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m⁶A-Mediated Translation Regulation

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INTRODUCTION

Soon after the first discovery that m⁶A was present in cellular mRNAs [1, 2] researchers speculated that m⁶A might play important roles in mRNA regulation. Due to a lack of methods for detecting this mark in individual mRNAs, however, it was difficult to determine the global impact of m⁶A on gene expression. The recent advent of next generation sequencing-based methods for transcriptome-wide m⁶A detection [3, 4], coupled with the identification of proteins that regulate m⁶A [5], have facilitated rapid growth in the number of studies investigating the function of m⁶A. A major theme that has emerged from these studies is that m⁶A can regulate multiple steps along the mRNA lifecycle, including splicing, export, stability, and translation. Here, we review recent discoveries in the area of m⁶A-mediated translation regulation and focus on the various mechanisms through which adenosine methylation contributes to translation control.

Prior to the global identification of m⁶A sites, only a small number of studies had directly investigated the role of m⁶A in protein production, with conflicting results. *In vitro* translation of m⁶A-containing dihydrofolate reductase (*DHFR*) mRNA showed slightly enhanced protein production in rabbit reticulocyte lysates (RRL) [6], and inhibition of m⁶A methylation with cycloleucine led to decreased levels of DHFR protein in cells [7], suggesting a positive role for m⁶A in translation regulation. Subsequent studies, however, found that exogenously expressed m⁶A-containing mRNAs in cells exhibited reduced translation compared to unmodified mRNA [8]. These studies used different methods and examined distinct mRNAs, so it was difficult to draw firm conclusions on how m⁶A contributes to translation regulation. Over forty years after the initial discovery of m⁶A, the factors that dictate whether m⁶A will directly impact the translation of a given mRNA are still incompletely understood, although we have substantially expanded our understanding of the multiple roles that m⁶A can play in regulating translation under unique cellular conditions.

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1. m⁶A-Mediated Regulation of Cap-Dependent Translation

1.1. Positive effects of m⁶A on translation

1.1.A. The role of YTHDF1: A major mechanism through which m⁶A regulates mRNAs is by recruiting m⁶A reader proteins. Among the first readers to be identified were members of the YT521B-Homology (YTH) domain-containing class of proteins [4]. This includes three highly similar cytosolic proteins: YTHDF1, YTHDF2, and YTHDF3; as well as YTHDC1 and YTHDC2, which are unique from the YTHDF proteins in terms of their size and the additional domains they contain.

The first of the YTH proteins to be directly linked to translation regulation was YTHDF1. Chuan He and colleagues showed that YTHDF1 knockdown in HeLa cells reduced the translation efficiency of YTHDF1 target mRNAs as measured by ribosome profiling [9]. This contrasted with YTHDF2, for which depletion caused only a small effect on translation efficiency and a substantial increase in mRNA abundance [10]. The translation efficiency of YTHDF1 targets was also decreased by depletion of the m⁶A methyltransferase, METTL3, suggesting that the effects of YTHDF1 depletion were due to recognition of m⁶A. Furthermore, YTHDF1 depletion increased the m⁶A/A ratio of mRNAs in the non-translatable mRNP pool and decreased the ratio of transcripts in the translatable pool [9], further suggesting that YTHDF1 causes global changes in the translation of methylated mRNAs. Recently, YTHDF1 has also been shown to promote translation in post-mitotic neurons following nerve injury [11], suggesting that YTHDF1-mediated protein production is a mechanism utilized by diverse cell types.

Mapping of global translation initiation site (TIS) occupancy using quantitative translation initiation sequencing (QTI-seq [12]) revealed an increase in TIS occupancy after YTHDF1 knockdown, suggesting that YTHDF1 increases the rate of translation initiation [9]. Subsequent affinity purification and mass spectrometry experiments revealed that YTHDF1 binds many proteins directly involved in translation, including several subunits of the translation initiation factor, eIF3. This finding led He and colleagues to propose a model in which YTHDF1 binds to m⁶A residues in 3'UTRs and recruits eIF3 to the 5' end of the mRNA (Figure 1). They hypothesize that this interaction is mediated by mRNA looping, in which eIF4G bridges interactions with eIF4E and poly(A) binding protein (PABP) to bring the 5' and 3' ends of the transcript in close proximity, forming a closed loop [13]. Further support for this model comes from the finding that YTHDF1 has the greatest effect on promoting translation when tethered to mRNAs that require initiation factors as well as eIF4G-mediated mRNA looping [9].

The looping model is an attractive model for explaining how RBPs that bind in 3'UTRs promote translation [14]. However, it relies on PABP binding to the poly(A) tail to bring the distal 3'UTR close to the mRNA 5' end, whereas most m⁶A sites are located in proximal 3'UTRs. Thus, understanding the factors that enable proximal 3'UTR-bound YTHDF1 to gain access to eIF3 via mRNA looping will be an important area of future study.

