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Dynamic RNA modifications in gene expression regulation

Ian A. Roundtree[1,2], **Molly E. Evans**[1], **Tao Pan**[1,*], and **Chuan He**[1,3,4,*]

[1]Department of Biochemistry and Molecular Biology, and Institute for Biophysical Dynamics, The University of Chicago, 929 East 57th Street, Chicago, IL 60637, USA

[2]Medical Scientist Training Program, The University of Chicago, 924 East 57th Street, Chicago, IL 60637, USA

[3]Department of Chemistry, The University of Chicago, 929 East 57th Street, Chicago, IL 60637, USA

[4]Howard Hughes Medical Institute, The University of Chicago, 929 East 57th Street, Chicago, IL 60637, USA

Abstract

Over one hundred types of chemical modifications have been identified in cellular RNAs. While the 5' cap modification and the poly(A) tail of eukaryotic messenger RNA play key roles in regulation, internal modifications are gaining attention for their roles in mRNA metabolism. The most abundant internal modification is N^6-methyladenosine (m^6A), and identification of proteins that install, recognize, and remove this and other marks have revealed roles for mRNA modification in nearly every aspect of the mRNA lifecycle, as well as in various cellular, developmental, and disease processes. Abundant noncoding RNAs such as transfer RNAs, ribosomal RNAs and spliceosomal RNAs are also heavily modified and depend on the modifications for their biogenesis and function. Our understanding of the biological contributions of these different chemical modifications is beginning to take shape, but it's clear that in both coding and noncoding RNA, dynamic modifications represent a new layer of control of genetic information.

Introduction

Modifications to RNA species have been well documented for over 50 years. In addition to canonical A, C, G, and U residues, modified nucleotides were discovered in abundant cellular RNAs as early as 1960 (Cohn, 1960). Sequencing of the first biological RNA in 1965, the alanine transfer RNA from yeast (Holley et al., 1965), identified 10 modifications including pseudouridine (Ψ). mRNA transcripts are known to contain modifications such as a 5' cap, which contributes to transcript stability, pre-mRNA splicing, polyadenylation, mRNA export, and translation initiation. The poly(A) tail at the 3' end facilitates nuclear

*Correspondence: chuanhe@uchicago.edu (C.H.), taopan@uchicago.edu (T.P.).

export, translation initiation and recycling, and promotes mRNA stability, largely through the association of the poly(A)-binding protein family.

Shortly after the discovery of the cap and tail modifications, internal modifications on mRNA were identified, including the most abundant internal modification of mRNA and long-noncoding RNA (lncRNA), N^6-methyladenosine (m^6A) (Adams and Coy, 1975; Desrosiers et al., 1974; Dubin and Taylor, 1975; Perry et al., 1975). m^6A was found to accelerate pre-mRNA processing and mRNA transport in mammalian cells (Camper et al., 1984; Finkel and Groner, 1983), and is essential for mammals. These observations suggested previously unrecognized regulatory roles of this mRNA modification that may impact various cellular processes. Analogous to the diverse chemical marks on histone tails, recent studies also reveal diverse internal modifications within eukaryotic mRNA, including additional methylations of adenosine to form N^1-methyladenosine (m^1A) and N^6,2'-O-dimethyladenosine (m^6Am), as well as cytosine methylation to 5-methylcytosine and its oxidation product 5-hydroxymethylcytosine (hm^5C) (Figure 1).

tRNAs contain the largest number of modifications with the widest chemical diversity. Eukaryotic tRNAs contain on average 13 modifications per molecule ranging from base isomerization, base and ribose methylations to elaborate addition of ring structures. tRNA modifications contribute to the efficiency and fidelity of decoding, as well as folding, cellular stability, and localization. Human rRNA contains >210 modifications including 2'-*O*-methyls, pseudouridines, and base methylations. Ribosomal RNAs present a striking example of how chemical modifications support functions as without internal pseudouridines and 2'-*O*-methylated sugars, rRNA biogenesis is blocked. Human small nuclear RNA (snRNA) contains > 50 modifications including 2'-*O*-methyls, pseudouridines, and base methylations. Some of these modifications are known to be important in the RNA splicing reaction.

In this review we summarize the chemical modifications of coding and noncoding RNA with a focus on introducing the underlying regulatory mechanisms and their biological consequences. Modifications on the 5' cap and 3' poly(A) tail of mRNA have been extensively reviewed elsewhere and will not be discussed.

Revealing Internal mRNA Modifications – The 'Epitranscriptome'

Recent advances in studying RNA modifications have benefited tremendously from improved methods for detection with both analytical chemistry and high-throughput sequencing. Though we aim to provide a conceptual overview of the methods upon which recent progress in the field is based, readers can refer to a recent comprehensive review of techniques in studying RNA modifications (Helm and Motorin, 2017).

Adenosine Methylations

We've known for some time about the abundance of site-specific internal modifications. For example, using P^{32}-labeled cellular RNA and thin layer chromatography, Lavi et al. estimated the abundance of m^6A in poly(A) selected species from both nuclear and cytoplasmic compartments to be about one per 700–800 nucleotides. Non-polyadenylated,

non-ribosomal RNA was also found to contain significant amounts of the internal methylation, with m^6A occurring every 1,800–3,000 nucleotides (Lavi et al., 1977). Digestion of mRNAs with RNases revealed that the modification is largely confined within a G(m^6A)C (~70%) or A(m^6A)C (~30%) sequence, suggesting that the deposition is selective among mRNA sequences (Wei and Moss, 1977; Wei et al., 1976), and that only a portion of consensus sequence motifs bear detectable methylation.

N^6-methyladenosine (m^6A)—m^6 A is chemically stable; however, due to the low cellular abundance of mRNA, methods to determine the precise modification sites and the modification fractions at these sites hindered biological studies for decades. Recently, two advances have fueled investigations into the function of internal mRNA modifications. First was the identification of an enzyme, fat-mass and obesity-associated protein (FTO) that catalyzes the oxidative demethylation of m^6A in nuclear RNA (Jia et al., 2011), providing evidence that reversible RNA modifications serve regulatory roles (He, 2010). A second m^6A demethylase of the same family, Alkbh5, affects mouse fertility and spermatogenesis (Zheng et al., 2013).

The second advance came with the use of high-throughput sequencing that provided transcriptome-wide maps of modification sites in both mRNA and long noncoding RNAs (lncRNA) at ~200 nucleotide resolution (Dominissini et al., 2012; Meyer et al., 2012), offering the first view of the m^6A 'epitranscriptome', and revealing distributions of m^6A mainly in the coding and 3' untranslated regions with a significant enrichment just upstream of the stop codon. Cross-linking approaches have since increased the resolution of this m^6A map, allowing for near single-base resolution determination of methylation sites in mRNA and noncoding RNAs (Chen et al., 2015; Linder et al., 2015). Attempts to determine modification fraction have been made by using a ligation-based method termed SCARLET, which provides single-base resolution of candidate m^6A sites as well as a quantitative modification fraction, albeit in a low-throughput manner (Liu et al., 2013), and an m^6A-selective reverse transcriptase from *Thermus thermophilus* for high-throughput, base resolution quantification of m^6A modification status (Harcourt et al., 2013). Although m^6A accounts for 0.2–0.6% of all adenosines in mammalian mRNA, attempts at quantifying modification fractions transcriptomes-wide have yielded semi-quantitative information at the cost of resolution (Molinie et al., 2016), highlighting a continued need for method development in m^6A-sequencing technology.

N^1-methyladenosine (m^1A)—Unlike m^6A, methylation at the N^1 position of adenosine occurs on the Watson-Crick interface and generates a positively charged base. Although m^1A modification is less abundant than m^6A in both human and mouse tissues, this modification carries a positive charge and thus can dramatically alter protein-RNA interactions and RNA secondary structures through electrostatic effects. m^1A maps uniquely to positions near the translation start site and first splice site in coding transcripts, and correlates with upregulation of translation in general (Dominissini et al., 2016; Li et al., 2016). This modification can be removed by ALKBH3, and is responsive to various types of cellular stress (Dominissini et al., 2016; Li et al., 2016). m^1A may affect translation through

facilitating non-canonical binding of the exon-exon junction complex at 5' UTRs devoid of 5' proximal introns (Cenik et al., 2017).

m^1A blocks Watson-Crick base pairing and thus most reverse transcription (RT). Partial read-through of m^1A could create mutations that mark the modification sites; however, mutations could be severely under-represented during library preparation due to abortive reverse transcription at or adjacent to the m^1A site or poor amplification of short ligation products (Hauenschild et al., 2015). Modifications with similar properties, as well as methods that install biochemical handles in the Watson-Crick interface will face similar challenges.

