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Chemical Modifications in the Life of an mRNA Transcript

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Abstract

Investigations over the past eight years of chemical modifications on messenger RNA (mRNA) have revealed a new level of posttranscriptional gene regulation in eukaryotes. Rapid progress in our understanding of these modifications, particularly, *N*⁶-methyladenosine (m⁶A), has revealed their roles throughout the life cycle of an mRNA transcript. m⁶A methylation provides a rapid mechanism for coordinated transcriptome processing and turnover that is important in embryonic development and cell differentiation. In response to cellular signals, m⁶A can also regulate the translation of specific pools of transcripts. These mechanisms can be hijacked in human diseases, including numerous cancers and viral infection. Beyond m⁶A, many other mRNA modifications have been mapped in the transcriptome, but much less is known about their biological functions. As methods continue to be developed, we will be able to study these modifications both more broadly and in greater depth, which will likely reveal a wealth of new RNA biology.

Keywords

ribonucleotide modifications; epitranscriptome; *N*⁶-methyladenosine; RNA metabolism

1. INTRODUCTION

The transcription of DNA into the messenger RNA (mRNA) transcripts that relay genomic information to the cytoplasm requires numerous processing steps to ensure proper gene expression. Through its life cycle, an mRNA transcript is transcribed from a genomic locus, capped, spliced, and polyadenylated. It then must be exported to the cytoplasm for storage or translation (Figure 1). Other types of RNA in the cell, such as transfer RNA (tRNA) and ribosomal RNA (rRNA), require extensive chemical modification throughout processing and maturation, and in certain cases, these additional moieties are essential for their proper function (90). The discovery that mRNA is polyadenylated provided a simple method to isolate these less abundant RNA species (24, 33). Through metabolic labeling studies, it quickly became clear that mRNA, like rRNA and tRNA, can also be modified (94). Later work identified two methylated species in mouse L cell mRNA: *N*⁶-methyladenosine (m⁶A)

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at a frequency of approximately 1–2 per 1,000 nucleotides, and 2′ *O*-methylated nucleosides (2′ OMe) (26,94,95). A few m⁶A sites were more precisely localized in SV40 virus mRNA (15), bovine prolactin mRNA (48), and Rous sarcoma virus RNA (56), eventually leading to the identification of the RR(m⁶A)CH ([G>A>U][G>A]m⁶AC[U>A>C]) motif, which has been validated in numerous studies since (57). A large 800-kDa enzyme complex responsible for *N*⁶-methylation was initially purified (12), and soon after, MT-A70 (now known as METTL3) was identified as the main *S*-adenosylmethionine (SAM)-binding constituent (13). Anti-m⁶A antibodies also enabled the identification of additional m⁶A sites in spliceosomal RNAs (14).

Although extensive work in plants and other systems continued (35), almost two decades passed before more detailed work was undertaken to characterize the distributions and functions of m⁶A and other modifications in mRNA. Two sets of studies drove the resurgent interest in this field. First, fat mass- and obesity-associated protein (FTO), a member of the nonheme Fe(II) and α -ketoglutarate-dependent dioxygenase AlkB family of proteins, and ALKBH5 were discovered to be mRNA m⁶A demethylases, suggesting that mRNA modifications could be reversible and regulated (45, 55, 139). Soon after, detailed characterization of the m⁶A mRNA methyltransferase complex revealed its key components: METTL3, METTL14, and WTAP (81, 97). Second, the transcriptome-wide distribution of m⁶A was mapped using antibody-based enrichment of m⁶A-containing RNA fragments followed by high-throughput sequencing (28, 89). Together, these breakthroughs allowed researchers both to perturb m⁶A by manipulating enzyme levels and to map its distribution throughout the transcriptome, enabling for the first time more detailed dissection of the biological functions of m⁶A in cells and animals.

Though many of the more than 140 RNA modifications that have been uncovered across archaea, bacteria, and eukarya were first annotated in more abundant tRNA or rRNA (11), the mapping of thousands of m⁶A sites in mRNA prompted a reexamination to determine what other modifications might be regulating the transcriptome. To date, pseudouridine (Ψ) (16, 84, 103), *N*¹-methyladenosine (m¹A) (29, 73), 5-methylcytidine (m⁵C) (51, 52, 62, 102, 109), and 2′ OMe (23) have also been mapped in mammalian mRNA samples (Table 1). In addition, the 5′ cap harbors 7-methylguanosine (m⁷G) (1, 122), and the cap-adjacent nucleotides can be 2′ *O*-methylated (1, 122) or *N*⁶,2′ *O*-methylated (m⁶A_m) (86, 121), thereby regulating transcript stability and translation.

Even though additional modifications will likely be identified, it remains a challenge to map low-abundance modifications on mRNA, particularly at high resolution. In general, mapping techniques utilize an enrichment strategy (such as an antibody) or specific chemical reactivity of the modification in question, or a combination of these two, to map modification sites via high-throughput sequencing. Though effective, this strategy is limited by the lack of single-base resolution information on exact sites of modification and quantitative information on the modification fraction of each site. For instance, antibody-based strategies are simple and easy to implement if specific antibodies are available, but enrichment peaks can span 100 nucleotides, precluding detailed analysis of individual sites. Antibody specificity is always a potential issue that must be carefully tested, particularly with respect to cross-reactivity with closely related chemical species. Furthermore, the

requirement for sufficient input RNA often precludes studies of rare cell populations and patient samples. An additional challenge beyond simply mapping mRNA modifications remains identifying the cellular machinery that regulates them, a critical step in beginning to understand their biological functions. In this respect, m⁶A is by far the most well-characterized modification in mRNA: Enzymes that both install and remove m⁶A, as well as m⁶A-binding proteins, are known, and with an antibody-based enrichment strategy that is simple to implement owing to the high abundance of m⁶A in mRNA, low-resolution mapping of m⁶A in a variety of systems becomes straightforward.

These tools have allowed for extensive characterization of m⁶A in numerous biological contexts, elucidating roles for m⁶A throughout mRNA transcript processing and translation. Two mechanisms through which m⁶A regulates cell biological functions have emerged: (a) coordinated transcriptome turnover through mRNA decay and (b) stimulus-induced translation of specific pools of transcripts in response to cellular and environmental signals. Here, we provide an overview of our current understanding of the roles of chemical modifications in mRNA processing, stability, and translation and how these themes contribute to the maintenance of cell state, embryonic development, stress responses, cancer progression, and viral infection. Though the majority of our mechanistic understanding of mRNA functions is derived from m⁶A studies, we also discuss what is known about the roles of other mRNA modifications and highlight current challenges in the field.