1.1.B. The role of YTHDF3: YTHDF3 was the last of the three YTHDF proteins to be functionally characterized, and recent studies now suggest that YTHDF3 cooperates with

YTHDF1 to promote translation. First, affinity purification-based approaches revealed that YTHDF1 and YTHDF3 share over 150 common interacting proteins [15, 16]. Many of these are related to translation, including several of the proteins that make up the small and large ribosomal subunits. Furthermore, YTHDF3 knockdown leads to decreased translation of mRNAs that are bound by both YTHDF1 and YTHDF3, suggesting that these two proteins cooperate to regulate translation [15, 16]. Indeed, reporter assays show that tethering both YTHDF3 and YTHDF1 to the 3'UTR causes a greater increase in translation efficiency than tethering of YTHDF1 alone [15]. Interestingly, tethering YTHDF3 alone fails to increase reporter translation, suggesting that it does not promote translation directly.

Although YTHDF3 may not be sufficient to promote translation of methylated mRNA, it does appear to facilitate translation enhancement when bound to mRNAs with YTHDF1 (Figure 1). Unlike YTHDF1, YTHDF3 does not bind eIF3 [15], so the mechanism of YTHDF3-mediated translation may involve the recruitment of ribosomal subunit proteins or other components of the translation machinery. It is unclear whether the interaction between YTHDF1 and eIF3 plays a role in YTHDF1/3-mediated translation.

In addition to its interactions with YTHDF1, YTHDF3 also binds to YTHDF2. This latter interaction appears to accelerate mRNA decay [15]. In fact, depletion of all three YTHDF proteins causes a greater increase in m⁶A/A ratios of cellular mRNA than depletion of any one protein or combination of two proteins alone [15], which suggests that all three YTHDF proteins contribute to mRNA decay. This is consistent with previous studies which have reported that the YTHDF proteins all have similar functions [17, 18].

A current model predicts that YTHDF1 and YTHDF3 bind to methylated mRNAs before YTHDF2 in order to promote mRNA translation, followed by degradation. This is based on pulse-chase metabolic labeling studies which suggest that YTHDF1 and YTHDF3 target nascent mRNAs before YTHDF2 [9, 15]. The mechanisms that enable this, however, are unknown. Recent studies have shown that EJC components interact with YTHDFs 1 and 3 [15], which may facilitate the transfer of nascent mRNAs to these proteins before the pioneer round of translation, after which their bound mRNAs are eventually transferred to YTHDF2 to facilitate degradation. YTHDF3 may further contribute to this regulation by controlling the binding specificity of YTHDFs 1 and 2, as YTHDF3 depletion increases the binding of YTHDF1 and YTHDF2 to non-target mRNAs and reduces their binding to target transcripts [15]. Further studies will be needed to understand the factors that control how YTHDF proteins recognize target mRNAs and promote translation or degradation, as well as how the interactions among the YTHDF proteins themselves facilitate these effects.

1.1.C. Other YTH proteins: YTHDC2 is the largest of the YTH domain-containing proteins and the only member of this family to contain helicase domains. Recent studies indicate that YTHDC2 increases the translation efficiency of a small subset of mRNAs, including transcripts such as *HIF-1α*, which have highly structured 5'UTRs [19]. This suggests that YTHDC2 may use its helicase domain to unwind secondary structures and enable translation. However, further research will be needed to determine the contribution of the YTHDC2 helicase domains, as well as m⁶A, to such a mechanism. Notably, other studies have confirmed the ability of YTHDC2 to promote translation efficiency but have also

shown that it accelerates mRNA decay [20, 21]. Thus, whether YTHDC2 primarily functions by promoting mRNA translation or degradation, and the factors that regulate its function, remain to be determined.

YTHDC1 is primarily expressed in the nucleus and has been shown to impact nuclear processes such as alternative splicing and *XIST*-mediated gene silencing. Thus, it is unlikely that this protein uses m⁶A recognition to directly regulate translation. However, recent studies have shown that YTHDC1 depletion effects alternative polyadenylation [22], so it may indirectly influence translation efficiency through the gain or loss of 3'UTR regulatory elements.

1.1.D. The role of METTL3: In addition to YTHDF1-mediated recruitment of eIF3 to methylated mRNAs, METTL3 has been shown to promote translation through interactions with eIF3 (Figure 1). Gregory and colleagues [23] found that depletion of METTL3 decreased the translation of a subset of m⁶A-containing mRNAs and led to a global alteration in the cellular polysome profile. Surprisingly, the translation-promoting effect of METTL3 was independent of its methyltransferase activity, as tethering a catalytically inactive form of METTL3 to the 3'UTR of a reporter mRNA had a similar effect on translation enhancement to the wild type version [23]. The translation-promoting effects of METTL3 were also not dependent on YTHDF1, suggesting that METTL3 promotes protein production independently of YTHDF1-eIF3 interactions. Indeed, co-immunoprecipitation experiments revealed that METTL3 binds both nuclear and cytosolic cap-binding complexes, as well as eIF3 [23]. The eIF3h subunit in particular appears to mediate METTL3 interactions with cap-binding proteins and translation initiation factors [24]. Collectively, these data suggest that METTL3 can recruit the translation initiation complex to target mRNAs through direct interactions with eIF3.

