



HHS Public Access

Author manuscript

Nat Rev Mol Cell Biol. Author manuscript; available in PMC 2015 April 10.

Published in final edited form as:

Nat Rev Mol Cell Biol. 2014 May ; 15(5): 313–326. doi:10.1038/nrm3785.

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

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Abstract

*N*⁶-methyladenosine (m⁶A) 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 m⁶A mapping studies, which have shown that m⁶A 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’), m⁶A demethylating enzymes (‘erasers’) and m⁶A 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 proteins¹. 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 physiology². 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 (m⁶A)^{3,4}. In addition, the characterization of adenosine methyltransferases and m⁶A 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 m⁶A in regulating important cellular pathways and processes, highlighting the potentially broad role for m⁶A

in regulating mRNA fate (**Box 1**). In this Review, we discuss the discovery of the m⁶A modification, including early studies that first revealed the existence of m⁶A as well as more recent studies that have profiled the features of m⁶A on a global scale. We also analyse the strengths and limitations of current methods used to study m⁶A 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 m⁶A in human disease. Last, we discuss the questions that are driving m⁶A research and the implications that their answers might have for our understanding of RNA biology.

Serendipitous discovery of m⁶A

The finding that polyadenylated RNA contains m⁶A 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 year⁹⁻¹². 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 [³H]-methionine, the methyl source in most biochemical reactions, had become commercially available with high specific activity. [³H]-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, N⁶-methyladenosine (m⁶A)^{7, 8}. In most studies, m⁶A was the only modified base that was detected, although one study also reported the presence of 5-methylcytosine, although at a fourth of the abundance of m⁶A^{12, 13}. Radioactive methyl groups were also found within what seemed to constitute an extended cap structure (**Box 2**)¹⁴. The finding of m⁶A in RNAs was not unprecedented, as m⁶A was known to be in bacterial tRNA and other noncoding RNAs¹⁵⁻¹⁹.

However, these initial studies provided the first evidence that m⁶A is present in mammalian poly(A) RNA, as well as mRNA encoded by diverse viruses²⁰⁻²⁴. Subsequent studies showed that m⁶A 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 m⁶A in poly(A) RNA derived from mRNA. Based on this, m⁶A might potentially be present at a level of ~3-4 m⁶A residues per mRNA⁷⁻¹². This measurement relies on the assumption, which is now known to be incorrect, that m⁶A 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 m⁶A

Despite the interesting possibility that mRNA is regulated by adenosine methylation, there were very few studies that further investigated the original findings on m⁶A. The initial interest in m⁶A was largely abandoned due to considerable reluctance to accept the idea that this modification is biologically relevant. One concern was that m⁶A could have arisen from contamination from small amounts of known m⁶A sources²⁵, such as ribosomal RNA²⁶ 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) tracts²⁹⁻³¹. 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 contaminant³², which supported the idea that m⁶A 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 species³³⁻³⁶.

In addition to the concern about a contamination artifact, another concern was that mutation of specific m⁶A sites did not result in a change in RNA fate in cells. For example, point mutations that depleted m⁶A 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 m⁶A has a minimal impact on mRNAs.

Another problem was that few endogenous mRNAs were shown to contain m⁶A. If m⁶A was indeed highly prevalent, it should be seen in numerous cellular mRNAs. Although m⁶A was frequently detected in viral mRNAs³⁹, the only endogenous mammalian mRNA shown to contain m⁶A was the *prolactin* mRNA^{40, 41}. Therefore, it was plausible that m⁶A was preferentially introduced into foreign mRNAs. The prevalence of m⁶A in poly(A) mRNA might reflect the incorporation of m⁶A into a small number of hypermethylated cellular mRNAs.

Addressing these issues was a formidable challenge, as no methods existed that could globally detect m⁶A sites in the transcriptome. Identifying m⁶A is challenging, as m⁶A reverse transcribes to a T during reverse transcription, and because m⁶A is not susceptible to chemical treatments that might facilitate its detection. This inability to identify endogenous m⁶A-containing mRNAs further dampened interest in investigating the functional roles of this putative mRNA modification.

Improved detection of m⁶A

The major breakthrough that resulted in the acceptance of m⁶A as a highly prevalent mRNA modification was the development of novel tools and methods for detecting m⁶A in mRNAs (**Box 3**). For example, the m⁶A immunoblotting technique showed that m⁶A-containing poly(A) RNAs span a broad range of sizes, ranging from less than 500 bases to over 7 kb³. This resembles the distribution expected for mRNAs, suggesting that mRNAs are the m⁶A-containing species in these blots.

m⁶A immunoblots also allow changes in m⁶A levels to be measured. For example, the intensity of the m⁶A smear in the immunoblot differs markedly when comparing RNA derived from different tissues and cancer cell lines, with the highest m⁶A levels in the brain, heart and kidney³. m⁶A-containing mRNAs are low in the fetal brain, but increase during development, achieving a maximal level in the adult brain³. These data argued against initial theories that m⁶A 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 m⁶A immunoblotting analysis revealed that each of these mRNAs is endogenously methylated in cells³.

The methylated transcriptome

The methylated transcriptome was first defined using the MeRIP-Seq technique and an essentially equivalent approach, termed m⁶A-Seq (**Box 4**)^{3, 4}. These are next-generation sequencing techniques that involve selective immunoprecipitation and sequencing of m⁶A-containing RNA fragments³. MeRIP-Seq led to the identification of 13,471 m⁶A peaks derived from 4,654 genes in the mouse brain. Analysis of HEK293T cell RNA led to the identification of 18,756 m⁶A peaks from 5,768 genes. Highly similar numbers were obtained in HepG2 cells, which showed 12,769 m⁶A peaks among 6,990 mRNAs⁴. These results provided the first unequivocal demonstration that m⁶A is a widespread mRNA modification, and thus laid to rest many of the previous doubts whether m⁶A was indeed a prevalent modification in mRNA.