2. CHEMICAL MODIFICATIONS IN mRNA PROCESSING

In early studies of m⁶A, treatment of cells with methylation inhibitors increased bovine prolactin intron-containing precursor mRNA (pre-mRNA) four- to six-fold, suggesting a possible connection between m⁶A methylation and splicing (17). Given the essential roles of chemical modifications in tRNA and rRNA processing, the possibility that modifications could influence mRNA processing has been actively explored. m⁶A methylation occurs cotranscriptionally, possibly driven by an interaction between the m⁶A methyltransferase complex and RNA Polymerase II (60, 107), supporting the notion that modifications installed early in the life of a transcript can impact later processing and export steps.

To be translated efficiently in the cytoplasm through canonical cap-dependent mechanisms, transcripts must be capped at the 5' end and polyadenylated at the 3' end (Figure 1) (108). The 5' cap consists of a guanine nucleotide methylated by RNMT at the 7-position (m⁷G) and connected through a reverse 5'-to-5' triphosphate linkage (96, 114), often followed by a 2'OMe (67). The m⁷G-containing cap prevents mRNA degradation from the 5' end and recruits translation initiation machinery through eukaryotic translation initiation factor 4E (eIF4E) binding (46). 2'OMe at the +1 position has been implicated in self/non-self recognition by the immune system, which viruses can exploit to evade detection (22).

When found at the cap +1 position, A_m can be m⁶A methylated to form m⁶A_m, which exists in roughly 10% of mRNAs in most cells and renders transcripts resistant to decapping by the enzyme DCP2 (86). Recent work nicely shows that cap m⁶A_m is a biologically relevant FTO substrate in cells that modulates transcript stability (86), but the regulation and functional significance of this modification remain unclear. However, other studies challenge the

suggestion that m⁶A_m, rather than internal m⁶A, is the primary substrate for FTO in cells. FTO-mediated m⁶A demethylation has been clearly demonstrated in subsets of FTO-sensitive acute myeloid leukemia (AML) (76), and inhibition of FTO by R-2HG elevates internal m⁶A levels on *MYC* transcripts in some AML lines (110). Notably, m⁶A_m levels in these cell lines are exceedingly low, accounting for only 3–5% of total m⁶A. In neurons, the *GAP43* transcript, which is essential for axon elongation, is initially m⁶A methylated before being transported to axons (134). After transport, it is demethylated by a local pool of FTO to facilitate protein production, a clear example of cytoplasmic, dynamic demethylation that regulates protein translation. FTO-mediated internal m⁶A demethylation has also been reported during the DNA damage response (127). Thus far, one laboratory could not observe FTO-mediated internal m⁶A removal (86), perhaps owing to less sensitive detection, whereas others have shown mRNA m⁶A demethylation mediated through FTO in diverse biological pathways. Recent work in hepatitis C virus (HCV) has also shown that depletion of *METTL3* and *METTL14* increases viral particle production, whereas depletion of *FTO* decreases it (39). In this case, the substrate is clearly internal m⁶A, as HCV viral RNA does not have a 5' cap. FTO could possess diverse roles that are not limited either to m⁶A and m⁶A_m or to mRNA substrates, issues that future work will need to address.

Another essential step in mRNA processing is the addition of the 3' poly(A) tail. Although initial mapping studies suggested that m⁶A sites are strongly enriched around the stop codon (28, 89), higher-resolution mapping uncovered numerous m⁶A sites at the beginning of the last exon (59). m⁶A appears to be particularly prevalent in long last exons, indicating that m⁶A may affect proximal alternative polyadenylation site usage and bias a transcript toward using a more distal polyadenylation site. Differential use of polyadenylation sites can influence transcript stability, export, translation, and localization (111), thus potentially connecting m⁶A to multiple facets of posttranscriptional and translational regulation by influencing polyadenylation site selection.

Once processed, many transcripts must make their way out of the nucleus for translation in the cytoplasm. The nuclear m⁶A-binding protein YTHDC1 plays a role in splicing (discussed below), but it also possesses additional functions in mRNA export. Fractionation of nuclear and cytoplasmic mRNA revealed accumulation of nuclear m⁶A-containing YTHDC1 target transcripts upon YTHDC1 depletion (99), suggesting a role in export that may be mediated through direct interaction with SRSF3. Recent work has also uncovered that m⁵C in mRNA, installed by NSUN2, mediates mRNA nuclear export through ALYREF binding (130). Though nuclear export is considered a broadly required, essential step toward translating mRNA into protein, recent work suggests that export may be selective in some cases (125). Future work will need to address if and how mRNA modifications contribute to these processes.

Equally important in the regulation of gene expression is the proper maintenance and/or induction of gene silencing. A classic example is transcriptional silencing of genes on the X chromosome through the long noncoding RNA *XIST*, which carries at least 78 m⁶A sites that appear to be important for its silencing function (92). These m⁶A sites are recognized by YTHDC1, and loss of gene silencing from loss of m⁶A methylation is rescued by YTHDC1 tethering, suggesting that this m⁶A-binding protein is also central to *XIST* function. One of

the preferred consensus sequences for the m⁶A methyltransferase complex, GGAC, is also enriched in primary-microRNA (pri-miRNA) (but not pre-miRNA) sequences, suggesting a role in miRNA processing. Profiling of m⁶A sites revealed that miRNAs are indeed marked by m⁶A and that depletion of *METTL3* leads to a global reduction in mature miRNA levels (3).

Early m⁶A mapping studies suggested an association between m⁶A and alternative splicing on the basis of splicing differences observed upon depletion of *METTL3* (28). Although the generality of m⁶A-mediated splicing regulation is a legitimate question (60), examples in multiple organisms suggest that m⁶A can influence splicing in specific instances. In *Drosophila melanogaster*, m⁶A controls sex-specific alternative splicing events through the m⁶A-binding protein YT521-B (44, 68). Flies lacking the *METTL3* homolog *Ime4* are viable, but demonstrate flight and locomotion defects as well as a bias toward maleness. m⁶A is required for female-specific alternative splicing of *Sex lethal (Sxl)*, which normally inhibits ectopic dosage compensation and female lethality. Even though *Ime4* loss does not globally affect splicing, approximately 100–200 genes demonstrate alternative splicing patterns.

In mammals, numerous RNA-binding proteins, including those of the HNRNP family, are required for proper mRNA processing. HNRNPC is broadly involved in mRNA splicing and processing and was pulled out of cellular extract owing to its preferential binding to the m⁶A-containing *MALAT1* hairpin (82). m⁶A methylation destabilizes A-U base-pairing in the hairpin, exposing the HNRNPC binding sequence to enhance binding (Figure 2). Intriguingly, these m⁶A-switch regions may be involved in alternative splicing, but given that they account for only ~7% of HNRNPC binding sites, m⁶A is likely just one of several mechanisms through which HNRNPC regulates splicing.