How does METTL3 recruit eIF3 to target mRNAs? METTL3 does not bind the cap itself, but binds to internal m⁶A residues [25]. Recent mechanistic studies have shown that METTL3 increases the translation of reporter mRNAs only when it is tethered to sites near the stop codon [24]. Furthermore, electron microscopy analysis of METTL3-containing polysomes indicates that METTL3 binds in close proximity to cap binding proteins. Together, these data suggest that METTL3 promotes translation through an mRNA looping mechanism [24]. Although over 4,000 mRNAs have reduced translation efficiency following METTL3 depletion [24], PAR-CLIP and MeRIP-Seq analyses suggest that METTL3 binds only a subset of methylated consensus sites [23, 26]. Therefore, determining the factors that regulate which m⁶A sites are recognized by METTL3 to promote translation will be an important area of future research.

In addition to interacting with eIF3, METTL3 has also been shown to promote translation through methylation of the coding sequence (CDS) of select target mRNAs (Figure 1). Kouzarides and colleagues [27] found that the transcription factor, CEBPZ, recruits METTL3 to the promoters of select active genes. When they looked at the distribution of m⁶A in mRNAs produced from METTL3-bound genes, they observed an enrichment in CDS m⁶A methylation which was reduced after removing METTL3 from transcription start sites by CEBPZ depletion [27]. In contrast to previous studies, they found that depletion of

METTL3 increased global translation efficiency. However, for mRNAs produced from genes that had METTL3 bound at their promoter, there was reduced translation efficiency. This was corroborated by reporter assays in which forced recruitment of METTL3 to a reporter mRNA promoter increased translation without affecting mRNA levels [27]. Tethering of a catalytically inactive form of METTL3 failed to have this effect, indicating that the increased translational efficiency required m⁶A. Thus, CDS m⁶A deposition by promoter-bound METTL3 appears to enhance translation of a subset of mRNAs.

How does CDS m⁶A enhance protein production? Intriguingly, GAN codons exhibited increased ribosome occupancy on METTL3-bound genes after METTL3 depletion [27]. This suggests that CDS m⁶A residues prevent ribosome stalling at GAN codons (Figure 1). Thus, CEBPZ-mediated recruitment of METTL3 to the promoters of select active genes may increase mRNA translation by relieving ribosome pausing. Some of the target genes that undergo this form of regulation have been implicated in the pathology of acute myeloid leukemia, which is consistent with the growing literature implicating the m⁶A machinery in this disease [28]. Whether this mechanism of translation regulation is widespread in other cancers, and the degree to which it controls protein production in noncancerous cell types, will be important areas of future research. Additionally, since this mechanism of translation enhancement through CDS methylation contrasts with the inhibitory effects observed in other studies (below), the role of CDS m⁶A in promoting versus inhibiting translation will be an important area of investigation.

1.2. Negative effects on translation

1.2.A. Impact of m⁶A on translation dynamics: In addition to the translation-promoting effects of m⁶A, emerging evidence suggests that this mark can also negatively impact protein production. Single-molecule measurements of ribosome dynamics in an *E. coli* translation system show that the presence of m⁶A in the CDS slows translation elongation [29]. The mechanism for this slowed elongation appears to involve m⁶A-induced influences on tRNA selection. Specifically, m⁶A residues disrupt tRNA accommodation, leading to reduced translation kinetics (Figure 1). One potential outcome of this mechanism could be that m⁶A in the CDS slows down translation rates, potentially leading to alterations in protein folding or nascent peptide recognition in the cell and possibly influencing protein localization [29].

Although it remains to be determined whether this precise mechanism of m⁶A-mediated ribosome stalling is conserved in eukaryotes, recent studies suggest that CDS m⁶A-mediated translation inhibition is not restricted to bacterial systems. For instance, m⁶A residues in the CDS are selectively correlated with decreased protein levels in xenopus oocytes [30]. Recent studies in mammalian cells also support an inhibitory effect of CDS m⁶A on translation [31]. Specifically, Slobodin *et al* discovered that slowed RNA polymerase increases mRNA methylation, causing a particularly strong m⁶A enrichment within the coding sequence and reduced protein production from reporter mRNAs [31]. Using synthetic mRNAs, they found that the greater the amount of m⁶A in a transcript, the stronger the translation inhibition *in vitro* [31]. This is consistent with previous studies using methylated mRNAs introduced into

cultured cells [8], although potential effects of m⁶A in untranslated regions in these assays cannot be discounted.

Although these findings suggest that high levels of m⁶A in coding sequences inhibit protein production in eukaryotic cells, it will be important to determine whether this is through a tRNA-accommodation and ribosome stalling mechanism as observed in bacteria [29]. Furthermore, since m⁶A is known to accumulate in long internal exons, it will be interesting to determine whether long internal exon-containing mRNAs are particularly prone to m⁶A-mediated ribosome stalling. Finally, the effects of translation inhibition versus enhancement will need to be further studied. Most likely, codon identity and the position of m⁶A within a codon are important factors regulating how m⁶A influences ribosome kinetics [27, 29].

1.2.B. Effects of m⁶A reader proteins: Recent studies uncovering additional m⁶A binding proteins have implicated known translation regulators in contributing to m⁶A-dependent translational control. FMRP, the protein product of the gene that is disrupted in fragile X syndrome, has long been known suppress the translation of target mRNAs [32]. Intriguingly, FMRP exhibits preferential binding to m⁶A-containing RNA *in vitro*, and FMRP target mRNAs are enriched for m⁶A in cells [33, 34]. FMRP overexpression causes a global reduction in translation, although the degree to which methylated versus unmethylated mRNAs are translationally suppressed, and the mechanisms that regulate FMRP recognition of m⁶A, remain to be determined. FMRP does show sequence specificity when binding m⁶A [33], suggesting that there could be subsets of FMRP target mRNAs for which FMRP binding and translational suppression are dictated by the presence of m⁶A.