However, m⁶A 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 m⁶A might be an obligate feature of all mRNAs as a result of biogenesis or processing. Importantly, the distribution of m⁶A along the length of transcripts was nonrandom, suggesting that m⁶A 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 regions³, further suggesting that m⁶A has conserved regulatory roles.

Importantly, although many transcripts were methylated in both brain and HEK293 cells, others were methylated in only one tissue³. 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 m⁶A peaks were typically localized to the same region of the transcript.

Interestingly, not all m⁶A peaks detected by MeRIP-Seq were found in mRNAs. In the brain for example, although 94.5% of m⁶A peaks were found within mRNAs, the rest mapped to several classes of (long) noncoding RNAs³. These data indicate that noncoding RNAs are also important targets of m⁶A.

MeRIP-Seq also demonstrated that different m⁶A peaks exhibit markedly distinct m⁶A 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 m⁶A peaks based on this method shows that m⁶A stoichiometry varies considerably, with the majority of m⁶A peaks likely exhibiting low stoichiometry³. This approach can be used to determine mRNAs with high stoichiometry m⁶A, which are therefore most likely to be influenced by methylation pathways in any given cell type.

Asymmetric m⁶A distribution in mRNAs

A striking result from the MeRIP-Seq analysis was the finding that m⁶A residues are enriched in specific regions of the mRNA^{3, 4} (Figure 1). The majority of m⁶A peaks are located in the vicinity of the stop codon, with a roughly even distribution both upstream and downstream of this site³. Additionally, a subset of mRNAs contains m⁶A residues in their 5' untranslated regions (UTRs)^{3, 4}. Typically, the 5'UTR of a transcript is relatively short; thus, the concentration of m⁶A residues within the 5'UTR is strikingly high compared with other regions of the transcript⁴. Interestingly, the fraction of cellular mRNAs that have m⁶A in the 5'UTR seems to exhibit tissue-specific differences^{4, 43}, indicating that regulation by 5'UTR m⁶A 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 m⁶A than do other tissues. Thus far, no tissues have been identified which completely lack m⁶A, although the overall abundance of m⁶A within RNA appears to vary across different tissue types³.

m⁶A residues are also found in the 3'UTR outside of the region adjacent to the stop codon³. However, these m⁶A residues were less abundant than the m⁶A residues near the stop codon. Furthermore, m⁶A 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, m⁶A was not found in poly(A) tails³, which is consistent with early metabolic labelling studies²⁵.

The enrichment of m⁶A 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'UTR⁴⁴. Many other mRNA-binding proteins bind to the 3'UTR of mRNAs⁴⁵, which is also the most structured portion of the molecule⁴⁶. Moreover, few mRNA-binding proteins show preferential binding to the 5'UTR⁴⁵. Thus, the enrichment of m⁶A 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 m⁶A 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 FIRE⁴⁷ 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 m⁶A)³.

These motifs were found in nearly 90% of all peaks³, and importantly, other motifs may exist for a smaller subset of m⁶A 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 GACG⁵¹. Furthermore, mutation of GAC sequences to GAU was sufficient to block adenosine methylation³⁸. The sequences discovered by FIRE analysis of the MeRIP-Seq dataset show that these m⁶A motifs apply to the majority of cellular m⁶A-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 m⁶A-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 m⁶A 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 m⁶A consensus motif, GAC, is found roughly every 64 nts in RNA, which suggests that an average 2000-nt long mRNA would have ~30 m⁶As distributed evenly along the length of the transcript. However, most transcripts lack an m⁶A altogether³, and in m⁶A-modified transcripts m⁶A is usually found near the stop codon. Thus, the enrichment of m⁶A 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, m⁶A peaks are typically found within unstructured regions of RNA⁴, 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 m⁶A 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 m⁶As within the open reading frame, but could account for some of the defining features of m⁶A distribution in transcripts. Indeed, in agreement with a tethering model, MeRIP-Seq data indicate that most m⁶A peaks reflect a cluster of m⁶A residues³. Additionally, previous studies showed that m⁶A residues were clustered in the Rous

sarcoma virus transcript³⁹. 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 m⁶A at single-nucleotide resolution will be important to determine whether the distribution of m⁶A in mammalian mRNAs is compatible with a clustering model.

Enzymes that mediate m⁶A methylation

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 methylation⁵². Nuclear preparations were capable of methylating RNA in a SAM-dependent manner in vitro, and the substrate specificity matched the m⁶A consensus motif established by biochemical methods⁵¹. 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*-dimethyladenosine⁵¹, unlike various Lys methyltransferases, which can produce ‘higher order’ methylated forms of Lys, such as di- and tri-methylated Lys⁵³. Subsequent efforts focused on purifying the catalytic subunit of the methyltransferase complex⁵⁴. The catalytic component was detected by its ability to crosslink to [³H]-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 function⁵⁴. 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, METTL14⁶⁰. METTL14 is highly similar to METTL3 and is a predicted methyltransferase⁶¹⁻⁶³. Subsequent studies confirmed that both METTL3 and METTL14 form complexes in cells and that purified METTL14 also selectively methylates GAC sequences in vitro⁶¹⁻⁶³. 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 m⁶A peaks in more than half of all m⁶A-containing mRNAs in mouse embryonic stem cells⁶³, 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 m⁶A levels in mRNA⁶¹. WTAP seems to interact with numerous cellular proteins, thus, despite its name, many of its functions are unrelated to WT1⁶⁵. WTAP, which lacks methyltransferase domains or activity, was shown to bind to the METTL3—METTL14 complex^{61, 62}, and induce its localization to nuclear speckles⁶¹. 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 m⁶A levels⁶¹. 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 sequences⁶¹.

However, early studies also detected methyltransferase activity in cytosolic extracts, which supports the idea that at least some methylation could occur in the cytoplasm⁵¹. Cytoplasmic methylation would imply a role for m⁶A in the control of mRNA 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 staining⁶⁷, and immunostaining for the *Drosophila* homologue, *IME4*, indicated some immunoreactivity in the cytoplasm⁵⁹. 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 m⁶A within transcripts also supports the idea that mRNA methylation might occur in the cytosol. MeRIP-Seq analysis of m⁶A localization in the transcriptome shows that m⁶A residues are highly enriched around stop codons. Cellular recognition of stop codons is mediated solely by ribosomes bound to release factors⁷⁰. 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 processes⁷¹. As ribosome function is predominantly cytosolic⁷², a ribosome-mediated methylation pathway would indicate a cytosolic location for methylation, at least for stop-codon proximal m⁶A residues.

