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Mechanisms of epitranscriptomic gene regulation

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Abstract

Chemical modifications on RNA can regulate fundamental biological processes. Recent efforts have illuminated the chemical diversity of post-transcriptional ("epitranscriptomic") modifications on eukaryotic mRNA, and have begun to elucidate their biological roles. In this review, we discuss our current molecular understanding of epitranscriptomic RNA modifications and their effects on gene expression. In particular, we highlight the role of modifications in mediating RNA-protein interactions, RNA structure, and RNA-RNA base pairing, and how these macromolecular interactions control biological processes in the cell.

1. Introduction

Chemical modifications on macromolecules play an important role in biological processes. These modifications not only diversify the chemical functionality afforded to proteins and nucleic acids by the basic building blocks of life (i.e. amino acids and nucleotides), but also provide a mechanism for regulating molecular function in a dynamic and reversible manner through enzyme-mediated transformations. This is perhaps no more evident than in the >150 structurally distinct post-transcriptional modifications that have been described on cellular RNA[1]. These modifications, which range from simple base and ribose methylation installed by a single enzyme to more exotic modifications requiring dedicated biosynthetic pathways[2], serve to expand the somewhat limited chemical diversity of building blocks (i.e. A, C, G, and U) available to canonical RNA polymers, and play important roles in regulating RNA folding and structure, base pairing, and RNA-protein interactions[3].

RNA modifications are most prevalent on structured, non-coding RNA[3]. The most heavily modified RNA is transfer RNA (tRNA), with on average 13 modifications per ~76mer RNA molecule[4]. Due to tRNA abundance, high modification stoichiometry, and ease of biochemical reconstitution and structural analysis, tRNA modifications are among the best understood and most extensively studied RNA post-transcriptional modifications[5]. Indeed, we now know most of the tRNA modification enzymes in bacteria and simple eukaryotes[2], and understand the involvement of many modifications in processes such as decoding, tRNA folding and structure, and recognition by tRNA-aminoacyl-synthetase enzymes[4,6,7]. In contrast, less is known about the extent of chemical modification on messenger RNA (mRNA), and how modifications on internal mRNA sequences (in contrast to the better

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CONFLICT OF INTEREST

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studied modifiations at the 5' and 3' ends of the transcript) may function in the absence of well-defined RNA secondary and tertiary structure. Spurred by advances in next-generation RNA sequencing approaches, numerous chemical modifications on mRNA have been now identified and mapped across transcriptomes[8], including N^6-methyladenosine (m^6A), pseudouridine (Ψ), N^6,2′-O-dimethyladenosine (m^6Am), N^1-methyladenosine (m^1A), 5-methylcytidine (m^5C), 5-hydroxymethylcytidine (hm^5C), N^4-acetylcytidine (ac^4C), N^7-methylguanosine (m^7G), inosine (I), and 2′-O-methylated nucleotides (Fig. 1). The study of the role that these modifications play in mRNA behavior, gene expression and higher-order biological processes has led to the emergence of the field of RNA epigenetics or "epitranscriptomics"[8,9].

Several mechanisms have been proposed by which modifications may affect mRNA properties. A number of modification sites have been mapped to the coding region of mRNA transcripts where they could function to alter tRNA selection rules and/or modulate the efficiency of ribosomal translation[10]. Such a mechanism has been well established for inosine, which base pairs preferentially with C and can efficiently recode translation[11]. This property has also enabled mapping of inosine sites by RNA mutational analysis[12]. In contrast, dramatic effects on base pairing have not been observed with other epitranscriptomic modifications, such as m^6A and m^5C, which both behave similarly to the corresponding unmodified base with regards to their capacity to form canonical Watson-Crick pairs[13]. Further, modification sites can be found in intronic regions[14] or within the 5'-UTR[15], or 3'-UTR[16,17]. Therefore, additional functional mechanisms such as perturbation of local RNA structure or recruitment of modification-specific RNA-binding proteins ("readers") are likely to be important. In this review, we describe the mechanisms underlying the function of the best-characterized epitranscriptomic mRNA modifications, focusing on their role in modulating RNA-protein and RNA-RNA interactions.

2. Protein readers of epitranscriptomic modifications

2.1 N^6-methyladenosine

N^6-methyladenosine (m^6A) was one of the first epitranscriptomic modifications to be mapped transcriptome-wide[16,17]. It is also the most abundant internal modification that has so far been identified in mammalian mRNA, with ~ 10,000 annotated sites in humans, and estimated to consitute 0.5% of all adenosine bases[18-21]. Most m^6A sites reside within the consensus motif RRACH (R = A, G; H = A, C, U)[22] and are installed by a methyltransferase complex containing the methyltransferase like 3 (METTL3), methyltransferase like 14 (METTL14), and other protein adaptors including WTAP, KIAA1429/VIRMA, RBM15/15B, CBLL1/HAKAI, and Zc3h13.[21,23-29] N^6-methyladenosine can also be reverted to adenosine via oxidative demethylation by fat mass and obesity-associated protein (FTO) and alkylation repair homolog protein 5 (ALKBH5), which are Fe(II)-dependent and 2-oxoglutarate-dependent dioxygenases[19,20]. The action of these enzymes indicates that m^6A is reversible and dynamic in nature, however the physiologically relevant substrates of these proteins have been debated. FTO and ALKBH5 are primarily nuclear, although the localization of FTO does vary in different cell lines[30], suggesting that demethylation of m^6A

mRNA sites in the cytosol may be a rare phenomenon[31]. Interestingly, FTO has been shown to demethylate the related modification m^6A_m[32] and to act upon snRNA[33].