Other adenosine modifications—Adjacent to the 5' cap, the second base in many mRNAs can be 2'-O-methylated. A portion of these bases also bear m^6A methylation to form N^6,2'-O-dimethyladenosine (m^6Am), deposited by a yet unidentified methyltransferase. This modification (Schibler and Perry, 1977) was confirmed from transcriptome-wide m^6A-seq (Linder et al., 2015) and has a low overall abundance. The m^6A portion of this modified nucleoside was known to be a substrate of FTO (Fu, 2012), with a recent study highlighting that m^6Am stabilizes mRNA by preventing DCP2-mediated decapping and microRNA-mediated mRNA degradation (Mauer et al., 2017).

Additional modifications of adenosine, such as further base methylation of m^6A to N^6,N^6-dimethyladenosine ($m^{6,6}A$), or the deposition of bigger, more elaborate chemical groups have been identified in eukaryotic RNA but have yet to be characterized within coding transcripts (Machnicka et al., 2013).

Cytosine Modifications

5-methylcytosine (m^5C)

Like m^6A, methylation at the 5 position of cytosine in mRNA was discovered more than 40 years ago (Desrosiers et al., 1974; Dubin and Taylor, 1975), though in significantly lesser abundance. Capitalizing on bisulfite methodology utilized for 5-methylcytosine identification in DNA (Schaefer et al., 2009), m^5C sites were mapped in human mRNA and lncRNA species. The distribution of these modified bases appears to favor untranslated regions, particularly the binding sites for Argonaute proteins I–IV (Squires et al., 2012). The tRNA m^5C methyltransferase NSUN2 has been identified as the methyltransferase responsible for m^5C methylation in several mRNAs and lncRNAs (Hussain et al., 2013; Khoddami and Cairns, 2013)). m^5C is recognized by the mRNA export adaptor protein ALYREF, suggesting a role in nuclear export of m^5C-containing transcripts (Yang et al., 2017). Of note, this study reports a strong bias for m^5C sites 100 nucleotides beyond translation initiation sites, unlike the relatively even distribution previously observed using similar sequencing technologies.

5-hydroxymethylcytosine (hm^5C)

As with 5-methylcytosine in DNA, m^5C in RNA can be oxidized by Tet-family enzymes to 5-hydroxymethylcytosine (hm^5C) (Fu et al., 2014). In *Drosophila melanogaster*, which lacks DNA hydroxymethylation, hm^5C is present in greater than 1,500 mRNAs. hMeRIP-seq

revealed the presence of this modification largely in exonic and intronic regions of protein-coding transcripts, dependent on the presence of the only known *Drosophila* Tet ortholog (Delatte et al., 2016). The abundance and potential roles of hm^5C in mammals will be interesting to monitor in the future, as will potential precursors and derivatives of this modification in relevant RNA species.

Isomerization of Uridine

Pseudouridine (Ψ), isomerization of the uridine base, is the most common modification in cellular RNA and an abundant component of rRNA and tRNA (Cohn, 1960). However, its presence in mRNA was largely ignored until recently when PseudoU-seq established the presence of Ψ in greater than 200 human and yeast mRNAs (Carlile et al., 2014). Using a similar protocol, Ψ-seq, identified >300 Ψ-modified mRNAs in human and an additional 41 in yeast (Schwartz et al., 2014a). The Ψ/U ratio was quantified to be ~0.2–0.7% in mammalian cell lines and tissues, and a chemical labeling and pull-down method (CeU-seq) identified over 2,000 sites in human mRNA, suggesting this modification is far more prevalent than previously appreciated (Li et al., 2015). Ψ sites are dynamically regulated by the Pus family enzymes, which catalyze the isomerization in response to stress conditions such as heat shock. Ψ is known to affect the secondary structure of RNA, and the function of Ψ in altering stop codon read-through may also be biologically relevant (Fernandez et al., 2013; Karijolich and Yu, 2011).

Ribose Modification

In addition to base modifications, methylation of the ribose 2’ hydroxyl exists at the second and third nucleotide in many mRNAs and as an abundant modification in tRNA and rRNA (Schibler and Perry, 1977). The 2’ hydroxyl group frequently participates in contacts forming higher order RNA structures; its methylation could have profound impact on RNA-protein interactions and RNA secondary structures. 2’-*O*-methylation (2’-OMe or Nm) sites in abundant RNA species such as rRNA have been mapped taking advantage of its higher resistance to alkaline-mediated hydrolysis compared to unmodified nucleosides (Marchand et al., 2016).

A new approach (Nm-seq) based on ribose sensitivity to periodate cleavage allows for enrichment of 2’-OMe in low abundant RNA species such as mRNAs, providing a highly sensitive single-base method for detection. Nm-seq uncovered over 7,000 potential methylation sites in human mRNA revealing a consensus sequence and enrichment within three amino acid codons and extensive ribose methylation in all four bases. The same approach has been used to map these sites in rRNA (Dai et al., 2017; Zhu et al., 2017).

In order to understand the critical roles of posttranscriptional modifications in mRNA, we must identify the abundance, sequence context, and cellular dynamics of these distinct entities. While recent advances have made progress on this front, methods to provide highly sensitive, quantitative, single-base resolution of RNA modifications remain a crucial goal for the field.

Dynamic control of the ‘Epitranscriptome’ by Methyltransferases and Demethylases

The epitranscriptome presents a dynamic layer of information, shaped largely by the enzymatic activities of methyltransferases or pseudoU synthases and demethylases. The deposition of m^6A in mammalian mRNA is catalyzed by a heterodimer of METTL3 and METTL14, and regulated by the association of a subunit protein WTAP (Liu et al., 2014; Wang et al., 2014b). Recent crystal structures of the METTL3/METTL14 complex have revealed that only METTL3 appears to possess a functional active site within the complex while METTL14 may largely function as a structural scaffold (Sledz and Jinek, 2016; Wang et al., 2016a, 2016b). Analysis of the binding sites of this complex suggests that methylation occurs preferentially in coding sequences and 3’UTRs. Additionally, a significant portion of binding sites fall within intronic sequences, suggesting that deposition of m^6A in mRNA takes place co-transcriptionally, perhaps mostly within nuclear speckles (Liu et al., 2014; Ping et al., 2014). Proteomic analysis of these core methyltransferase components by IP-MS revealed an additional factor, KIAA1429, which is critical in mediating full activity of the complex (Schwartz et al., 2014b), and is necessary for proper establishment of the cellular m^6A profile (Figure 2A).

m^6A methylation can be removed passively from the transcriptome via degradation of modified RNA or via active demethylation by m^6A demethylases FTO or ALKBH5, both belonging to the AlkB family of dioxygenases known to demethylate N-methylated nucleic acids (Figure 2A). These proteins oxidatively demethylate m^6A *in vitro*, and contribute to m^6A levels in cellular mRNA (Jia et al., 2011; Zheng et al., 2013). FTO has also been shown to demethylate m^6Am, adjacent to the mRNA 5’ cap (Fu, 2012; Mauer et al., 2017) as well as internal m^6A, impacting mRNA metabolism. The m^6A demethylation activity of ALKBH5 critically impacts mRNA nuclear export and spermatogenesis, and both enzymes participate in the various disease mechanisms related to cancer (Cui et al., 2017; Li et al., 2017b; Zhang et al., 2016a, 2017). A recent study discovered that the METTL3-METTL14 complex is rapidly recruited to the DNA damage site created by UV irradiation where it mediates local RNA m^6A methylation. This process facilitates recruitment of DNA damage repair polymerase κ, and can be reversed by FTO within a short period of time (Xiang et al., 2017). These studies are building a framework for understanding how methyltransferases and demethylases actively control methylation dynamics in homeostatic and acute responses to cellular stimuli.

m^1A deposition in tRNA is largely dependent on secondary structure (Takuma et al., 2015). m^1A in mRNA occurs in structured, GC-rich regions and tRNA methyltransferases with moonlighting activity in mRNA may be responsible for this modification in coding transcripts (Dominissini et al., 2016; Ozanick et al., 2005). Methyltransferases for both 2’-*O*-methylations at the 5’-cap have been identified (Belanger et al., 2010; Langberg and Moss, 1981), although no enzyme for internal ribose modifications nor an active demethylation process has been reported. The methyltransferase responsible for further methylation of A_m to m^6A_m adjacent to the 5’ cap is also unknown.