Could other methyltransferases drive m⁶A formation?

The possibility that additional methyltransferases have a role in adenosine methylation is supported by the fact that a small number of m⁶A peaks do not seem to contain the AAC or

GAC consensus site^{3,4}. Furthermore, m⁶A can be found at the first position in many mRNAs after the m⁷G cap, resulting in a m⁷Gpppm⁶Am 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 N⁶ position of adenosine (see **Supplementary Table 1**). Importantly, the residues following the methylated adenosine can be any base⁵⁰. Because of the lack of a cytosine following and a guanosine immediately preceding the adenosine residue, these m⁶A residues differ from the GAC and AAC consensus motifs. Thus, methylation of these noncanonical m⁶A 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 function⁷³. The adenosine methyltransferase activity that targets the U6 small nuclear RNA, which is different from METTL3²⁸, could potentially target certain m⁶A 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 fractions⁷⁴. One fraction, similarly to METTL3, methylated diverse RNA templates; however, a separate activity selectively labelled RNA containing a 5' cap⁷⁴. 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 methyltransferases may also contribute to m⁶A formation in cells.

m⁶A demethylation pathways

An important recent discovery was the finding that endogenous enzymes can demethylate m⁶A. The presence of m⁶A erasers suggests that the effects of m⁶A can potentially be reversed, and that cellular demethylation pathways might be a mechanism of dynamic regulation of m⁶A within mRNAs. Notably, these erasers are not expressed in yeast, pointing to the potential role of passive RNA degradation as a mechanism to remove m⁶A in certain cell types⁷⁵. Whether m⁶A 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 m⁶A 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 DNA⁷⁷. A subsequent study demonstrated that FTO could also demethylate 3-methyl uracil (m3U) in single-stranded RNA⁸⁰. Interestingly, m3U is a minor constituent of ribosomal RNA⁸¹, potentially linking FTO to ribosome function.

Furthermore, m⁶A in RNA has been shown to be an additional substrate for FTO. One study showed that FTO demethylated m⁶A with a catalytic efficiency that was substantially higher than the activity of FTO towards 3mU⁵. Overexpression of FTO in HeLa cells reduced the level of m⁶A in purified poly(A) RNA by ~18%, whereas *FTO* knockdown increased m⁶A levels by 23% in poly(A) mRNA. Consistent with this, another study showed that FTO overexpression greatly reduced the levels of m⁶A in cellular RNA³, and the size of bands produced were consistent with mRNA being a target of this enzyme. Thus, m⁶A 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 m⁶A levels are generally unaffected in most mRNAs in *FTO* knockout brain⁴². However, for a small subset of mRNAs, m⁶A peaks are markedly higher in the *FTO*-knockout tissue than in wild-type⁴². Thus, these data demonstrate that FTO demethylates mRNAs, although only a few m⁶A-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 m⁶A residues.

FTO was originally proposed to target m⁶A in nuclear RNAs⁵. Nuclear m⁶A-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 speckles⁵. 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 m⁶A intermediates. Demethylation of m⁶A involves a hydroxylation reaction to form *N*⁶-hydroxymethyl-adenosine (hm⁶A) (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 (f⁶A), in analogy to the diverse oxidized forms of cytosine generated by the FTO-homolog Tet proteins⁸⁴. It is not clear yet whether these labile oxidized intermediates of m⁶A 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 m⁶A residues. Rhein, a

bioactive component of rhubarb, is a moderately selective FTO inhibitor⁸⁵. 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 m⁶A demethylase is ALKBH5, an FTO homologue of the AlkB family⁶. A systematic test of each of the nine mammalian AlkB homologues showed that ALKBH5 catalyses the demethylation of an m⁶A-containing RNA with nearly as high a rate as FTO, and showed specificity for demethylation of m⁶A over various other methylated nucleotides in single-stranded RNA⁶.

ALKBH5 seems to affect mRNA export pathways. In *ALKBH5* knockdown cells, increased levels of poly(A) mRNA were detected in the nucleus⁶, 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 testes⁶, 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 m⁶A-deficiency in lower organisms relate to gametogenesis and meiosis^{57, 59, 64, 75}, suggesting a possibly evolutionarily conserved function for m⁶A in these processes.

It will be important to determine if ALKBH5 demethylates mRNA, noncoding RNAs, or both. *ALKBH5* knockdown increased m⁶A levels in poly(A) mRNA by ~9%, whereas ~50X overexpression of ALKBH5 reduced m⁶A levels by ~29%⁶. The subtle changes in m⁶A levels in cellular poly(A) mRNA suggests that this enzyme, similarly to FTO⁴², only demethylates certain m⁶A residues in mRNAs. Selected mRNAs were tested for their susceptibility to ALKBH5-mediated demethylation, which showed that in some cases, the m⁶A level increased in *ALKBH5* knockdown cells⁶. 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 nucleus⁶, 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 enriched⁷⁷, ALKBH5 is predominantly expressed in testis, with substantially lower levels in other tissues⁶. 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 m⁶A action

Based on the localization of methyltransferase and demethylase enzymes, m⁶A may be introduced and removed in either the nucleus or the cytosol. Similarly, m⁶A 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 m⁶A that begin to tease apart these compartment-specific m⁶A roles.

Protein recruitment

m⁶A 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 m⁶A-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 domain⁸⁷. A YTH-domain containing protein, MRB1, was also found to be a m⁶A-binding protein in yeast⁷⁵. The RNA-binding protein HuR (also known as ELAVL1) was also detected in m⁶A-RNA pulldowns from mammalian lysates⁴. Several other proteins were shown to bind m⁶A using yeast extracts⁷⁵. However, since these studies used a pulldown approach, it was not clear which of these proteins directly bind to m⁶A or are instead part of a m⁶A-binding ribonucleoprotein complex. Also, it is not clear if these proteins bind m⁶A in living cells.