SAM is the methyl donor for most cellular methylation reactions, and it is critical that cells tightly regulate its levels. SAM synthetase is encoded by the *MAT2A* gene, which accumulates with a retained intron in the nucleus and is degraded under SAM-replete conditions. Rapid binding and m⁶A methylation of *MAT2A* by METTL16 promote intron retention (93). Upon SAM depletion, however, METTL16 cannot efficiently methylate *MAT2A*, and as such, its dwell time on the transcript increases and facilitates splicing through specific regions conserved across vertebrates. Notably, the conserved hairpins on which *MAT2A* is methylated bear striking similarity to U6 small nuclear RNA (snRNA), which has long been known to be m⁶A methylated. Cross-linking and analysis of cDNA suggest that METTL16 binds additional mRNA, long noncoding RNA, and noncoding RNA targets, but whether METTL16 methylates these targets and the potential functional consequences of that methylation remain to be characterized (120). Though the function of U6 snRNA methylation remains unclear, its central role in splicing leaves open the possibility of another connection between m⁶A methylation and splicing.

Though most m⁶A-binding YTH-family proteins are primarily cytoplasmic, the fifth family member, YTHDC1, is nuclear, and recent studies have shed light on its putative function. Xiao et al. conclude that YTHDC1 alters splicing by favoring exon inclusion through direct interaction with SRSF3 and via competition with another splicing factor, SRSF10 (128). A

recent study mapping m⁶A in nascent RNA is consistent with this model and finds that cotranscriptional deposition of m⁶A near splice junctions promotes faster splicing (83). Another study confirmed that SRSF3 interacts with YTHDC1, but the splicing changes observed upon depletion of YTHDC1 were limited and were not strongly correlated with the presence of m⁶A (99). Additional results from this work suggest roles for YTHDC1 in mRNA export. Splicing is a fundamental nuclear process that is affected by numerous pre-mRNA processing steps, including nuclear export and retention. It is likely that m⁶A influences mRNA splicing, directly or indirectly, through these various mechanisms.

However, the reported changes in splicing patterns upon knockdown of the methyltransferase *METTL3* (28, 82, 138) and the presence of m⁶A methylation at splice sites (128, 138) have been questioned (60). Recent analysis of chromatin-associated, nuclear, and cytoplasmic nascent RNA from HeLa cells suggests that m⁶A is deposited cotranscriptionally regardless of whether splicing has occurred and that m⁶A is not deposited particularly close to splice sites (60). This work also concluded that m⁶A is not frequently deposited in introns, whereas other studies suggest that it could be quite prevalent in introns (83) and that the METTL3/METTL14 methyltransferase complex binds to intronic regions (81). These discrepancies will require further studies of m⁶A methylation throughout transcription, including a thorough characterization of how specific sites are selected for m⁶A methylation (as only approximately 20% of consensus sequences are methylated); the cause-and-effect relationships between m⁶A and splicing will also need to be dissected. The examples of m⁶A-mediated splicing changes described above are critical in response to developmental cues and cellular signals, which are dynamic and context specific. It is likely that m⁶A-mediated regulation is not a general feature of constitutive splicing, but instead is a mechanism through which the cell can alter splicing in response to specific stimuli. This is notably similar to m⁶A-mediated regulation of transcript turnover and translation, discussed in detail below, which is induced in response to specific signals.

3. REGULATION OF mRNA STABILITY

Transcript half-life can be a major contributing factor in determining protein expression levels, and rapid changes in transcript stability in response to stimuli can be critical to mounting the appropriate cellular response. mRNA modifications can influence transcript stability by a variety of mechanisms, through their effects on local RNA structure and their ability to recruit binding proteins to specific transcripts. The m⁶A-binding protein YTHDF2 targets several thousand transcripts, whose half-lives increase upon YTHDF2 knockdown (117). The extent of stabilization is correlated with the number of m⁶A sites. YTHDF2 may direct these transcripts to mRNA decay sites and can recruit the CNOT/CCR4/CNOT1 complex to promote deadenylation and decay (Figure 2) (31). YTHDF2 colocalizes with DCP1a, a marker of P-bodies, which have been associated with mRNA decay, among other functions (117). This mode of m⁶A-mediated mRNA degradation may represent a fundamental mechanism for coordinated transcriptome turnover, with critical roles that have already been demonstrated in embryonic development (53, 137) and cellular differentiation (37, 71, 132, 135). During zebrafish development, for example, knockout of *ythdf2* results in delayed clearance of m⁶A-methylated transcripts, delaying activation of zygotic genes during the maternal-to-zygotic transition (137). Later in development, m⁶A methylation on

endothelial genes *notch1a* and *rhoca* is required for their YTHDF2-mediated decay during the endothelial-to-hematopoietic transition, and loss of YTHDF2 prevents specification of hematopoietic stem/progenitor cells (135). *Ythdf2* knockout mice are infertile because germ cells fail to undergo meiosis (53), and numerous cell types require the assistance of m⁶A to decay specific transcripts for proper differentiation (37, 71, 132, 135).

m⁶A is not purely a mark for RNA degradation, however. In the case of MLL-rearranged AML, high expression of the demethylase FTO reduces m⁶A levels on the key *ASB2* and *RARA* transcripts and, yet, reduces their half-lives (76). A YTHDF2-mediated mechanism would likely manifest as an increase in half-life upon reduction of m⁶A levels; expectedly, knockdown of *YTHDF2* had no effect on *ASB2* and *RARA* transcript levels. The insulin-like growth factor 2 mRNA-binding proteins (IGFBP1, IGFBP2, and IGFBP3) represent a newly identified class of m⁶A-binding proteins that may confer increased stability to m⁶A-containing transcripts (50). This may occur through interactions with IGFBP effectors, such as HUR, which could protect IGFBP-bound transcripts from degradation. Only approximately 30% of IGFBP target genes overlap with YTHDF2 target genes, suggesting that these proteins may regulate distinct pools of transcripts. It remains to be determined, however, how selectivity is achieved, because both YTHDF2 and the IGFBPs recognize a canonical m⁶A sequence motif, GGACU. They have relatively different expression profiles, suggesting that their functions may predominate in different tissues or under different cellular conditions. It is clear, however, that the effect of m⁶A on transcript stability depends on a multitude of factors, likely including sequence context, cell state and/or environmental conditions, and the expression of m⁶A demethylases and m⁶A-binding proteins.

Though m⁶A is arguably the best-characterized modification from a mechanistic standpoint, it is likely not the only one that influences transcript stability. Using a chemical modification strategy to induce reverse transcription stops, hundreds of Ψ sites have been mapped in yeast and mammalian transcriptomes (16, 75, 84, 103). In yeast, some of these sites can be attributed to Pus7p activity, which induces pseudouridylation of 265 sites upon heat shock (103). Under heat shock conditions, pseudouridylated genes are expressed at approximately 25% higher levels in wild-type strains than in a Pus7p mutant strain (whereas nonpseudouridylated transcripts are expressed at similar levels), suggesting that Ψ may have an effect on transcript stability. Notably, however, there is no discernible correlation between the number of Ψ sites and the extent of the expression change, so whether this effect is direct remains unclear.