Studies of m^6A have shown that this modification can regulate many different aspects of RNA biology including mRNA stability, splicing, nuclear export and translation[8]. Most of these roles are thought to be mediated by recognition of m^6A-modified transcripts by m^6A-specific RNA-binding proteins, or reader proteins (Fig. 2). The first m^6A readers to be identified were the YT521-B homology (YTH) proteins YTHDF2 and YTHDF3[17]. The defining feature of these proteins is the YTH domain, which binds specifically to m^6A-modified RNA sequences, and which is also found in 3 other human proteins – YTHDF1, YTHDC1, and YTHDC2. Structural and biochemical studies of YTH domains together with m^6A-modified substrates have indicated that discrimination of the methylated base is facilitated by an aromatic pocket lined with 3 Trp residues[34-37]. Interestingly, despite making contacts with residues surrounding the m^6A site, YTH domains (particularly those from YTHDF1-3) do not show strong sequence bias and appear to function as general m^6A-binding modules, although studies have shown a preference for the predominant m^6A-containing GGACU consensus sequence[38-41]. In addition to YTH domain proteins, several diverse RNA-binding proteins have been identified as m^6A readers through affinity proteomics and crosslinking and immunoprecipitation (CLIP) approaches. These include eukaryotic translation initiation factor 3 (eIF3), ELAV-like protein 1 (ELAVL1), leucine-rich PPR motif-containing protein (LRPPRC), fragile X mental retardation protein 1 (FMR1), insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs), hnRNP proteins and proline rich coiled-coil 2A (Prrc2a) (Fig. 2)[15,42-45]. While most of these proteins possess established RNA-binding domains (e.g. KH domain, RRM domain), in the absence of detailed structural and biochemical data the specificity of m^6A binding and the mechanism by which these proteins recognize the methylated base are largely unknown. Nevertheless, the existence of m^6A readers containing multiple distinct RNA binding motifs suggests multiple possible solutions to m^6A recognition.

The identification and characterization of m^6A readers has paved the way for understanding the role of m^6A modifications in diverse biological processes. A number of studies have established that m^6A-modified mRNAs show accelerated turnover and enhanced translation[38-41]. These effects, which have been shown to play important roles in modulating gene expression during early development, differentiation, immune response, stress response, and disease progression[46-58], appear to be primarily mediated by the YTH-domain proteins. YTHDF2 was the first YTH-domain protein to be characterized in a biological context, and promotes RNA decay through multiple mechanisms including localization of m^6A-modified mRNAs to processing bodies (sites of RNA decay in the cytoplasm)[41] and recruitment of deadenylase[59] and endonuclease enzymes[60]. YTHDF2 has a modular architecture whereby its N-terminal domain is responsible for interactions with effector proteins and its C-terminal YTH domain selectively recognizes m^6A-modified mRNAs. YTHDF1, which shares 67% sequence similarity with YTHDF2, does not appear to play a role in RNA degradation but instead enhances translation by interacting with translation initiation factors[40]. Interestingly, while YTHDF1 and YTHDF2 appear to affect different molecular processes, they share many target transcripts in common, as revealed by CLIP analysis,[39] and possess similar *in vitro* affinity and specificity for m^6A-modified

sequences[61]. YTHDF3, which has been less well studied than YTHDF1 and YTHDF2, appears to have dual roles in promoting degradation and translation in concert with YTHDF1 and YTHDF2.[38,39] While the aforementioned work has established a model whereby YTHDF proteins have distinct biological roles, 2 recent studies from Jaffrey[62] and Hanna[63] have called this model into question by proposing that YTHDF1/2/3 proteins are functionally redundant and all mediate mRNA decay but have no role in mRNA translation. The recent work, which includes both independent experiments and re-analysis of previously published data, stands in sharp contrast to previous studies, particularly regarding the finding that YTHDF1/3 do not promote mRNA translation. Notably, YTHDF1 has been shown to promote translation in multiple different biological contexts[40,49,64] and its N-terminal domain has been used as a translational activator in artificial tethering assays[40,65], suggesting that it can regulate translation in certain systems. Further investigation will be needed in order to fully eludicate the role of YTHDF proteins in diverse biological processes.

The biological effects of m^6A are not limited to mature mRNAs in the cytoplasm and can also regulate pre-mRNA processes occurring in the nucleus. YTHDC1 is a nuclear YTH-domain protein that reads m^6A and regulates the splicing of modified transcripts through interactions with the splicing factor SRSF3.[66] This m^6A-YTHDC1-SRSF3 interaction promotes exon inclusion; without m^6A or YTHDC1, other splicing factors cause differentially spliced transcripts. In Drosophila, YT521-B (the homolog of YTHDC1) recognizes m^6A in the intron of Sxl and mediates the female specific alternative splicing of Sxl.[46-48] In mouse, YTHDC1 is essential for embryo viability and germline development; the loss of YTHDC1 in oocytes leads to extensive altered splicing, polyadenylation, and 3′ UTR length of mRNA.[67] YTHDC1 can also promote nuclear export of m^6A-methylated mRNAs by recruiting the binding of nuclear export adapter protein SRSF3 and export receptor NXF1.[68] Similarly, FMRP can promote the nuclear export of m^6A-modified transcripts[69], together with its roles in translation and stability regulation in the cytoplasm. Finally, m^6A reading events in the nucleus can extend to non-coding RNAs including lncRNA[25] and miRNA[70], as well as the recent demonstration that chromosome-associated regulatory RNAs (carRNA) are controlled by m^6A modification and YTHDC1 recognition[71].