YTHDF1, YTHDF2 and YTHDF3 binding to m⁶A has been validated *in vitro*^{86, 88}. The binding affinity was relatively weak, ranging from 400 nM to 1.2 μM^{86, 88}. Although this binding is relatively weak compared with established sequence-specific RNA-protein interactions⁸⁹, it is possible that these proteins function by binding transiently to m⁶A.

Do these proteins bind m⁶A in living cells? Transcriptome-wide methods for monitoring binding sites of RNA-binding proteins in living cells, such as HITS-CLIP, iCLIP, and PAR-CLIP⁹⁰⁻⁹² 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 resolution⁹⁰. The major criteria for establishing that a putative m⁶A-binding protein is a bona fide interactor is that the binding sites of the protein need to precisely match m⁶A residues in the transcriptome. An m⁶A-binding protein might physiologically function to bind to m⁶A in rRNA or in ncRNAs (such as U6), and not mRNA; therefore overexpression of an m⁶A-binding protein can lead to nonphysiological binding to mRNA. For this reason, putative m⁶A-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 m⁶A 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 m⁶A in the mRNA. Single-nucleotide resolution m⁶A mapping techniques will therefore be needed to

confidently identify m⁶A-binding proteins. Additionally, it is important to demonstrate that any putative m⁶A-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 μ M and >1,000 μ M, respectively⁹³. Similarly, L3MBT1, which binds methyl-Lys in histone proteins, exhibits a ~80-fold increase in affinity between the nonmethylated and methylated forms (6 μ M and >500 μ M respectively)⁹⁴. Thus, the methyl group on adenosine may introduce >20-fold binding selectivity.

Conformational changes

m⁶A 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 destabilized⁹⁵ (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 interactions⁹⁶. Thus, subtle alterations in binding and basepair stability could contribute to some of the effects of m⁶A 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 N⁶ 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 MEN β and MALAT1⁹⁷. Interestingly, MALAT1 also contains m⁶A on the basis of MeRIP-Seq analysis³. Thus, methylation could act as a trigger to disrupt RNA structures that depend on hydrogen bonding of both N⁶ hydrogens on adenosine.

m⁶A effects on mRNA fate and function

The mechanisms through which m⁶A regulates cellular mRNAs are still in the process of being uncovered. However, studies to date have provided important insights into our understanding of how m⁶A contributes to the processing, localization, and functional roles of mRNAs in cells.

Effects on mRNA splicing

One of the earliest proposed roles for m⁶A was as a regulator of splicing (Figure 3c). This was initially based on studies that characterized the m⁶A content of the pre-mRNA in the nucleus, compared to the m⁶A content in mature cytoplasmic mRNA. In these studies, pre-mRNA was found to be methylated at ~4 m⁶A residues per mRNA, whereas the mature mRNA was methylated at ~2 m⁶A residues per mRNA³². These studies documented that

methylation occurs in the nucleus, and suggested that the removal of introns resulted in the loss of total m⁶A 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 introns⁶¹. 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 m⁶A 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 m⁶A was preferentially found in exons, and only ~5-7% of m⁶A 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, m⁶A 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 m⁶A within intronic sequences needs to be more thoroughly examined by performing MeRIP-Seq on premRNA, one possibility is that m⁶A is targeted to specific intronic regions in order to influence splicing efficiency.

The function of m⁶A 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 m⁶A and exhibit METTL3 binding than mRNAs that are expressed as only one spliced isoform^{4, 61}. Additionally, m⁶A is more likely to be found in introns and exons that undergo alternative splicing⁴. Thus, m⁶A appears to contribute to alternative splicing of mRNA. However, a mechanistic relationship between the presence of m⁶A and splicing events is not yet established.

Regulation of mRNA translation

There are conflicting results as to whether m⁶A affects protein translation *in vitro*. Early studies using a rabbit reticulocyte lysate system showed that translation of m⁶A-containing *DHFR* mRNA was increased by about 50% compared with mRNA⁹⁸. 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 transcripts⁹⁹ (Figure 3d). Thus, the effects of m⁶A 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 m⁶A 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 m⁶A stabilizes mRNA⁴ (Figure 3e). Loss of methylation correlated with reduced expression levels of transcripts that contain m⁶A⁴. This effect was most prominent for mRNAs that contain m⁶A in introns. These studies support the general idea that m⁶A is needed for normal mRNA expression levels. A possible explanation for these data is that

m⁶A 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 m⁶A levels influence mRNA abundance and protein production. In these mice, five mRNAs that have been tested had increased m⁶A 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 unchanged⁴².

Furthermore, other reports suggest that m⁶A promotes mRNA degradation^{63, 86}. One study focused on YTHDF2, a mammalian protein that has been identified to bind to m⁶A using pulldown approaches⁸⁶. YTHDF2 was shown to promote mRNA degradation of thousands of cellular transcripts⁸⁶. 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 m⁶A. In some transcripts, the PAR-CLIP site seems to overlap with the m⁶A peak⁸⁶. Further analysis will be required to define the specific identity and number of m⁶A-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 cells⁶³. In knockdown cells, many *METTL3*- and *METTL14*-target mRNAs showed a modest increase in stability, suggesting that m⁶A functioned to induce mRNA stability. The authors proposed that m⁶A displaces HuR, a known mRNA stabilizer. Indeed, *in vitro* HuR binding to its typical U-rich binding site was impaired by adjacent m⁶A residues⁶³. Previous studies had suggested that HuR might be an m⁶A-binding protein⁴; however, this more recent analysis argued that m⁶A reduces HuR binding, thereby facilitating mRNA degradation.