The tRNA methyltransferase NSUN2 has been identified as a mediator of m^5C in nearly 300 mRNAs by miCLIP (Hussain et al., 2013), though fewer coding transcripts were identified as targets using other methods (Khoddami and Cairns, 2013; Squires et al., 2012). m^5C can be oxidized in *Drosophila* by a conserved Tet ortholog CG43444 (dTet) to generate hm^5C in mRNA (Delatte et al., 2016). The potential of hm^5C for further oxidation and eventual decarboxylation provides m^5C a plausible route to reversibility, although evidence for this has yet to be reported.

Notably, RNA modification enzymes commonly exhibit substrate promiscuity. For example, Ψ in mRNA can be attributed in part to several pseudouridine synthase (PUS) enzymes conserved across eukaryotic genomes (Carlile et al., 2014; Li et al., 2015; Schwartz et al., 2014a), and previously described as tRNA and rRNA modifiers. Perturbations of Ψ sites in response to environmental stimuli suggest that mRNAs are indeed physiological targets of these enzymes. The installation of a carbon-carbon bond between the base and sugar upon isomerization to Ψ however, suggests that this modification is not readily reversible.

Mammalian mRNA carries additional modifications at low abundance; some of these modifications are observed in rRNA, tRNA and other non-coding RNAs, and may be byproducts of enzymes which recognize shared sequences motifs or structural features across transcripts. While certain mRNA modifications provide a fitness advantage (Ma et al., 2017), the field will continue to benefit from biochemical characterization of nucleic-acid modifying enzymes to further uncover their biological roles.

Properties of mRNA Modifications – Structure and Function

Chemical modifications in RNA affect the transcripts by altering charge, base pairing potential, secondary structure and protein-RNA interactions. These properties in turn shape the outcome of gene expression by modulating RNA processing, localization, translation, and eventual decay.

m^6A, the most common modification in mRNA, occupies an exocyclic amine which participates in Watson-Crick base pairing. Watson-Crick base pairing of m^6A with opposite U would force rotation of the carbon-nitrogen bond to display the methyl group at the *anti* conformation, which destabilizes the RNA duplex to locally unstructured transcripts (Roost et al., 2015). This effect also modulates secondary structure *in vivo* (Spitale et al., 2015), and predisposes these unstructured regions for recognition by proteins such as HNRNPC and HNRNPG (Liu et al., 2015, 2017; Zhou et al., 2016).

The m^6A modification directly recruits m^6A-specific proteins of the YTH domain family (Dominissini et al., 2012). These proteins bridge methyl-selective RNA binding with a myriad of cellular processes, and produce m^6A-dependent regulation of pre-mRNA processing, microRNA (miRNA) processing, translation initiation and mRNA decay (Figure 2B). Within the nucleus, several proteins bind precursor RNAs with selectivity for m^6A. YTHDC1 (also known at YT521-B) promotes inclusion of alternative exons via interactions with members of the splicing related SR-protein family (Xiao et al., 2016), and affects X-chromosome silencing (Patil et al., 2016). Three members of the HNRNP (Heterogeneous

Nuclear RiboNucleoProtein) family also function to regulate the processing of m^6A-modified transcripts. HNRNPA2B1, along with METTL3, co-regulates alternative splicing events as well as the generation of miRNAs from methylated precursors (Alarcon et al., 2015), while HNRNPC and HNRNPG mediate splicing outcomes on methylated transcripts by recognizing and binding to m^6A-dependent structural switches (Liu et al., 2015, 2017).

Mature mRNAs with m^6A methylation are subject to regulation in the cytoplasm by the remaining YTH family proteins with documented selectivity for m^6A. YTH Domain Family 1 (YTHDF1) associates with initiating ribosomes, delivering its target mRNAs for enhanced translation efficiency in HeLa cells (Wang et al., 2015). A second YTH family protein, YTHDF2, directly recruits the CCR4-NOT deadenylase complex and accelerates degradation of methylated transcripts (Du et al., 2016; Wang et al., 2014a). While accelerated decay globally shapes the profile of methylated mRNAs, some transcripts exhibit increased half-lives upon m^6A methylation. This suggests additional pathways for stabilization of these mRNAs, potentially through additional effector proteins (Wang et al., 2014b). Under heat shock conditions, YTHDF2 localizes to cell nuclei where it directs cap-independent translation of heat shock response transcripts (Zhou et al., 2015). Under these same conditions, the 43S preinitiation complex is recruited to 5’ UTR m^6A sites via interactions with the eIF3 subunit (Meyer et al., 2015). Both m^6A at the 5’ UTR and m^6Am near the cap appear to correlate with increased translation. YTHDF3 mediates translation along with YTHDF1 by interaction with a common set of ribosomal proteins, as well as decay of mRNA targets by associating directly with YTHDF2 (Li et al., 2017a; Shi et al., 2017), and may play additional cell-type specific roles and functions based on m^6A location within transcripts.

Aside from depositing m^6A, the methyltransferase complex may also function as a protein scaffold in RNA-processing and metabolism (Schwartz et al., 2014b; Xiao et al., 2016; Zheng et al., 2013). METTL3 enhances translation of bound RNA independent of its catalytic activity by direct recruitment of eIF3 to the translation initiation complex (Lin et al., 2016). Other components of m^6A machinery may similarly perform roles beyond their enzymatic functions by affecting nuclear organization and protein occupancy on mRNAs.

m^1A is a unique base methylation because it blocks Watson-Crick base paring and introduces a positive charge. In mRNAs, m^1A exists within highly structured 5’ UTRs, suggesting that it may function to alter predicted secondary structure (Dominissini et al., 2016; Li et al., 2016). Within loop structures, this charge may serve to stabilize interactions with the phosphate backbone of RNA. m^1A methylation in transcripts correlates with increased translation, perhaps due to accessibility or direct recruitment of initiation and elongation factors. The positive charge of this modification makes it amenable to specific protein-RNA interactions and unique RNA-RNA interactions, the biological impact of which is currently unknown.

The patterns of m^5C distribution on mRNA with respect to cis-acting regulatory motifs and miRNA/RISC binding sites suggest that this modification may be involved in post-transcriptional regulation of mRNA metabolism (Squires et al., 2012). NSUN2-medited methylation is required for the processing of noncoding vault RNAs (vtRNAs) into small

vault RNAs (svRNAs), but downstream consequences in coding transcripts have not emerged as a result of this defect (Hussain et al., 2013). Recently, ALYREF was shown to recognize m^5C in mRNA via a methyl-specific RNA-binding motif, and regulate the export of bound transcripts in an NSUN2-dependent manner (Yang et al., 2017), while hm^5C, derived from Tet-dependent oxidation of m^6C, preferentially marks mRNAs within coding regions and favors translation of *Drosophila* transcripts (Delatte et al., 2016; Fu et al., 2014).

Similar to m^6A methylation, installation of Ψ in mRNA can encode additional information. Compared to U, Ψ can coordinate an additional water molecule allowing for hydrogen bonding to the adjacent phosphate backbone, which could rigidify regions containing Ψ in duplex and single-stranded RNA. This property also enhances the base stacking of Ψ by favoring a 3'-endo conformation of ribose, further restricting the flexibility of the residue (Charette and Gray, 2000). The unique properties of Ψ allows for complete read-through when placed within nonsense codons, generating a protein product with a C-terminal extension (Fernandez et al., 2013; Karijolich and Yu, 2011). Despite these intriguing effects Ψ could exert on mRNA structure and translation, the function of this modification in mRNA in mediating biological processes has yet to be reported. However, the modification can have profound effects on rRNA as discussed below.

2'-*O*-methylation of RNA can have dramatic effects on structure and stability, as the modifications masks the hydrophilic hydroxyl that largely defines the macromolecule. 2'-OMe residues function to enhance duplex stability of RNA-RNA hybrids (Yildirim et al., 2014), and could thus promote stability and efficacy of RNA-based therapeutics. In *vivo*, 2'-OMe is typically found in unstructured CDS regions of mRNA, with half of all sites falling within 50 nucleotides of the nearest splice site, suggesting a link between structural elements and mRNA processing outcomes. Interestingly, 2'-OMe sites concentrate to six codons that encode three amino acids: glutamate, lysine, and glutamine, perhaps dependent on the RNA-binding properties of specific methyltransferase (Dai et al., 2017). This suggests that 2'-OMe in mRNA may affect translation efficiency, a concept that has been demonstrated in modified bacterial mRNA (Hoernes et al., 2016). This modification plays important roles in other RNA species.

