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The Proteins of mRNA Modification: Writers, Readers, and Erasers

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Abstract

Over the past decade, mRNA modifications have emerged as important regulators of gene expression control in cells. Fueled in large part by the development of tools for detecting RNA modifications transcriptome-wide, researchers have uncovered a diverse epitranscriptome which serves as an additional layer of gene regulation beyond simple RNA sequence. Here, we review the proteins that write, read, and erase these marks, with a particular focus on the most abundant internal modification, m^6A. We describe the discovery of the key enzymes that deposit and remove m^6A and other modifications and discuss how our understanding of these proteins has shaped our views of modification dynamics. We also review current models for the function of m^6A reader proteins and how our knowledge of these proteins has evolved. Finally, we highlight important future directions for the field and discuss key questions that remain unanswered.

Keywords

Epitranscriptome; RNA modifications; m^6A; reader protein; methyltransferase; demethylase

1. INTRODUCTION

Cellular RNAs contain over 150 different chemical modifications, most of which reside in non-coding RNAs such as rRNA and tRNA. While the presence of modifications in these abundant RNA species has been known for decades, much of our understanding of post-transcriptional modifications in mRNAs has emerged within just the past several years. These studies have been fueled by new technologies that have enabled the detection and quantification of mRNA modifications and the precise mapping of modification sites transcriptome-wide. These efforts have revealed a growing “epitranscriptome” comprising diverse modifications of varying abundance.

Chemical modifications to mRNAs were initially discovered in the 1970s, when researchers identified m^6A and m^5C from bulk hydrolyzed nucleosides isolated from oligo(dT)-purified cellular RNA (1, 2). This work revealed m^6A to be the most abundant internal modification, and subsequent studies determined that it was present in a GAC or AAC consensus sequence

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(3, 4). However, due to a lack of methods for profiling m^{6}A transcriptome-wide, it was several decades before individual methylated mRNAs throughout the transcriptome would be discovered. Then, in 2012, the development of antibody-based approaches (MeRIP-seq/m^{6}A-seq) for global m^{6}A detection revealed thousands of methylated mRNAs and identified a distinct distribution of m^{6}A within long internal exons and near the 5' end of terminal exons (5, 6). These studies, in addition to the discovery that m^{6}A could be reversibly modified through the action of demethylating enzymes (7), sparked a renewed interest in mRNA modifications.

In this review, we provide an overview of the writers, readers, and erasers of mRNA modifications, with a primary focus on m^{6}A. We review the recent advances in our understanding of how these proteins are regulated and their impact on gene expression and cellular function, and we highlight links to human disease. Finally, we discuss key findings as well as controversies and describe emerging areas of importance.

2. WRITERS OF MRNA MODIFICATIONS

2.1. The m^{6}A methyltransferase complex: Discovery of the core METTL3/METTL14 dimer

m^{6}A is deposited co-transcriptionally by a large methyltransferase complex (MTC) (Figure 1a). Efforts to identify the factors responsible for m^{6}A formation began in the early 1990s, when researchers demonstrated that nuclear extracts from HeLa cells were capable of methylating substrate RNAs in vitro (8). A large complex was subsequently purified from HeLa cell nuclear extracts which had multiple components that were required for methyltransferase activity (9, 10). Within this complex, a 70 kilodalton protein with *S*-adenosyl-methionine (SAM) binding activity was identified and subsequently cloned from HeLa cell cDNA (9). This protein, MT-A70 (10), was found to be catalytically active in vitro, expressed in many tissues, and predominantly localized to the nucleus (9).

MT-A70 belongs to the methyltransferase-like (METTL) superfamily of proteins with SAM-binding domains and is now known as METTL3. METTL14, another member of this family with homology to METTL3, has since been demonstrated to be a critical component of the MTC. Initial studies indicated that METTL14 had methyltransferase activity in vitro (11). However, later work showed that this activity was due to co-purification of METTL3, which forms a strong dimer with METTL14 (12). Structural studies further confirmed that only METTL3 binds to SAM, while METTL14 contributes to substrate recognition and stabilizes the interaction between METTL3 and RNA (12–14) (Figure 1a). Thus, although METTL14 does not have methyltransferase activity, it is required for target RNA methylation. Indeed, multiple studies have shown that depletion of either METTL3 or METTL14 alone results in a strong decrease in the abundance of m^{6}A methylation in cellular mRNAs (11, 15).

In addition to its m^{6}A methyltransferase activity, METTL3 has also been shown to have methyltransferase-independent functions. In cancer cells, METTL3 promotes the translation of a subset of mRNAs by binding to the 3'UTR and mediating interactions with eIF3h through mRNA looping (16, 17) (Figure 1b). Through this mechanism, METTL3 enhances the translation of oncogenic transcripts including *EGFR*, *TAZ*, and *BRD4* and promotes cellular transformation and tumor growth (16, 17).

2.2. Additional MTC components

In 2008, FIP37 was identified in a yeast two-hybrid screen for interactors of MTA, the m^{6}A methyltransferase in *Arabidopsis thaliana* (18). Subsequent studies showed that the yeast homolog of FIP37, MUM2, also interacts with the yeast m^{6}A methyltransferase IME4 and contributes to m^{6}A deposition (19). *FIP37*/*MUM2* are homologs of mammalian Wilms' tumor 1-associated protein (*WTAP*), which in 2014 was shown to bind in complex with METTL3 and METTL14 and to be indispensable for MTC-mediated m^{6}A methylation in mRNAs (11, 15, 20). WTAP regulates the localization of the MTC by recruiting it to nuclear speckles, sites of high transcriptional activity (11, 15, 20) (Figure 1a).

The discovery of other MTC components was facilitated by proteomics studies that sought to identify interacting partners of METTL3, METTL14, and WTAP (15, 21, 22). These studies identified KIAA1429, also known as VIRMA, as an interacting partner of WTAP. This was consistent with previous work showing a similar interaction in *Drosophila* (23). Biochemical studies further demonstrated that depletion of VIRMA leads to a significant decrease in m^{6}A in poly(A) RNA, indicating its importance for a functional MTC (21). In addition to VIRMA, Horiuchi and colleagues identified RBM15 as a WTAP interactor (22). Later, it was confirmed that RBM15 and its paralog RBM15B interact with the MTC in a WTAP-dependent manner (24). Depletion of RBM15/15B results in a substantial loss of m^{6}A in poly(A) RNA and in the long noncoding RNA *XIST*, confirming a functional role for RBM15/15B in the methylation of cellular RNAs. Identification of RBM15/15B binding sites using iCLIP revealed that these proteins bind U-rich regions near methylated, but not unmethylated, DRACH motifs (D=A,G,U; R=A,G; H=A,C,U), which are preferred consensus motifs where m^{6}A methylation occurs (24). This suggests that RBM15/15B may help target the MTC to a subset of sites near U-rich sequences (Figure 1a).

HAKAI, an E3 ubiquitin ligase, was also identified within the MTC in proteomics studies (22). Not only has this association been confirmed for the HAKAI homologs in *Arabidopsis* and *Drosophila*, but depletion of the HAKAI homolog in each species leads to a reduction of m^{6}A in mRNA (25–27), demonstrating an evolutionarily conserved role in methylation. Interestingly, although HAKAI is an E3 ubiquitin ligase, its catalytic activity is not required for its role in regulating adenosine methylation in flies (26), suggesting that it most likely does not regulate MTC activity by ubiquitination. Indeed, in contrast to its described role as an E3 ligase, Hakai has been shown to stabilize the homologs of core MTC components METTL3, METTL14, and WTAP in flies (26).

The zinc finger protein ZC3H13 is an additional MTC component that was identified using immunoprecipitation and mass spectrometry (21). Knockdown of ZC3H13 leads to reduced m^{6}A deposition and, like other MTC components, it is critical for mESC self-renewal (21, 28). Mechanistically, ZC3H13 regulates the MTC by promoting the nuclear localization of WTAP, VIRMA, and HAKAI (28).

2.3 m^{6}A methylation specificity

Early biochemical studies using enzymatic digestion of radiolabeled cellular RNA indicated that m^{6}A occurs within a GAC or AAC consensus sequence (3, 4). More recent

transcriptome-wide m^{6}A mapping studies have further identified the preferred extended DRACH motif (5, 6). However, only a small proportion of DRACH motifs contain m^{6}A, suggesting that other factors contribute to methylation specificity. Methylation of specific residues may be primarily guided by *cis*-elements within the RNA, giving rise to a hard-wired "m^{6}A code" that is dictated primarily by sequence and, to a lesser extent, RNA structure (29). Additionally, gene architecture appears to contribute to m^{6}A distribution, as m^{6}A is depleted near exon junctions but enriched within long internal exons and in the beginning of terminal exons, the latter giving rise to the appearance of stop codon-proximal enrichment which is a characteristic feature of m^{6}A across diverse cell types and species (5, 6, 30, 31).