In both of these studies, the effect of m⁶A 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 m⁶A. This is important, as the stoichiometry of m⁶A in the vast majority of m⁶A-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 m⁶A and microRNAs

Analysis of the MeRIP-Seq dataset revealed a strong correlation between the presence of m⁶A and microRNA-binding sites³. 67% of 3'UTRs that contain m⁶A peaks also contain at least one TargetScan-predicted microRNA-binding site. Because ~30% of genes have microRNA-binding sites in their 3'UTRs¹⁰⁰, this is a significantly enhanced association. Importantly, the m⁶A peak and microRNA site did not overlap. In general, the m⁶A 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'UTRs⁴⁴.

The strong association between m⁶A 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 m⁶A than mRNAs that contain sites for microRNAs that are not abundant in brain³. 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 m⁶A mapping techniques have resolved many of the longstanding concerns regarding the physiological relevance of m⁶A and have renewed interest in this mRNA modification. Currently, an understanding of the function of m⁶A 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 m⁶A 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 m⁶A mapping is needed to define the specific m⁶A residues that are bound to putative m⁶A-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 resolution⁷⁵. This approach was used to map m⁶A 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 m⁶A antibodies and analysing the crosslinking site using methods that have been highly optimized for HITS-CLIP and related techniques⁹⁰⁻⁹². Last, the inclusion of control RNAs with known m⁶A stoichiometry could be used as standards to obtain absolute measures of m⁶A stoichiometry. These strategies will be essential to more precisely define the specific modified m⁶A residues in the transcriptome and the degree of methylation at specific adenosines within a transcript.

A major goal will be to determine how m⁶A affects the fate of mRNA. In addition, there is a need to understand whether all m⁶As 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 m⁶A-binding proteins, and the demonstration that endogenous protein binds mRNAs at m⁶A residues. This requires, in part, mapping of m⁶A at higher resolution than is currently available. The demonstration that m⁶A-binding proteins are indeed binding m⁶A should in turn enable the characterization of the mechanisms through which m⁶A regulates mRNAs.

One of the exciting aspects of m⁶A 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 m⁶A demethylation are particularly important. However, evidence for dynamic reversibility of m⁶A in mRNA has not been established. The recent identification of mRNAs that are targeted by FTO⁴² 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, m⁶A 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 m⁶A-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 m⁶A 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 transformation¹⁰³. Furthermore, in yeast, induction of meiosis coincides with increased expression of the METTL3 homologue IME4 and concomitant m⁶A accumulation^{58, 104}. IME4 also affects the developmental response to nutrient starvation in yeast⁶⁴. 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 m⁶A.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank O. Elemento and members of the Jaffrey laboratory for helpful comments and suggestions. This work was supported by NIH grant R01 DA037150 (S.R.J.) and the Revson Senior Fellowship in Biomedical Sciences to K.M.

Biography

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Samie R. Jaffrey is a professor of pharmacology at Weill Cornell Medical College, New York, USA. He received his M.D. and Ph.D. at Johns Hopkins University and completed his postdoctoral studies in the laboratory of Solomon H. Snyder at Johns Hopkins University. His laboratory studies dynamic regulation of mRNA methylation and the role of m⁶A in cellular mRNA regulation. His laboratory also develops novel tools for imaging RNAs in living cells in order to study RNA trafficking and RNA processing events.

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.

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Box 1: Physiological processes linked to m⁶A

Obesity

Diverse mutations within intron 1 of human *FTO* have been found and are associated with increased body weight¹⁰⁵. 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 alleles¹⁰⁵. *FTO*^{-/-} mice have lower body mass¹⁰⁶, whereas *FTO* overexpression leads to increased food intake and obesity¹⁰⁷. 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 cocaine⁴². 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 cancer¹⁰². 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 morphology⁶. 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*^{-/-} mice⁶; however, whether hypermethylation of these mRNAs underlies their alternative expression pattern remains to be determined.

Stem cell differentiation

m⁶A is required for self-renewal capacity of human embryonic stem cells and is found in critical pluripotency regulators⁶³; dynamic changes in m⁶A levels are seen during stem cell differentiation⁶³.

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 m⁶A 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 meiosis⁶⁴ 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 Slz1⁶⁴. The MIS complex localizes to the nucleolus during meiosis, and is required for the proper time course of meiosis⁷⁵.

Plant development

m⁶A contributes to plant embryonic development, and is required for normal growth patterns, apical dominance, and floral development^{57, 110}

Drosophila melanogaster oogenesis

m⁶A is required for Notch signaling during oogenesis based on phenotypes seen in *D. melanogaster* deficient in the adenosine methyltransferase IME4⁵⁹.

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 factor¹¹¹. Lack of this methyl group markedly reduces translational efficiency in standard *in vitro* translation reactions¹¹².

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-0¹¹³. 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 METTL3¹¹⁴. 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 position¹¹⁵. 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 base¹¹⁶. 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 m⁶A

m⁶A immunoblotting. An important advance in establishing the prevalence of m⁶A in mRNA was the development of m⁶A-specific antibodies. The first m⁶A-specific antibody was described in 1988²⁷. It was initially used in studies to immunoprecipitate small nucleolar RNAs²⁷ and was commercialized by Synaptic Systems (Gottingen, Germany). A second antibody was used to study m⁶A in bacterial DNA¹¹⁷. Both these antibodies only recognize oligonucleotides containing m⁶A.³

m⁶A antibodies can be used to generate m⁶A immunoblots, which allows m⁶A-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 m⁶A-specific antibody. m⁶A immunoblots can also be used to determine if m⁶A 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-m⁶A immunoblotting³, providing a simple way to probe the presence of m⁶A within individual endogenous mRNAs in cells.

m⁶A-sensitive ligation reaction. m⁶A can be detected in single target mRNAs based on the reduced ability of m⁶A-containing mRNA to template a ligation reaction¹¹⁸. The m⁶A-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 m⁶A 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 m⁶A instead of an A. The difference in the rates can be used to predict the m⁶A stoichiometry in the target RNA.

m⁶A-sensitive reverse transcription. Reverse transcription of m⁶A is markedly impaired when using *Tth* DNA polymerase, which can function as a reverse transcriptase¹¹⁹. The slowed reverse transcription can be detected by the reduced level of incorporated thymidine in a DNA primer¹¹⁹.