Increasing evidence suggests that other m^6A readers can compete with YTH-domain proteins to regulate RNA transcript behavior. FMRP was identified as a selective m^6A reader that stabilizes its target transcripts and inhibits translation[42,44,72], acting in an opposing manner to YTHDF1-3[44,72]. While FMRP does bind to m6A-modified RNAs *in vitro*[42,44], its specificity for methylated transcripts is modest, and its cellular effects may be due to overlap of FMRP binding sites with m6A sites, rather than direct m6A recognition[72]. Additionally, IGF2BPs bind to GG(m^6A)C motifs in mRNAs and increase stability and translation of modified transcripts.[45] In mouse neural cells, Prcc2a was identified to bind m^6A-modified mRNAs and stabilize its target transcripts[43]. These findings indicate that distinct m^6A readers may compete with one another for RNA binding and that the biological consequences of m^6A modification may consist of the cumulative sum of such protein-RNA interactions distributed over a set of transcripts. Alternatively, particular m^6A-protein interactions may dominate in certain biological contexts based on the expression of

individual protein factors or other regulatory features. Further studies are needed to elucidate the network of m^6A-reader interactions and how these individual binding events specify the ultimate fate of m^6A-containing mRNA.

2.2 N^1-methyladenosine (m^1A)

N^1-methyladenosinse (m^1A), which is methylated at the N^1 position of adenine instead of the N^6 position, is a well-established and abundant post-transcriptional modification on tRNA and rRNA[73]. While the vast majority of cellular m^1A is found on tRNA, it has recently also been detected in mRNA[74,75]. In contrast to most known epitranscriptomic modifications, m^1A is positively charged and is incompatible with canonical Watson-Crick pairing[74,75]. Further, it is chemically labile and can undergo Dimroth rearrangement to m^6A under alkaline conditions[76]. The occurrence of m^1A on mRNA has been highly controversial to date, with estimates from different studies ranging from thousands to only a handful of sites[74,75,77,78]. There are likely many reasons for this, including the chemical lability of the modification, its low abundance on mRNA, and the differences in mapping strategies and bioinformatic analysis platforms utilized by different groups[74,75,77-79]. Further, m^1A could show context-dependent distribution on mRNA. It is unlikely that m^1A modifications can be tolerated within the coding sequence given the dramatic effect of this modification on Watson-Crick pairing. Indeed, studies have mapped most mRNA m^1A modifications to the 5'-UTR of transcripts[74,75,77,78]. Writers and erasers of m^1A on mRNA appear to be the same enzymes that modulate modification levels on tRNA, including the TRMT6/61A methyltransferase complex[77,78] and the AlkB family demethylases ALKBH1 and ALKBH3[30,74,75,80]. Further studies will be necessary to comprehensively map m^1A modifications in diverse biological contexts and characterize relevant writer and eraser proteins acting upon mRNA.

While m^1A sequence maps have generated considerable controversy in the field[77,78], multiple studies of m^1A readers have converged on the finding that this mark is read by YTH-domain proteins, particularly the YTHDF1-3 family (Fig. 2)[81-83]. While this is somewhat surprising, given the preference of these proteins for m^6A-modified sequences, it is conceivable that the hydrophobic m^6A-binding pocket can also accommodate the proximal N^1-methyl group in a similar fashion. Recognition of m^1A by YTHDF proteins appears to have similar functional consequences as binding of these proteins to m^6A. A study from our group showed that in living cells, YTHDF2 knockdown increased the abundance of m^1A-modified mRNAs, whereas the knockdown of the m^1A eraser ALKBH3 resulted in global destabilization of m^1A-containing transcripts[83]. Further building the case for YTH-protein reading of m^1A, a recent report showed that YTHDF3 recognizes m^1A-modified IGF1R mRNA to promote its degradation, inhibiting migration, invasion, and proliferation of trophoblast.[82] Taken together, these findings demonstrate a role for m^1A in transcript turnover, likely in part through recruitment of YTHDF proteins[81-83]. Whether there are functional differences between m^1A and m^6A-mediated recruitment of YTH proteins remains to be seen, as well as whether unique m^1A reader proteins exist.

2.3 5-methylcytidine (m^5C)

5-methylcytidine (m^5C) is an abundant epitranscriptomic modification on eukaryotic mRNA. While less prevalent than m^6A, thousands of m^5C mRNA sites have been mapped and show enrichment in coding sequences and near the translation initiation site[84-87]. NSUN2, a tRNA m^5C methyltransferase, appears to be the major mRNA m^5C methyltransferase, but it is likely that other m^5C mRNA methyltransferases exist[84-88]. In contrast to the adenosine modifications discussed above, which occur on the Watson-Crick face, m^5C involves methylation on the opposite edge of the nucleobase at the C5 position, and therefore might be expected to have a less dramatic effect on RNA structure or on processes involving base pairing (i.e. decoding). This makes recognition by RNA-binding reader proteins a particularly appealing model for understanding its functional role, particularly, in the larger context of well-characterized reader proteins that bind to 5-methylcytosine bases on DNA such as methyl-binding domain (MBD) proteins[89]. Currently, our understanding of the effect of m^5C modification on mRNA transcripts is poorly developed, but recent studies identifying m^5C reader proteins have helped the field coalesce. One challenge in the study of m^5C and associated readers has been the lack of a clear consensus sequence for m^5C deposition sites, although recent work has begun to implicate sequence motifs within mRNA that resemble the well-established m^5C sites on tRNA[84,85]. Nevertheless, researchers seeking to identity reader proteins have pushed forward with m^5C-modified oligonucleotides mimicking individual high-confidence m^5C sites within the transcriptome. In 2017, Yang and co-workers identified Aly/REF export factor (ALYREF) as a direct reader of m^5C (Fig. 2)[87]. Using a combination of genetic knockdown and RNA imaging, they proposed that ALYREF could promote the nuclear export of m^5C-modified mRNA. HNRPNK has also been implicated as a nuclear m^5C reader that can regulate chromatin state through its interactions with chromatin-associated RNAs and various transcriptional regulators[90]. While ALYREF and HNRPNK may function as nuclear m^5C readers, several groups have characterized the transcriptional and translational regulator YBX1 as a cytoplasmic m^5C reader protein through its cold shock domain (CSD) (Fig. 2). Similar to other epigenetic and epitranscriptomic readers that bind to methylated epitopes, YBX1 recognizes the C5-methyl group through a hydrophobic pocket containing aromatic residues[91,92]. YBX1 stabilizes its mRNA clients by interacting with accessory proteins (ELAVL1,HUR in humans, Pabpc1a in zebrafish)[91,92], supporting a role for m^5C in mRNA stability.