Other reader proteins can also negatively impact protein production by sequestering methylated mRNAs away from the translatable pool. The YTHDF2 protein, for instance, influences the amount of m⁶A-containing mRNA in polysome fractions by recruiting mRNAs to p-bodies and eventually facilitating their degradation [10]. YTHDF1 and YTHDF3 also appear to contribute to mRNA storage [15], suggesting that all of these proteins may play a role in mRNA sequestration to influence translation.

2. m⁶A-Mediated Control of Cap-Dependent Translation

2.1 Direct interactions between m⁶A and eIF3—Most translation events in the cell occur through recognition of the cap by the eIF4F proteins. This cap-dependent translation is thought to predominate protein production at steady-state. However, it has long been known that many cellular states, such as apoptosis, mitosis, and the activation of cellular stress response pathways, globally suppress cap-dependent translation through a variety of mechanisms [35]. Despite global reduction in translation, however, a select number of mRNAs retain the ability to be translated and are often translationally upregulated. Several proposed mechanisms for such cap-independent translation events have been introduced, and although our understanding of this mode of translation is far from complete, emerging evidence indicates that m⁶A is capable of regulating some forms of cap-independent translation (Figure 3).

The first indication that m⁶A facilitates cap-independent translation came from the discovery that m⁶A residues in 5'UTRs enable ribosome loading and start site recognition on synthetic

mRNAs in the absence of cap-binding proteins [36]. *In vitro* translation assays [36] and expression of exogenous methylated mRNAs in cells [37] further demonstrated that methylation of 5'UTRs could promote robust translation in the absence of cap-binding proteins. Cells subjected to heat shock stress showed a global upregulation of 5'UTR m⁶A, suggesting that this mode of m⁶A-mediated cap independent translation is important during the cellular stress response [36, 37]. Indeed, hypermethylation of the 5'UTR of *HSP70*, a transcript known to be translationally upregulated following heat shock, led to increased translation of this mRNA in an m⁶A-dependent manner [36, 37]. Chaperones such as HSP70 are important regulators of stress-induced proteostasis, and thus require mechanisms to evade cap-dependent translational suppression during stress. The finding that transcripts encoding many stress-response proteins are hypermethylated following heat shock and other stresses [36–39] suggests that this may be a conserved mechanism for controlling the levels of many stress-response proteins (Figure 3).

Insights into the mechanism of m⁶A-mediated cap-independent translation came from the finding that eIF3 preferentially binds to m⁶A-containing RNA [36]. PAR-iCLIP analysis of eIF3 binding sites in cells further revealed that eIF3 binds to sites of 5'UTR methylation in cells [36]. Thus, unlike the mechanism of eIF3-mediated translation initiation which involves YTHDF1 or METTL3 recruitment of eIF3 following recognition of m⁶A sites near the stop codon, eIF3 here binds directly to m⁶A sites in 5'UTRs and facilitates recruitment of the translation machinery (Figure 2). This mode of translation also appears to depend on an accessible 5' end of the mRNA and a scanning-based mechanism, which explains why only m⁶A residues in 5'UTRs, and not located internally, can promote this mode of translation initiation [36]. 5'UTR m⁶As are therefore distinct from classical viral IRES elements since m⁶A promotes recruitment of ribosomal preinitiation complexes to the 5' end of mRNA, rather than enabling internal ribosome entry.

One question that remains is how m⁶A residues in the 5'UTR are selectively increased following cellular stress. Qian and colleagues [37] propose a model in which m⁶A residues are protected from demethylation by stress-induced nuclear localization of the m⁶A reader, YTHF2. Binding of YTHDF2 prevents m⁶A residues from being demethylated by FTO, which they found prefers to demethylate m⁶A residues in 5'UTRs. However, binding affinities of YTHDF2 for m⁶A are relatively low [40, 41], suggesting that its interactions with m⁶A are transient, so further investigation of how YTHDF2 interactions may be stabilized during stress to warrant protection from demethylation are needed.

Additionally, recent studies have challenged the idea that m⁶A is the preferred substrate for FTO. Jaffrey and colleagues demonstrated that FTO preferentially demethylates m⁶A_m, an extended cap modification which occurs when the first transcribed nucleotide is methylated at both the 2' position of the ribose and the N⁶ position of the adenine base [42]. Since m⁶A and m⁶A_m are both detected by MeRIP-Seq, a perceived 5'UTR m⁶A demethylation could in fact be caused by loss of m⁶A_m. Thus, careful analysis should be done to confirm that m⁶A_m demethylation is not responsible for the perceived 5' end preference for m⁶A demethylation and that FTO-dependent effects on translation are not caused by m⁶A_m. Single-nucleotide resolution mapping methods which distinguish m⁶A from m⁶A_m [43] have shown that stress-induced 5'UTR hypermethylation does indeed occur through m⁶A [36],

but the potential contribution of m^6A_m to stress-induced translation has not been explored. Indeed, m^6A_m has been linked to increased translation efficiency [41] and has been shown to promote mRNA stability [40], so teasing apart the effects of FTO-mediated m^6A_m demethylation from those of m^6A targeting will be important going forward. Notably, m^6A_m is estimated to occur in up to 30% of mRNAs [44]. However, recent studies suggest that FTO preferentially targets m^6A_m residues in snRNAs as opposed to mRNAs [45, 46]. Thus, further work will be needed to determine the degree to which FTO targets m^6A_m in mRNAs under different cellular conditions, such as in response to cellular stress.