A major question has been how m^{6}A is preferentially deposited at specific DRACH motifs. Yue et al. demonstrated that VIRMA depletion leads to selective loss of m^{6}A sites from the vicinity of the stop codon in HeLa cells (21). This effect was not seen with depletion of METTL3, METTL14, or WTAP, suggesting that VIRMA may help confer methylation specificity near the 5' end of the 3'UTR. However, it remains unclear how VIRMA mediates this specificity, and more studies are needed to determine the mechanism and how generalizable this is across cell types. It is possible that VIRMA bridges interactions between the MTC and transcription-associated proteins, or that it somehow recognizes specific gene architecture elements.

Crosstalk with chromatin marks may be another mechanism for m^{6}A specificity. Studies in mESCs have shown that the MTC is recruited through METTL14 to the H3K36 trimethylation mark (H3K36me3), which is enriched at genomic regions near m^{6}A (32) (Figure 1a). Depletion of the H3K36 methyltransferase SETD2 leads to reduced m^{6}A at approximately 46% of co-regulated H3K36me3/m^{6}A sites, indicating that this may be a mechanism for guiding the MTC to a substantial proportion of RNAs destined for methylation, but also suggesting that other factors exist (32). Indeed, several transcription factors, including SMAD2/3, CEBPZ, and p53, have been shown to bind and recruit the MTC to methylate specific target RNAs (33–35) (Figure 1a). In addition, RNA polymerase II (RNA pol II) transcription rate affects methylation frequency, with slower transcription resulting in more methylation and decreased mRNA stability (36) (Figure 1a). This is reminiscent of other co-transcriptional processes like splicing and 3' end formation that are also altered by transcription rate (37).

In general, m^{6}A patterns are relatively static across tissues and even species (38), but there are transcripts that exhibit distinct tissue and cell type-specific methylation profiles, even when accounting for differences in gene expression (38, 39). Variability in the expression of specific transcription factors or abundance of H3K36me3 may explain some of this variability. Studies using single-cell m^{6}A profiling have shown that many more consensus motifs are methylated in mRNAs than previously thought based on m^{6}A mapping in bulk cells (40). Most of these sites occur rarely throughout a population of cells, but some RNAs contain high-frequency sites that are methylated in nearly all cells. Thus, chromatin marks and transcription-associated proteins may help recruit the MTC to sites of nascent transcription to facilitate methylation at any suitable motif in the region, but in some cases,

there may be other unknown factors that contribute to consistent methylation at the same adenosines in all cells.

Post-translational modifications to MTC components are additional factors that may control methylation. For instance, several lysine residues within METTL3 are susceptible to SUMOylation, which represses its methyltransferase activity (41). Additionally, methylation of arginine residues within the C terminus of METTL14 promotes its interactions with RNA and RNA pol II, leading to enhanced m^6A methylation (42). Post-translational modification of the MTC machinery remains a relatively underexplored area, so future studies are needed to better understand how modification of MTC components controls m^6A deposition.

2.4 METTL16: An m^6A methyltransferase with key roles in cellular metabolism

Although METTL3 is responsible for methylating the majority of cellular mRNAs, a small number of sites are targeted for methylation by METTL16. In addition to depositing m^6A in the U6 snRNA (43, 44) and the long noncoding RNAs *MALAT1* and *XIST* (44, 45), METTL16 also methylates the *MAT2A* mRNA, which encodes the SAM synthetase, as part of a feedback mechanism that helps maintain cellular SAM levels (Figure 1c). When SAM levels are high, METTL16 methylates a conserved hairpin in the *MAT2A* 3'UTR, leading to intron retention which blocks the production of a functional protein. Depletion of SAM leads to reduced enzymatic turnover of METTL16 and increased occupancy on the *MAT2A* transcript, which in turn promotes *MAT2A* splicing and increased MAT2A protein production (43). In *C. elegans*, the METTL16 homolog METT-10 also regulates SAM levels by methylating the SAM synthase mRNA to regulate its splicing (46, 47). However, the mechanism differs from mammalian cells and involves direct inhibition of the U2AF35 splicing factor by 3' splice site m^6A (46, 47). Nevertheless, this suggests an evolutionarily conserved role for METTL16 as a regulator of SAM homeostasis.

Although both METTL3 and METTL16 use SAM as a methyl donor (Figures 1a,c), they recognize target RNAs through different mechanisms. While METTL3 targets DRACH motifs, METTL16 methylates adenosines within a distinct sequence motif (UAC<u>A</u>GAGAA) that resides within a hairpin structural element (44–46). Depletion of METTL16 causes a significant reduction of total m^6A levels in poly(A) RNA and loss of m^6A in thousands of mRNAs (43, 48). However, due to its role in regulating SAM levels, it is difficult to uncouple METTL16-mediated methylation from changes due to reduced METTL3 activity, and METTL16 is likely to methylate only a few hundred mRNAs or less (48). This is consistent with the fact that the vast majority of m^6A sites in mRNAs exist within the DRACH motif (3–6). Interestingly, like METTL3, METTL16 has also been shown to have methyltransferase-independent functions by promoting translation initiation (48) (Figure 1b). METTL16 is predominantly localized to the cytoplasm; therefore, while it clearly directs the nuclear methylation of a subset of cellular RNAs, a major function of METTL16 may be to control gene expression through translation regulation.

2.5 Writers of other mRNA modifications

Pseudouridine (ψ) is a frequent internal mRNA modification, comprising 0.3-0.4% of uridines in mammalian cells (49). ψ profiling studies have identified several hundred to

several thousand ψ sites in human and yeast mRNAs (49–52). Several pseudouridine synthase enzymes have been shown to catalyze ψ formation in mRNAs, but PUS1 and PUS7 are the predominant pseudouridine synthases in both mammals and yeast (50–52) (Figure 2). Loss of these enzymes leads to alterations in splicing and 3' end processing, suggesting a role in regulating co-transcriptional mRNA processing (52). Additionally, pseudouridylation of stop codon uridines can promote translational readthrough, providing a potential mechanism for premature stop codons to bypass nonsense-mediated decay (53).

In contrast to ψ and m^6A, some of the additional internal mRNA modifications that have been identified are less abundant. N^1-methyladenosine (m^1A) is deposited in mammalian tRNAs by a dimer of TRMT61A and TRMT6 (54) (Figure 2). While there is evidence that this complex can add m^1A to mammalian mRNAs (55), the extent to which mRNAs contain m^1A remains an active area of research. Some studies have reported hundreds or thousands of sites, while others suggest that m^1A is present at only a small number of residues, including in mitochondrial mRNAs, and mostly at low stoichiometry (55–58). Similarly, N^4-acetylcytidine (ac^4C) is present in human rRNA and tRNAs, but reports of its abundance in mRNAs have been variable, with mass spectrometry estimates varying by an order of magnitude. NAT10 has been identified as the ac^4C acetyltransferase (Figure 2), and a recent study using a chemical reduction approach to map ac^4C transcriptome-wide identified over 7,000 sites, most of which were eliminated in *NAT10* knockout cells (59–61). Interestingly, most of these sites are found in the 5' UTR and are associated with increased translation initiation (59). Further studies examining the distribution of ac^4C and m^1A and whether they differ across cell types or cellular conditions will be important. Additionally, although challenging, measures of stoichiometry for these and for all modifications will be critical for a better understanding of their impact on cellular RNAs.

Like m^6A, the 5-methylcytidine (m^5C) modification was first observed in the 1970s in biochemical studies of polyadenylated RNA (62). This was reported in RNA from cultured hamster cells, but not in other mammalian cell lines (1, 3). In 2012, a bisulfite sequencing approach reported widespread m^5C in mammalian mRNAs (63). However, subsequent studies have found more variable numbers of sites, and mass spectrometry data show that m^5C is much less abundant than prevalent modifications like m^6A (64–67). NSUN2 has been identified as the primary m^5C methyltransferase in mammalian mRNAs (63, 68) (Figure 2). Importantly, NSUN2 is upregulated in many cancers, and mutations in NSUN2 can result in intellectual disability, although some of the underlying mechanisms likely relate to its tRNA methyltransferase activity (69, 70).

In addition to base modifications, methylation of the 2'-hydroxyl of the ribose backbone (N_m) is prevalent in tRNAs, rRNAs, and small nucleolar RNAs (snRNAs) (71). N_m is a low-abundance modification in mRNAs, and knowledge of specific transcripts that contain N_m has been limited by difficulties in detecting this mark transcriptome-wide. Dai and colleagues reported nearly 700 N_m sites in HEK293 cells and over 2,100 sites in HeLa cells (72); however, roles for Nm in mRNA regulation and its abundance in other cell types remain poorly understood. N_m in rRNAs is deposited by the methyltransferase fibrillarin (FBL) (73) (Figure 2), and a recent study demonstrated that FBL can also install N_m in the peroxidasin (PXDN) mRNA (74). This results in increased mRNA abundance, but decreased

translation efficiency, demonstrating a functional consequence of internal N_m within this transcript.