Our current understanding of m⁶A-mediated regulation of transcript stability centers primarily on the YTHDF2-dependent degradation mechanism described above, which directs coordinated transcriptome processing and turnover in multiple settings. This provides a regulated and efficient route to the decay of specific transcripts in response to cellular signals that proves to play important roles in numerous biological systems, and more such examples are likely to be uncovered as m⁶A continues to be studied more broadly. There are likely other stability control pathways to be uncovered, with respect to both other m⁶A-binding proteins and transcript modifications. Indeed, there is evidence that m⁶A may be involved in RNA interference and miRNA-mediated transcript degradation in response to cellular stresses (2, 64).

It has become clear that in addition to the extensive transcriptional control required to direct cells and organisms through differentiation and development, specific pools of transcripts must also be decayed in a timely manner to effectively suppress the expression of pluripotency factors (Figure 3a). Layers of complexity are added to this problem by the intriguing possibility that m⁶A in different contexts may also mediate increased transcript stability, likely driven by the interplay among changing expression levels of m⁶A methyltransferases and demethylases and m⁶A-binding proteins under different conditions. How other mRNA modifications will contribute to this paradigm remains unclear. For instance, from a chemical perspective, 2'OMe enhances RNA stability, making it more resistant to alkaline hydrolysis and nucleolytic attack (66). However, whereas its distribution in mRNA was recently uncovered using a chemical strategy (23) and it is known that C/D-box small nucleolar RNA-guided Fibrillarin installs it on rRNA (63, 113), its function in mRNA and any potential effect on stability remain to be elucidated.

4. TRANSLATIONAL CONTROL THROUGH mRNA MODIFICATIONS

4.1. Recruitment of Translation Machinery by RNA Modifications

Changes in transcript stability can indirectly influence protein production, but recent work has highlighted that mRNA modifications can also more directly recruit translation machinery. The mRNA cap structure that is so critical for efficiently translating many transcripts contains a modification in its own right: m⁷G binds directly to eIF4E, a component of the eIF4F complex that recruits the 43S preinitiation complex (108) and the CBP20/CBP80 complex, which is involved in mRNA processing, export, and translation (54). In an in vitro assay with purified components, a reporter containing an m⁶A-methylated β -globin 5' untranslated region (UTR) with a short coding sequence was successfully translated even in the absence of the normally essential group-4 eIFs (88). m⁶A in the 5' UTR can directly bind eIF3 (Figure 2), and through ABCF1, it mediates ternary complex binding to facilitate cap-independent translation (21). Notably, in contrast to translation mediated by an internal ribosome entry site, the same pool of transcripts can undergo both cap-dependent and cap-independent translation, shifting between the two depending on cellular status. This mechanism is likely crucial for cells to respond to stress, when cap-dependent translation is compromised. Upon heat shock, newly transcribed RNAs are m⁶A methylated in the 5' UTR (141). These m⁶A sites are recognized by YTHDF2 that translocates from the cytoplasm to the nucleus upon heat shock, limiting accessibility to the FTO demethylase such that these sites remain methylated. Upon export to the cytoplasm, these methylated transcripts, including *Hsp70*, can then be translated in a cap-independent manner, allowing for increased *Hsp70* production under conditions where cap-dependent translation is compromised (Figure 3b). By this mechanism, m⁶A can coordinate stimulus-induced translation of transcripts that allows the cell to respond to heat shock. It remains to be seen whether this mechanism is relevant in response to other stresses or cellular signals.

Association between proteins bound to the cap on the 5' end and the poly(A) tail on the 3' end is a well-established paradigm of translational regulation. Many mRNA 3'-end-binding proteins, such as poly(A) binding protein, regulate translation by such a "looping" mechanism (108). The m⁶A-binding protein YTHDF1 binds to the 3' ends of transcripts

and enhances the translation efficiency of its targets, possibly through such a looping interaction between YTHDF1 and eIF3 cap-binding components (Figure 2) (118). The molecular details of YTHDF1 function remain unclear, but another m⁶A-binding protein, YTHDF3, may be involved (70, 106). FMR1 was recently found to preferentially bind m⁶A. It also has some shared targets with YTHDF1 but inhibits translation (34). YTHDF1 and FMR1 may compete for some of the same targets, shifting the balance between translational enhancement and inhibition under different conditions. However, although YTHDF1-mediated translation upregulation has been robustly demonstrated in HeLa cells, its roles in promoting translation in other cells such as MCF7 and HEK293 cells have not been observed (107). The possibility that the effect may depend on cell type and context led us to investigate more deeply the functions of YTH-domain-containing proteins. mRNA methylation-dependent regulation most likely occurs in response to cellular and environmental cues to allow for rapid responses through specific sets of transcripts. Thus far, YTHDF2-mediated mRNA decay seems to play more significant roles during differentiation and may be less relevant under other growth conditions. Similarly, YTHDF1-mediated regulation of translation may occur in a cell- and stimulus-specific manner, as recently demonstrated in adult mouse dorsal root ganglion in response to injury (124).

Circular RNAs (circRNAs) were long thought to be rare splicing by-products; however, detailed analysis of high-throughput sequencing data has recently revealed that they may be more prevalent (101). A subset of circRNAs are m⁶A methylated, apparently also by the METTL3/METTL14 methyltransferase complex, but interestingly many circRNA m⁶A peaks appear in exonic regions that are not methylated in mRNA (140). m⁶A methylation of a GFP-containing circRNA reporter promotes translation through a mechanism involving eIF4G2 and YTHDF3 (131). Even though this represents an artificial system and the broader functions of circRNAs remain unclear, this study did uncover polysome-bound circRNAs as well as circRNA-derived peptides through proteomics studies. Thus, some circRNAs could be translated, and m⁶A could modulate circRNA function through YTHDF3.

In addition to the temporal regulation of translation in response to stimuli, m⁶A may also represent a simple mechanism for spatial control of protein translation. Localized translation of growth-associated protein 43 (GAP43) is essential for axon elongation. *GAP43* transcripts are initially m⁶A methylated but must be demethylated by a local pool of FTO in the axon to facilitate protein production (134). This report is an early example of a specific mRNA modification regulating localized translation. Because RNA localization coupled to local translation is critical for establishing and maintaining cell polarity in a variety of settings (87), we anticipate that more such examples will surface in the future.

4.2. Alternative Decoding Through mRNA Modifications

RNA modifications on mRNA as well as tRNA and rRNA have the ability to directly influence mRNA decoding during peptide synthesis. Modifications in the anticodon loop of tRNAs can influence codon-anticodon interactions and can thereby introduce codon usage bias to enhance translation of transcripts with high levels of those codons, particularly in response to cellular stress (10, 41). Similarly, modifications on transcripts may alter translation efficiency and/or decoding. In an AML model, promoter-bound METTL3,

recruited by the transcription factor CEBP2, methylates transcripts within coding regions with a preference for specific codons (7). This methylation can relieve stalled ribosomes, thereby increasing translation efficiency.