2.3. m^6A -mediated regulation of translation initiation during the ISR—A major mechanism of translation regulation in response to stress is through the Integrated Stress Response (ISR). This conserved eukaryotic mechanism involves the activation of one of four kinases (HRI, PKR, PERK, and GCN2), all of which phosphorylate the alpha subunit of eIF2 on serine 51 [47]. eIF2 α phosphorylation reduces the ability of eIF2 to combine with GTP and met-tRNA_i to form the ternary complex (TC). Thus, eIF2 α phosphorylation suppresses global cap-dependent translation by limiting the availability of the TC. The ISR is activated by diverse stresses, including heme depletion, viral infection, endoplasmic reticulum stress, and amino acid deprivation, thereby representing a conserved mechanism for maintaining cellular homeostasis under a variety of cellular conditions [47].

One of the downstream effects of eIF2 α phosphorylation is the upregulation of ATF4, a transcription factor which activates genes important for the ISR. The *ATF4* 5'UTR contains two upstream open reading frames (uORFs), one of which (uORF2) is overlapping and out of frame with the *ATF4* main ORF [48]. Thus, under normal conditions, ATF4 protein production is limited by translation of uORF2. However, during stress, *ATF4* translation is increased due to “leaky scanning,” a process in which ribosomes scanning the 5'UTR fail to initiate at uORF2 due to limited TC availability; these ribosomes therefore continue scanning and eventually initiate translation at the downstream main ORF [49].

Qian and colleagues [38] found that m^6A is an additional factor regulating the translation of *ATF4* during stress. In their efforts to understand how amino acid starvation influences the proteins that recognize *ATF4* mRNA, they identified the m^6A demethylase ALKBH5 as a protein exhibiting modestly increased binding to *ATF4* after starvation [38]. This led them to speculate that m^6A might be participating in ATF4 translation during stress. Indeed, m^6A profiling revealed that *ATF4* is methylated in uORF2, and that this methylation is decreased after starvation in an ALKBH5- and FTO-dependent manner. Both ALKBH5 and FTO depletion decreased ATF4 protein expression after amino acid deprivation, and METTL3 or METTL14 depletion enhanced starvation-induced ATF4 production, suggesting that m^6A negatively impacts ATF4 protein production during stress. Importantly, an *ATF4* reporter mRNA harboring a point mutation that prevents methylation of the uORF2 site was resistant to the effects of ALKBH5 or FTO depletion and showed enhanced expression following starvation, further supporting the idea that removal of uORF2 m^6A enables *ATF4* translation in response to amino acid deprivation [38].

To understand the mechanism of m^6A -mediated regulation of *ATF4*, the authors performed ribosome toeprinting assays and found that the presence of m^6A in uORF2 increases

ribosome retention at the upstream start codon. They propose a model in which the presence of m⁶A hinders ribosome scanning and promotes translation initiation at upstream uORFs (Figure 2).

They find that amino acid starvation upregulates ALKBH5 and FTO, which they hypothesize leads to increased *ATF4* ORF translation by alleviating the m⁶A-mediated retention of ribosomes at uORF2. Although reporter assays showed that blocking m⁶A in uORF2 increased the translation of the main ORF, this effect was only observed under starvation conditions, indicating that the proposed m⁶A-mediated *ATF4* translation mechanism also likely relies on reduced TC availability (Figure 3). Understanding the interplay between these two models to control translation initiation during stress will be an important area of research moving forward.

Stress-induced changes in m⁶A may be influencing TIS selection of other mRNAs as well. Global profiling of m⁶A and TIS occupancy during starvation showed that 5'UTR m⁶A was associated with increased TIS signal at non-AUG start codons [38]. One example is the stress-response mRNA, *GADD45G*, which displayed a pattern of starvation-induced m⁶A reduction and increased CDS translation similar to *ATF4*. Understanding the factors that regulate m⁶A-dependent TIS selection during stress will be critical for determining how prevalent this mechanism of translation is likely to be.

2.2 ABCF1-associated translation regulation—In addition to activating the phosphorylation of eIF2 α , amino acid deprivation suppresses protein synthesis through inhibition of mTORC1, a master translation regulator that, among other things, influences the assembly of the eIF4F complex and thus its ability to bind the 5' cap [50]. Surprisingly, a substantial amount of protein production persists following inhibition of mTORC1 or phosphorylation of eIF2 α [25]. This led Qian and colleagues [25] to speculate that other widespread mechanisms exist to facilitate cap-independent translation. They found that METTL3 depletion enhanced the sensitivity of cells to the mTORC1 inhibitor, torin1, causing nearly 80% reduction in global protein synthesis compared to less than 50% reduction observed with torin1 alone. This effect could be rescued by overexpression of catalytically active METTL3, but not by an inactive form, suggesting that m⁶A contributes to a substantial amount of translation that persists following inhibition of the eIF4F proteins.