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 m⁶A on thin-layer chromatography to detect m⁶A. This technique provides excellent measures of m⁶A 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 m⁶A 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 m⁶A relative to A during reverse transcription. This approach detects differences in the rate of incorporation of thymidine opposite m⁶A 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.

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Box 4: MeRIP-Seq profiling of m⁶A across the transcriptome

MeRIP-Seq³ enables transcriptome-wide profiling of m⁶A by subjecting m⁶A-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 resolution⁷⁵ (see the figure, part a). m⁶A-containing RNAs are then immunoprecipitated with m⁶A-specific antibodies, which results in selective enrichment of RNA fragments containing m⁶A. After sequencing, the reads are mapped to the genome, and m⁶A peaks are identified. The pre-immunoprecipitation RNA fragments are also sequenced to provide an RNA-Seq dataset which is incorporated during peak calling.

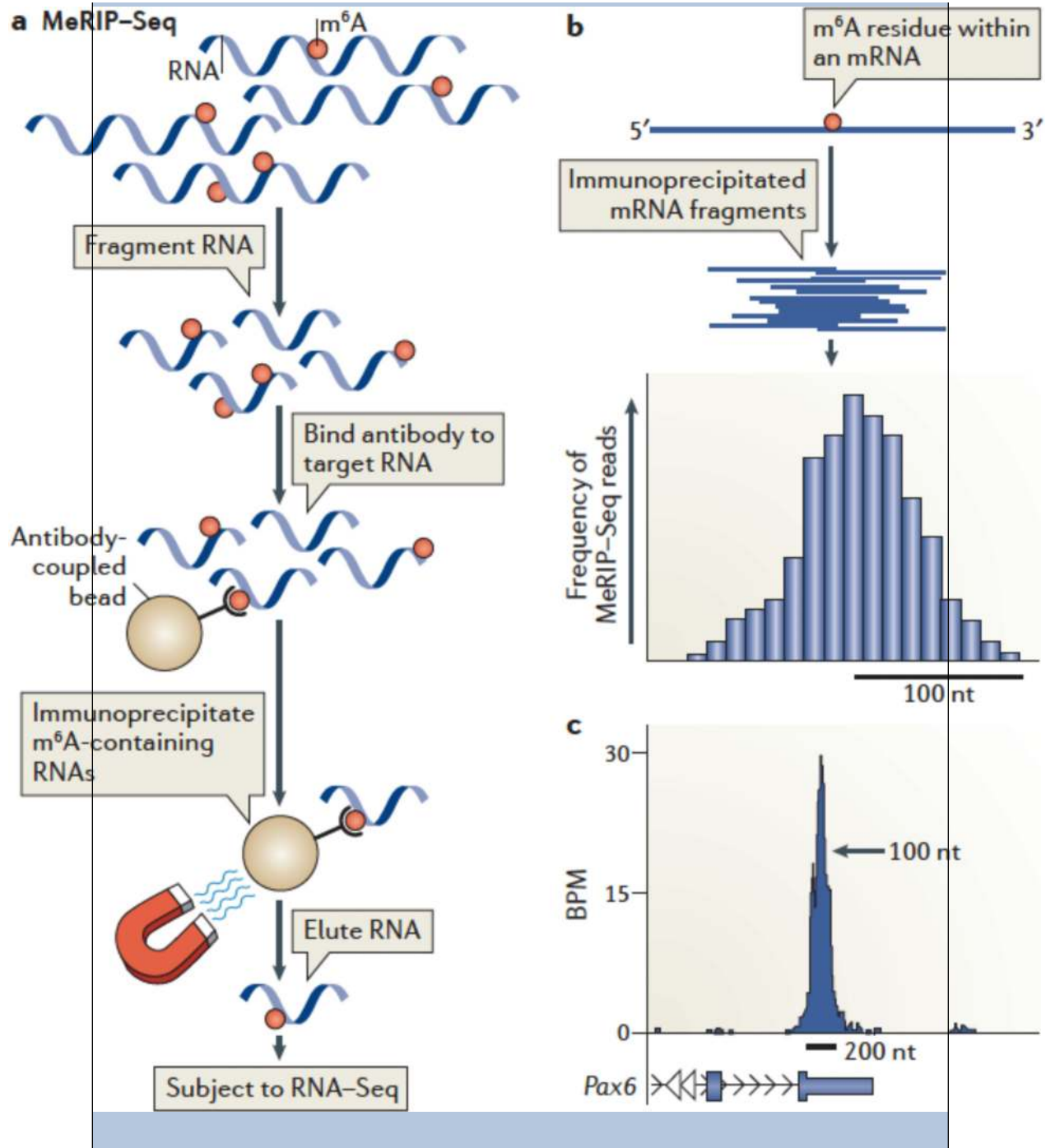
Typically MeRIP-Seq experiments are performed with two different m⁶A-specific antibodies, and peaks found in both datasets are considered “true” m⁶A peaks. This is important, as antibodies can exhibit low-level RNA binding in an m⁶A-independent manner⁷⁵. Only peaks found in both datasets are considered ‘true’ m⁶A peaks.

MeRIP-Seq generates m⁶A peaks rather than specific m⁶A sites; thus, the localization of an m⁶A residue in an mRNA can only be approximated. This is because MeRIP-Seq provides ~100 nt-long RNA fragments that can contain m⁶A anywhere within the fragment. Multiple different fragments that contain the same m⁶A 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 m⁶A residue (see the figure, part b).

Prediction of the methylated m⁶A within a peak is possible, but challenging, because the nonrandom nature of RNA fragmentation¹²³ leads to asymmetric peaks and thus difficulty in determining the peak midpoint. Closely clustered m⁶A residues can also result in broadening or asymmetry of m⁶A peaks. Nevertheless, the center of the peak often aligns with the location of the predicted m⁶A consensus G-m⁶A-C³, consistent with the idea that the peak derives from a m⁶A residue at the midpoint.

A typical m⁶A 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 m⁶A residue.