In vitro translation experiments reveal that Ψ may be able to function in a similar way, suppressing translation termination by isomerizing uridines in stop codons to Ψ (58). An engineered reporter/guide RNA system in *Saccharomyces cerevisiae* confirms that this mechanism can function in the context of a cell, but the broader functional relevance of this potential recoding remains unclear. Particularly intriguing, however, is the possible clinical application of such a system, given that many genetic diseases are attributed to premature termination codons that compromise the production of functional protein. Inducing translation machinery to read through premature stop codons by modifying transcripts might provide a means to improve read-through and, thus, functional protein production for therapeutic benefit. In contrast, 2'OMe in coding regions can induce a steric clash that delays EF-Tu GTPase activation, stalling translation (20). In vitro translation studies of other modifications, including m¹A, m⁶A, m¹G, m²G, and m⁶G, suggest that the presence of these modifications on mRNA can also modulate tRNA accommodation and decoding, sometimes in a position-dependent manner within a codon (19, 20, 133).

Modifications on tRNA and rRNA, critical components of translation machinery, have long been known to influence protein production via many mechanisms. Proper processing and maturation of these RNAs require numerous modifications, and misregulation can affect the amount and composition of available translation machinery in the cell. In the ribosome, rRNA modifications tend to occur on conserved residues in functional regions such as the decoding site where tRNAs translate mRNA sequence and near the catalytic peptidyl transferase center (104). Similarly, tRNA modifications, particularly in the anticodon loop, can influence translation speed and fidelity as well as codon usage (41, 47). We now know that mRNA modifications can play similar roles, particularly in decoding, and it will be interesting to see whether there is direct interplay between mRNA and tRNA modifications (particularly involving the anticodon loop) or whether they represent parallel or redundant mechanisms of translational regulation in the cell.

5. m⁶A REGULATES CELLULAR DIFFERENTIATION

As research on mRNA modifications expands into different systems, it is becoming clear that coordinated transcriptome turnover and translational regulation represent general mechanisms by which m⁶A in mRNA contributes to cell fate and differentiation. Throughout embryonic development, cells must undergo numerous transitions, responding to signals to both differentiate and proliferate in a spatiotemporally controlled manner. This requires extensive coordination of transcription, transcriptome processing and turnover, and translation to ensure that each cell expresses the appropriate complement of genes for the appropriate period of time.

The first clue that such coordination does indeed occur came from knockout of the m⁶A methyltransferase Mettl3 in mouse embryonic stem cells. Knockout cells are unable to differentiate and instead maintain a naive pluripotent state (Figure 3a). Many transcripts

essential for maintaining pluripotency, including *Nanog*, are m⁶A methylated and have increased half-lives upon *Mettl3* knockout. This hyper naive pluripotent state is resistant to differentiation and eventually results in embryonic lethality in the animal (8, 37). Many features observed in the mouse are conserved in human embryonic stem cells, including methylation of pluripotency-specific factors and the inability of *METTL3*^{-/-} embryonic stem cells to differentiate (8).

Although we have far from a complete understanding of how m⁶A methylation is involved in embryonic development, the early lethality caused by *Mettl3* knockout in mice suggests essential contributions. This may be connected to the many functions of m⁶A that have been uncovered in the mammalian germline. Even prior to detailed characterization of the METTL3/METTL14 methyltransferase complex, knockout mice of the m⁶A demethylase *Alkbh5* were found to have smaller testes and impaired spermatogenesis, possibly owing to aberrant gene expression (139). Similarly, *Ythdc2*^{-/-} mice are viable but infertile, likely a result of aberrant upregulation of genes specific to spermatogonial stem cells, such that germ cells fail to undergo meiosis (6, 49, 126). Although *Ythdc2* is also expressed in oocytes, its role in female fertility remains to be characterized. A second m⁶A-binding protein, Ythdf2, is highly expressed in both testes and oocytes, and when deleted in oocytes, it prevents the necessary elimination of a subset of transcripts required to maintain gene dosage during maturation (53). *Mettl3*^{-/-} and *Mettl14*^{-/-} mice also have spermatogenesis defects (79), but the fact that *Ythdf2*^{-/-} mice exhibit less severe phenotypes suggests that additional m⁶A-mediated mechanisms are likely involved in the early stages of mammalian development.

This theme extends to many other stem cell types, where decay of specific transcripts is critical for differentiation. When transferred to lymphopaenic mice, *Mettl3*^{-/-} naive T cells fail to undergo homeostatic expansion and maintain a naive state (71). This is partially attributed to a failure of m⁶A-methylated *Socs1*, *Socs3*, and *Cish* transcripts to be decayed, inhibiting IL-7-mediated STAT5 activation. Conditional knockout of *Mettl14* in the nervous system results in extended cortical neurogenesis into the postnatal stages, resulting in a delay in production of neuronal subtypes (132). Loss of m⁶A on pools of transcripts required for neural stem cell maintenance, cell cycle progression, and neuronal differentiation prevents timely transcript turnover, lengthening cell cycle progression. Consistent with this, recent work in *Ythdf2*^{-/-} mice also found loss of neural stem cell progenitor proliferation resulting from delayed transcript clearance, leading to impaired cortical development (72). Notably, many neuronal lineage genes are already expressed in stem cells, but until required, they are suppressed by m⁶A-dependent decay, suggesting that m⁶A may be involved in transcriptional prepatterning in the spatiotemporal regulation of gene expression (Figure 3a).

The field has now uncovered numerous systems where m⁶A is critical for cellular differentiation. Interestingly, in the majority of these cases, the phenotype is attributed to loss of m⁶A on specific transcripts that need to be degraded for differentiation to proceed, but we suspect this is only one factor contributing to the observed phenotypes. m⁶A-mediated translational regulation has yet to be found to contribute to these phenotypes. Coordinated transcriptome turnover through m⁶A methylation has emerged as a new facet of gene expression regulation during cell differentiation, but nuclear roles of m⁶A may also be critical to the observed phenotypes (7, 119).

6. RNA MODIFICATIONS IN HUMAN DISEASE

6.1. mRNA Methylation and Demethylation in Cancer

Given the strong ties between mRNA modifications and stem cell fate, it is no surprise that connections to cancer are emerging as well. Gene expression analysis in human AML samples found that FTO is highly expressed in a subset of MLL-rearranged AML subtypes. High levels of the FTO demethylase accelerate leukemogenesis in these AML cells, at least in part owing to FTO/m⁶A-mediated expression regulation of *ASB2* and *RARA*, two key genes that are upregulated during hematopoiesis (76). High expression of FTO downregulates *ASB2* and *RARA* by reducing their m⁶A levels. Notably, this is in contrast to the observation that, in many cases, a reduction in m⁶A levels on a specific transcript results in upregulation of gene expression due to decreased decay rates. A new class of m⁶A-binding proteins, IGF2BP1–3, that may mediate this effect has also been identified (50). This dependence of subsets of AMLs on the FTO demethylase may be exploited, as FTO can be effectively inhibited by R-2-hydroxyglutarate (R-2HG). R-2HG is a reported oncometabolite, produced by the neomorphic activity of mutant isocitrate dehydrogenase enzymes. In AMLs with high levels of FTO demethylase, however, R-2HG inhibits FTO activity and thus elevates m⁶A levels on proliferation-promoting transcripts such as *MYC*, thereby reducing *MYC* mRNA half-life through a YTHDF2-dependent mechanism (110). Hyperactivated *MYC*, however, overwhelms FTO-mediated regulation, so R-2HG does not inhibit proliferation in cells with high *MYC* expression. These two studies highlight the complexity of m⁶A-mediated regulation of gene expression, as even when looking at the same general class of disease (AML), we can observe opposite effects of m⁶A on the stability of different transcripts and on cell proliferation.