To understand the proteins that might mediate stress-induced translation, the authors once again explored *HSP70*, an mRNA known to be hypermethylated following heat shock. They found that ABCF1, a protein previously linked to TC recruitment [51, 52], exhibited slightly enriched binding to *HSP70* mRNA following heat shock stress. ABCF1 knockdown diminished stress-induced *HSP70* translation and, like METTL3, sensitized cells to torin1 treatment, supporting the idea that ABCF1 contributes to eIF4F-independent translation. Moreover, ribosome profiling showed a correlation between changes in translation efficiency of mRNAs following either ABCF1 or METTL3 depletion, suggesting that they act on many of the same mRNAs to control translation [25].

What is the mechanism through which ABCF1 controls cap-independent translation of methylated mRNAs? The data suggest that ABCF1 may help recruit the ternary complex

during stress conditions. However, the contribution of m⁶A to this process is unclear. Based on *in vitro* crosslinking assays, ABCF1 is unlikely to be directly recognizing m⁶A [36]. Many mRNAs are not affected by METTL3 and ABCF1, which is consistent with the fact that other mechanisms (e.g., eIF3-dependent mechanisms) mediate m⁶A-directed cap-independent translation during stress.

Additionally, many of the effects of ABCF1 on m⁶A-mediated cap-independent translation may be indirectly occurring through METTL3 (Figure 3). ABCF1 depletion reduces translation of METTL3, which has m⁶A in its 5'UTR. This suggests a potential positive feedback mechanism in which 5'UTR methylation of METTL3 enables its ABCF1-mediated translation during stress, which in turn may facilitate 5'UTR methylation and cap-independent translation of other transcripts [25]. Several other components of the m⁶A processing machinery also have m⁶A sites within their mRNAs [3, 4] so it will be interesting to explore whether these proteins are part of similar m⁶A-dependent feedback mechanisms.

The mechanisms through which m⁶A promotes stress-induced translation are diverse (Figure 3). In one case, m⁶A in the 5'UTR can directly recruit eIF3. This is dependent on 5' end accessibility and scanning [36]. However, m⁶A also appears to increase ribosome retention, potentially through slowed scanning, which leads to enhanced translation at upstream initiation sites [38]. Although multiple studies indicate that 5'UTR methylation promotes translation following various stressors [25, 36–38], m⁶A residues outside of the 5'UTR are also likely to regulate gene expression during cellular stress. For instance, He and colleagues found that tethering of YTHDF1 to the 3'UTR of a reporter mRNA speeds its translational recovery after sodium arsenite treatment [9]. They suggest that YTHDF1 may stabilize the formation of stalled initiation complexes on mRNAs in stress granules, enabling YTHDF1-bound transcripts to quickly resume translation after the stress has been removed. Interestingly, all of the YTHDF proteins have been found to localize to stress granules [53–55], suggesting that they may all act in some capacity to modify the response of methylated mRNAs during stress. Other m⁶A binding proteins may play similar roles. Indeed, recent studies have identified IGF2BP proteins as additional m⁶A readers which can facilitate the storage and stabilization of mRNAs in stress granules and promote translation [56]. Thus, the contribution of m⁶A to mRNA storage and translation in response to stress, and how unique reader proteins facilitate these responses through m⁶A recognition, will be important areas of future study.

3. m⁶A-Mediated Translation of Viral and Unique Cellular RNAs

3.1 Viral RNA—Viral RNAs were among the first methylated transcripts to be identified [57–60], and m⁶A has been shown to have both positive and negative impacts on viral replication [61]. Although the precise mechanisms through which m⁶A mediates viral gene expression are still being explored, several recent studies have revealed potential roles for m⁶A in regulating both host and viral RNAs after viral infection.

Studies of HIV have reported that methylation of viral RNA positively regulates viral replication [18, 62, 63]. This appears to involve YTHDF proteins, whose expression enhances viral RNA expression and protein production [18]. Intriguingly, all three YTHDF proteins were shown to promote translation when tethered to a reporter mRNA, which

suggests overlapping rather than distinct functions of these readers [18]. m⁶A residues in SV40 viral RNA have also been shown to increase viral protein production and positively influence viral replication [64]. These effects were dependent on the m⁶A-binding activity of YTHDF2; however, the precise mechanism through which YTHDF2 impacts SV40 viral protein production remains to be determined.

In addition to its roles in viral RNA regulation, m⁶A also appears to be controlling the expression of host mRNAs during viral infection. Studies of both HIV and ZIKA virus have shown that viral infection modulates host mRNA methylation levels to regulate gene expression [62, 65]. Intriguingly, this viral-induced host mRNA methylation includes the upregulation of m⁶A residues in cellular 5'UTRs [65]. Thus, similar to other cellular stresses [36–38], viral infection may be an additional stimulator of 5'UTR hypermethylation-directed translation regulation. Indeed, viral infection is one activator of the ISR [47]. However, the contribution of host 5'UTR hypermethylation to protein production during viral infection remains to be determined.