Research has demonstrated that diversity in mRNA and noncoding modifications can tune nearly every aspect of mRNA function. m^6A, being the most abundant and well-studied mRNA modification, highlights the ability of a small chemical modification to alter fundamental properties. Unsurprisingly, these properties are closely connected, as recent work linking Pol II transcription status, m^6A deposition, and translation efficiency has shown (Slobodin et al., 2017). As work on more rare modifications continues, we will likely discover new proteins and mechanisms that amplify chemical changes into more profound biochemical and cellular consequences.

Different modes in reading RNA modifications

Cellular factors that mediate the outcomes of modified RNA messages are crucial to our understanding of the biological roles of mRNA modifications. The "reading" of a RNA modification can come in several different forms. The modification could be directly

recognized by a binding pocket as shown by the binding of m^{6}A by the YTH domain proteins (Li et al., 2014; Xu et al., 2014, 2015; Zhu et al., 2014), indirectly recognized through a structural switch (Liu et al., 2015), or by other “reading” mechanisms which also warrant consideration.

One factor neglected in almost all RNA modification studies is the solvation effect (or hydrophobic effect) (Noeske et al., 2015). Hydrophobic modifications induces solvation penalty in water; interactions of hydrophobic RNA modifications with hydrophobic protein side chain residues could reduce solvation penalty, and this contribute to the observed *in vivo* selectivity of RNA-binding proteins, such as SFSF and HNRNP family proteins, which lack obvious modification-specific binding domains (Figure 2B). Certain tRNAs tend to preferentially associate with translation machinery when modified in the anti-codon loop, in which the reduction of solvation penalty of the hydrophobic adducts may contribute to the preferential ribosome binding (Agris, 2008, 2015; Agris et al., 2007). Distinguishing properties that enable recognition by one or more of these mechanisms are still largely unknown, but may account for variable outcomes observed for modified RNAs.

m^{6}A mRNA modification in development and disease

As we learn more about relationships between RNA modification and transcript properties, we are now in a position to understand how these subtle chemical changes affect essential physiology. In mouse embryonic stem cells (mESCs), depletion of either Mettl3 or Mettl14 reduces m^{6}A methylation and increases transcript stability (Wang et al., 2014b). Loss of *Mettl3* delays turnover of self-renewal factors, preventing differentiation into downstream lineages (Batista et al., 2014). *Mettl3*$^{-/-}$ mice are not viable, and cells derived from early embryos are unable to resolve their naïve pluripotency due to extended transcript lifetime in the complete absence of methylation (Geula et al., 2015). In each case, methylation appears to be critical in embryo development and cell differentiation due to its role in governing stability of key regulatory transcripts. Together, these results suggest a most critical role of m^{6}A in marking groups of transcripts for coordinated metabolism in response to cellular signaling and/or environmental cues.

During early embryo development, the composition and utilization of the cellular transcriptome must be responsive to temporal cues. Master transcriptional factors (TFs) activate hundreds of transcripts, which in turn shape the cellular mRNA pool. RNA modifications may be selectively deposited to a group of transcripts during transcription activation by selected TFs. The modification provides an additional “identity” to these transcripts for their coordinated translation and decay, thus facilitating coordinated transcriptome utilization and switching. As such, we propose that mRNA m^{6}A methylation offers a mechanism to facilitate rapid transcriptome turnover during cell differentiation (Figure 3). To test this hypothesis, we have investigated a prototype of transcriptome switching during early embryo development in vertebrates: the maternal to zygotic transition (MZT) in zebrafish. Maternal transcripts are rapidly degraded and the transcriptome is replaced by newly synthesized zygotic mRNA during MZT (Li et al., 2013). In zebrafish a portion of maternal mRNAs are m^{6}A methylated, and rapidly cleared by Ythdf2. In the absence of Ythdf2, this clearance is delayed, preventing timely initiation of MZT resulting

in prolonged developmental delay (Zhao et al., 2017). The diverse chemical marks now known to exist in mRNA offer the possibility to simultaneously mark multiple groups of transcripts in response to different cues. For example, in *Drosophila*, methylation is not essential for viability, but is critical for sex determination and neuronal functions (Haussmann et al., 2016; Lence et al., 2016).

Methylation-dependent processes can also be controlled via active demethylation. FTO, initially identified by genome-wide association studies for diabetes predisposition, is required for proper splicing in route to adipogenesis (Frayling et al., 2007; Wu et al., 2017; Zhang et al., 2015; Zhao et al., 2014), while ALKBH5, is required for spermatogenesis in mice (Zheng et al., 2013). These transitions also impact tumorigenesis. FTO for example, demethylates genes in the 5' UTR under normal conditions, but fails to associate under heat shock conditions because of YTHDF2 binding (Zhou et al., 2015).

Defects in NUDT16-mediated RNA decapping are known to occur in leukemia (Anadon et al., 2017), as are several examples of defects within the internal epitranscriptome of mRNA. Various cancers show altered levels of either FTO or ALKBH5, and the perturbations in transcript methylation cause widespread deregulation of their targets (Cui et al., 2017; Li et al., 2017b; Zhang et al., 2016a, 2016b, 2017). In some cases, inhibition of these demethylases with small molecule inhibitors could reduce cancer progression, as with the naturally occurring oncometabolite 2-hydroxyglutarate (2HG), which may inhibit FTO and/or ALKBH5 and lead to observed benign outcomes (Brat et al., 2015; Chou et al., 2011; Eckel-Passow et al., 2015; Patel et al., 2012; Yan et al., 2009). Studies of aberrant RNA methylation in human diseases such as cancer are fast evolving and will further aid our understanding of roles of RNA modifications in human physiology.

Modifications in Abundant Noncoding RNAs

Thus far, this discussion of RNA modification has focused on messenger RNAs. Although the studies of modifications in tRNAs and rRNAs set the stage for their study in mRNAs, the functional impact of tRNA/rRNA modifications has been challenging to discern. The new wave of studies involving noncoding RNAs more broadly has renewed and heightened interest in understanding the function and dynamics of modifications in these "classical" RNAs. For example, well-studied ncRNAs such as let-7 miRNA, XIST, and MALAT1 contain numerous chemical modifications that contribute to their respective roles in cancer (Esteller and Pandolfi, 2017).

Transfer RNA (tRNA)

tRNAs are the most heavily modified RNA species with regards to both number, density and diversity. Nearly 1 in 5 nucleotides are modified in mammalian tRNA, and over 50 unique modifications have been identified in eukaryotes (Kirchner and Ignatova, 2015). The modifications range from simple thiolations and base or sugar methylations to extensive addition of sugars, amino acids, and complex organic adducts. These diverse modifications are catalyzed by a myriad of nuclear and cytoplasmic enzymes, which can act at a single site in a single tRNA or at multiple sites across several tRNA species. Complex modifications often require step-wise installation by a cascade of enzymes (e.g. wybutosine and mcm^5s^2U)

or methylation followed by deamination of the same base, a form of RNA editing (Rubio et al., 2017).

The anti-codon loop is one hot-spot of modification. Modifications of the anti-codon loop aid in translation by preventing frameshifting, expanding codon recognition, and strengthening the codon-anticodon interaction (Figure 4A). Almost every tRNA is modified either at position 34 or position 37 or both, corresponding to the wobble position and the nucleotide 3' of the anticodon. Position 34 is important for accurate and efficient decoding; modification at this first anticodon (wobble) position can restrict (e.g. mcm^5U34) or expand (e.g. cmo^5U34 or I) the decoding of a tRNA species by affecting the conformational dynamics of the anticodon stem loop or the tRNA-mRNA Watson Crick base-pairing (Agris, 2008). Position 37 is also heavily modified. Perhaps the best example of this modification is the presence of wybutosine at position 37 of phenylalanine tRNA. Extensive aromatic stacking of yW37 confers conformational stability of the loop, and prevents pairing with U33 to keep the anticodon open. This lack of flexibility can also prevent four-base anticodon pairing and is necessary to prevent frameshifting (Stuart et al., 2003). While yW37 is present in mammals, it is notably absent in Drosophila, which has led to the suggestion that certain organisms may utilize frameshifting as a mechanism to increase coding diversity (Waas et al., 2007). Similar stabilization strategies occur with other tRNA modifications, such as modifications of A37 to i^6A or t^6A to direct codon specific translation and maintain translational accuracy and efficiency.