In addition to internal N_m, the first transcribed nucleotide adjacent to the 5' cap is also 2′-*O*-methylated by HMRT1 in mammalian cells (75, 76). 20-30% of these are adenosine residues that are additionally methylated at the N^6 position by PCIF1 to form N^6,2'-O-dimethyladenosine (m^6A_m) (77–79) (Figure 1a). Recent mapping studies in mammalian cells have found between 800-3,000 m^6A_m-containing mRNAs (78–80), with possible differences between cell types (79). Some studies have indicated increased stability of m^6A_m-containing mRNAs (78, 80, 81), possibly by inhibiting 5' decapping (81). m^6A_m may also play roles in translation, with some studies reporting a modest increase in translation of m^6A_m containing mRNAs (80, 81). However, other studies have found no change in translation efficiency upon PCIF1 depletion (78). Expression of a GFP mRNA with A_m or m^6A_m at the 5' end in PCIF knockout cells showed decreased translation of the m^6A_m-containing transcript compared to A_m mRNA (79). These discrepancies may indicate biological context-dependent effects of m^6A_m, since different cell lines were used across studies. This is supported by evidence that m^6A_m:A_m ratios can vary widely across tissues in mice (15:1 in brain, 2:1 in liver) (82). However, this will need to be further explored to better understand the functions of m^6A_m.

3. m⁶A READER PROTEINS

m^6A exerts many of its biological functions through the regulation of RNA:protein interactions, including the recruitment of m^6A "reader" proteins. Reader proteins can either directly recognize m^6A or bind indirectly to RNAs through m^6A-mediated structural changes. In the past decade, several studies have employed biochemical assays to identify and characterize m^6A reader proteins. Although these proteins can recognize methylated RNAs through a variety of RNA binding domains (RBDs), the most robust and specific interaction with m^6A is mediated through the YT521-B homology (YTH) domain.

3.1 YTH domain-containing proteins

The first m^6A reader proteins were identified with RNA pulldown assays using methylated and unmethylated RNA baits followed by mass spectrometry (6). This revealed two members of the YTH (YT521-B homology) domain-containing family of proteins: YTHDF2 and YTHDF3, as well as the RNA binding protein ELAVL1 (also known as HUR) as proteins with enhanced binding to m^6A.

The YTH domain was first identified in the splicing factor YT521-B (now known as YTHDC1) as a novel RNA-binding domain (83, 84). Homologous YTH domain-containing proteins have been identified in diverse organisms, from yeast and plants to mammals, suggesting important functions throughout evolution. Interestingly, while the *Saccharomyces cerevisiae* YTHDF homolog MRB1 binds to m^6A, the *Schizosaccharomyces pombe* YTH domain protein Mmi1 does not, and the presence of m^6A actually reduces its affinity for RNA (85, 86). In mammals, there are five YTH domain-containing proteins: YTHDF1, 2, and 3 (also called DF1, DF2, and DF3), YTHDC1 (DC1), and YTHDC2 (DC2). All share the highly conserved YTH domain and are capable of binding m^6A, although studies

suggest that YTHDC2 exhibits lower specificity for m^{6}A and may bind non-methylated regions (discussed below). The YTH proteins also differ in size, domain organization, and subcellular localization: while the DF proteins are predominantly cytoplasmic with shared domain architectures, DC1 is nuclear, and DC2 exhibits a nucleocytoplasmic localization with several distinct domains not found in the other YTH proteins (Figure 3a).

3.2 m^{6}A recognition by the YTH domain

The YTH domain is a highly conserved domain of approximately 130 residues that adopts an open α/β fold containing six β-sheets surrounded by α-helices (87–91). The methylated adenosine is lodged in an aromatic cage consisting of three tryptophans (DF1, DF2, DF3), or two tryptophans and a leucine residue (DC1, DC2). The N^6-methyl moiety is nested within a hydrophobic pocket and faces the tryptophan residue forming the base of the cage, and interactions with the adenine base are stabilized by hydrogen bonds between the nucleobase and the sidechains and backbones of neighboring residues (87, 89–91) (Figure 3b).

The reported binding affinities of the YTH domain for methylated RNA are relatively low, with in vitro measurements generally ranging from 100nm to 3μM for the DFs and DC1, and a ~5 to 25 fold lower affinity reported for DC2 (89–93). However, the YTH domain has a 10 to 100-fold higher affinity for m^{6}A versus unmodified A (88, 91, 94). This suggests that YTH proteins may only transiently interact with their targets, or that cooperative interactions with other RBPs are required to stabilize the interaction.

The YTH domain can recognize m^{6}A relatively indiscriminately, as mutation of m^{6}A-adjacent nucleotides at positions −2, −1 and +1 does not significantly alter binding of the YTH domain in vitro (89, 92). An exception is the YTH domain of DC1, which shows a slight preference for G at the −1 position (89, 91, 92). One limitation to in vitro binding assays is that they often use RNAs of fixed length and sequence, and the binding conditions may not mimic the environment of the cell. Methylated RNA may need to be in a favorable conformation to enable m^{6}A recognition by the YTH domain. For example, DF2 preferentially binds to m^{6}A in single-stranded regions, or when flanked by a 5’ bulge when in a duplex (94). In contrast, when m^{6}A is in a Watson-Crick conformation, it remains inaccessible for recognition by the YTH domain. Significantly, 11-30% of all m^{6}A sites are predicted to be in regions inaccessible for binding (94). Similarly, the accessibility of sites can also be impaired by binding of other RBPs. For example, the high stoichiometry m^{6}A sites found in the 18S and 28S rRNAs are not bound by YTH domain proteins in CLIP-seq studies, likely because they are buried within the ribosome (24).

3.3 Functions of YTH domain-containing proteins.

YTHDC1.—DC1 was initially characterized as a spliceosome-interacting protein localized to nuclear speckles (95, 96), and it is unique among the YTH proteins in that it is exclusively nuclear. This localization is driven by a nuclear localization sequence as well as low complexity regions which are required for DC1 condensation in nuclear speckles (Figure 3a) (96–98).

Consistent with its nuclear localization, DC1 regulates several steps of pre-mRNA processing (Figure 3c). Studies in HeLa cells have shown that DC1 is bound competitively by the splicing proteins SRSF3 and SRSF10 to regulate exon inclusion (97) and nuclear export (99). In mouse oocytes, DC1 can interact with CPSF6, SRSF3, and SRSF7 to regulate splicing and alternative polyadenylation of m^{6}A-modified mRNAs (100). DC1 was also found to promote the stability of RNA at sites of double-strand DNA breaks following recruitment and activation of METTL3 (101). Additionally, DC1 binds to the highly methylated long noncoding RNA (lncRNA) *XIST* and is essential to *XIST*-mediated gene silencing (24).

The functions of DC1 suggest that it binds to chromatin-associated RNAs rapidly after m^{6}A deposition. For example, in mESCs, DC1 is required for maintenance of stemness and silencing of transposons through the recruitment of SETDB1 and H3K3me3 deposition (102), and in HEK293T cells, m^{6}A and DC1 contribute to the removal of the repressive H3K9Me2 marks through the recruitment of KDM3B (103). DC1 also contributes to transcription regulation by facilitating the degradation of m^{6}A-containing chromatin-associated regulatory RNAs (carRNAs) and limiting their ability to promote open chromatin (104).

DC1 plays important roles in ESCs and is essential for embryonic development (100), so it will be important going forward to understand how DC1 activity is regulated. One possibility is that DC1 function is influenced by the activation of specific signaling pathways. For instance, DC1 contains multiple phosphorylation sites, and its localization to nuclear speckles can be influenced by activation of the Src family kinase FYN (96).

YTHDC2.—DC2 exhibits a unique domain composition compared to the other YTH proteins. DC2 is the largest of the YTH proteins and contains several conserved protein domains in addition to its YTH domain, including ankyrin repeats, an oligonucleotide-binding fold, and DEAD-like helicase domains (Figure 3a). The cellular functions of DC2 are still being elucidated, but it has been shown to be important for spermatogenesis and oogenesis through its regulation of mRNA stability and translation (105–107). CLIP-based studies have revealed that DC2 has a unique binding pattern characterized by enrichment in introns and intergenic regions and within the coding sequence of mRNAs (24), which is distinct from the distribution of m^{6}A and the binding sites of other YTH proteins. Additionally, DC2 binding sites show poor overlap with mapped m^{6}A sites (24, 108), suggesting that DC2 may not function exclusively as an m^{6}A reader. Indeed, DC2's YTH domain is dispensable for its biological function in spermatogenesis, and DC2 can bind target RNAs in poly(U) sequences in the absence of its YTH domain (108, 109). Consistent with this, the binding affinity and specificity of DC2 toward m^{6}A is much lower than for other YTH proteins (92). Further studies will be necessary to determine the extent of m^{6}A-dependent and m^{6}A-independent RNA regulation by DC2.