3.2 circRNAs—Circular RNAs (circRNAs) are a unique class of RNAs which have recently attracted attention due to the discovery that thousands of circRNAs are detectable in cells [66]. circRNAs are generated through pre-mRNA back-splicing and have been hypothesized to have a variety of functions in gene expression regulation, including acting as sponges for miRNAs and decoys for cellular RBPs [67]. However, recent studies have suggested that some circRNAs are capable of producing proteins [68, 69], adding another potential mechanism of gene expression control mediated by this unique class of RNAs. Since circRNAs constitute a covalently closed loop and lack 5' and 3' ends, the potential mechanism for their translation necessitates the requirement for cap-independent methods.

Two groups recently demonstrated that a substantial number of circRNAs contain m⁶A [70, 71]. Yang et al serendipitously discovered that m⁶A can promote circRNA translation after noticing that the presence of m⁶A consensus motifs correlated with translation of circRNA reporters [70]. They further demonstrated that protein production from m⁶A-containing circRNA reporters is impacted by METTL3 and METTL14 levels, suggesting that the presence of m⁶A contributes to circRNA translation.

m⁶A circRNA translation is inhibited by depletion of YTHDF3, suggesting additional roles for this protein in regulating m⁶A-mediated translation outside of its contribution to cap-dependent m⁶A translation [70]. Co-immunoprecipitation studies reveal that YTHDF3 interacts with eIF4G2, a protein known to mediate some forms of IRES-driven translation by directly binding to IRES elements and recruiting the translation machinery [72]. eIF4G2 depletion reduces translation from circRNA reporters but not linear mRNA reporters, suggesting a selective role for eIF4G2—potentially through interactions with YTHDF3—in mediating m⁶A circRNA translation [70] (Figure 2). eIF3A, which recognizes m⁶A to promote cap-independent translation of linear mRNAs [36], also has this effect; however, whether eIF3 directly binds m⁶A to mediate circRNA translation remains to be determined.

How prevalent is m⁶A-mediated circRNA translation? Two recent studies report very different estimates of the total number of methylated circRNAs (ranging from 85 to over

1,400), which may reflect differences in the methods used to identify methylated circRNAs in each dataset [70, 71]. Nevertheless, these studies indicate that adenosine methylation is a prominent feature of at least a subset of circRNAs. Moreover, the finding that potentially hundreds of circRNAs are located in polysomes suggests that circRNA translation could be more widespread than originally thought [70]. Whether m⁶A is the major driver of circRNA translation, and what the function of m⁶A-produced circRNA peptides is in the cell, remains to be determined.

Finally, m⁶A residues are found in many other types of ncRNAs as well and have been shown to influence non-coding RNA structure [73, 74] and lncRNA function [75]. There is emerging evidence that a small proportion of lncRNAs may be translated in cells [76, 77]. Whether m⁶A contributes to lncRNA translation and whether such mechanisms rely on cap-dependent or cap-independent translation will be intriguing areas of future study.

3.3 Local translation—Although many studies investigating the effects of m⁶A on translation have focused on protein synthesis throughout the cell, it is well-appreciated that many forms of localized protein synthesis occur which have important impacts on cell function [78, 79]. Recent studies have begun to explore the role of m⁶A in regulating local protein synthesis. Using dorsal root ganglia (DRG) neurons cultured in microfluidic devices that enable fluidic separation of cell bodies from axons, Yu et al [80] showed that m⁶A immunoreactivity is elevated by neurotrophin exposure, a stimulus known to elicit local protein synthesis in axons [81]. Depletion of FTO in axons led to increased axonal m⁶A immunoreactivity and defects in axon elongation. Furthermore, FTO overexpression inhibited the axonal translation of GAP-43, a protein which contributes to neurite outgrowth [82, 83]. This effect was also seen with point mutation of m⁶A sites in exogenously overexpressed *GAP-43*, suggesting that m⁶A was the reason for the inhibited translation.

Since m⁶A antibodies also detect m⁶A_m, it is necessary to determine whether FTO depletion-induced changes in immunoreactivity are due to m⁶A or m⁶A_m, and whether m⁶A sites in transcripts other than *GAP-43* contribute to local translation. Nevertheless, these studies provide early hints that m⁶A regulation could impact local protein production in neurons. Local translation is particularly important for highly polarized cells, such as neurons, in which axons or dendrites can project hundreds of microns from the cell body. Indeed, recent studies have shown that m⁶A is present in thousands of mRNAs located at synapses [84], raising the exciting possibility that methylation may control the local translation of many neuronal mRNAs. The degree to which m⁶A contributes to local translation and whether such regulation relies on cap-dependent or cap-independent mechanisms remains to be determined.

CONCLUSIONS

The studies performed to date suggest that m⁶A can play diverse roles in translation regulation. Although m⁶A-mediated translation enhancement appears to influence many mRNAs under a variety of cell types and contexts, there are conflicting reports of the effects of m⁶A methyltransferase depletion on global translation [9, 23, 25, 27, 85]. Additionally, there are many methylated mRNAs which reside in mRNP and translationally suppressed

fractions [10]. Thus, enhancement of protein synthesis is just one of many functions of m⁶A in mRNA. Further complicating the picture is that METTL3 itself can regulate translation independently of methylation [23], making it difficult to determine how much translation regulation following loss of METTL3 is due to m⁶A. What is clear, however, is that m⁶A has complex and diverse effects on translation regulation which is dependent on its location within a transcript, the proteins that recognize it, and the cellular environment. Intriguingly, in contrast to mammalian cells, the vast majority of cytoplasmic m⁶A-containing mRNAs in yeast are associated with ribosome fractions during early mitosis, suggesting that m⁶A may have a greater impact on translation in yeast compared to mammalian systems [86].