Outside of the anticodon loop, modifications are known to influence the structure of tRNA. The clearest example is human mitochondrial $tRNA^{Lys}$. tRNAs lacking m^1A9 do not fold into the canonical cloverleaf structure; instead, these hypomodified tRNAs adopt an elongated structure due to A9-U64 base pairing that extends the acceptor stem. The methylation of A9 is sufficient to induce the cloverleaf folding by disrupting this base pairing (Helm et al., 1999). Although completely unmodified tRNA has been shown to be less stable than fully modified tRNA, the study of the effects of individual modifications on tRNA structure is not straightforward in most instances and remains to be elucidated. One recent example highlights the effect of m^5C in tRNA stability, in which $NSUN2^{-/-}$ cells accumulate 5' tRNA fragments and have an impaired translational response to cellular stress (Blanco et al., 2016). Deficiency in NSUN2 results in microcephaly and other neurological disorders in humans and mice though this tRNA modification based mechanism, in which NSUN2 deficient brains become susceptible to oxidative stress (Blanco et al., 2014).

Besides translation and structure, tRNA modifications have been shown to have a wide variety of functions in many aspects of tRNA biogenesis and function. Modifications can act as quality control in the biosynthesis of tRNAs. For example, yeast $tRNA^{iMet}$ lacking m^1A58 are targeted for degradation in the nucleus (Kadaba et al., 2006), and $tRNA^{Val(AAC)}$ lacking m^7G46 and additional modifications are targeted for rapid tRNA decay as well (Alexandrov et al., 2006). Modification at the wobble position in yeast tRNAs has also been shown to affect ribosome A-site loading (Rezgui et al., 2013). In *Leishmania*, a wobble modification can affect the subcellular localization of $tRNA^{Glu}$; tRNAs carrying mcm^5U are imported into the mitochondria whereas tRNAs carrying mcm^5s^2U are not (Kaneko et al., 2003).

Until recently, tRNA modification was thought to be stoichiometric and static. However, recent studies in yeast and human tissue culture have shown that tRNAs can be partially modified and these modifications are dynamic. The application of recently developed sequencing methods revealed that partial modification can occur at several m^1A, N^1-methylguanosine (m^1G), and N^3-methylcytidine (m^3C) sites in tRNA and among different tRNA species in human cell culture (Clark et al., 2016). In yeast, stress can modulate the overall levels of modifications such as m^5C, 2'-O-methylcytidine (Cm), and N^2,N^2-dimethylguanosine ($m^{2,2}G$) as measured by LC/MS-MS, with different stresses causing different up- or downregulated patterns of modification (Chan et al., 2010). For instance, a dynamic m^5C34 modification in $tRNA^{Leu(CAA)}$ was shown to enhance the translation of mRNAs enriched with UUG codons under oxidative stress (Chan et al., 2012).

ALKBH1 is the first tRNA demethylase identified in human cells (Liu et al., 2016). ALKBH1 is responsible for the demethylation of m^1A58 in the TΨC-loop of tRNA; it regulates translational initiation by adjusting the levels of $tRNA_i^{Met}$, as well as translational elongation through adjusting the affinity of a dozen tRNA species to the elongation factor eEF1A. The related demethylase ALKBH3 (Ougland et al., 2004; Ueda et al., 2017) has also been characterized, although the cellular targets of ALKBH3 are still under investigation. With the first identified tRNA demethylases, the field has opened to the discovery that more demodification enzymes may exist to regulate modification status in response to cellular signaling or stress in order to reprogram tRNA stability and translation.

Ribosomal RNA (rRNA)

rRNA is also extensively modified. Approximately 2% of rRNA nucleotides are modified, corresponding to over 100 sites of modification in yeast and over 200 sites in humans. While the number of modifications is large, the diversity of modifications is small; most modifications are 2'-*O*-methylation of the sugar and Ψ (~50 each in yeast, and ~100 each in humans), although around 10 base modifications have also been identified in both humans and yeast (Sharma and Lafontaine, 2015; Sloan et al., 2016).

Modifications in eukaryotic rRNA are primarily installed through nucleolar RNA-dependent mechanisms that rely on a guide RNA to direct protein enzymes to the site of modification. Box C/D small nucleolar RNAs (snoRNAs) direct the installation of 2'-*O*-methylations while Box H/ACA snoRNAs direct the installation of Ψ (Reviewed in Watkins and Bohnsack, 2012). RNA-independent mechanisms exist in yeast, but are responsible for only two modifications. Pus7 installs Ψ at position 50 in 5S RNA and Spb1 installs the 2'-O-methylation at position 2922 in 25S RNA (Sloan et al., 2016). Stand-alone proteins install base modifications throughout the ribosome. SnoRNA-guided pseudouridylations and 2'-*O*-methylations are thought to occur co-transcriptionally or in early stages of ribosome biogenesis (Kos and Tollervey, 2010; Turowski and Tollervey, 2015), while base modifications likely occur later in ribosome biogenesis. However, as most modifications are buried within the ribosome, the modifications must occur before significant folding of the rRNA and the maturation of the ribosome (Figure 4B).

Modifications are not randomly distributed throughout rRNA. Instead, the modifications cluster around functional sites in the rRNA, including the decoding site and the peptidyl

transfer center (PTC), suggesting their functional relevance (Decatur and Fournier, 2002). Functional studies of these modifications in yeast have shown that deletion of a single modification rarely has an effect on cell growth or phenotype, although some exceptions exist. Instead, preventing the formation of two or more modifications within a cluster is typically required to observe a measurable phenotype. Cumulatively, rRNA modifications have been shown to affect cell growth and drug sensitivity as well as ribosome biogenesis, abundance, structure, and activity (Reviewed in Sloan et al., 2016).

Certain Ψ and 2'-*O*-methylation modifications have been shown to occur at substoichiometric levels; that is, specific sites within rRNA are only partially modified. In yeast, over 10% of sites are modified less than 85% (Taoka et al., 2016) while in human cells, about one-third of 2'-O-methylations are substoichiometric (Krogh et al., 2016). In one case, the cause of a fractional 2'-*O*-methylation appears to be the abundance of the snoRNA responsible for its installation (Buchhaupt et al., 2014); however, for most modifications, the cause of partial modification has not yet been determined. Fractional modifications contribute to ribosome heterogeneity, and ribosomes with different modification status could have distinct functions and serve to translate a subset of mRNAs as potentially "specialized" ribosomes, which may depend on rRNA modification status. This could be useful in response to stress; a cell stress could impact rRNA modifications and therefore function of the ribosome. Indeed, two yeast rRNA Ψ residues have been shown to be induced by post-diauxic growth (Carlile et al., 2014) and heat-shock (Schwartz et al., 2014a). While the exact function of these inducible Ψ bases has not been determined, these observations support the idea that rRNA modifications are dynamic and could serve to alter ribosome function.

Despite the existence of partial and inducible modifications, the likelihood that demodification enzymes exist to alter rRNA modification fraction from mature ribosomes seems unlikely under normal growth conditions. As noted above, most of the modifications are buried within the ribosome and would not be easily accessible to a demodification enzyme. It is certainly possible that rRNA modification levels could be dynamically modulated, but any changes in the modification level of rRNA would likely have to occur before ribosome assembly is complete. Changes in the installation machinery or a demodification enzyme that acts early in ribosome biogenesis could serve to modulate rRNA modification levels (and potentially rRNA function). Potential demodification may also be useful to mark damaged ribosomes under cellular stress to facilitate their degradation.

Small nuclear RNA (snRNA)

Finally, spliceosomal RNAs (snRNAs) are also extensively modified. Like rRNA, the predominant modifications in mammalian snRNA are Ψ and 2'-*O*-methylations in addition to a few base modifications (Massenet and Branlant, 1999; Reddy and Busch, 1988). The number of modifications per snRNA varies, with U2 having the highest number of modifications (the human U2 has over 20 ψ, 2'-*O*-methylations, and a single base methylation, m^6A). Additionally, snRNA components of the minor spliceosome also contain ψ and 2'-*O*-methylations, though much fewer than the major snRNAs (Karijolich and Yu, 2010). Installation of ψ and 2'-*O*-methylations has only been shown to occur in an RNA-

dependent manner in humans. The modification enzymes are identical to those responsible for rRNA modification, and rely on common structural components found in the two RNA species. However, as modification typically occurs in Cajal bodies, these snRNAs are referred to as Small Cajal body-specific RNAs or scaRNAs (reviewed in Meier, 2016). In yeast, both RNA-independent and RNA-dependent mechanisms of modification exist to install Ψ.