5-methylcytosine on RNA can also be oxidized to 5-hydroxymethylcytosine and 5-formylcytosine[93-95], although the prevalence of these oxidative modifications on mRNA has not been firmly established. These modifications may serve as an added layer of regulation on interactions between m^5C and its reader proteins, which may be unable to bind in their presence. Just as well, dedicated readers for these 5-methylcytidine derivative may exist, as has been shown for the analogous modifications on DNA[96].

3. Anti-readers of epitranscriptomic modifications

While most studies of epigenetic and epitranscriptomic readers have focused on proteins that bind specifically to the modified epitope, an alternative mechanism by which RNA

modifications can affect biological function is by abrogating interactions between RNA binding proteins and their preferred unmodified sequence motifs, We refer to such proteins that bind preferentially to unmodified sequences over their modified counterparts as “anti-readers”. In principle, many RNA-binding proteins that bind sequence specifically could exhibit this property since modified bases may be incompatible with recognition in the same way that a sequence mutation can modulate the interaction affinity[97]. Further, since modified bases constitute a small fraction of the transcriptome, it is likely that the majority of RNA-binding proteins have evolved to recognize unmodified sequence motifs. In practice, however, only a small number of proteins have been identified as anti-readers of methylated bases, which may speak to the promiscuity of their RNA binding sites with regards to nucleobase structure.

Using affinity proteomics and biochemical characterization with recombinant protein, our group and Vermeulen, Carrell, He and co-workers identified the stress granule protein G3BP1 as an anti-reader of m^{6}A (Fig. 3)[42,44]. G3BP1 binds 10-fold worse to an oligonucleotide containing the major m^{6}A motif GGm^{6}ACU, as compared to the corresponding unmodified sequence lacking m^{6}A methylation[42]. Cellular studies of G3BP1-mRNA interactions showed that a substantial amount of G3BP1 RNA binding sites overlap with known m^{6}A sites, and demonstrated that G3BP1 promotes mRNA stability[44]. Together, these findings suggest that m^{6}A could act as a molecular switch that could regulate the recruitment of proteins with opposing functions, such as YTHDF2 to promote RNA decay or G3BP1 to maintain RNA stability. Additionally, G3BP1 is a critical stress granule protein, and other stress granule proteins USP10 and CAPRIN1 were identified as potential anti-readers of m^{6}A.[42,44] This hints that m^{6}A could be involved in the trafficking of mRNAs to stress granule by abrogating interactions with G3BP1 – which would be predicted to exclude certain mRNAs from recrtuiment to these structures. Interestingly, a number of studies have suggested that m^{6}A-modified mRNAs and m^{6}A readers are enriched in stress granules.[98,99] Therefore, more comprehensive investigation is needed to dissect the potential role of m^{6}A in stress granule assembly.

Similar to m^{6}A, m^{5}C can also repel certain RNA-binding proteins. Frye and co-workers demonstrated that NSUN2-mediated m^{5}C methylation at cytosine 69 of vault RNA 1.1 (VTRNA1.1) inhibits binding of the splicing factor SRSF2, leading to different splicing patterns in the presence or absence of m^{5}C (Fig. 3)[100]. Together, NSUN2 and SRSF2 orchestrates the maturation of the vault RNAs and produce distinct small-vault RNAs[100]. Further, m^{5}C methylation has been shown to abrogate binding of the chromatin-modifying PRC2 complex to the XIST and HOTAIR lncRNAs, which both contain multiple m^{5}C sites[101]. Now that m^{5}C sites on mRNA are becoming better characterized, it is likely that such anti-reader mechanisms function in this context as well. It is also plausible that other RNA modifications could act in a similar way to inhibit RNA recognition by RNA-binding proteins. For instance, m^{1}A introduces a positive charge and alters the hydrogen bonding pattern of the Watson-Crick face. This could serve to repel RNA-binding proteins via charge-charge repulsion and/or altered hydrogen bonding. The identification and characterization of anti-readers of RNA modifications will be important to further our understanding of epitranscriptomic RNA regulation.

