SM, Roberts RJ, Manley JL. Sequence specificity of the human mRNA N6-adenosine methylase in vitro. Nucleic Acids Res. 1990; 18:5735–41. [PubMed: 2216767]
52. Bokar JA, Rath-Shambaugh ME, Ludwiczak R, Narayan P, Rottman F. Characterization and partial purification of mRNA N6-adenosine methyltransferase from HeLa cell nuclei. Internal mRNA methylation requires a multisubunit complex. J Biol Chem. 1994; 269:17697–704. [PubMed: 8021282]
53. Martin C, Zhang Y. The diverse functions of histone lysine methylation. Nat Rev Mol Cell Biol. 2005; 6:838–49. [PubMed: 16261189]

54. Bokar JA, Shambaugh ME, Polayes D, Matera AG, Rottman FM. Purification and cDNA cloning of the AdoMet-binding subunit of the human mRNA (N6-adenosine)-methyltransferase. RNA. 1997; 3:1233–47. [PubMed: 9409616] [Identifies METTL3 as a key component of the m6A methyltransferase complex.]
55. Rottman FM, Bokar JA, Narayan P, Shambaugh ME, Ludwiczak R. N6-adenosine methylation in mRNA: substrate specificity and enzyme complexity. Biochimie. 1994; 76:1109–14. [PubMed: 7748945]
56. Narayan P, Ludwiczak RL, Goodwin EC, Rottman FM. Context effects on N6-adenosine methylation sites in prolactin mRNA. Nucleic Acids Res. 1994; 22:419–26. [PubMed: 8127679]
57. Zhong S, et al. MTA is an Arabidopsis messenger RNA adenosine methylase and interacts with a homolog of a sex-specific splicing factor. Plant Cell. 2008; 20:1278–88. [PubMed: 18505803]
58. Clancy MJ, Shambaugh ME, Timpte CS, Bokar JA. Induction of sporulation in Saccharomyces cerevisiae leads to the formation of N6-methyladenosine in mRNA: a potential mechanism for the activity of the IME4 gene. Nucleic Acids Res. 2002; 30:4509–18. [PubMed: 12384598]
59. Hongay CF, Orr-Weaver TL. Drosophila Inducer of MEiosis 4 (IME4) is required for Notch signaling during oogenesis. Proc Natl Acad Sci U S A. 2011; 108:14855–60. [PubMed: 21873203]
60. Havugimana PC, et al. A census of human soluble protein complexes. Cell. 2012; 150:1068–81. [PubMed: 22939629]
61. Ping XL, et al. Mammalian WTAP is a regulatory subunit of the RNA N^6-methyladenosine methyltransferase. Cell Res. 2014 [Identifies, together with reference 62, WTAP as additional components of the mammalian m6A mRNA methyltransferase complex.]
62. Liu J, et al. A METTL3-METTL14 complex mediates mammalian nuclear RNA N(6)-adenosine methylation. Nat Chem Biol. 2014; 10:93–5. [PubMed: 24316715]
63. Wang Y, et al. N(6)-methyladenosine modification destabilizes developmental regulators in embryonic stem cells. Nat Cell Biol. 2014; 16:191–8. [PubMed: 24394384] [Indentifies, together with references 61 and 62, METTL14 as an adenosine methyltransferase and a component of the m6A mRNA methyltransferase complex. Reference 63 also shows that that m6A in mouse embryonic stem cells is required for stem cell differentiation.]
64. Agarwala SD, Blitzblau HG, Hochwagen A, Fink GR. RNA methylation by the MIS complex regulates a cell fate decision in yeast. PLoS Genet. 2012; 8:e1002732. [PubMed: 22685417]
65. Horiuchi K, et al. Identification of Wilms' tumor 1-associating protein complex and its role in alternative splicing and the cell cycle. J Biol Chem. 2013; 288:33292–302. [PubMed: 24100041]
66. Narayan P, Rottman FM. An in vitro system for accurate methylation of internal adenosine residues in messenger RNA. Science. 1988; 242:1159–62. [PubMed: 3187541]
67. Uhlen M, et al. Towards a knowledge-based Human Protein Atlas. Nat Biotechnol. 2010; 28:1248–50. [PubMed: 21139605]
68. Leach RA, Tuck MT. Methionine depletion induces transcription of the mRNA (N6-adenosine)methyltransferase. Int J Biochem Cell Biol. 2001; 33:1116–28. [PubMed: 11551827]
69. Leach RA, Tuck MT. Expression of the mRNA (N6-adenosine)-methyltransferase S-adenosyl-L-methionine binding subunit mRNA in cultured cells. Int J Biochem Cell Biol. 2001; 33:984–99. [PubMed: 11470232]
70. Petry S, Weixlbaumer A, Ramakrishnan V. The termination of translation. Curr Opin Struct Biol. 2008; 18:70–7. [PubMed: 18206363]
71. Czaplinski K, et al. The surveillance complex interacts with the translation release factors to enhance termination and degrade aberrant mRNAs. Genes Dev. 1998; 12:1665–77. [PubMed: 9620853]
72. Dahlberg JE, Lund E. Does protein synthesis occur in the nucleus? Curr Opin Cell Biol. 2004; 16:335–8. [PubMed: 15145360]
73. Bujnicki JM, Feder M, Radlinska M, Blumenthal RM. Structure prediction and phylogenetic analysis of a functionally diverse family of proteins homologous to the MTA70 subunit of the human mRNA:m(6)A methyltransferase. J Mol Evol. 2002; 55:431–44. [PubMed: 12355263]
74. Tuck MT. Partial purification of a 6-methyladenine mRNA methyltransferase which modifies internal adenine residues. Biochem J. 1992; 288(Pt 1):233–40. [PubMed: 1445268]