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Summary

- In 2012, two independent studies demonstrated that *N*⁶-methyladenosine (m⁶A) 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 m⁶A 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 m⁶A 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 m⁶A demethylases exist.
- m⁶A likely functions by recruiting m⁶A binding proteins which influence RNA processing and regulation. Although a small number of m⁶A binding proteins have been identified, much work remains to understand the full repertoire of m⁶A binding proteins and how they contribute to mRNA regulation.
- Although many functions likely exist for m⁶A, studies so far suggest that it plays a role in splicing regulation and mRNA stability

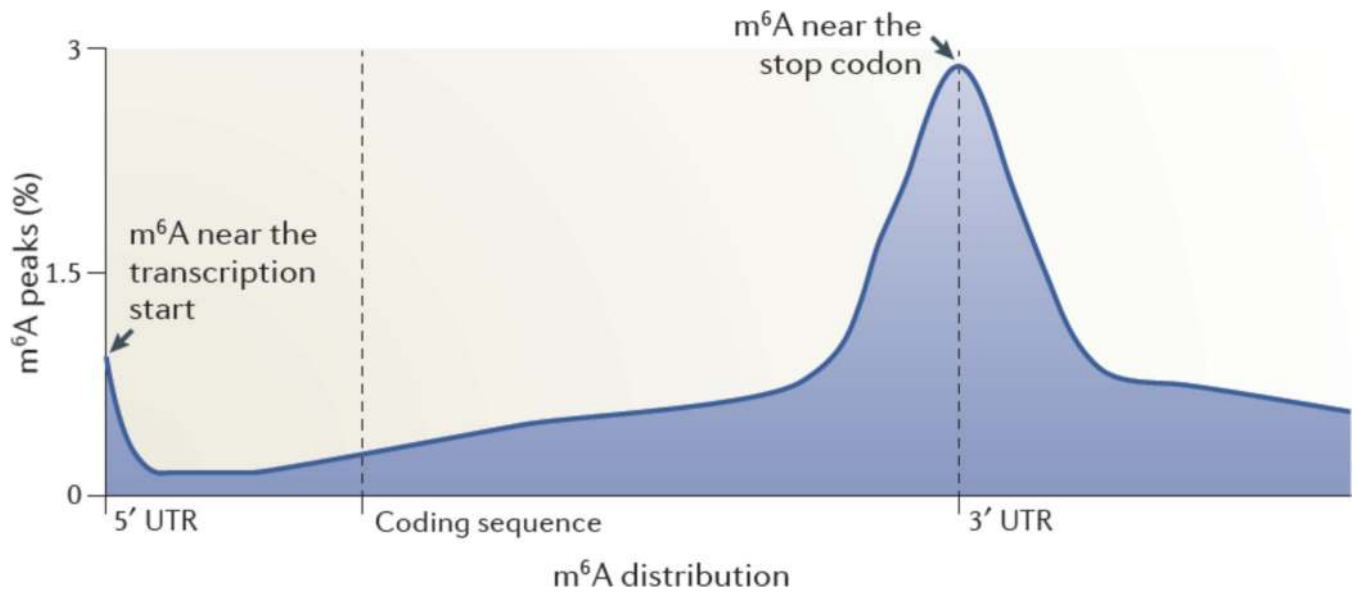
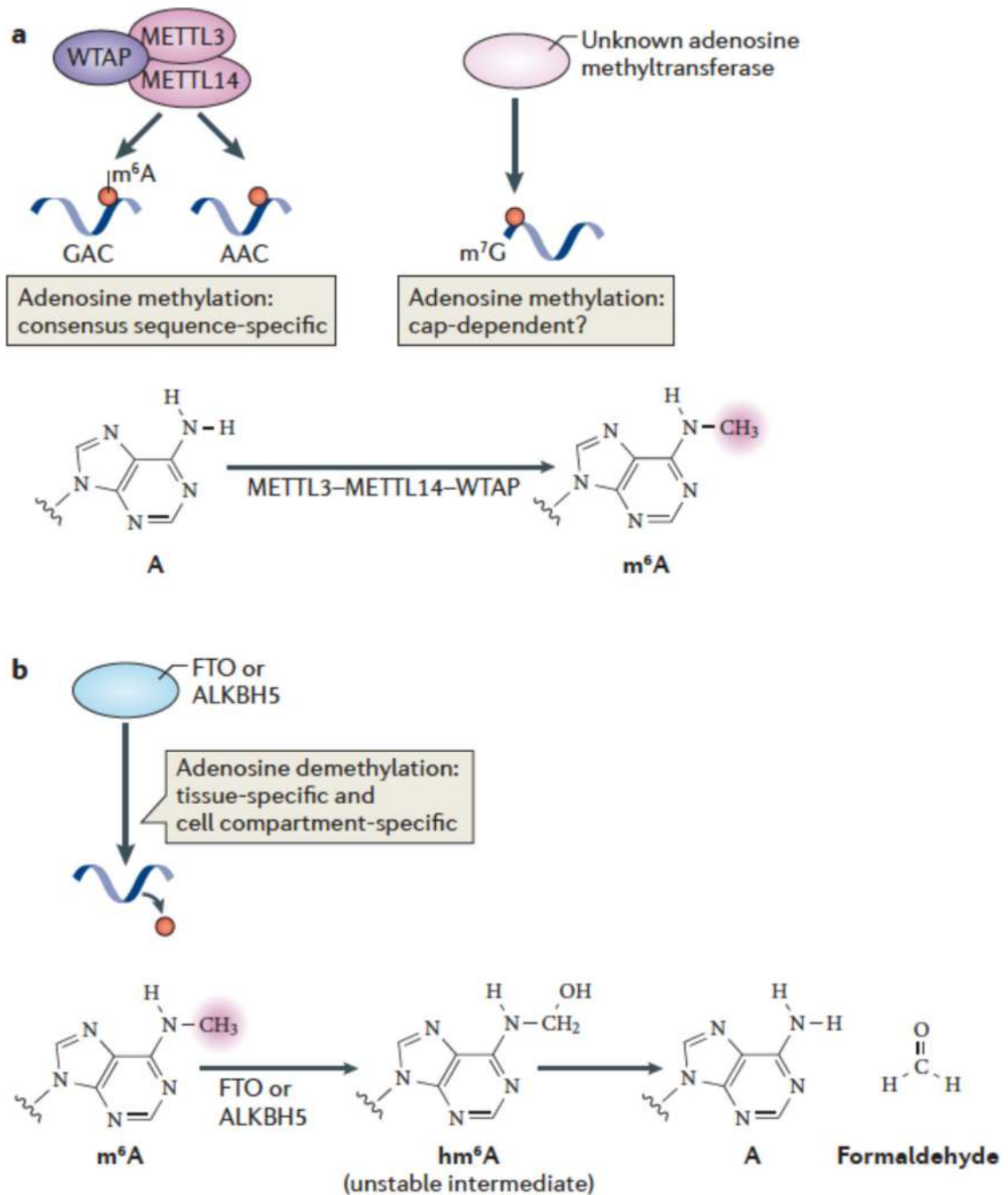