Similar to rRNA modifications, the modifications cluster to functional regions of the snRNA, especially in base-pairing regions and around the nucleotides responsible for branch-site recognition (Reddy and Busch, 1988). As U2 has the most RNA modifications, the functions of these modifications have been studied in depth. A few 2-'*O*-methylations at the 5' end of U2 were shown to be individually required for spliceosome assembly while Ψ at the 5' end exhibit a cumulative positive effect on assembly (Dönmez et al., 2004). Pseudouridines at the branch point pairing region in U2 have also been shown to affect the structure around the branch point adenosine in mRNA (Lin and Kielkopf, 2008).

So far, pseudouridylation of human snRNA has not shown to be inducible, but two inducible Ψs have been identified in yeast U2 snRNA. These modifications are not present under normal growth conditions; however, nutrient stress or heat shock can induce installation of these modifications (Wu et al., 2010). Ψ93 is installed by a snoRNA-dependent mechanism and is only present under nutritional stress. Ψ56 is installed in an RNA-independent mechanism by the Pus7 enzyme and is present under both nutritional and heat stress. The RNA sequence contexts of these inducible modifications resemble, but do not perfectly match constitutive Pus7 sites. Mutation to match the conserved consensus sequence results in stoichiometric modification, suggesting that imperfection in selectivity may hold a key to regulatory function. However, the exact molecular mechanism for these induced pseudouridylations has not been determined.

Partial modifications of snRNA have not been reported as quantitative information on 2'-*O*-methylations and Ψ in snRNA has yet to be determined. Additionally, no demodification enzymes have been reported to act on snRNA. Despite this, the dynamics of snRNP assembly and disassembly do not rule out the possibility that some of these modifications can be removed in response to a change in cellular conditions.

Concluding remarks

The diverse landscape of RNA modification has revealed itself as a critical entity for post-transcriptional gene regulation. Reversible mRNA methylation offers a tunable mechanism to achieve regulatory and cellular complexity beyond what can be achieved by primary sequence or secondary structure alone. Most directly, mRNA methylation in the form of N^6-methyladenosine provides a selectivity mark that is decoded by evolutionarily conserved proteins of the YTH family as well as other RNA-binding proteins through different reading mechanisms.

Fundamental mechanisms that take advantage of m^6A methylation promote incorporation of methylated transcripts into canonical pathways for RNA metabolism. These pathways

accelerate processing, translation initiation, and eventual decay of m^{6}A-modified mRNA during cell differentiation. The result of this selection is increased protein production within limited time frames – an outcome perfectly suited, and indeed required, for developmental and differentiation processes. Functions of the m^{6}A could be diverse in different cell types and different biological processes. Components of the m^{6}A regulatory network could be mutated or deregulated in certain types of cancer, and the mechanisms that underline these pathologies are current areas of investigation and potential areas of intervention.

Mechanisms for achieving selectivity in m^{6}A-dependent gene regulation remain a mystery. Components of the methyltransferase targeting system likely exist to limit methylation to a defined, reproducible subset of consensus sequences in response to various signals. Similarly, binding modes of effector proteins must select appropriate RNA substrates and protein binding partners to exert their required function within the cell, and demethylases may execute removal of methylations of target transcripts within specific time windows and cellular locations (Figure 5). Modes of regulation in these areas are likely context-specific and are important areas of future exploration. Additional mRNA modifications further define the ‘epitranscriptome’, increasing potential modes for selectivity in post-transcriptional regulation, and many uncharacterized modifications are still under investigation.

Modifications are particularly abundant in functional RNA species such as tRNA and rRNA. In tRNA, modification is necessary for biogenesis, function, and stability, and perturbations to these modifications have been linked to numerous human diseases, including cancer, neurological disorders, and mitochondrial-linked disorders (reviewed in Torres et al., 2014). Since some tRNA modifications have been shown to be partial, reversible, and responsive to stresses, these modifications and their dynamic properties will be highly relevant in biological regulation. ALKBH1, the first tRNA demethylase discovered, leads to developmental defects in neurons in mouse models, suggesting currently unappreciated regulatory functions. Furthermore, the roles of modification in tRNA fragments, which have been shown to be widely used for regulating gene expression, have yet to be explored (Kirchner and Ignatova, 2015). rRNA modification is similarly ubiquitous, and defects in modification have been linked to dyskeratosis congenita, a human disease that affects pseudouridylation. Sites of partial modification are known to exist in rRNA. Inducible rRNA and snRNA sites have also been identified.

In summary, although RNA modifications have been known for decades, recent advances have revealed functions in nearly every class of cellular RNAs. In messenger RNA, modification can affect protein production by modulating splicing, translation, and decay rates through various mechanisms. Functional RNAs such as tRNA and rRNA often require modification for proper biogenesis and stability, but also utilize base alterations to tune structure and function. Modifications of all RNA species have been linked to various diseases, the full pathology of which has yet to be elucidated. Nonetheless, future studies will not only advance our understanding of this layer of biological regulation, but further our understanding of human health and disease.

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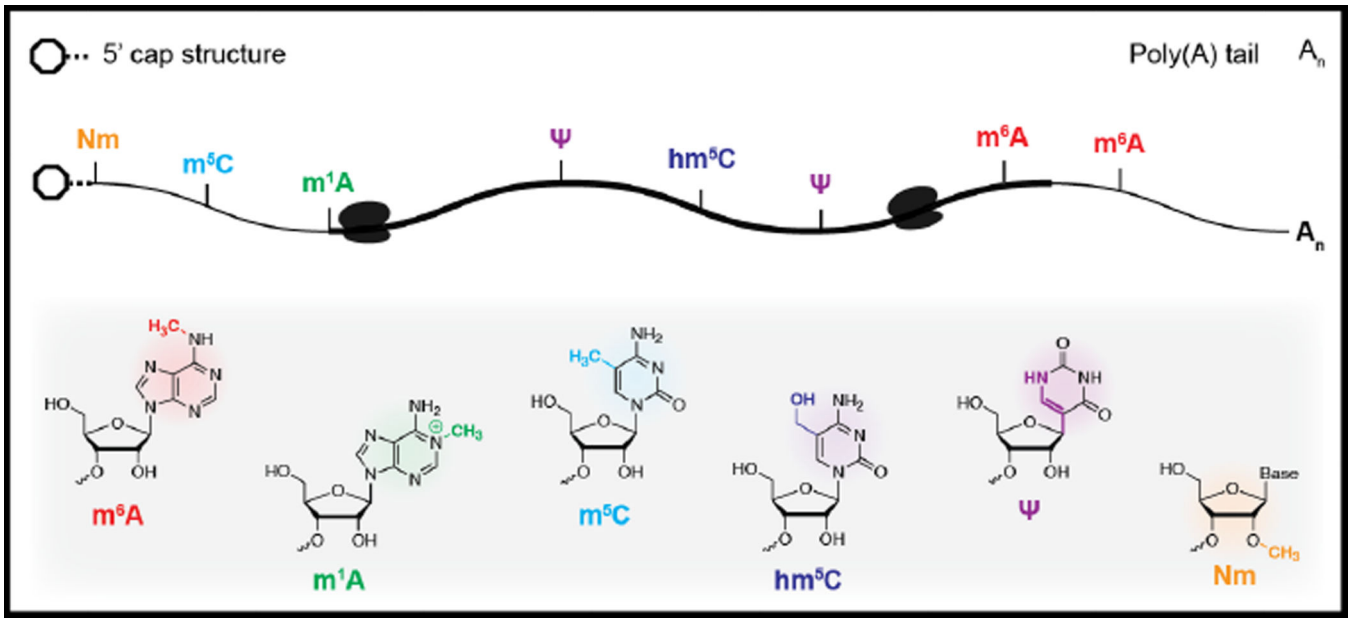

Figure 1. Chemical modifications in eukaryotic messenger RNA

A schematic representation of common chemical modifications in eukaryotic mRNA transcripts. Several of these modifications map uniquely to the mRNA cap structure, 5' or 3' untranslated regions, or the coding region (bold) of the transcript.