75. Schwartz S, et al. High-resolution mapping reveals a conserved, widespread, dynamic mRNA methylation program in yeast meiosis. Cell. 2013; 155:1409–21. [PubMed: 24269006]
76. Sanchez-Pulido L, Andrade-Navarro MA. The FTO (fat mass and obesity associated) gene codes for a novel member of the non-heme dioxygenase superfamily. BMC Biochem. 2007; 8:23. [PubMed: 17996046]
77. Gerken T, et al. The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. Science. 2007; 318:1469–72. [PubMed: 17991826]
78. Falnes PO, Johansen RF, Seeberg E. AlkB-mediated oxidative demethylation reverses DNA damage in Escherichia coli. Nature. 2002; 419:178–82. [PubMed: 12226668]
79. Trewick SC, Henshaw TF, Hausinger RP, Lindahl T, Sedgwick B. Oxidative demethylation by Escherichia coli AlkB directly reverts DNA base damage. Nature. 2002; 419:174–8. [PubMed: 12226667]
80. Jia G, et al. Oxidative demethylation of 3-methylthymine and 3-methyluracil in single-stranded DNA and RNA by mouse and human FTO. FEBS Lett. 2008; 582:3313–9. [PubMed: 18775698]
81. Maden BE, Corbett ME, Heeney PA, Pugh K, Ajuh PM. Classical and novel approaches to the detection and localization of the numerous modified nucleotides in eukaryotic ribosomal RNA. Biochimie. 1995; 77:22–9. [PubMed: 7599273]
82. Cheung MK, Gulati P, O'Rahilly S, Yeo GS. FTO expression is regulated by availability of essential amino acids. Int J Obes (Lond). 2012
83. Vujovic P, et al. Fasting induced cytoplasmic Fto expression in some neurons of rat hypothalamus. PLoS One. 2013; 8:e63694. [PubMed: 23671692]
84. Ito S, et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science. 2011; 333:1300–3. [PubMed: 21778364]
85. Chen B, et al. Development of cell-active N6-methyladenosine RNA demethylase FTO inhibitor. J Am Chem Soc. 2012; 134:17963–71. [PubMed: 23045983]
86. Wang X, et al. N^6-methyladenosine-dependent regulation of messenger RNA stability. Nature. 2014; 505:117–20. [PubMed: 24284625] [Shows that the YTHDF2 m6A-binding protein can destabilize target mRNAs by recruiting them to cellular mRNA decay sites.]
87. Zhang Z, et al. The YTH domain is a novel RNA binding domain. J Biol Chem. 2010; 285:14701–10. [PubMed: 20167602]
88. Fu Y, et al. FTO-mediated formation of N(6)-hydroxymethyladenosine and N(6)-formyladenosine in mammalian RNA. Nat Commun. 2013; 4:1798. [PubMed: 23653210]
89. Yang X, Li H, Huang Y, Liu S. The dataset for protein-RNA binding affinity. Protein Sci. 2013
90. Zhang C, Darnell RB. Mapping in vivo protein-RNA interactions at single-nucleotide resolution from HITS-CLIP data. Nat Biotechnol. 2011; 29:607–14. [PubMed: 21633356]
91. Konig J, et al. iCLIP reveals the function of hnRNP particles in splicing at individual nucleotide resolution. Nat Struct Mol Biol. 2010; 17:909–15. [PubMed: 20601959]
92. Sugimoto Y, et al. Analysis of CLIP and iCLIP methods for nucleotide-resolution studies of protein-RNA interactions. Genome Biol. 2012; 13:R67. [PubMed: 22863408]
93. Botuyan MV, et al. Structural basis for the methylation state-specific recognition of histone H4-K20 by 53BP1 and Crb2 in DNA repair. Cell. 2006; 127:1361–73. [PubMed: 17190600]
94. Li H, et al. Structural basis for lower lysine methylation state-specific readout by MBT repeats of L3MBTL1 and an engineered PHD finger. Mol Cell. 2007; 28:677–91. [PubMed: 18042461]
95. Kierzek E, Kierzek R. The thermodynamic stability of RNA duplexes and hairpins containing N6-alkyladenosines and 2-methylthio-N6-alkyladenosines. Nucleic Acids Res. 2003; 31:4472–80. [PubMed: 12888507]
96. Hibio N, Hino K, Shimizu E, Nagata Y, Ui-Tei K. Stability of miRNA 5'terminal and seed regions is correlated with experimentally observed miRNA-mediated silencing efficacy. Sci Rep. 2012; 2:996. [PubMed: 23251782]
97. Brown JA, Valenstein ML, Yario TA, Tycowski KT, Steitz JA. Formation of triple-helical structures by the 3'-end sequences of MALAT1 and MENbeta noncoding RNAs. Proc Natl Acad Sci U S A. 2012; 109:19202–7. [PubMed: 23129630]

98. Heilman KL, Leach RA, Tuck MT. Internal 6-methyladenine residues increase the in vitro translation efficiency of dihydrofolate reductase messenger RNA. Int J Biochem Cell Biol. 1996; 28:823–9. [PubMed: 8925412]

99. Kariko K, et al. Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. Mol Ther. 2008; 16:1833–40. [PubMed: 18797453]

100. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005; 120:15–20. [PubMed: 15652477]

101. Yeo GS. FTO and obesity: a problem for a billion people. J Neuroendocrinol. 2012; 24:393–4. [PubMed: 22248259]

102. Garcia-Closas M, et al. Genome-wide association studies identify four ER negative-specific breast cancer risk loci. Nat Genet. 2013; 45:392–8. 398e1–2. [PubMed: 23535733]

103. Tuck MT, James CB, Kelder B, Kopchick JJ. Elevation of internal 6-methyladenine mRNA methyltransferase activity after cellular transformation. Cancer Lett. 1996; 103:107–13. [PubMed: 8616802]

104. Bodi Z, Button JD, Grierson D, Fray RG. Yeast targets for mRNA methylation. Nucleic Acids Res. 2010; 38:5327–35. [PubMed: 20421205]

105. Loos RJ, Bouchard C. FTO: the first gene contributing to common forms of human obesity. Obes Rev. 2008; 9:246–50. [PubMed: 18373508]

106. Fischer J, et al. Inactivation of the Fto gene protects from obesity. Nature. 2009; 458:894–8. [PubMed: 19234441]

107. Church C, et al. Overexpression of Fto leads to increased food intake and results in obesity. Nat Genet. 2010; 42:1086–92. [PubMed: 21076408]

108. Iles MM, et al. A variant in FTO shows association with melanoma risk not due to BMI. Nat Genet. 2013; 45:428–32. 432e1. [PubMed: 23455637]

109. Fustin J-M, et al. RNA-Methylation-Dependent RNA Processing Controls the Speed of the Circadian Clock. Cell. 2013; 155:793–806. [PubMed: 24209618] [Demonstrates that inhibition of adenosine methylation alters the circadian period of target mRNAs and disrupts mRNA processing.]