YTHDF proteins.—The DF proteins include three cytoplasmic proteins sharing 65-70% sequence identity and a similar global domain organization. The YTH domain is found near the C-terminus and is preceded by a low complexity region of ~350-400 residues which are P/Q/N-rich (Figure 3a). Low complexity regions are known to contribute to protein

condensation and liquid-liquid phase separation (LLPS), and consistent with this, the low complexity domain is required for LLPS of DF proteins (110) and mediates their association with stress granules (111) and P-bodies (112). Initially, it was proposed that DF proteins have distinct and partially overlapping functions: DF1 promotes translation of its target mRNAs (113), DF2 promotes their degradation (112) and DF3 promotes both functions (114). However, this model has recently been challenged by a unified model for DF protein function, which proposes that all three DF proteins act redundantly to promote mRNA decay with no effect on translation (93, 115, 116). Interestingly, single-molecule analysis of the RNA targets of DF proteins has revealed that individual mRNAs can be bound more than once by different DF proteins throughout their lifetime (117). This suggests that DF proteins may not immediately promote decay every time they bind to their target RNAs. The precise function of DF proteins has been examined in only a handful of cell types and experimental systems, and future experiments will be necessary to understand the full extent of DF-mediated regulation.

mRNA decay.—DF2 was initially implicated in m^6A-mediated mRNA instability following the observation that the abundance of its target mRNAs was increased following DF2 depletion (112). DF2 was also found to traffic to P-bodies and contribute to mRNA deadenylation and decay (112) (Figure 3c). This effect is consistent with metabolic labeling studies performed in the 1970s, which suggested selective instability of m^6A-containing mRNAs (118), as well as with the finding that *Mettl3* depletion leads to increased mRNA abundance (119, 120). Subsequent work identified a direct interaction between DF2 and the CNOT1 scaffolding subunit of the CCR4-NOT deadenylase complex (121). Interestingly, this study found no direct interaction between CNOT and either DF1 or DF3 and showed that tethering of DF1 or DF3 to a reporter mRNA caused deadenylation, but the magnitude was substantially reduced compared to tethering of DF2 (121). These results suggest that all three DF proteins can mediate mRNA degradation, but that the effect is strongest for DF2. This is consistent with recent studies in mESCs and HeLa cells which demonstrate that the loss of all three DF proteins causes a greater increase in methylated mRNA abundance compared to loss of any single DF protein alone, and that depletion of DF2 has a more pronounced effect on mRNA stability compared to depletion of DF1 or DF3 (93, 115). This is also consistent with the finding that DF2-bound RNAs are less likely to be subsequently bound by other DF proteins compared to DF1- or DF3-bound RNAs, potentially because DF2 targets are more efficiently processed for decay (122).

In addition to recruitment of the CCR4-NOT deadenylase complex, DF2 can interact directly with HRSP12 to recruit RNase P/MRP and elicit endonucleolytic cleavage and degradation of target mRNAs (123) (Figure 3c). DF2 can also promote mRNA decapping through interactions with UPF1 and recruitment of PNRC2 and DCP1A (124). These interactions are not detected with DF1 or DF3, again suggesting that DF2 has a more prominent role in mRNA decay.

Translation control.—A role for DF1 in promoting translation was initially demonstrated in ribosome profiling studies in HeLa cells, which showed that DF1 depletion leads to reduced translation efficiency of its target mRNAs (113). DF1 was also shown to interact

with eIF3a/b and eEF2 to promote translation initiation and elongation of methylated mRNAs (113, 125). However, other studies have failed to observe global effects on translation following loss of DF1 and suggest a more limited role for m^6A and DF proteins in translational control (93, 110, 115, 126). It is likely that the translation-promoting function of DF1 is influenced by cell type or cellular conditions. For instance, several studies support a role for DF1 in promoting translation in neurons (127–129), and the positive effect of DF1 tethering on translation is most evident during stress recovery (113). In addition to DF1, DF3 has been reported to promote translation of its target mRNAs, but it appears to do so in cooperation with DF1 (114, 130). Future studies examining cell type- and context-dependent effects of DF1/DF3-mediated translation regulation and the mechanisms of their cooperative function will be important for a more complete understanding of how DF proteins impact protein production.

Biological context.—Many studies of DF proteins have been performed in immortalized cells at steady state. It is therefore important to consider how factors like expression level, local subcellular concentration, and cell type-specific interactions may differentially influence DF proteins when proposing general models for their function. For instance, studies in the nervous system have shown that perturbation of distinct DF proteins results in drastically different phenotypes (131). In the developing brain, DF2 is essential for neuronal progenitor cell differentiation and methylated mRNA clearance (132). In contrast, DF1 is important for axonal guidance (133), and in mature neurons it is required for learning and memory through translational control of its target RNAs (127). Moreover, DF1 and DF3, but not DF2, are required for proper synapse morphology (128). Additionally, DF2 and DF3, but not DF1, contribute to the localization of the methylated mRNAs *Camk2a* and *Map2* to neurites in hippocampal neurons (122). Furthermore, depletion of *Mettl3* or *Mettl14* in mature neurons does not lead to a global increase in m^6A-modified mRNAs, suggesting that mRNA decay is not the primary function of m^6A in neurons (122, 134, 135). This is an important distinction from the effects of m^6A observed in studies in mESCs and other dividing cells and suggests that DF protein function depends on context-dependent factors.

How could DF proteins mediate these differential effects? Analysis of proximity labeling datasets in HeLa cells suggests that DF1, DF2, and DF3 interact with a similar set of proteins (93). In contrast, other studies have reported unique protein interacting partners with the different DF proteins (123, 124, 136). The low complexity region is where most differences occur among the DFs and is important for these unique interactions (123, 124, 136). It is therefore possible that while many DF binding partners are conserved among the three proteins, subtle differences in DF protein sequence can enable unique interactions which then promote distinct functions in certain contexts.

It is also possible that post-translational modifications (PTMs) of DF proteins enable selective regulation of these otherwise highly similar proteins. All three DF proteins contain several phosphorylation sites within their low complexity regions (137). Phosphorylation of these regions could prevent protein condensation in RNA granules and could be an important mechanism to control their activity in response to stress or cellular stimuli. Other modifications have also been described in DF proteins. For example, DF2 is ubiquitinated in a cell-cycle dependent manner, which influences its stability (138). Additionally, stress-

dependent SUMOylation of the DF2 C-terminus was found to increase its affinity toward m^{6}A-modified mRNAs (139). Interestingly, the DF2 SUMOylation sites that have been reported are not found in DF1 and DF3. Going forward, it will be important to understand which PTMs are found in the various DF proteins and whether they are differentially regulated in a cell-type or context-specific manner.

3.4 Other direct m^{6}A-binding proteins

In addition to the YTH domain-containing proteins, several other RNA-binding proteins have been identified as m^{6}A readers (6, 140–143). For example, subunits of the translation initiation factor complex eIF3 can bind to m^{6}A sites within 5'UTRs to promote cap-independent translation of cellular mRNAs in response to stress (143). Additionally, eIF3 is required for the translation of m^{6}A-containing circRNAs, further indicating a role for m^{6}A in cap-independent translation, although this mechanism also involves YTHDF3 and eIF4G2 (144).

IGF2BP proteins (IGF2BP1, EGF2BP2 and IGF2BP3) have also been identified as m^{6}A readers. These proteins recognize m^{6}A through their K homology (KH) domains and act to promote mRNA stability and translation (142) (Figure 3a). IGF2BP binds RNA at sequences similar to the m^{6}A consensus sequence, but interestingly these sites are enriched throughout 3'UTRs as opposed to near the stop codon as is typically observed for m^{6}A (142). Moreover, the selectivity of IGF2BP proteins for m^{6}A is much lower than that of YTH proteins (approximately 3-fold increase in binding affinity for m^{6}A compared to A) (142). Another possibility is that IGF2BP proteins are indirect m^{6}A readers that bind RNA through m^{6}A-dependent structural changes (see below), as has been demonstrated for IGF2BP3 (145).

The fragile X mental retardation protein (FMRP) is another protein that displays preferential m^{6}A binding (140). Similar to the IGF2BP proteins, FMRP exhibits a modest preference for binding m^{6}A compared to A (~2.5-fold increase in binding affinity) (146, 147), and evidence from CLIP-based analyses suggests that only a subset of FMRP targets are methylated (146, 148). Nevertheless, FMRP has been shown to regulate some mRNAs through m^{6}A. Many of these roles have been demonstrated in neurons, where FMRP contributes to mRNA export (147, 148), neuronal differentiation (147), and mRNA decay (146). However, FMRP can also directly interact with DF2, and at least part of its function in the control of mRNA stability could be through this interaction (146). Future studies are needed to better understand the m^{6}A-dependent and m^{6}A-independent functions of FMRP.