Going forward, understanding the proteins that bind to m⁶A to influence translation and the temporal and spatial constraints on when they interact with m⁶A will be necessary. For instance, how do highly similar proteins like the YTHDF proteins coordinate and compete for m⁶A recognition? Are their roles unique, or are there contexts in which they have redundant functions? Additionally, recent studies suggest that many non-YTH domain-containing proteins may preferentially bind to methylated RNA, some of which have been shown to influence translation [33, 34, 36, 56]. Therefore, investigating the contribution of these other m⁶A readers to m⁶A-mediated protein production will be important for a more complete understanding of the mechanisms that control m⁶A-dependent translation enhancement or inhibition.

Additionally, studies that use FTO manipulation to uncover effects of adenosine methylation on translation regulation should directly examine whether the observed effects are caused by m⁶A or m⁶A_m. This is particularly important given that immunoblot and pulldown assays using m⁶A antibodies cannot distinguish between m⁶A and m⁶A_m. While it is clear that 5'UTR m⁶A promotes translation, the contribution of m⁶A_m, which is also located in the 5'UTR, has not been explored.

Finally, the discovery that m⁶A residues promote cap-independent translation in a variety of cellular contexts has been an important addition to our understanding of stress-induced translational responses in the cell. 5'UTR m⁶A residues can recruit eIF3 to facilitate translation [36], and recent studies suggest that 5'UTR methylation may also influence ribosome scanning and translation initiation through unknown mechanisms [38]. Uncovering the factors that regulate these distinct modes of m⁶A action in 5'UTRs will be important areas of future research. In addition to during times of stress, cap-independent translation is utilized in other circumstances, such as during mitosis or apoptosis [87–89]. Thus, it will be interesting to determine whether m⁶A residues in 5'UTRs also impact translation during these conditions. As we further refine our understanding of the effects of m⁶A on translation regulation, we will undoubtedly gain new insights into the factors that control both cap-dependent and cap-independent translation in cells.

Supplementary Material

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Acknowledgements

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Highlights

- m⁶A regulates both cap-dependent and cap-independent translation in cells
- Various m⁶A reader proteins control protein production through distinct mechanisms
- m⁶A regulates the translation of select mRNAs in response to cellular stress

Figure 1. Regulation of cap-dependent translation by m⁶A.

m⁶A influences multiple aspects of cap-dependent translation. In the CDS, m⁶A residues have both positive and negative impacts on translation: while m⁶A can impair tRNA accommodation to slow translation elongation, it also relieves ribosome stalling at GAN codons. In the 3'UTR, m⁶A sites promote translation through recruitment of various reader proteins. YTHDF1 binds m⁶A residues near the proximal 3'UTR and promotes translation through interactions with eIF3, likely through an mRNA looping mechanism. YTHDF3 interacts with YTHDF1 to enhance its translation-promoting effect. Whether this YTHDF1/3-mediated translation enhancement involves interactions between YTHDF1 and eIF3 or between YTHDF1/3 and the ribosome are unknown (indicated by the question mark). The m⁶A methyltransferase, METTL3, also recognizes m⁶A sites in 3'UTRs and increases translation efficiency through eIF3. This does not require the methyltransferase activity of METTL3.

Figure 2. Mechanisms of m^6A -mediated cap-independent translation.

m^6A promotes cap-independent translation in a variety of contexts. Top: m^6A residues in the 5'UTR slow ribosome scanning to promote translation initiation at non-canonical upstream initiation sites. Middle: 5'UTRs of select mRNAs are hypermethylated after cellular stresses such as heat shock, which leads to cap-independent translation through direct binding of m^6A by eIF3. Bottom: Methylation of circRNAs promotes their translation in a YTHDF3- and eIF4G2-dependent mechanism. Potentially thousands of circRNAs contain m^6A , although the proportion of these that promote translation remains to be determined.

Figure 3. m⁶A influences cap-dependent translation in response to activation of several stress-response pathways.

Diverse cellular stresses cause global suppression of cap-dependent translation and concomitant enhancement of cap-independent translation. m⁶A is one mechanism through which cap-independent translation is achieved. (A) Several cellular stress states cause hypermethylation of select 5'UTRs. These m⁶A residues are directly recognized by eIF3, which recruits the translation machinery to promote translation of chaperone and other proteins. (B) Activation of the Integrated Stress Response leads to eIF2α phosphorylation and reduced TC availability. In parallel, removal of 5'UTR m⁶A residues disfavors

translation initiation at upstream sites and promotes protein production at downstream ORFs, such as those in *ATF4* and *GADD45G*. (C) mTORC1 inhibition reduces eIF4F availability, but m⁶A permits cap-independent translation persistence, potentially through recruitment of the translation machinery facilitated by ABCF1. Additionally, ABCF1 promotes METTL3 production, potentially contributing to a positive feedback loop in which increased METTL3 further enhances 5'UTR methylation. The factors controlling such a feedback mechanism, and whether ABCF1 directly contributes to m⁶A-mediated cap-independent translation, are not completely understood (indicated by question marks).

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