110. Bodi Z, et al. Adenosine Methylation in Arabidopsis mRNA is Associated with the 3' End and Reduced Levels Cause Developmental Defects. Front Plant Sci. 2012; 3:48. [PubMed: 22639649]

111. Gu M, Lima CD. Processing the message: structural insights into capping and decapping mRNA. Curr Opin Struct Biol. 2005; 15:99–106. [PubMed: 15718140]

112. Muthukrishnan S, Both GW, Furuichi Y, Shatkin AJ. 5'-Terminal 7-methylguanosine in eukaryotic mRNA is required for translation. Nature. 1975; 255:33–7. [PubMed: 165427]

113. Shatkin AJ, et al. 5'-Terminal caps, cap-binding proteins and eukaryotic mRNA function. Biochem Soc Symp. 1982; 47:129–43. [PubMed: 6765492]

114. Schibler U, Perry RP. The 5'-termini of heterogeneous nuclear RNA: a comparison among molecules of different sizes and ages. Nucleic Acids Res. 1977; 4:4133–49. [PubMed: 600792]

115. Ishikawa M, Murai R, Hagiwara H, Hoshino T, Suyama K. Preparation of eukaryotic mRNA having differently methylated adenosine at the 5'-terminus and the effect of the methyl group in translation. Nucleic Acids Symp Ser (Oxf). 2009:129–30.

116. Marcotrigiano J, Gingras AC, Sonenberg N, Burley SK. Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell. 1997; 89:951–61. [PubMed: 9200613]

117. Kong H, et al. Functional analysis of putative restriction-modification system genes in the Helicobacter pylori J99 genome. Nucleic Acids Res. 2000; 28:3216–23. [PubMed: 10954588]

118. Dai Q, et al. Identification of recognition residues for ligation-based detection and quantitation of pseudouridine and N6-methyladenosine. Nucleic Acids Res. 2007; 35:6322–9. [PubMed: 17881375]

119. Harcourt EM, Ehrenschwender T, Batista PJ, Chang HY, Kool ET. Identification of a selective polymerase enables detection of N(6)-methyladenosine in RNA. J Am Chem Soc. 2013; 135:19079–82. [PubMed: 24328136]

120. Liu N, et al. Probing N6-methyladenosine RNA modification status at single nucleotide resolution in mRNA and long noncoding RNA. RNA. 2013; 19:1848–56. [PubMed: 24141618]

121. Levene MJ, et al. Zero-mode waveguides for single-molecule analysis at high concentrations. Science. 2003; 299:682–6. [PubMed: 12560545]

122. Vilfan ID, et al. Analysis of RNA base modification and structural rearrangement by single-molecule real-time detection of reverse transcription. J Nanobiotechnology. 2013; 11:8. [PubMed: 23552456]

123. Roberts A, Trapnell C, Donaghey J, Rinn JL, Pachter L. Improving RNA-Seq expression estimates by correcting for fragment bias. Genome Biol. 2011; 12:R22. [PubMed: 21410973]

Box 1: Physiological processes linked to m^{6}A

Obesity

Diverse mutations within intron 1 of human *FTO* have been found and are associated with increased body weight [105]. Carriers of one or two copies of the mutant allele are on average 2.6 lb or 6.6 lb, respectively, heavier than individuals with normal alleles[105]. *FTO*$^{-/-}$ mice have lower body mass[106], whereas FTO overexpression leads to increased food intake and obesity [107]. The intronic mutations in humans likely influence *FTO* gene expression, although the underlying mechanisms remain poorly understood.

Synaptic signaling

FTO-knockout mice have impaired dopamine release, reduced dopaminergic receptor responses and an altered locomotor response to cocaine [42]Select mRNAs involved in dopaminergic signalling pathways are hypermethylated in these mice, which may underlie their neurobiological and behavioural phenotypes. However, the consequences of mRNA hypermethylation on transcript stability and protein production are complex, and further studies are needed to determine the effects of FTO-induced demethylation in specific neuronal subtypes and signalling pathways.

Cancer

Recent genome-wide association studies have demonstrated that intron 1 *FTO* mutations are also risk factors for estrogen receptor negative breast cancer[102]. Additional studies have identified mutations within intron 8 of *FTO* which lead to increased melanoma risk independently of any effects on body mass index (BMI)[108]. These studies identify for the first time a set of *FTO* SNPs which are not associated with BMI but predispose to cancer. Although it remains to be determined how these intron 8 mutations affect the demethylase activity of FTO, they are postulated to influence *FTO* mRNA expression levels,.

Sperm development

Consistent with the high expression level of ALKBH5 in testes, *ALKBH5* knockout mice exhibit reduced testicular size and abnormal morphology [6]. They also have impaired spermatogenesis and increased apoptosis, which probably contributes to the reduced fertility observed in these mice. This phenotype may be explained in part by the altered expression level of genes in the spermatogenesis and P53 apoptotic pathways observed in *ALKBH5*$^{-/-}$ mice[6]; however, whether hypermethylation of these mRNAs underlies their alternative expression pattern remains to be determined.

Stem cell differentiation

m^{6}A is required for self-renewal capacity of human embryonic stem cells and is found in critical pluripotency regulators[63]; dynamic changes in m^{6}A levels are seen during stem cell differentiation[63].

Circadian periods

The global methylation inhibitor 3-deazaadenosine (DAA) causes an extension of the normal circadian period both in cells and in mice. Moreover, MeRIP-Seq analysis shows that m^{6}A decreases in several clock genes following DAA treatment in cells. This correlates with prolonged nuclear retention in clock transcripts *Per2* and *Arntl*[109]. Similar results were also observed after *METTL3* knockdown. Thus, adenosine methylation may influence the cyclic expression of mRNAs encoding clock genes.