While the m^{6}A-binding activity of many m^{6}A readers is conserved across cell types (140), it is likely that cell type- and tissue-specific readers exist. For example, PRCC2A has been identified as a reader in oligodendrocytes, where it binds RNAs at DRACH motifs and displays a binding pattern in mRNAs that matches the distribution of m^{6}A (149). PRRC2 plays an important role in oligodendrocyte differentiation and myelination by stabilizing the *Olig2* mRNA in an m^{6}A-dependent manner (149). Similarly, RBM45 is a brain-enriched m^{6}A-binding protein that influences splicing and neuronal differentiation (150). It will be interesting to see if PRRC2, RBM45, and other novel readers contribute to m^{6}A-mediated regulation in other cell types, or if there is cell type-specific specialization in reader protein function. Additionally, m^{6}A likely plays important roles in RNA regulation by repelling

RBPs. For example, the stress granule protein G3BP1 exhibits preferential binding to non-methylated RNA in some sequence contexts, which in turn impacts RNA stability (140).

3.5 Indirect readers

In addition to direct recognition of m^6A by RBPs, m^6A can influence RNA:protein interactions by altering RNA structure. m^6A can destabilize RNA duplexes by 0.5-1.7 kcal/mol through both a non-favorable trans conformation when paired with U and through improved stacking in its unpaired conformation (151). Thus, m^6A has the potential to act as a so-called "m^6A-switch", where it alters local RNA structure to enable, enhance, or prevent the binding of some RBPs (Figure 3c). The first example of this mechanism was the binding of heterogeneous nuclear ribonucleoprotein C (HNRNPC) to a stem-loop within the lncRNA *MALAT1* (152). The presence of m^6A within this region destabilizes the stem-loop and exposes an HNRNPC binding site. Global analysis revealed nearly 2,800 binding sites of HNRNPC that are altered when m^6A is depleted, resulting in changes in alternative splicing of target RNAs (152). Similar observations have since been made for HRNRNPG (153) and HNRNPA2B1 (154), suggesting that the m^6A-switch mechanism may play an important role in splicing regulation in the nucleus. Additionally, global profiling of m^6A-dependent structural changes has shown that many changes are conserved from the nucleus to the cytoplasm, suggesting that m^6A-dependent structural changes are likely to impact mature mRNAs as well (145). Indeed, m^6A can indirectly impact RNA recognition by HuR, which in turn mediates miRNA-mediated mRNA targeting to control mESC self-renewal (155).

3.6 Readers of other modifications

Readers of modifications other than m^6A have also been identified, although in general these remain less well understood (Figure 2). For example, a recent study identified the metabolic enzyme HRSP12 as an m^1A reader that can cooperate with YTHDF2 and m^6A to direct RNA degradation (156). However, it remains unclear how HRSP12 recognizes m^1A, as it lacks homology for known RNA binding domains. Other studies have identified several m^5C readers, including ALYREF, which plays a role in mRNA export (68), YBX1, which stabilizes target RNAs (157), and RAD52, which displays increased binding for m^5C at sites of DNA repair (158). The mechanism by which these proteins recognize m^5C differs among the proteins, including the involvement of a critical lysine residue in ALYREF and a cold-shock domain in YBX1 (68, 157). Thus, unlike the YTH domain proteins that bind m^6A, currently identified m^5C readers appear to utilize distinct domains to recognize m^5C.

Readers of Ψ are also poorly understood. In *S. cerevisiae*, methionine aminoacyl $tRNA^{Met}$ synthetase (MetRS) not only binds to Ψ in tRNAs, but also in mRNAs, where it controls the translation of specific transcripts (159). Additionally, Martinez et al. recently found that Ψ sites in pre-mRNAs are enriched for splicing factor binding sites (52). However, direct binding by these proteins was not characterized, and it is possible that they bind indirectly to Ψ-modified RNAs.

Much of the impact of mRNA modifications on gene expression is due to their influence on RNA:protein interactions. Thus, it will be important going forward to identify reader proteins for individual modifications and to determine how specific modified residues alter

RNA recognition either directly or indirectly. For many modifications, such studies are still in their infancy. For instance, in vitro RNA pulldown studies identified TBL3 as a potential reader protein for ac^4C (160), but this interaction and potential functional implications have yet to be validated in cells.

4. ERASER PROTEINS

4.1. FTO

Shortly before the first transcriptome-wide m^6A mapping studies were published, FTO was discovered as an m^6A demethylase which could remove m^6A from RNA (7). This was an important finding for the still nascent epitranscriptomics field, since it suggested that chemical modifications could potentially be susceptible to dynamic regulation. FTO is a member of the Fe(II) and 2-oxoglutarate-dependent dioxygenase family of proteins, which encode homologs of the bacterial ALKB enzyme that function in DNA and RNA repair (161). In addition to removing m^6A from RNA in vitro, FTO was shown to influence m^6A levels in mRNAs in cells and in vivo (7, 162). Subsequent studies shed light on the mechanism through which FTO demethylates m^6A and found that it does so through the production of N^6-hydroxymethyladenosine and N^6-formyladenosine intermediates (163). FTO lacks general sequence specificity for m^6A, but its demethylation activity can be influenced by m^6A-dependent conformational changes, such that much higher activity is seen when m^6A is in a single stranded or hairpin structure comparted to a duplex (164, 165). This structural preference may explain why many m^6A sites do not seem to be impacted by FTO. Indeed, FTO knockout does not lead to dramatic hypermethylation of all m^6A sites (162, 166) and its overexpression in cells often has only small effects on lowering global m^6A levels (167).

An important shift in our understanding of FTO came in 2017, when Mauer et. al. reported that FTO can demethylate m^6A_m (81) (Figure 4). This study further showed that FTO removes m^6A_m much more efficiently than m^6A in vitro and preferentially targets m^6A_m over m^6A in cells. These findings reshaped the view of FTO as exclusively an m^6A demethylase and prompted additional studies into the true targets of FTO in cells.

The substrate preference of FTO appears to be affected in part by differences in expression levels and subcellular localization of FTO across different cell types. Initial reports characterized FTO as a nuclear protein (7), but subsequent studies have shown that it can be localized to the cytoplasm in some cell types (168, 169). FTO has also been reported in neuronal synaptic compartments and in axons of cultured neurons, where it may mediate local demethylation of select mRNAs (128, 170) (Figure 4b). Analysis of FTO demethylation in various immortalized cell lines and subcellular regions revealed that FTO has a preference for demethylating m^6A in the nucleus and m^6A and m^6A_m in the cytoplasm in polyadenylated RNA, as well as m^1A in tRNA (168) (Figure 4a,b). Thus, in some cell lines such as HeLa and HEK293T, where FTO is largely nuclear, the effects of FTO depletion on m^6A_m are less pronounced, perhaps because access to m^6A_m is blocked by the nuclear cap binding complex. Indeed, forced expression of FTO in the cytoplasm leads to higher levels of m^6A_m demethylation compared to m^6A (81, 168).

Dynamic regulation of FTO localization may be a mechanism for cells to selectively regulate m^6A and m^6A_m levels. Alteration of FTO subcellular localization has been observed in the brain in response to starvation and fear conditioning (171, 172). FTO post-translational modifications may also impact its subcellular localization in response to stress (173). More research is needed to determine whether other stimuli influence nuclear and cytoplasmic FTO levels and if this corresponds to selective demethylation of m^6A or m^6A_m.

Recent studies also suggest that mRNAs may not be the primary targets of FTO: in line with the predominantly nuclear localization of FTO in many cell types, small nuclear RNAs (snRNAs) have been identified as FTO targets, with FTO depletion causing increased m^6A_m in snRNAs (166, 174) (Figure 4a). snRNAs are an important class of splicing regulatory transcripts, and reduction in FTO has been shown to result in altered splicing, potentially mediated by its impact on snRNA expression (166, 174, 175). Additionally, m^6A in long-interspersed element-1 (LINE1) RNAs has been shown to be targeted by FTO in mESCs (176) (Figure 4a). Loss of FTO causes elevated LINE1 methylation, leading to repression of LINE1 RNA and altered chromatin state and gene expression, which impacts oocyte maturation and embryonic development (176). Thus, removal of m^6A and m^6A_m in nuclear non-coding RNAs appears to be an important aspect of FTO's demethylase activity.