The m⁶A methyltransferase complex components METTL3 and METTL14 have also been implicated in AML in three independent studies (7, 116, 123). Promoter-bound METTL3 can be recruited by the CEBP2 transcription factor to methylate transcript coding regions, enhancing translation efficiency by relieving stalled ribosomes (7). Higher levels of METTL3 protein expression in AML may also inhibit hematopoietic stem/progenitor cell differentiation through an m⁶A-induced boost in translation of c-*MYC*, *BCL2*, and *PTEN* transcripts (116). Consistent with this, silencing *METTL14* promotes differentiation of both hematopoietic stem/progenitor cells and AML cells, likely through targets such as *MYC* and *MYB* (123). In these three studies, m⁶A appears to promote expression and translation of oncogenic transcripts, but recent work on glioblastoma reveals that ALKBH5-mediated demethylation enhances *FOXMI* expression and ALKBH5 depletion is sufficient to inhibit tumorigenesis (136). Thus, although m⁶A-mediated gene expression regulation can be hijacked to activate oncogenes, the mechanisms will likely vary depending on the type of cancer and the genetic background in which it occurs.

6.2. mRNA Modifications in Viral Infection

Around the same time it was initially described in mRNA in the 1970s, m⁶A methylation was detected in RNA from numerous viruses, including B77 avian sarcoma virus, Rous sarcoma virus, simian virus 40, influenza, and adenovirus (9, 15, 27, 43, 65). As with the recent explosion in work on mRNA modifications, however, the functions of these

modifications have only recently begun to be elucidated. Viruses hijack host cell machinery during infection and replication. HIV-1 uses host tRNA^{Lys3} as a primer for minus-strand strong-stop synthesis and then exploits m¹A methylation at position 58 in tRNA^{Lys3} to properly terminate plus-strand strong-stop synthesis (38, 98).

As our understanding of the machinery that regulates m⁶A methylation of mRNA has improved, it has become clear that viruses can also utilize this machinery for their own purposes and that hosts may exploit viral RNA modifications to suppress infection. Three studies looking at m⁶A in HIV-1 found multiple m⁶A peaks in HIV-1 RNA that are critical for infection (61, 77, 112). Whereas the influence of the YTH-domain-containing proteins remains at odds among the three studies (85), depletion of the *METTL3/METTL14* methyltransferase complex consistently inhibits viral replication and release.

Recent work on HCV has yielded some intriguing mechanistic insights into how viruses might utilize m⁶A machinery. Depletion of *METTL3* and *METTL14* increases viral particle production; conversely, depletion of the demethylase *FTO* decreases it (39). Upon infection, YTHDF1, YTHDF2, and YTHDF3 localize to the lipid droplets that function as sites of viral assembly. Depleting these proteins increases viral particle production and release, likely via a specific region of the HCV genome that, when depleted of m⁶A, increases viral titer. Intriguingly, this region is conserved across many flaviviruses, suggesting that this mechanism of m⁶A-mediated regulation of viral replication may be conserved across this family of viruses. Similar effects were seen in Zika virus, another member of the *Flaviviridae* family (78).

Though current studies have been limited primarily to m⁶A, it is becoming clear that viruses easily co-opt the mechanisms used by the host cell to regulate the transcriptome through mRNA modifications. This may involve mislocalization of mRNA modification machinery to sites of viral replication, which may, in turn, expose this machinery to host substrates that it would not normally encounter. Host cells may also utilize RNA modifications to respond to viral infection. When differences in host transcript methylation are measured, it will be interesting to disentangle whether transcripts are differentially methylated as a regulated response to viral infection or whether they represent nonspecific methylation events induced by mislocalization of m⁶A machinery.

7. TECHNICAL CHALLENGES SPARK CONTROVERSY: m¹A AND m⁵C

The 5' UTR is a critical regulatory region in regulating transcript translation (46, 69), so it was particularly intriguing when two studies showed that m¹A could be found on the 5' ends of thousands of mRNA transcripts, near the first splice site (29, 73). These studies failed to identify a preferred sequence motif. Yet, Dominissini et al. (29) found that m¹A tends to occur in regions with higher GC content and more predicted structure and that proteins derived from transcripts with m¹A near the start codon tend to have slightly elevated protein expression levels relative to non-m¹A transcripts. Importantly, however, the average stoichiometry of m¹A methylation appears to be 20%, so it cannot be discerned from the proteomics data whether the elevated protein levels are derived from the methylated or unmethylated transcript pools. Both studies (29, 73) also found that m¹A, like many other

RNA modifications, is dynamic in response to stresses such as heat shock and nutrient starvation, suggesting that m¹A may represent a way to regulate protein translation. Suggesting a functional connection that has yet to be elucidated, a recent computational study identified a common class of transcripts depleted of introns in the 5' UTR that share features such as more extensive secondary structure, a greater dependence on eIF4E for translation, endoplasmic reticulum–proximal ribosome association, noncanonical exon-exon junction complex deposition, and m¹A methylation (18). While this study suggests that m¹A may be a characteristic feature of a group of functionally related transcripts, it remains correlative and the common functional connection remains unknown.