Yeast meiosis

mRNA methylation in *Saccharomyces cerevisiae* selectively occurs during meiosis[64] and is mediated by a core RNA methyltransferase complex. This complex, termed MIS, comprises Ime4 (an orthologue of mammalian METTL3), Mum2 (an orthologue of the mammalian Wilm's-tumor-1-associated protein WTAP) and Slz1[64]. The MIS complex localizes to the nucleolus during meiosis, and is required for the proper time course of meiosis[75].

Plant development

m^{6}A contributes to plant embryonic development, and is required for normal growth patterns, apical dominance, and floral development [57, 110]

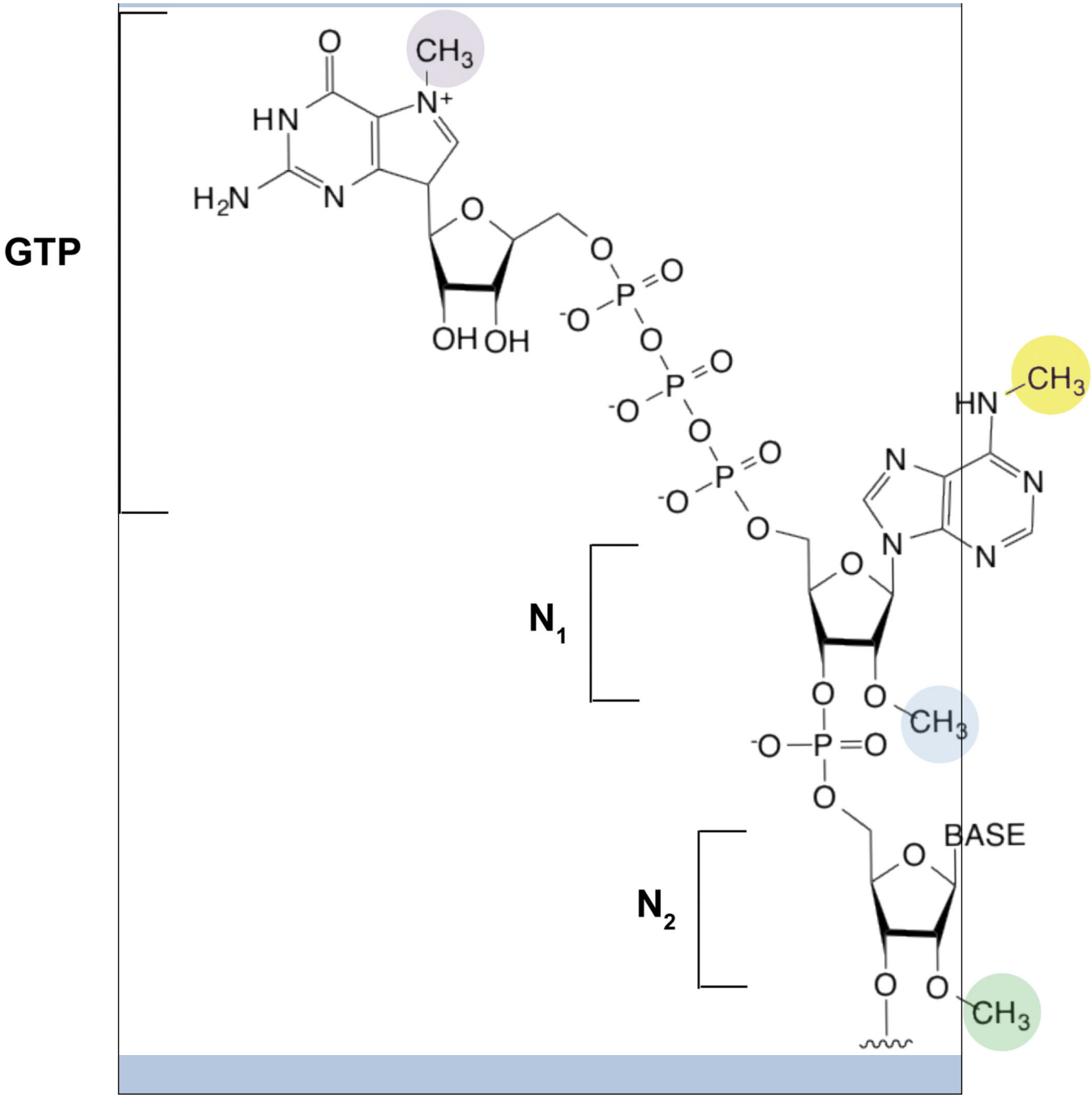
Drosophila melanogaster *oogenesis*

m^{6}A is required for Notch signaling during oogenesis based on phenotypes seen in *D. melanogaster* deficient in the adenosine methyltransferase IME4[59].

Box 2: Methyl code of the 5’ mRNA cap

The mRNA cap is introduced in the nucleus by the attachment of a GTP to the first encoded nucleotide (N_1) at the 5’ end of a transcript. The GTP is attached by an unusual linkage, in which the 5’ hydroxyl of the GTP is connected by three phosphates to the 5’ hydroxyl of the first nucleotide[12, 111] (see the figure). The most well-characterized methyl group is found on the N^7 position of the essential guanosine (purple). N^7 methylation is required for mRNA export and subsequent recognition of the cap by the eIF4E translation initiation factor[111]. Lack of this methyl group markedly reduces translational efficiency in standard *in vitro* translation reactions[112].

The first two nucleotides (N_1 and N_2) of the mRNA can also be methylated on the N_1 2’ hydroxyl (blue), to form ‘cap-1’ or on both the N_1 and N_2 2’ hydroxyl (green) to form “cap-2.” 5’ caps which lack methyl groups on either N_1 or N_2 2’ hydroxyls are referred to as cap-0[113]. The first nucleotide in mRNA is frequently an adenosine, and this can be additionally methylated at the N^6 position (yellow)[14, 113]. The N^6 methylation appears to follow the 2’-O-methylation and is likely mediated by a methyltransferase other than METTL3[114]. Thus, when considering the cap as an extended structure comprising the m^7G and the first encoded nucleotides, five different methylated forms of the cap can occur ($m^7GpppCm$, $m^7GpppGm$, $m^7GpppUm$, $m^7GpppAm$ and m^7Gpppm^6Am). The function of these differentially methylated forms of the 5’ cap is unclear. However, mRNAs containing synthetic caps with m^6A at the N_1 position enhance translation beyond the level seen with caps containing nonmethylated nucleotides at the N_1 position[115]. Additionally, structural studies suggest that eIF4E recognizes m^7G but not the N_1 nucleotide, and therefore its binding to the cap is unaffected by the methylation state of the first base[116]. Thus, other proteins may recognize the methylation states of the N_1 and N_2 nucleotides. The squiggly line refers to the position of the ribose sugar to which the base is attached.