Figure 1. Spatially distinct pools of m⁶A in mRNAs

Metagene analysis of MeRIP-Seq data shows that m⁶A is enriched in discrete regions of mRNA transcripts³. In this approach, each m⁶A 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 m⁶A residues along the entire mature transcript body. Shown is an idealized metagene profile based on published MeRIP-Seq datasets³. m⁶A residues can be found at the first encoded residue, often as a dimethylated nucleotide (m⁶Am). m⁶A is also found at each of the other indicated positions in mRNAs, with a particularly prominent enrichment near the stop codon in mRNAs.



(b) FTO and ALKBH5, the two mammalian m⁶A mRNA demethylases identified to date, catalyze methyl group removal from the N⁶ 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 N⁶-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.

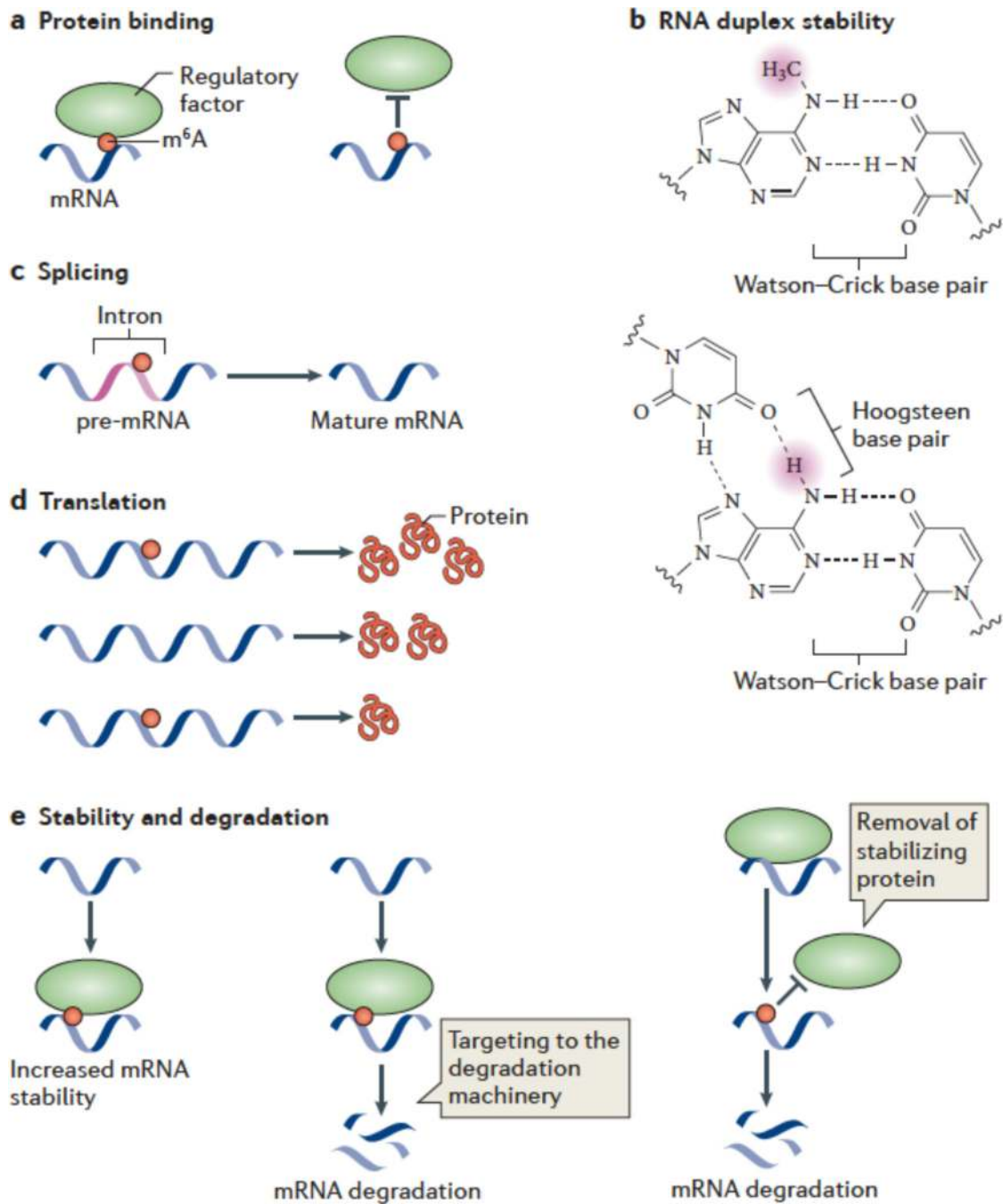


Figure 3. Mechanisms and functions of m⁶A

(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, m⁶A is capable of pairing with uridine. Since a free proton at the N⁶ position is still available for hydrogen bonding, N⁶-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.

m⁶A interferes with the formation of base triples. As is shown in this U•A-U base triple, two free protons are required at the N⁶ 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, m⁶A 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, m⁶A might stabilize mRNAs by binding to proteins that promote transcript stability.