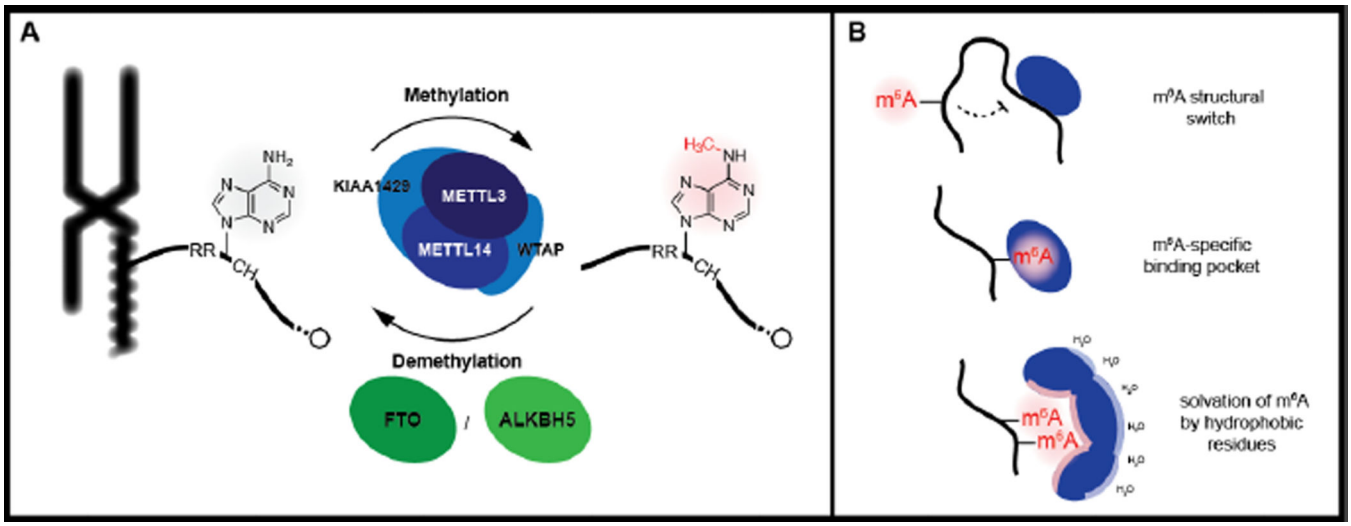

Figure 2. Active m^{6}A methylation, demethylation, and downstream consequences for protein-RNA interactions

(A) m^{6}A is installed co-transcriptionally by a complex consisting of METTL3, METTL14, WTAP, and KIAA1429. Each of these components binds mRNA and is required for complete methylation, but only METTL3 contributes to the catalytic activity of the complex. (B) m^{6}A methylation affects protein-RNA interactions through multiple mechanisms. Methylation can perturb the secondary structure of mRNA, exposing or masking potential RNA-binding motifs (top). Selective m^{6}A-binding proteins exhibit increased affinity for methylated mRNAs, and in turn incorporate these transcripts into various steps of mRNA metabolism (middle). Methylation itself introduces hydrophobic moieties. In the case of m^{6}A, association with hydrophobic amino acid side chains or low complexity regions of proteins may assist in solvation of the modified base (bottom).

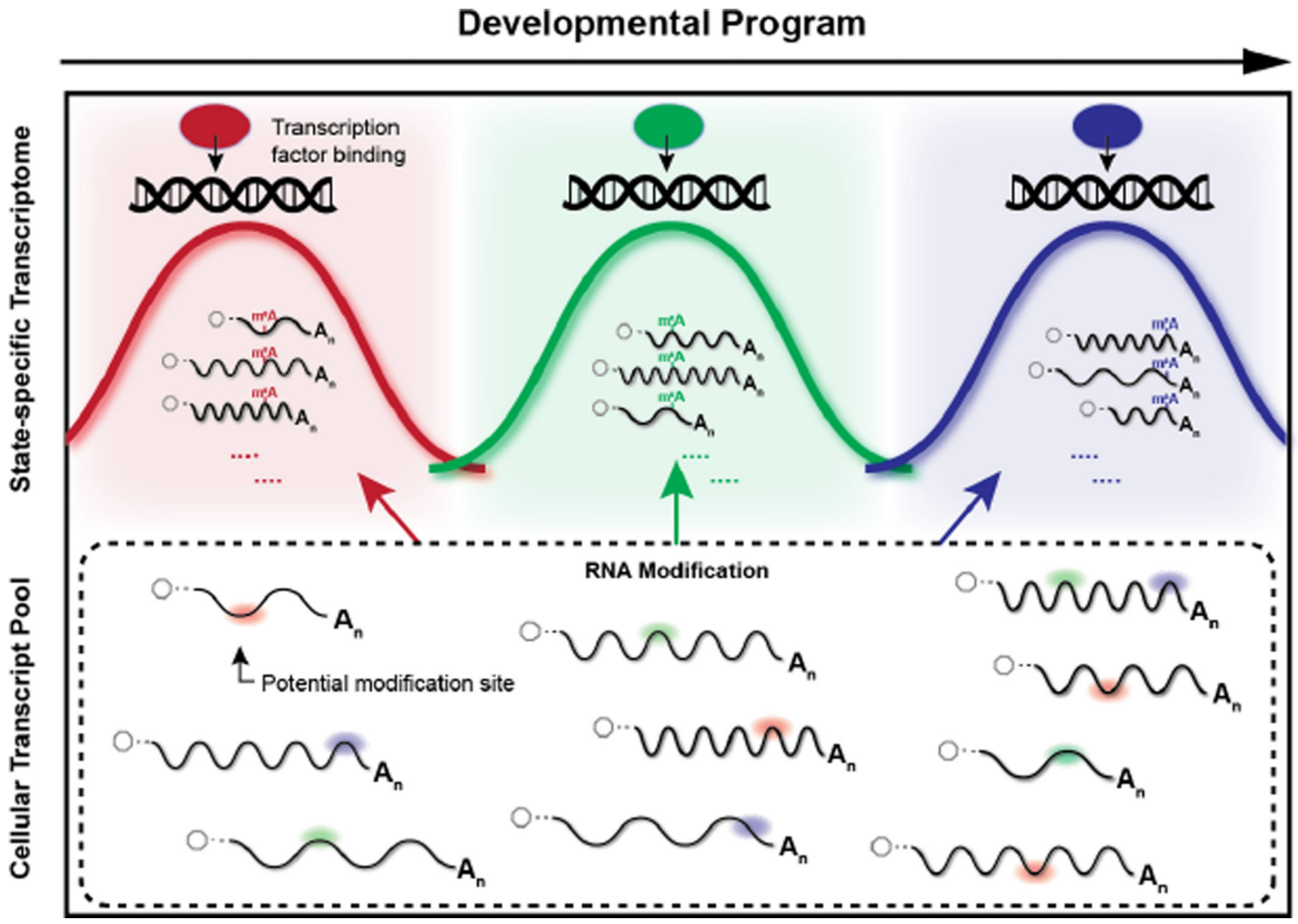

Figure 3. RNA modification groups transcripts for cellular processes

Developmental programs require rapid switching of the cellular transcriptome to bring about phenotypic changes. Recruitment of transcription factors can alter the composition of the RNA pool by introducing required transcripts to the existent mRNA population. In order to accomplish more rapid transitions, cells utilize the existing pool of mRNA, tuning their expression accordingly. One way to differentiate distinct groups of mRNAs from a diverse cellular pool is by post-transcriptional modification by m^6A, which marks transcripts for incorporation into pathways for translation and decay. Additional modifications may lend greater diversity to such mechanisms.