Box 3: Techniques for detecting m^{6}A

m^{6}A immunoblotting. An important advance in establishing the prevalence of m^{6}A in mRNA was the development of m^{6}A-specific antibodies. The first m^{6}A-specific antibody was described in 1988[27]. It was initially used in studies to immunoprecipitate small nucleolar RNAs[27] and was commercialized by Synaptic Systems (Gottingen, Germany). A second antibody was used to study m^{6}A in bacterial DNA[117]. Both these antibodies only recognize oligonucleotides containing m^{6}A. [3].

m^{6}A antibodies can be used to generate m^{6}A immunoblots, which allows m^{6}A-containing RNAs to be identified based on their size. RNAs are fractionated by denaturing gel electrophoresis and transferred to a membrane; the blot is probed with an m^{6}A-specific antibody. m^{6}A immunoblots can also be used to determine if m^{6}A is present in a specific transcript. For example, biotinylated oligonucleotides have been used to pull down mRNAs that hybridize to specific target mRNAs, followed by anti-m^{6}A immunoblotting[3], providing a simple way to probe the presence of m^{6}A within individual endogenous mRNAs in cells.

m^{6}A-sensitive ligase reaction. m^{6}A can be detected in single target mRNAs based on the reduced ability of m^{6}A-containing mRNA to template a ligation reaction[118]. The m^{6}A-containing mRNA acts as a splint to catalyse a T4 DNA ligase-directed ligation of two oligonucleotides, which hybridize to the RNA so that the 3' end of one of the oligonucleotides is complementary to the putative m^{6}A in the RNA. If the 3' nucleotide is a G, which can form a nonstandard G-A interaction, the ligation reaction is significantly slower than if the complementary nucleotide is an m^{6}A instead of an A. The difference in the rates can be used to predict the m^{6}A stoichiometry in the target RNA.

m^{6}A-sensitive reverse transcription. Reverse transcription of m^{6}A is markedly impaired when using *Tth* DNA polymerase, which can function as a reverse transcriptase[119]. The slowed reverse transcription can be detected by the reduced level of incorporated thymidine in a DNA primer[119].

SCARLET. Another approach, termed SCARLET (site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography [TLC]), utilizes targeted nuclease digestion and radiolabeling steps combined with the well-known altered migration of m^{6}A on thin-layer chromatography to detect m^{6}A. This technique provides excellent measures of m^{6}A stoichiometry and can potentially be used to recover any base in the transcriptome to determine whether it contains a modification. However, since detection of the m^{6}A requires TLC, it cannot be adapted to a transcriptome-wide approach.

PacBio sequencing. The PacBio instrument uses a novel fluorescence detection technology which relies on 'zero-mode waveguides[121] to detect the altered kinetics of base incorporation opposite to an m^{6}A relative to A during reverse transcription. This approach detects differences in the rate of incorporation of thymidine opposite m^{6}A versus A when generating cDNA from an RNA template[43, 122]. However, this method

requires complex instrumentation as well as purification of the specific target mRNA, and therefore will benefit from methodological simplification.

Box 4: MeRIP-Seq profiling of m^{6}A across the transcriptome

MeRIP-Seq[3] enables transcriptome-wide profiling of m^{6}A by subjecting m^{6}A-containing RNA to next-generation sequencing. In MeRIP-Seq, RNA is fragmented into ~100-nt long fragments. Smaller fragments can also be generated to obtain finer resolution[75] (see the figure, part a). m^{6}A-containing RNAs are then immunoprecipitated with m^{6}A-specific antibodies, which results in selective enrichment of RNA fragments containing m^{6}A. After sequencing, the reads are mapped to the genome, and m^{6}A peaks are identified. The pre-immunoprecipitation RNA fragments are also sequenced to provide an RNA-Seq dataset which is incorporated during peak calling.

Typically MeRIP-Seq experiments are performed with two different m^{6}A-specific antibodies, and peaks found in both datasets are considered “true” m^{6}A peaks. This is important, as antibodies can exhibit low-level RNA binding in an m^{6}A-independent manner[75]. Only peaks found in both datasets are considered ‘true’ m^{6}A peaks.

MeRIP-Seq generates m^{6}A peaks rather than specific m^{6}A sites; thus, the localization of an m^{6}A residue in an mRNA can only be approximated. This is because MeRIP-Seq provides ~100 nt-long RNA fragments that can contain m^{6}A anywhere within the fragment. Multiple different fragments that contain the same m^{6}A residue will align as overlapping reads on the genome. The overlapping reads produce the appearance of a peak whose midpoint reflects the theoretical location of the m^{6}A residue (see the figure, part b).

Prediction of the methylated m^{6}A within a peak is possible, but challenging, because the nonrandom nature of RNA fragmentation[123] leads to asymmetric peaks and thus difficulty in determining the peak midpoint. Closely clustered m^{6}A residues can also result in broadening or asymmetry of m^{6}A peaks. Nevertheless, the center of the peak often aligns with the location of the predicted m^{6}A consensus G-m^{6}A-C[3], consistent with the idea that the peak derives from a m^{6}A residue at the midpoint.

A typical m^{6}A peak is ~200 nt wide at its base (see the figure, part c; showing UCSC Genome Browser plots of MeRIP-Seq reads of Pax6 mRNA. BPM= reads per base per million mapped reads Within the peak, reads cluster at the highest density near the middle of the peak, which is the presumed location of the m^{6}A residue.