4. Effects on RNA structure and ribosomal translation

In addition to the ability of epitranscriptomic modifications to modulate interactions between mRNAs and RNA-binding proteins, several complementary mechanisms have been proposed to provide a biochemical framework underlying the role of these modifications in gene expression regulation. These include effects on RNA secondary structure, which may directly or indirectly (through modulation of RNA-binding protein affinity) affect downstream processes as well as modulation of ribosomal decoding and translational elongation efficiency. While the effects of m^6A on mRNA have been attributed primarily to its recruitment of reader proteins, model studies of m^6A-modified oligonucleotides[102] as well as transcriptome-wide structure mapping[103] have shown that m^6A can also change local RNA structure. These effects appear to be mediated primarily by disrupted Watson-Crick base pairing as well as through enhanced stacking interactions that serve to stabilize unpaired, single-stranded regions[102]. While such structural modulation can have widespread effects on mRNA behavior, it has been specifically implicated in the regulation of protein binding through what has been termed an "m^6A switch" (Fig. 4A)[14]. Pan *et al.* proposed that these switches are ubiquitous throughout the transcriptome and can affect the binding of HNRNPC/G proteins on pre-mRNAs to control splicing and mRNA abundance[14,104]. In contrast to the YTH-domain proteins, HNRNPC does not appear to directly recognize the m^6A-modified base. Instead, methylation in the stem of an RNA hairpin structure can weaken the stem-loop structure and expose the single-stranded HNRNPC binding site, thereby promoting the recruitment of this protein in the presence of m^6A (Fig 4A)[14]. Further, a similar m^6A-switch like mechanism has been proposed for the binding of the nuclear m^6A reader HNRNPA2B1, which mediates methylation-dependent primary microRNA processing and affects alternative splicing[70,105].

While m^6A is an epitranscriptomic modification primarily found on mRNA, other epitranscriptomic modifications that are being actively investigated are predominantly found on structured RNAs such as tRNA and rRNA, in addition to their presence on mRNA. Pseudouridine (Ψ) has long been studied in non-coding RNA where it is known as the "fifth ribonucleotide"[106] and has been shown to stabilize RNA structure through the formation of a water-bridged hydrogen bond between the ΨNH1 proton and adjacent phosphate oxygen atoms as well as through increased stacking interactions[107,108]. High-throughput chemical mapping of Ψ sites, taking advantage of its unique reactivity with carbodiimide reagents, has revealed widespread pseudouridylation on mRNA[109-111]. Interestingly, a number of these sites are found within sequences predicted to adopt a characteristic "bulged stem loop" structural motif[112]. Currently, it is not known whether Ψ is involved in the stabilization and regulation of such structures in mRNA or whether this motif is simply a requirement for deposition of Ψ by pseudouridine synthase (PUS) enzymes. Similarly, 5-methylcytosine (m^5C), an epitranscriptomic mRNA modification, is primarily found in the variable loop (VL) of tRNA where it protects these molecules from stress-induced endonucleolytic cleavage[113]. Recent work suggests that m^5C modifications are predominantly found in stem-loop forming structures[84,85]. Studies have indicated that m^5C does not have a large effect on base pairing interactions but can induce structural distortions in the helical backbone[114].

Another mechanism by which mRNA modifications can affect gene expression is through the regulation of ribosomal translation[115]. In most cases, the mechanism underlying this process appears to involve alteration in the efficiency or specificity of tRNA selection, either resulting in modulation of the rate of ribosomal elongation or the incorporation of non-cognate amino acids opposite modified codons. This is analogous to the well-established role in decoding of different RNA modifications in the anticodon of tRNA (particularly at the "wobble position")[5]. The most striking example is the recoding of translation that occurs upon A:I editing, resulting in high efficiency incorporation of non-cognate amino acids opposite inosine-containing codons due to its propensity to base pair with C instead of T[11]. While RNA A:I editing has been studied extensively, our understanding of the effects of more recently discovered epitranscriptomic modifications on translational fidelity and efficiency is still in its infancy. Several studies relying on both *in vitro* and cellular experimentation have begun to reveal insights into this process, as we describe below.

Early studies by Yu and Ramakrishnan into the effects of artificial pseudouridylation on translation led to the remarkable finding that pseudouridine could convert stop codons into sense codons [116,117]. Structural analysis of the 30S ribosomal subunit in complex with the anticodon stem-loop of tRNA-Ser bound to the ΨAG codon indicated that the Ψ modification at the 1st position made possible normally forbidden purine-purine base pairs at the 2nd and 3rd codon positions, in which the purine bases in the codon adopt a *syn* conformation, rather than the more commonly observed *anti* conformation. Interestingly, while Ψ induced non-canonical pairing at adjacent base pairs, the Ψ residue itself is found in a standard Ψ-A base pair, and the underlying role of Ψ in inducing non-canonical pairing and recoding is not fully understood. While this work put forth an attractive hypothesis for the biological function of Ψ on mRNA, in the past decade since the initial report of Ψ recoding, few studies have been conducted to further expand on this finding. Further, epitranscriptomic Ψ maps have not shown enrichment of Ψ at stop codons, suggesting that such a mechanism may not be relevant to the majority of endogenous Ψ sites. More recently, using a reconstituted bacterial translation system and studies in human cells, Koutmou and co-workers have explored the effect of Ψ within coding sequences[118]. Their study demonstrates that Ψ increases the rate of amino acid substitutions (i.e. miscoding) and reduces the rate of translation elongation (Fig 4C). While these findings do connect with the prior work on Ψ recoding[116,117], the observed effects are more modest than previously claimed. In part, this may be due to sequence or structural-context dependent effects of Ψ in mRNA. Further work will be needed to comprehensively elucidate the role of this abundant epitranscriptomic mark in diverse biological systems.