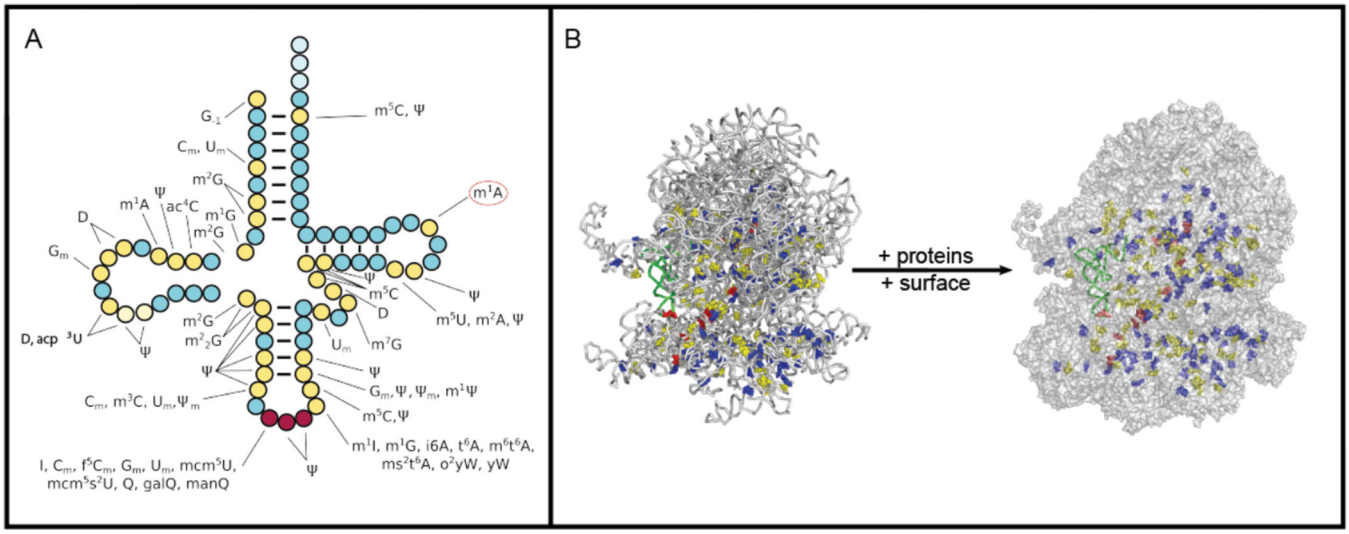

Figure 4. Landscape of tRNA and rRNA modifications

(A) All annotated mammalian tRNA modifications were pulled from Modomics (http://modomics.genesilico.pl/). Blue residues have no modification annotated while yellow residues are modified in at least one mammalian cytosolic tRNA (*Bos taurus Homo sapiens, Mus musculus, Oryctolagus cuniculus, Ovis aries, Rattus norvegicus*). The anticodon, a hot spot of modification, is highlighted in red. m^1A58, the first tRNA modification shown to be reversible, is circled. Modifications that occur in the variable loop were left out for simplicity. m^2G - N^2-methylguanosine; $m^2{}_2G$ - N^2N^2-dimethylguanosine; m^1G - 1-methylguanosine; ac^4C - N^4-acetylcytidine; m^1A - 1-methyladenosine; D - dihydrouridine; acp^3U - 3-(3-amino-3-carboxypropyl)uridine; m^3C - 3-methylcytidine; I - inosine; m^1I - 1-methylinosine; mcm^5U - 5-methoxycarbonylmethyluridine; mcm5s2U - 5-methoxycarbonylmethyl-2-thiouridine; Q - queuosine; galQ - galactosyl-queuosine; manQ - mannosyl-queuosine; f^5Cm - 5-formyl-2'-O-methylcytidine; t^6A- N6-threonylcarbamoyladenosine; ms^2t^6A - 2-methylthio-N6-threonylcarbamoyladenosine; m^6t^6A - N^6-methyl-N^6-threonylcarbamoyladenosine; i^6A - N^6-isopentenyladenosine; o^2yW - peroxywybutosine; yW - wybutosine; $m^1\Psi$ - 1-methylpseudouridine; Ψ_m - 2'-O-methylpseudouridine; m^7G - 7-methylguanosine; m^5C - 5-methylcytidine; m^2A - 2-methyladenosine; m^5U - 5-methyluridine.

B) All annotated *Homo sapiens* rRNA modifications recorded in Modomics (http://modomics.genesilico.pl/) were mapped onto the cryo-EM structure of the human ribosome (4ug0). An E- site tRNA (green) is shown for orientation. 2'-O-methylations are shown in blue, pseudouridylations are shown in yellow, and base modifications are shown in red. For simplicity, 2'-O-methyl-seudouridine is shown in blue. The rRNA ribbon diagram shows the prevalence of modification (~2% of total RNA residues). As the surface rendering incorporating ribosomal proteins shows, the large majority of the modifications are buried within the core of the ribosome.

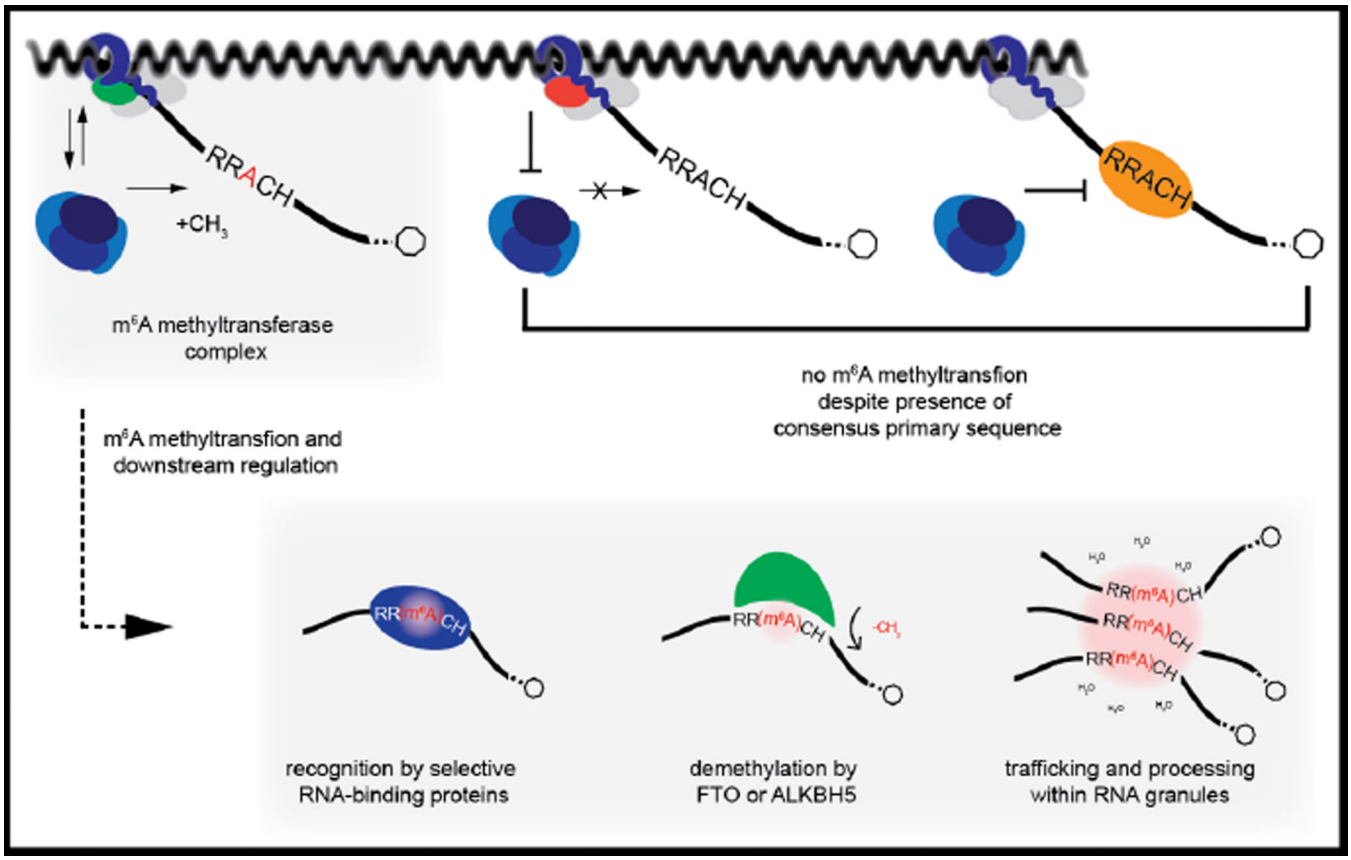

Figure 5. Mechanisms of selectivity in m^{6}A installation and downstream regulation

Mechanisms for selectivity in installation of m^{6}A are largely unknown, and cannot be predicted by primary sequence alone. Potential mechanisms include recruitment of methyltransferase components to nascent RNA by chromatin features associated with RNA Polymerase II (green), or exclusion from the transcription complex (red). RNA-binding proteins that occupy consensus sequences for m^{6}A may also prevent installation of the mark (orange). Once methylated, transcripts can be bound by RNA-binding that recognize modifications or secondary structure changes, or actively demethylated and no longer subject to regulation by m^{6}A-dependent pathways. Transcripts that are heavily decorated with methylations may face large solvation penalties, and benefit from trafficking within RNA granules.