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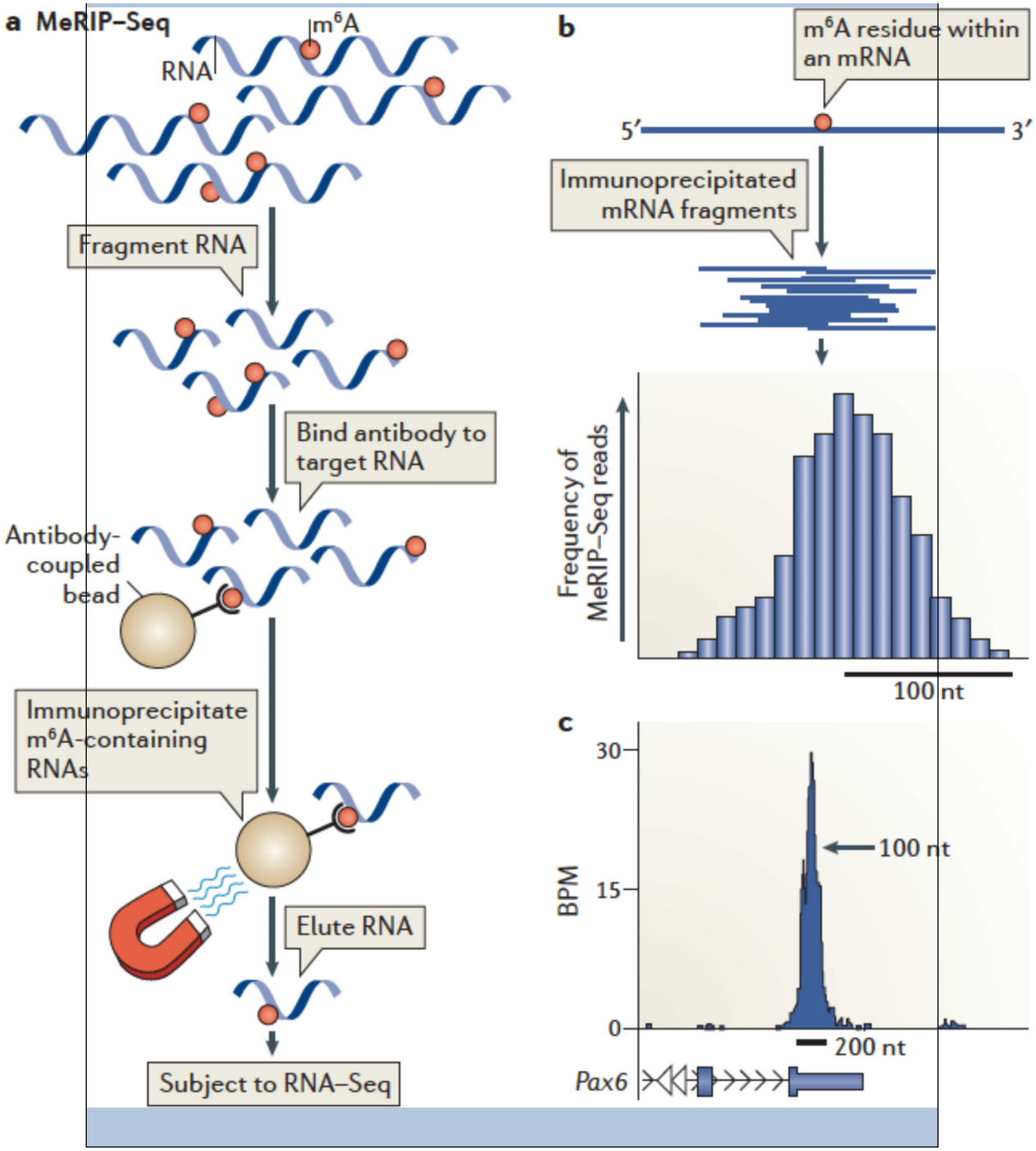

Summary

- In 2012, two independent studies demonstrated that N^6-methyladenosine (m^6A) is a widespread base modification in the mammalian transcriptome which exhibits a unique enrichment near the stop codon and in the UTRs of mRNAs.
- Recent studies have identified METTL3, METTL14, and WTAP as components of an m^6A methyltransferase complex. Further characterization of this complex will be needed to understand the dynamics and specificity of adenosine methylation in various classes of cellular RNA.
- FTO and ALKBH5 are the two m^6A demethylating enzymes identified to date. Based on studies of the mRNA targeting specificity and tissue-specific expression patterns of these enzymes, however, it is likely that additional m^6A demethylases exist.
- m^6A likely functions by recruiting m^6A binding proteins which influence RNA processing and regulation. Although a small number of m^6A binding proteins have been identified, much work remains to understand the full repertoire of m^6A binding proteins and how they contribute to mRNA regulation.
- Although many functions likely exist for m^6A, studies so far suggest that it plays a role in splicing regulation and mRNA stability