The effects of m^6A on ribosomal translation elongation demonstrate how modifications can function in different manners depending upon sequence and structural context. In reconstituted prokaryotic *in vitro* translation systems, m^6A impedes translational elongation by perturbing tRNA selection (Fig. 4B)[119]. Consistent with this, Qian and co-workers found that m^6A modifications *in vivo* correlate with ribosomal pausing[120]. Somewhat paradoxically, however, removal of these modifications by methyltransferase depletion results in further decreased translational efficiency, suggesting a positive effect of m^6A in promoting translation. To reconcile these somewhat contradictory findings, the authors suggest a model whereby m^6A functions to resolve RNA secondary structures (which would

otherwise impede ribosomal progression) through the action of the YTHDC2, a YTH-domain protein containing an RNA helicase domain (Fig. 4B)[120]. In contrast, m^6A residues found in unstructured regions of mRNA coding sequences likely have a net negative effect on translation[120]. As a further context-dependent function, m^6A in the 5'-UTR has been proposed to promote translation by mediating cap-independent translation through the direct recruitment of eIF3[15], although detailed structural and biochemical information on this interaction is lacking.

In addition to the modifications mentioned above, several other epitranscriptomic marks have been associated with translational regulation, including m^5C and N^4-acetylcytidine (ac^4C). Since manipulation of individual endogenous RNA modification sites *in vivo* is still an unsolved challenge, insights into the effects of these modifications have been primarily generated through *in vitro* translation assays using site-specifically modified synthetic mRNAs[121], or through cellular transfection of fully modified mRNA templates generated through *in vitro* transcription with modified NTP building blocks. Measurements using these artificial mRNAs can then be integrated with cellular/organismal assays that correlate translational phenotypes with annotated modification sites. Using these approaches, the presence of m^5C in the CDS has been associated with lower translation rate, though m^5C modifications on 5' and 3' UTRs had negligible correlation with translation.[84,122] The mechanistic basis of these effects are unclear, although it may be related to direct interactions of the modified base with the ribosome or modulation of RNA structure[114,115]. In contrast, ac^4C, which is one of the most recent epitranscriptomic modifications identified on mRNA, has been found to promote translation (Fig. 4D)[123]. It has been proposed that ac^4C modification found at the wobble sites in the CDS stabilize anticodon-codon interactions enabling efficient tRNA discrimination and decoding efficiency[123]. Such a mechanism is analogous to the known role of ac^4C in bacteria, where it is present in the anticodon of tRNA, and promotes proper pairing[124].

5. CONCLUSION

Epitranscriptomic modifications on mRNA have emerged as a new modality for gene expression regulation. Led by studies of m^6A, modifications have been shown to regulate diverse aspects of mRNA biology including splicing, nuclear export, stability, translation, and more[14,15,38-42,44,45,66,68,69,81-83,87,91,92,104,105,118-120,123]. Importantly, in several contexts, RNA modification-associated effects on gene expression have been shown to have functional consequences in higher order biological processes including development, cancer, immune activation, and learning/memory[46-57,91,92]. In this review, we have described our current understanding of the major molecular mechanisms underlying the role of epitranscriptomic RNA modifications including the recruitment of reader proteins, effects on intramolecular RNA structure, and modulation of codon-anticodon pairing interactions during ribosomal decoding. While these studies have focused on only a small number of known mRNA modifications, they have laid a conceptual framework for understanding the function of nucleobase modifications in the context of mRNA.

Moving forward, as additional modifications are identified, and we delve deeper into the biology of known modifications, there are several gaps that the field should work towards

addressing. First and foremost, we lack a general strategy for mapping modification sites transcriptome wide. Current approaches rely heavily on antibody-based detection, which is known to have inherent limitations with regards to specificity and generality, and can only be applied to one modification per experiment. Further, information regarding modification stoichiometry is difficult to ascertain using antibody-based enrichment approaches. While RNA bisulfite sequencing can provide stoichiometry information it is plagued by false positives due to RNA structure-mediated non-conversion, and is primarily used for m^5C mapping. Promising developments in single-molecule direct RNA sequencing[125] may ultimately lead to improvement in this area and have already been applied to m^6A with some success[126,127], although it is still too early to conclude whether such approaches will be applicable to the majority of RNA modifications. Given the importance of reliable modification maps for generating biological hypotheses concerning the function of modifications, this is an important step towards a comprehensive understanding of RNA modification biology. Second, as additional proteins are characterized as RNA modification readers, generating a systems level understanding of how different modification-reader interactions are regulated and interplay with one another in diverse biological contexts will be critical to understand functional consequences. Work towards this end has already provided insight into YTH-domain protein interactions with m^6A[128], but similar analyses need to be performed for other RNA modification reader proteins. Finally, as we make progress in uncovering transcriptome-wide RNA modification maps for diverse modifications, identifying the functional consequence of any individual site is still a major undertaking. Robust approaches for reconstituting and manipulating endogenous modifications sites in a specific fashion would be enabling for such studies.