Numerous studies have linked FTO to human disease. In 2007, genome-wide association studies (GWAS) found that mutations clustering within intron 1 of *FTO* were associated with increased body mass index and risk for obesity (177). Since then, multiple studies have found associations between genetic variants in *FTO* and obesity and related phenotypes (178). Importantly, some obesity-associated mutations within FTO have also been found to impact long-range genomic interactions with other genes such as *IRX3*, suggesting that the disease links of these mutations may not be mediated through the FTO protein itself (179). However, mice with either missense *Fto* mutations or gain/loss of FTO expression exhibit metabolic phenotypes and developmental defects, so it is likely that misregulation of the FTO protein is a contributing factor to obesity-related diseases (178).

In addition to its links to obesity and metabolism, FTO has been associated with several other diseases, including Alzheimer's disease (180, 181), neuropsychiatric disorders (182), and various cancers (178, 183). FTO expression is upregulated in some AML subtypes, where it promotes leukemogenesis and inhibits leukemia cell differentiation (184, 185). FTO can also be inhibited by the oncometabolite 2-hydroxyglutarate (2HG) and has been shown to mediate anti-tumor effects in 2HG-sensitive cells through the MYC/CEBPA signaling pathway (185). FTO has also been implicated in glioblastoma, as its inhibition impairs glioblastoma stem cell (GSC) growth and self-renewal and leads to decreased tumor growth in mice. This is accompanied by decreased expression of oncogenes such as *ADAM19* (186). Collectively, these studies point to FTO as a potential therapeutic target and suggest cell type-specific pathways that may be regulated by FTO to contribute to disease. However, FTO has been shown to play both positive and negative roles in cancer progression (183), so continued investigation of the cellular target RNAs of FTO will be important for determining its value as a therapeutic target in specific cell types. It will also be important to better

understand whether FTO's effects on a particular RNA's expression are mediated through m^6A or m^6A_m demethylation.

Finally, recent work has uncovered potential roles for FTO as a mechanism for increasing biomass and yield in plants. Early studies in *Arabidopsis* first demonstrated that m^6A is required for normal plant development (18, 187), indicating that proper m^6A regulation is required for plant growth. Although plants lack an FTO homolog, overexpression of human FTO causes m^6A demethylation and promotes chromatin accessibility and transcriptional activation, ultimately leading to substantial increases in plant growth (188). This is an exciting area of research with important implications for increasing crop yield.

4.2. ALKBH5

ALKBH5 was the second m^6A demethylase enzyme to be discovered and is also a member of the iron and 2-OG dependent family of dioxygenase enzymes (189). ALKBH5 localizes to nuclear speckles and carries out demethylation of nuclear RNA substrates, and ALKBH5 depletion has been shown to impact nuclear mRNA export (189). Structural studies have provided insights into the mechanism of m^6A binding and have shown that, like FTO, ALKBH5 prefers single stranded substrates (164, 190–192). However, unlike FTO, ALKBH5 does not have reported activity on m^6A_m, thus making it likely that m^6A is its only substrate (81) (Figure 4a).

The expression pattern of ALKBH5 across tissues is distinct from that of FTO. ALKBH5 is highly abundant in testis, and *Alkbh5* knockout mice have impaired spermatogenesis and decreased male fertility (189). *Alkbh5* knockout mice otherwise appear normal, suggesting that a primary function for *Alkbh5* is to mediate m^6A levels during gametogenesis. However, roles for ALKBH5 in other tissues may still be possible, especially through targeting of specific transcripts. For instance, conditional knockout of *Alkbh5* in bone marrow mesenchymal stem cells (MSCs) has been shown to regulate MSC differentiation and bone mass through regulation of *Prmt6* (193).

Several studies have linked altered levels of ALKBH5 with human disease. For instance, ALKBH5 is upregulated in breast cancer cells in response to a hypoxic environment, which alters m^6A levels and abundance of the pluripotency factor *NANOG* to promote breast cancer stem cell growth (194). ALKBH5 is also elevated in glioblastoma, where it impacts GSC proliferation through modulation of *FOXM1* levels (195). Consistent with roles for m^6A in AML progression, ALKBH5 levels are also increased in AML and associated with poor prognosis (183) (Figure 4b).

The discovery of m^6A demethylase enzymes suggests the possibility that demethylation of mRNAs in the cytoplasm can be a mechanism for dynamic m^6A regulation. As discussed above, there are several studies which suggest that this may be true for specific transcripts. However, an alternative model is that demethylases do not dynamically regulate cytoplasmic m^6A, but instead act to remove m^6A sites in the nucleus shortly after their formation in order to maintain these sites in a persistently unmethylated state. This model is supported by evidence from a nucleotide-resolution m^6A mapping study which found that knockout of either ALKBH5 or FTO causes new sites to emerge, instead of leading to hypermethylation

of existing sites. This suggests that demethylase-regulated sites are normally present only transiently and thus fail to be detected in wild type cells. However, this idea is countered by several studies which have found that ALKBH5 or FTO depletion is accompanied by only minor increases in m^6A (81, 162, 189). Going forward, it will be important to understand whether m^6A demethylation serves to "correct" initial transcript methylation or if it acts as a mechanism for dynamic regulation of RNAs. In either case, current evidence suggests that the impact of FTO and ALKBH5 on global m^6A is for the most part modest, but that demethylation of specific transcripts may have important outcomes for cellular function in distinct cellular states or cell types.

4.3. Erasers of other modifications

In addition to FTO and ALKBH5, other members of the ALKB family of dioxygenase enzymes have been found to demethylate modified RNAs. For instance, ALKBH1 can remove m^1A from tRNAs (196), and ALKBH3 can demethylate m^1A residues in mRNA and tRNA (57, 197) (Figure 2). Both of these enzymes have demethylase activity on other modifications as well (198). As discussed above, there have been conflicting reports regarding the prevalence of m^1A in cellular mRNAs. Although it seems clear that mitochondrial mRNAs can be m^1A-modified, it is unknown whether ALKBH3 acts on these transcripts.

The Ten-eleven translocation (TET) family of dioxygenase proteins (TET1, TET2, and TET3) have been well characterized for their ability to convert 5-methylcytosine in DNA to 5-hydroxymethylcytosine (5hmC) (199). Additionally, TETs are capable of converting m^5C to 5hmC in RNA (200, 201) (Figure 2). 5hmC is detectable in cellular mRNAs (201–203), suggesting that it is not a transient product but instead can have its own functions. Indeed, studies in flies indicate roles for 5hmC in promoting translation and regulating brain development (201), and cytosine hydroxymethylation in ESCs leads to reduced stability of pluripotency-related transcripts which may be important during differentiation (203).

5. CONCLUDING REMARKS

Although mRNA modifications were first detected nearly 50 years ago, recent technical advances in identifying modified nucleotides have fueled rapid progress in our understanding of their regulation and biological impact, particularly for m^6A. However, important questions remain. For modifications other than m^6A, m^6A_m, and pseudouridine, some discrepancies remain regarding their abundance in cellular mRNAs. The development of more reliable profiling methods will be important for determining the location and stoichiometry of these modifications and enabling consistency across studies. Researchers have made substantial progress in the development of novel modification detection methods over the past few years, including the use of direct RNA sequencing, which holds great promise for the detection of diverse modifications within individual RNA molecules. Still, there are substantial challenges that remain, in particular for mapping modifications with low abundance.

Although many core components of the m^6A MTC have been identified, our understanding of the precise mechanism that directs methylation of specific sites remains incomplete.

Some evidence suggests that sequence is the major determinant of methylation, with a slight contribution of RNA structure (29). Additionally, it is clear that gene architecture plays a role, as m^6A is depleted near exon boundaries and enriched in long internal exons and near the beginning of terminal exons (6, 30, 31). However, transcription factors and histone modifications also help recruit the MTC to specific transcripts (32–35), which suggests that in addition to RNA sequence elements, methylation of transcripts can be guided by cell- and context-specific factors. Additionally, it is important to note that much of our current knowledge of the factors driving mRNA methylation comes from studies in bulk cells. Our group recently developed a method for profiling m^6A in single cells, which revealed that mRNAs contain many m^6A sites, most of which occur relatively rarely throughout the population of cells (40). This suggests either high variability in methylation site selection between cells, or that the methylation of specific residues may not be as critical as methylation of transcript regions. Continued studies of m^6A in single cells, especially in tissues or in response to changing cellular states, will be critical for improving our understanding of m^6A distribution and regulation.

Another area of high importance is the study of m^6A reader proteins, including the YTHDF proteins. Work in mESCs as well as in HeLa and HEK293 cell lines suggests that all three DF family proteins interact with similar proteins and are functionally redundant in contributing to the degradation of transcripts (93, 115). However, other studies have shown that different DF proteins can have distinct interacting partners, and there is also a body of evidence for DF-specific effects in the nervous system. It will be important to consider the extent to which reader proteins are functionally redundant within different biological contexts. Furthermore, methods for dissecting single-molecule DF:protein interactions will be important for illuminating how the three DF proteins compete or cooperate in cells to bind to m^6A sites.