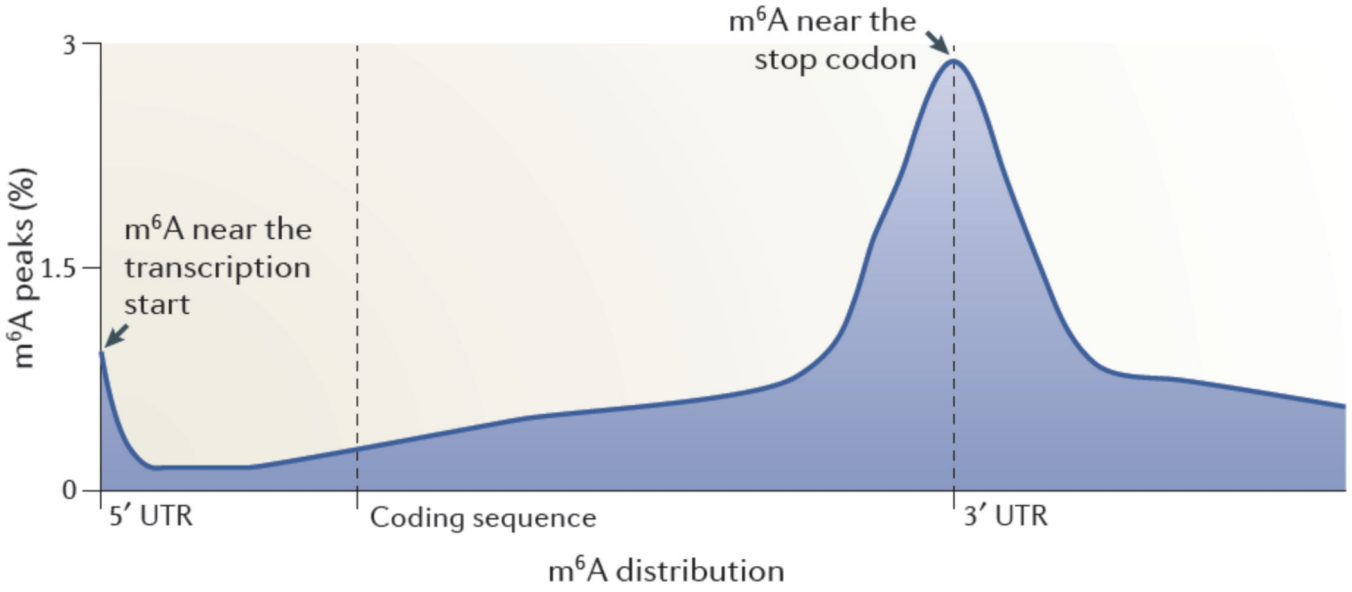

Figure 1. Spatially distinct pools of m^{6}A in mRNAs

Metagene analysis of MeRIP-Seq data shows that m^{6}A is enriched in discrete regions of mRNA transcripts[3]. In this approach, each m^{6}A peak in the transcriptome was mapped to a virtual transcript based on its position in the mRNA in which it is found. The metagene profile represents an overall frequency distribution of m^{6}A residues along the entire mature transcript body. Shown is an idealized metagene profile based on published MeRIP-Seq datasets[3]. m^{6}A residues can be found at the first encoded residue ,often as a dimethylated nucleotide (m^{6}Am). m^{6}A is also found at each of the other indicated positions in mRNAs, with a particularly prominent enrichment near the stop codon in mRNAs.

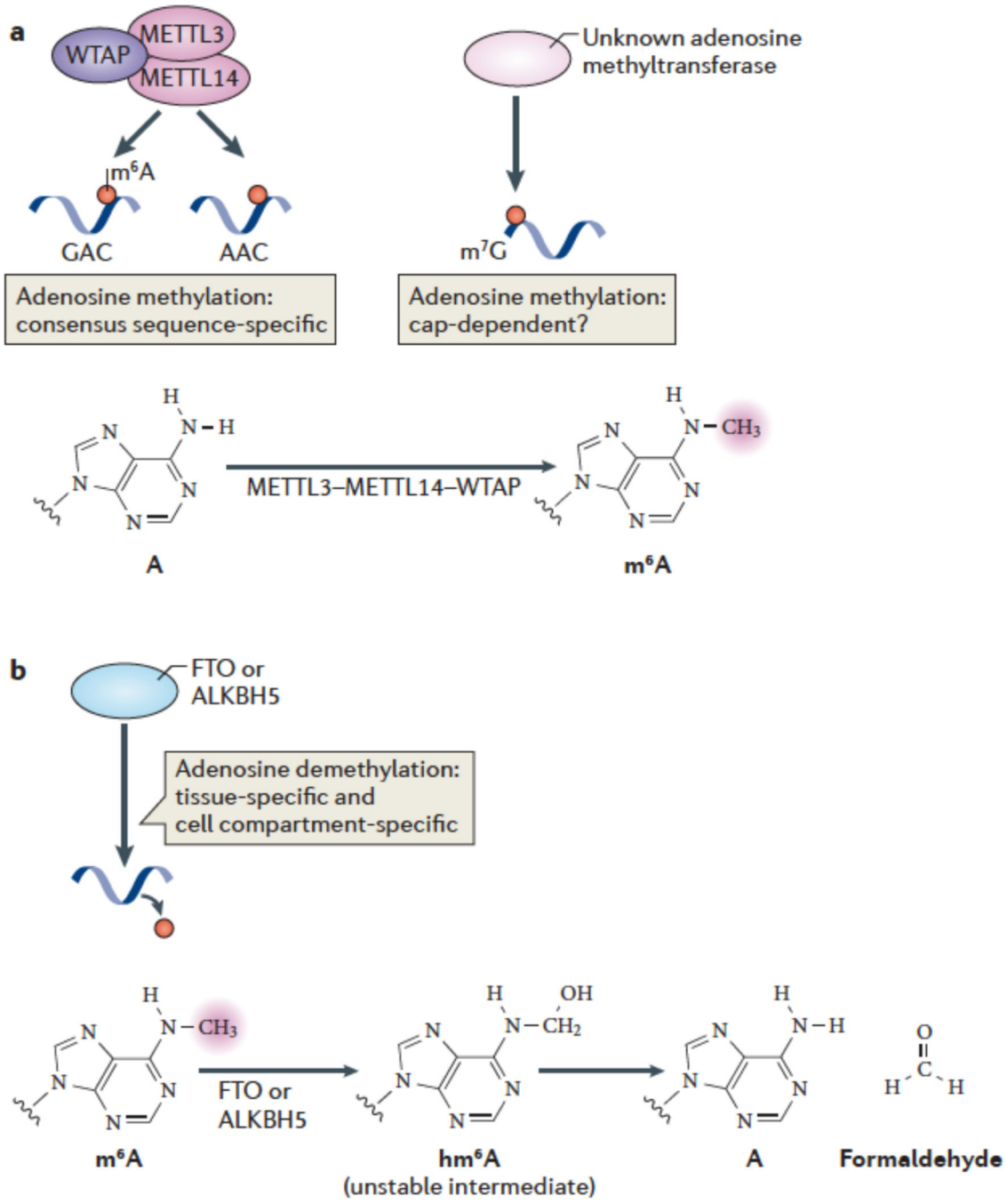

Figure 2. m^{6}A methylation and demethylation pathways

(a) METTL3 and METTL14 are two homologous m^{6}A methyltransferases that synergize to methylate adenosines in RNA. WTAP is an additional component of this complex which lacks methyltransferase activity but which has a strong influence on m^{6}A formation by interacting with METTL3/14. The central adenosines in the GAC and AAC motifs are the methylation sites of these enzymes. Additional methyltransferases may also contribute to m^{6}A formation in cells, such as those which direct N^6 methylation of adenosines associated with the cap structure. The generation of N^6-methyladenosine (m^{6}A) is shown.