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DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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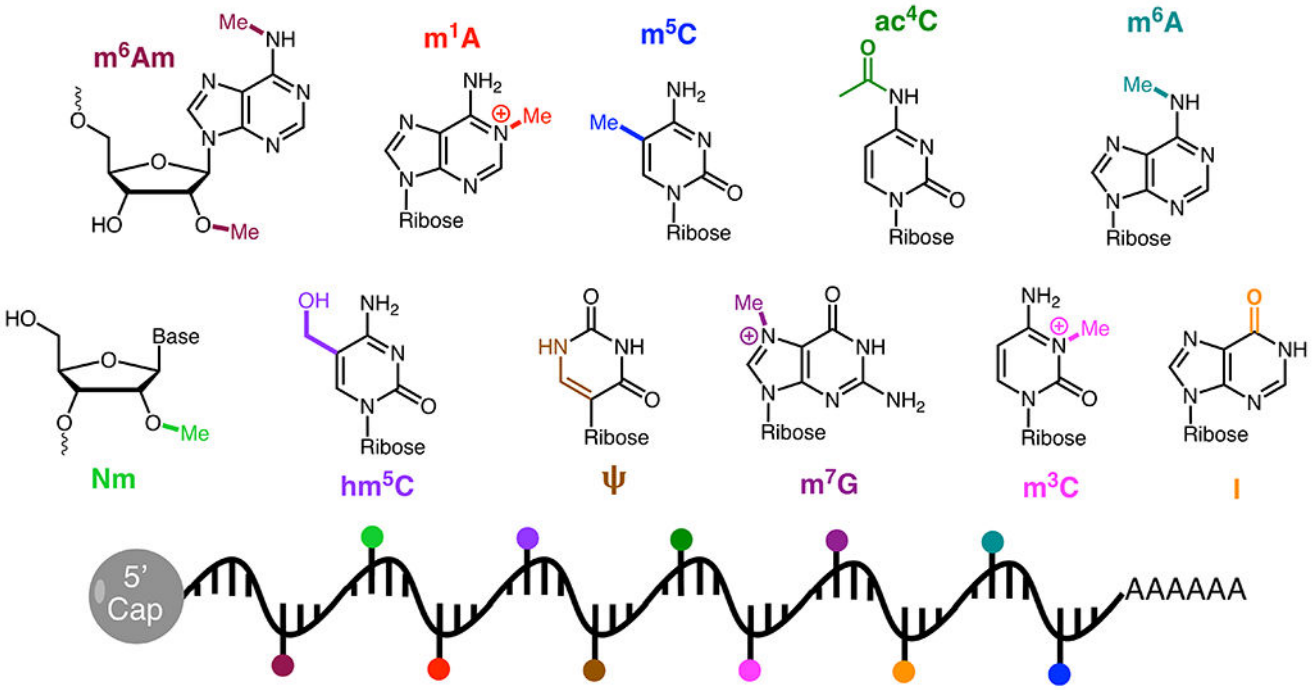

Figure 1.
Chemical structures of known epitranscriptomic mRNA modifications.

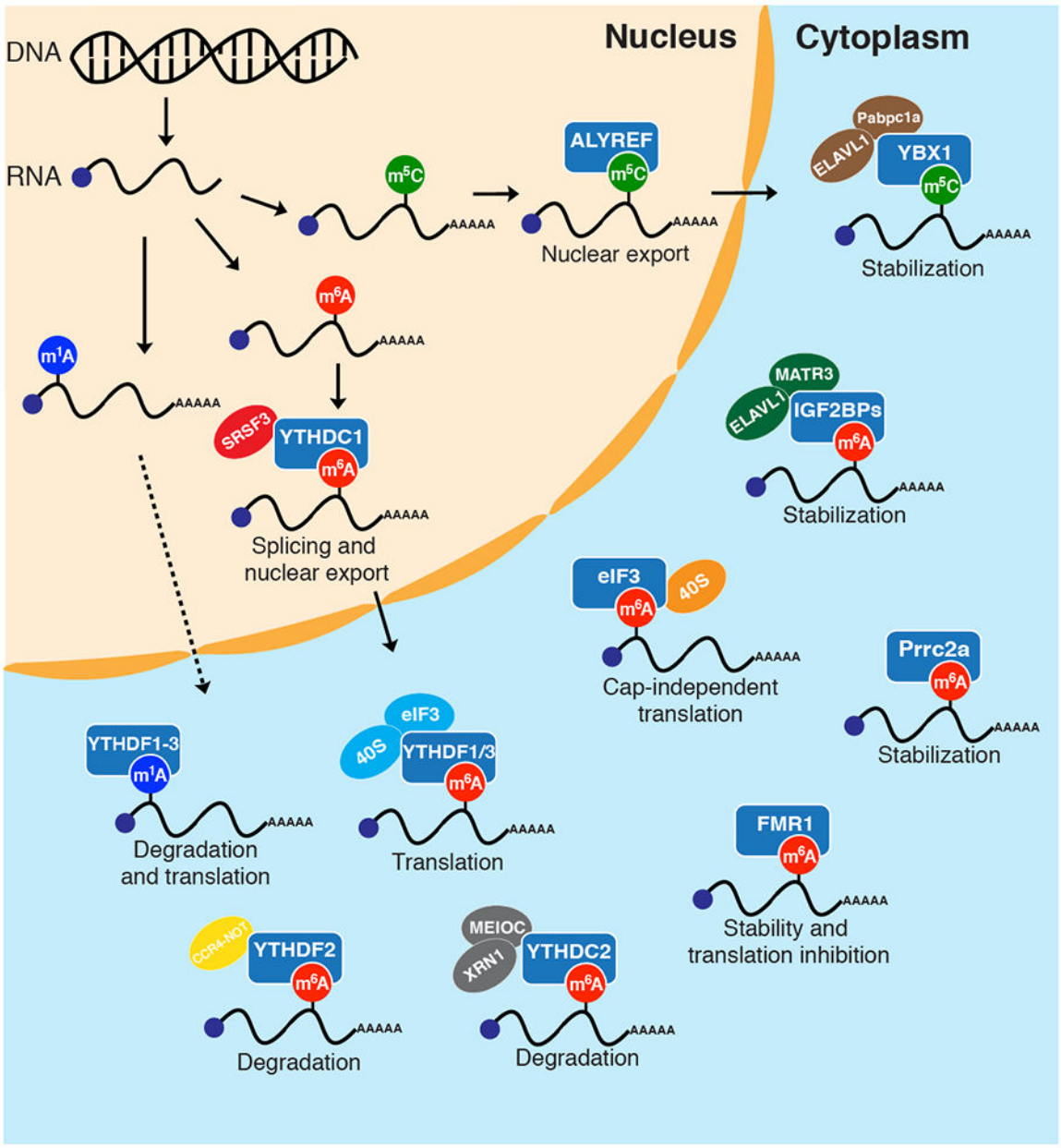

Figure 2.