Aiming to improve the resolution of m¹A maps using a thermostable group II intron reverse transcriptase (TGIRT) (91), two studies have opposing views of the role of m¹A in the transcriptome (74, 100). Both agree that a small subset of m¹A sites within a tRNA-like motif appear to be regulated by the TRMT6/61A tRNA m¹A methyltransferase and that m¹A may play some interesting roles in mitochondrial mRNA. To look at the transcriptome more broadly, Safra et al. (100) utilized TGIRT to introduce mutations at m¹A sites and the Dimroth chemical rearrangement to convert m¹A to m⁶A to reduce the mutation rate as a control. This method led them to identify only 10 sites in cytosolic mRNA transcripts, resulting in speculation on the prevalence and role of m¹A in mRNA (40). This work does suggest, however, that m¹A at these sites may inhibit translation. In contrast, utilizing the same TGIRT enzyme but under optimized conditions and using AlkB-mediated demethylation to reverse mutations, Li et al. (74) identified 740 m¹A sites. In large part, this discrepancy is likely due to the technical challenges involved in working with both TGIRT and m¹A. Suboptimal conditions for reverse transcription as well as the harsh conditions required for Dimroth rearrangement that cause RNA degradation likely dramatically reduced the ability of the study by Safra et al. (100) to identify less abundant sites. In addition, Li et al. (74) incorporated adaptors with unique molecular identifiers to eliminate PCR duplication during analysis. Thus, though the two studies implement conceptually similar methodologies, differences in actual execution likely affected the mutagenicity of the enzyme, RNA integrity, and data analysis in ways that could dramatically alter their conclusions (30). Furthermore, mass spectrometry–based quantification of m¹A in mammalian cell lines by three different laboratories revealed levels of m¹A at approximately 5–10% of the level of m⁶A in mRNA samples (29, 73, 129). This cannot be accounted for with the very few sites noted in Safra et al. (100). Moving forward, uncovering the functional roles of m¹A, particularly in stress responses where it is dynamic, will be critical.

m⁵C has garnered significant attention as an epigenetic mark in DNA, but it was also identified along with other modifications in early analyses of mRNA (32). Despite interest in mapping m⁵C sites in mRNA, the bisulfite sequencing–based methods that revolutionized DNA methylation studies require harsh conditions that are detrimental to RNA samples. Nevertheless, many groups have tried with mixed results to adapt the principles of bisulfite sequencing to RNA samples. Initial work uncovered approximately 10,000 m⁵C sites throughout the transcriptome using a bisulfite sequencing–based approach (109). Subsequent attempts leveraged a chemical analog (5-azacytidine) (62) or a mutated methyltransferase (NSUN2) (52) to capture modified RNAs, which dropped the number of identified m⁵C sites to range between a few hundred and 1,000. Of the known m⁵C RNA methyltransferases,

including DNMT2 and NSUN1–6 in mammals, only NSUN2 seems to have a broad substrate specificity that encompasses tRNAs, other noncoding RNAs, and a few mRNAs. Recently, a comparative study was undertaken to profile m⁵C sites in mouse embryonic stem cells and brain tissue utilizing a combination of bisulfite sequencing with validation of conversion by mass spectrometry and of specific targets by immunoprecipitation followed by quantitative PCR (4). This work went further than previous analyses, looking in more detail at the distribution of m⁵C sites along transcripts as well as overlaps with known RNA-binding-protein binding sites, revealing a distribution enriched around start codons. Recent work also suggests a role for m⁵C in nuclear export through ALYREF (130). An additional layer of complexity is introduced if Tet regulates m⁵C levels. In flies, Tet-mediated oxidation of m⁵C to 5hmC is critical in brain development (25), and in mammalian cells, Tet2 promotes myelopoiesis during infection (105).

The biological functions of m¹A, m⁵C, and other modifications in mRNA remain open questions, in large part because we do not understand the molecular mechanisms that underpin their regulation and function and because we are still limited by the sensitivity of sequencing technology. For instance, even though recent studies uncovered three enzymes that may methylate a very small portion of m¹A sites, most sites from the initial m¹A maps and the latest work by Li et al. (74) remain unaccounted for in terms of the methyltransferase responsible for installing them. The possibility that these modifications are installed by other tRNA and rRNA methyltransferases should continue to be tested. The resolution and robustness of sequencing methods should continue to be improved, but biochemical studies of m¹A, m⁵C, and other modifications also need to be undertaken so that molecular mechanisms can be uncovered and biological impact can be explored in a causative way. Although sequencing-based studies have been incredibly powerful in exposing the breadth of these chemical modifications in mRNA, they are inherently limited by their correlative nature, making function difficult to assess. Finally, it is critical to recognize that RNA could be localized to specific cellular compartments. As such, the overall modification levels could be quite low, but specific transcripts could be enriched in specific compartments or granules to impact cellular function. This is an understudied aspect of mRNA modifications that may be particularly relevant with less abundant modifications.

8. CHALLENGES AND FUTURE DIRECTIONS

The two examples highlighted above, m¹A and m⁵C, illustrate one of the major challenges in studying the epitranscriptome—the development of high-throughput, high-resolution approaches to map the broad range of chemical moieties that are likely harbored in the transcriptome. Currently, many approaches rely on enrichment with modification-specific antibodies. Although this strategy is relatively simple to implement, cross-reactivity is always a concern, and sites are not mapped at high resolution. Cross-linking-based methods can improve resolution (80), but they remain antibody based and do not overcome specificity issues. In some cases, specific chemical properties can be exploited to design higher-resolution mapping strategies. This type of strategy has been exploited to map Ψ, 2'OMe, and m⁵C, but it comes with its own risks. Recent work has focused on exploiting the mutagenicity of reverse transcriptase enzymes to map modifications at singlebase resolution by quantifying mutation rates. Two recent studies implemented one such enzyme, TGIRT

(91), to map m¹A sites (74, 100). As discussed above, however, technical differences led to dramatically different conclusions, underscoring the need for optimization and quality control. This strategy of developing new reverse transcriptases has been extended to m⁶A as well, though these enzymes have not yet been implemented widely (5, 42). To allow for direct detection of RNA modifications in long reads, researchers are also developing new RNA sequencing technologies such as the Oxford Nanopore and SMRT sequencing platforms (36, 115), which would eliminate many of the issues noted above. Moving forward, methods development will need to focus on a few key areas, namely improving resolution, generating quantitative data on modification fraction, and reducing the amount of input material required to enable profiling of rare cell populations and patient samples. Regardless of the mapping method used, increased emphasis should be placed on the validation of identified sites through orthogonal biochemical assays.

Although much focus is being placed on the development of sequencing methods, tools and assays that dissect the functions of these modifications in the cell will be equally important to develop to drive progress in the field. Developing new imaging tools and implementing biochemical methods to isolate RNA from specific subcellular compartments will help reveal how transcripts at specific cellular sites can impact function. We know many of the enzymes and regulatory proteins responsible for the regulation of m⁶A in mRNA. However, for other modifications, substantial work remains to identify enzymes with the relevant activity and to map their binding sites throughout the transcriptome and at specific subcellular locations. We also currently have limited ability to assess mRNA modification levels and function in cellular assays, further limiting our ability to probe directly the functional consequences of modifications in specific transcripts under changing cellular conditions. By leveraging the large amount of information we gain from high-throughput sequencing data on the sequence contexts of these modifications, it may be possible to develop novel imaging-based approaches and cellular reporters that will allow direct monitoring of modifications in real time.