(b) FTO and ALKBH5, the two mammalian m^6A mRNA demethylases identified to date, catalyze methyl group removal from the N^6 position of adenosine residues. FTO catalyzes oxidation of m6A in a reaction that requires O2, ascorbate, Fe(II) and 2-oxoglutarate. Oxidation of m6A generates CO2, succinate, and Fe(III). The product of this reaction is N^6-hydroxymethyladenosine, an unstable intermediate that spontaneously decomposes to adenosine and formaldehyde. The squiggly lines refer to the position of the ribose sugar to which the base is attached.

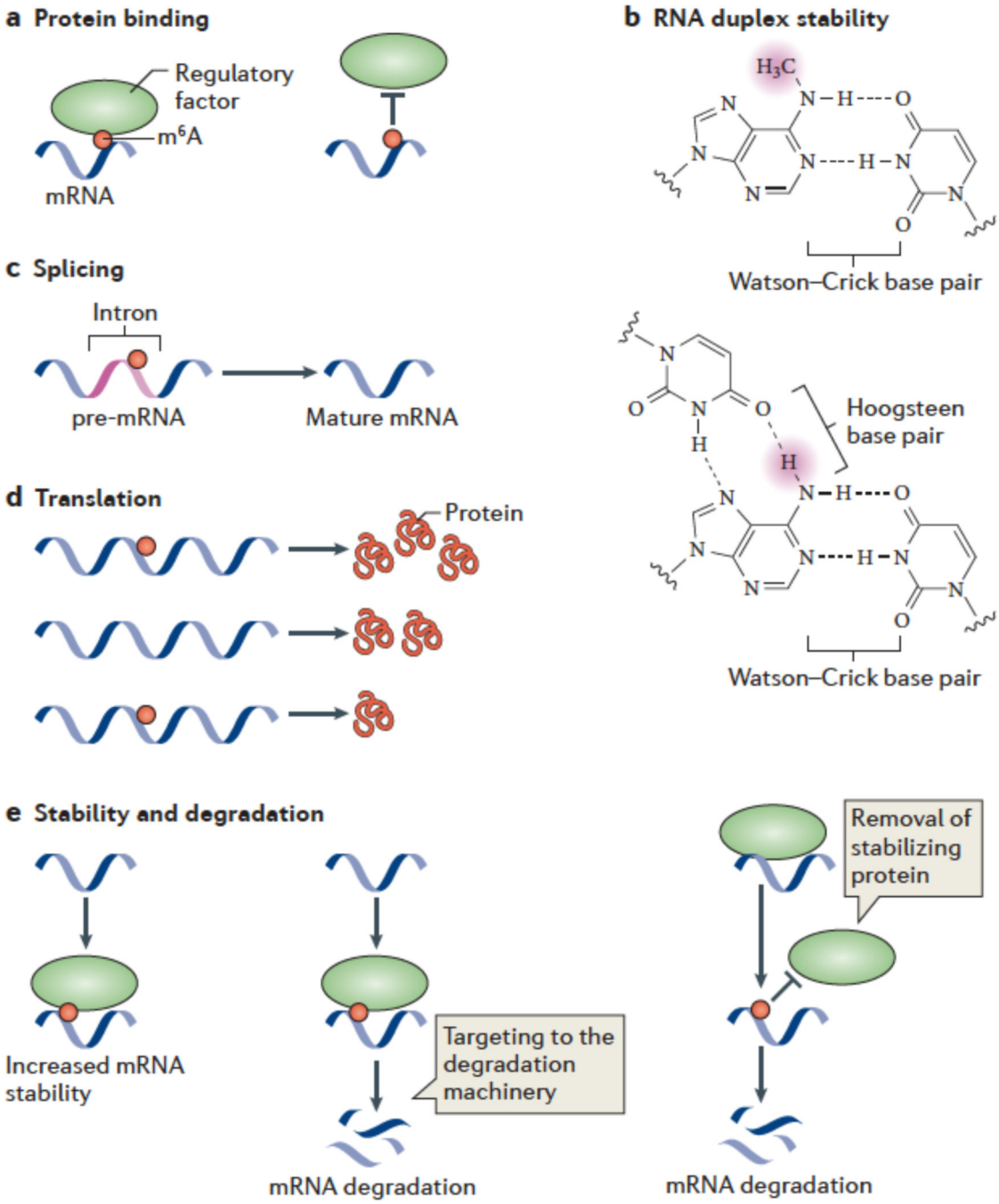

Figure 3. Mechanisms and functions of m^{6}A

(a,b) Several mechanisms have been ascribed to m6A. m6A is likely to have a role in facilitating or blocking RNA—protein interactions (a). m6A can also affect RNA by altering RNA structure or folding (b). In standard Watson-Crick base pairing, m^{6}A is capable of pairing with uridine. Since a free proton at the N^6 position is still available for hydrogen bonding, N^6-methyladenosine behaves like adenosine in its ability to base pair to uridine. The squiggly line refers to the position of the ribose sugar to which the base is attached.

m^6A interferes with the formation of base triples. As is shown in this U•A-U base triple, two free protons are required at the N^6 position for Hoogsteen base pairing on one face and Watson-Crick base pairing on the other. The presence of a methyl group in place of one of these protons blocks base-triple formation. Thus, m^6A can disrupt RNA structures dependent on base triples.

c-e) Great efforts are underway to determine the effects of m6A on mRNA fate and function. It has been suggested that m6A is targeted to specific intronic regions in order to influence splicing efficiency (c). Adenosine methylation may also lead to increased or reduced translation compared with unmethylated transcripts (d). m6A might promote mRNA degradation by recruiting proteins which target mRNAs toward the cellular degradation machinery. Alternatively, m^6A might stabilize mRNAs by binding to proteins that promote transcript stability.