Questions also remain about the functions of m^6A eraser proteins, in particular FTO. While m^6A was predicted to be a dynamic modification after the discovery of the FTO demethylase, the generality of this assumption has since been challenged. FTO is now known to play important roles in the demethylation of m^6A_m (81), and its effects on m^6A are likely to be transcript and perhaps cell type-specific. ALKBH5 is largely nuclear and plays its most important biological roles in male germ cell development (189). This suggests that m^6A may not be as generally dynamic—at least in the sense of widespread active removal—as previously thought. As with so many other aspects of m^6A, defining the cell types, cellular states, and specific transcripts that are targeted for demethylation will be important for a more complete understanding of the physiological importance of m^6A demethylation and for our views of m^6A dynamics.

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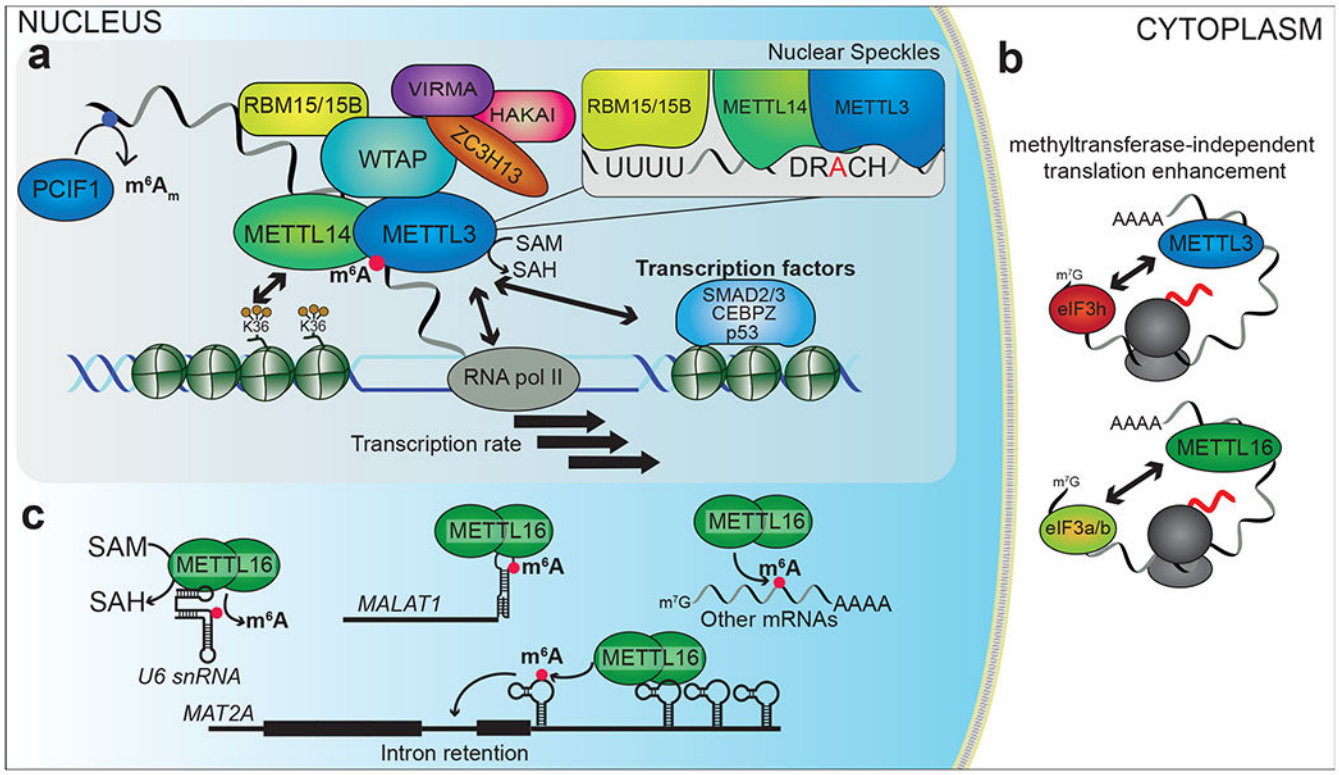

Figure 1. Regulation and function of m^{6}A writer proteins.

a) m^{6}A is deposited co-transcriptionally by a methyltransferase complex which includes METTL3 as the catalytic subunit and additional proteins METTL14, WTAP, RBM15/15B, VIRMA, HAKAI, and ZC3H13. This complex is recruited to RNAs through interactions with H3K36me3 histone marks, transcription factors, and RNA polymerase II (RNA pol II), and its activity can be influenced by transcription rate. METTL3 deposits m^{6}A within the DRACH consensus sequence, which is where the majority of m^{6}A sites within mRNAs reside. b) In the cytoplasm, METTL3 and METTL16 interact with the translation initiation machinery to promote mRNA translation independently of their methyltransferase activity. c) A subset of mRNAs can be methylated by METTL16, which requires distinct sequence and structural elements. METTL16 also deposits m^{6}A in snRNAs and lncRNAs. At the mRNA 5' end, PCIF1 deposits m^{6}A$_m$ adjacent to the cap.

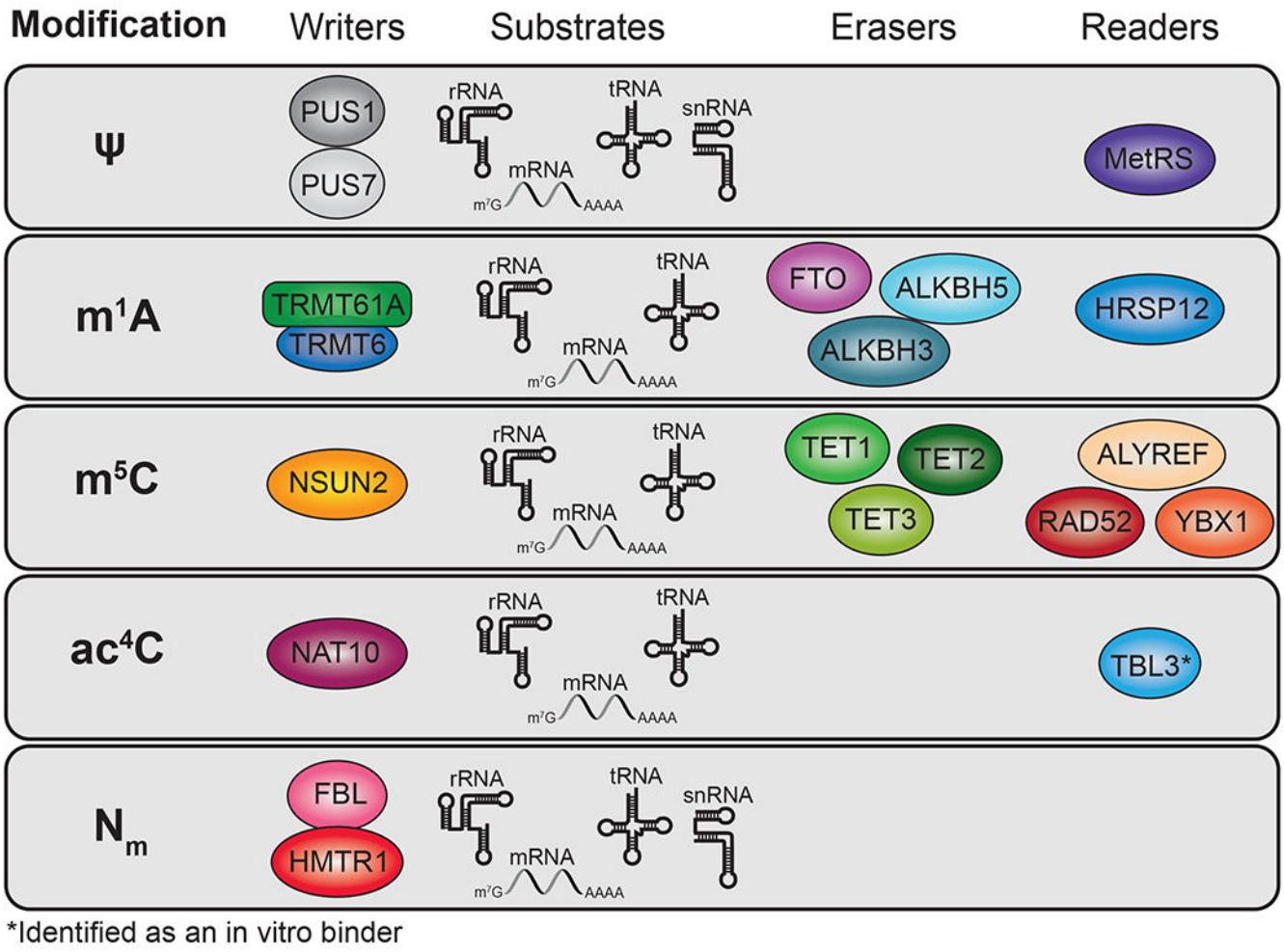

Figure 2. Writers, readers, and erasers of other mRNA modifications.