The past eight years have yielded tremendous progress toward understanding how chemical modifications on mRNA influence cell biology. Much of this work has been focused on m⁶A, revealing its essential roles in coordinated transcriptome turnover and stimulus-induced translation. Examples of these regulatory paradigms in action have now been found throughout embryonic development, in stem cell differentiation, and in stress responses, and they have been coopted during cancer progression and viral infection. Importantly, these examples have been found in numerous organisms including yeast, flies, zebrafish, mice, and humans, suggesting that these modes of regulation may be evolutionarily conserved across eukaryotes. It remains to be seen whether the other modifications more recently found in mRNA have similar functions and mechanisms or whether their distinct chemical properties endow them with entirely different functions. It is clear, however, that a wealth of new RNA biology that will reveal novel layers of gene expression regulation remains to be uncovered.

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Figure 1.

Messenger RNA (mRNA) modifications and mRNA processing. **1** mRNA transcripts are transcribed from their genomic locus by RNA Polymerase II (Pol II). As transcription proceeds, nascent transcripts are **2** capped, **3** spliced, and **4** polyadenylated so they can be **5** efficiently exported for protein translation. Though the canonical mRNA cap contains m^7G , m^6A_m at the cap-adjacent nucleotide can render transcripts resistant to decapping by DCP2. m^6A has been implicated in many aspects of mRNA processing, **6** translation, and **7** decay. m^5C may mediate mRNA export through ALYREF, whereas Ψ and $2'OMe$ may influence protein translation by altering codon-anticodon interactions.

Figure 2. Mechanisms of m⁶A-mediated regulation. m⁶A installation and removal are regulated by the nuclear METTL3/METTL14 methyltransferase complex and the demethylases FTO and ALKBH5, respectively. In the nucleus, HNRNPC and YTHDC1 likely regulate m⁶A-mediated splicing, processing, and export. In the cytoplasm, YTHDF1 enhances cap-dependent translation, eIF3 induces cap-independent translation through m⁶A binding, and YTHDF2 regulates m⁶A-dependent mRNA decay. YTHDC2 appears to affect both decay and translation. YTHDF3 may be involved in some of these processes, in particular, m⁶A-mediated translational regulation. Abbreviations: 40S, small ribosomal subunit; eIF3, eukaryotic initiation factor 3; FTO, fat mass- and obesity-associated protein; pre-mRNA, precursor mRNA.

Figure 3.

Mechanisms of m⁶A-mediated transcriptome turnover and translation. (a) m⁶A marks transcripts required for maintaining a pluripotent state in numerous stem cell types. For differentiation to proceed, these transcripts must be degraded, presumably to minimize continued production of pluripotency factors. Cell type-specific factors must then be transcribed and translated to guide cells to the proper cell fate. In stem cell populations, loss of m⁶A often manifests as a block in differentiation, as pluripotency factors remain expressed at high levels. In cancer, loss of m⁶A can result in elevated levels of oncogenic factors to drive cancer progression, but this is not a general mechanism in all cases. (b) m⁶A can regulate translation of specific pools of transcripts under stress conditions. Under normal conditions, cap-dependent translation is heavily relied on to produce protein in the cell, and m⁶A-binding proteins such as YTHDF1 may contribute in some cases. Under heat shock stress, however, cap-dependent translation is compromised, yet the cell needs to efficiently translate specific factors such as HSP70. By recruiting eIF3, m⁶A can facilitate cap-independent translation of key transcripts in the heat shock response. Abbreviations: 40S, small ribosomal subunit; DF1, YTHDF1; eIF3, eukaryotic initiation factor 3; M3/M14, METTL3/METTL14; PABP, poly(A) binding protein; Pol II, Polymerase II.

Table 1

mRNA modification structures, regulatory enzymes, features, and functions

mRNA modification	Regulatory enzymes and binding proteins	Features and known functions
m^6A N^6 -methyladenosine	Methyltransferases: METTL3, METTL14, METTL16, WTAP, KIAA1429, Ime4 (yeast, fly) Demethylases: FTO, ALKBH5 Binding proteins: YTHDF1–3; YTHDC1,2; HNRNPC; IGF2BP1–3; FMR1	Features: 5' UTR, CDS, 3' UTR, RRACH motif Functions: transcriptome turnover, translation regulation, cell differentiation, embryonic development, stress response
m^1A N^1 -methyladenosine	Methyltransferases: TRMT6/TRMT61A, TRMT61B, TRMT10C Demethylases: ALKBH1, ALKBH3	Features: 5' UTR; early CDS; GC-rich, structured regions; first splice site Functions: translational regulation during stress response?
m^5C 5-Methylcytidine	Methyltransferase: NSUN2 Demethylases: Tet (fly), TET2 Binding protein: ALYREF	Features: 5' UTR, CDS, 3' UTR Functions: nuclear export, brain development (5hmC), response to infection (5hmC)
Ψ Pseudouridine	Pseudouridine synthases: Pus1–4,6,7,9 (yeast); DKC1/dyskerin	Features: CDS; UGΨAR (Pus4) and GUΨCNANC (Pus7) motifs in yeast, GUΨC and UGΨAG motifs in human Function: translational regulation through recoding
$2'Ome$ 2'-O-methylation	Methyltransferases: C/D-box small nucleolar RNA-guided Fibrillarin in rRNA; enzyme unknown in mRNA	Features: Primarily U_m , in CDS; 5' cap +1, +2 nucleotide Functions: translational regulation, codon bias?

mRNA modification	Regulatory enzymes and binding proteins	Features and known functions
<p>m^7G</p> <p>7-Methylguanosine</p>	<p>Methyltransferase: RNMT Binding proteins: eIF4E, CBP20/CBP80</p>	<p>Feature: 5' cap Functions: mRNA processing and export, cap-dependent translation</p>
<p>m^6A_m</p> <p>$N^6,2'O$-methyladenosine</p>	<p>Demethylase: FTO</p>	<p>Features: 5' cap +1 nucleotide Function: transcript stability</p>

Abbreviations: ALKBH, alkylated DNA repair protein alkB homolog; CBP20/80, nuclear cap binding protein; CDS, coding sequence; DKC1, an H/ACA ribonucleoprotein complex subunit; eIF, eukaryotic translation initiation factor; FMR1, a synaptic functional regulator; FTO, fat mass- and obesity-associated protein; HNRNPC, heterogeneous nuclear ribonucleoproteins C1/C2; IGF2BP1–3, insulin-like growth factor 2 mRNA-binding protein 1–3; METTL, methyltransferase-like protein; mRNA, messenger RNA; NSUN2, tRNA [cytosine(34)-C(5)]-methyltransferase; Pus, pseudouridine synthase; TRMT6, tRNA [adenine(58)-N(1)]-methyltransferase noncatalytic subunit; TRMT10C, tRNA methyltransferase 10 homolog C; TRMT61A, tRNA [adenine(58)-N(1)]-methyltransferase catalytic subunit; TRMT61B, tRNA [adenine(58)-N(1)]-methyltransferase, mitochondrial; UTR, untranslated region; WTAP, Wilms tumor 1-associating protein; YTHDC1/2, YTH domain-containing protein 1/2; YTHDF1–3, YTH domain-containing family protein 1–3.