Protein readers of epitranscriptomic modifications. Different reader proteins bind to m^6A, m^5C, and m^1A and regulate cellular fate of modified RNAs.

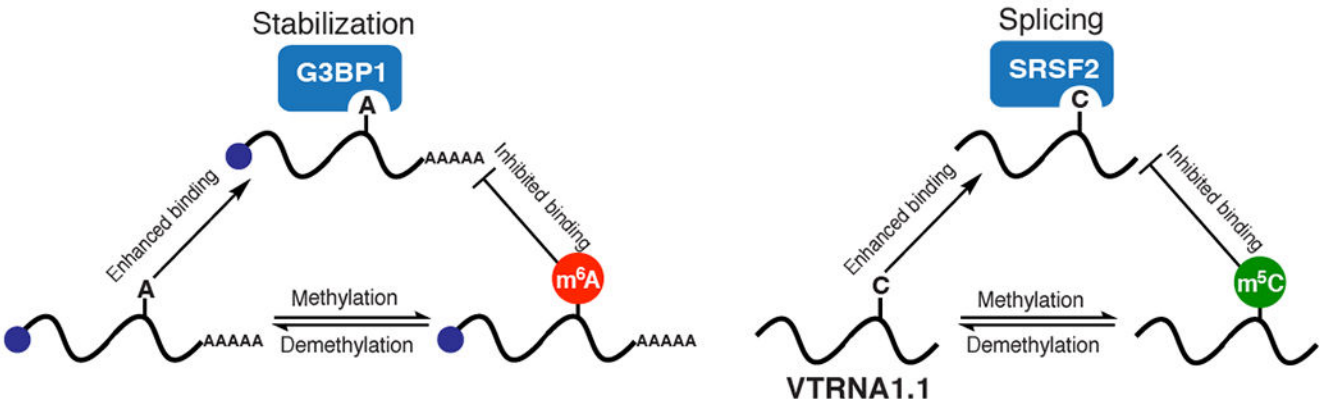

Figure 3.

Anti-readers of m^6A and m^5C. RNA-binding of G3BP1 and SRSF2 is significantly reduced with m^6A- and m^5C-modified RNAs, respectively.

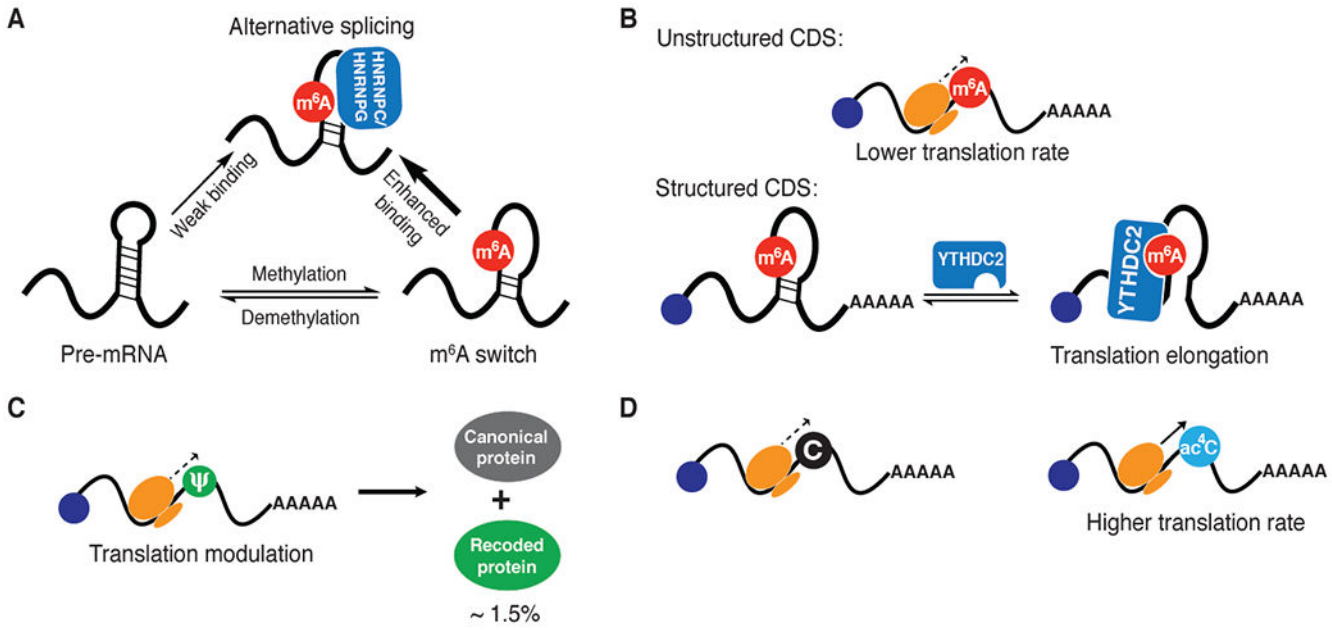

Figure 4.

Structural and translational effects of RNA modifications. A) m^6A-switch: m^6A destabilizes RNA duplex and exposes the opposite strand that is a binding target for HNRNPC/G. B) m^6A in CDS can impede translation but can recruit YTHDC2 to increase translational rate in structured regions. C) Pseudouridine can recode mRNAs during translation. D) N^4-acetylcytidine in CDS increases translation elongation.