Shown are the major regulatory proteins of pseudouridine, m^1A, m^5C, ac^4C, and Nm as well as the types of RNAs in which these modifications have been identified. Further characterization of many of these regulatory proteins will be important for understanding how these modifications are controlled in cells and their contribution to gene expression. In some cases, eraser and reader proteins have not yet been identified.

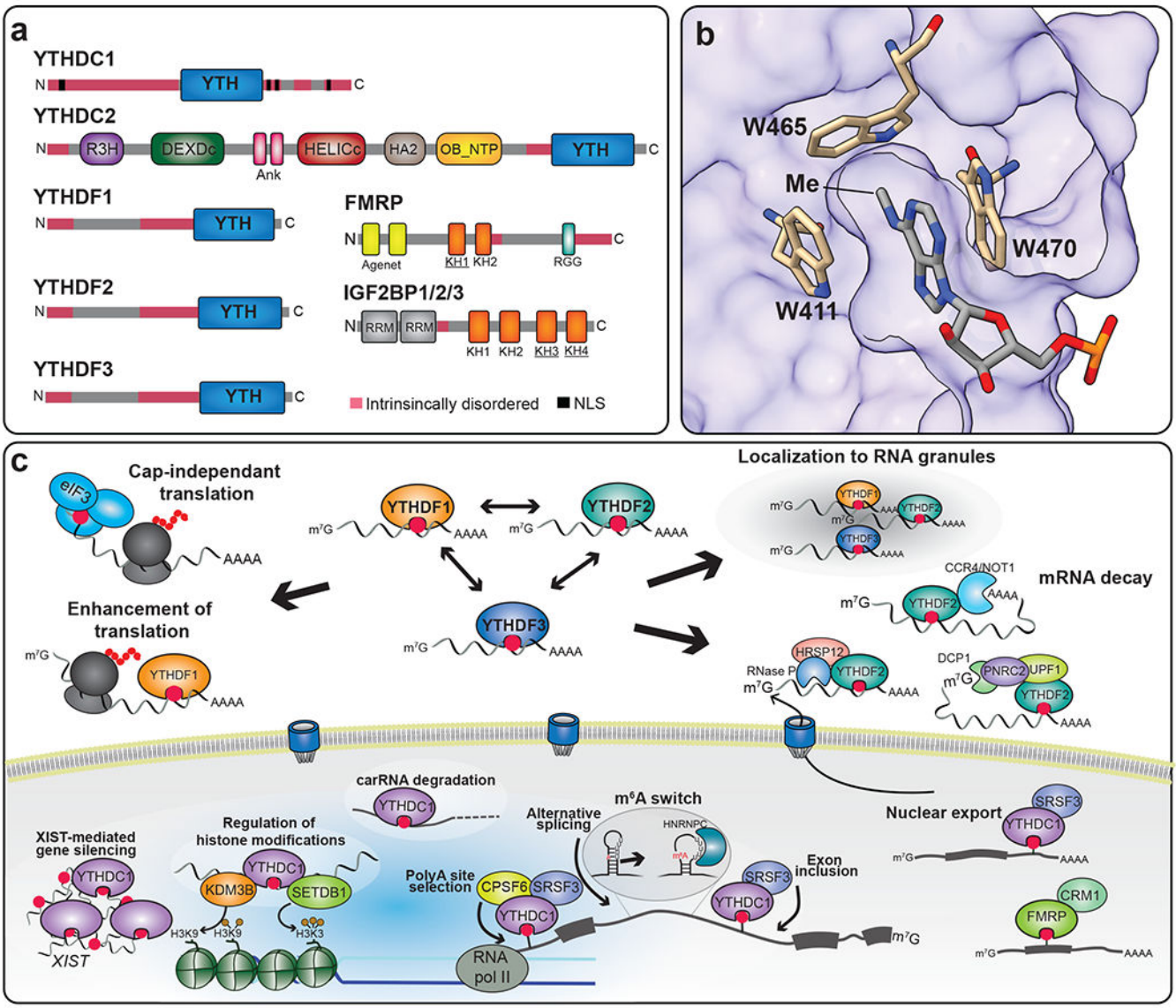

Figure 3. m^{6}A reader proteins have diverse functions and mechanisms of m^{6}A recognition.

a) Schematic showing the domain structures of direct m^{6}A binding proteins. YTHDF1/2/3, YTHDC1, and YTHDC2 recognize m^{6}A through the highly conserved YTH domain. K-homology (KH) domains mediate m^{6}A binding in FMRP and IGF2BP proteins. The KH domains required for m^{6}A recognition are underlined. (b) Surface representation of the YTH domain of human YTHDF1 in complex with m^{6}A (PDB: 4RCJ). m^{6}A is nested in a hydrophobic pocket of the YTH domain. The three tryptophan residues forming the aromatic cage surrounding m^{6}A are highlighted, as well as the position of the methyl moiety in m^{6}A. c) m^{6}A readers impact several aspects of RNA processing and function. In the nucleus, YTHDC1 regulates chromatin accessibility, promotes degradation of carRNAs, and binds to m^{6}A sites within the lncRNA *XIST* to promote gene silencing. YTHDC1 also interacts with splicing factors and controls alternative splicing, polyadenylation, and nuclear export. FMRP recognition of methylated transcripts also promotes their export. In the cytoplasm,

m^6A is recognized by eIF3 to promote cap-independent translation of mRNAs with 5'-UTR m^6A sites. YTHDF proteins can localize to stress granules and P-bodies, and all three proteins can promote mRNA decay. The role of YTHDF2 in mRNA destabilization is the most extensively studied and can include recruitment of various effector proteins to promote decapping, deadenylation, or endonucleolytic decay. Binding of YTHDF1 and YTHDF3 can also lead to increased translation of methylated mRNAs in some cell types.

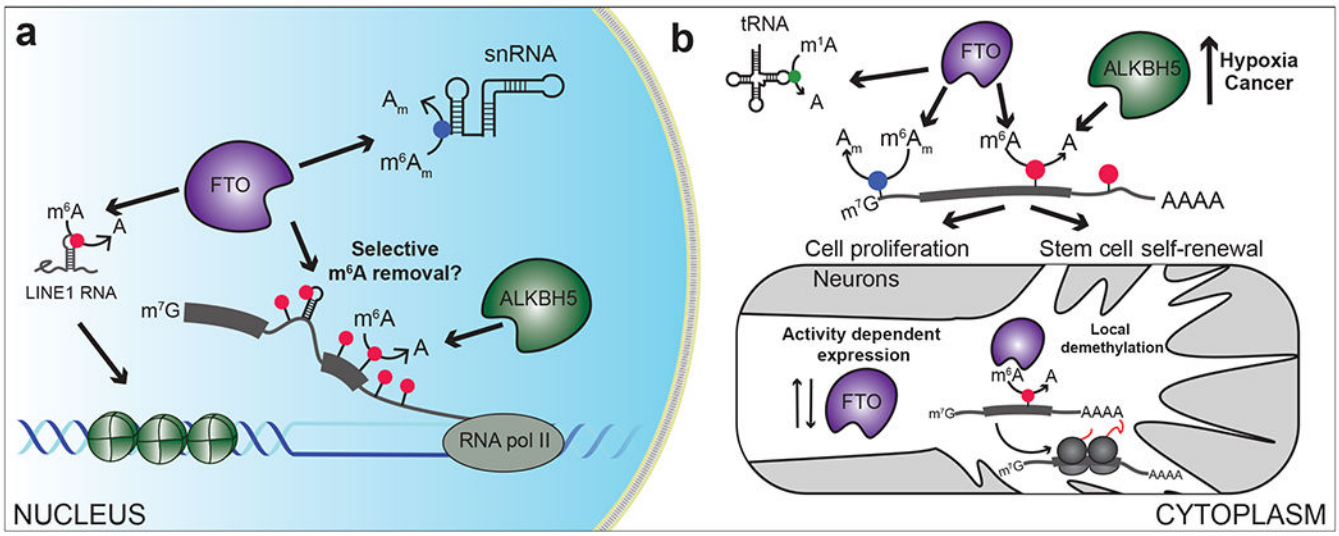

Figure 4. Targets of m^6A eraser proteins.

a) In the nucleus, FTO and ALKBH5 demethylate m^6A sites in mRNAs. FTO can also remove m^6A and m^6A_m in lncRNAs and snRNAs. b) In the cytoplasm, FTO can remove m^6A and m^6A_m from mRNAs and m^1A from tRNA. In the nervous system, FTO expression is regulated by activity and can promote m^6A demethylation of specific transcripts in axons. ALKBH5 expression is increased in hypoxic conditions and in cancer cells, resulting in decreased m^6A levels of specific mRNAs and enhanced cell proliferation and stem cell self-renewal.