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Small changes, big implications: the impact of m^{6}A RNA methylation on gene expression in pluripotency and development

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Abstract

In order to maintain a state of self-renewal, yet retain the ability to rapidly differentiate in response to external signals, pluripotent cells exert tight control over gene expression at many levels. Recent studies have suggested that N6-methyladenosine (m^{6}A) RNA methylation, one of the most abundant post-transcriptional modifications, is important for both pluripotency and differentiation. In this review, we summarize the current state of the m^{6}A field, with emphasis on the impact of writers, erasers and readers of m^{6}A on RNA metabolism and stem cell biology.

Keywords

Stem cells; pluripotency; development; m^{6}A methylation; RNA; post-transcriptional modification

1. Introduction

Transitions from one cell type to another require complex, carefully executed maneuvers to coordinate global changes in gene expression and ensure the desired outcome is achieved efficiently and appropriately. Although transcriptional changes are integral to most cell fate transitions, transcription takes time to induce or repress and must be coordinated with post-transcriptional regulation to facilitate rapid remodeling of the transcriptome in response to extracellular cues[1–6].

Cells utilize several levels of post-transcriptional regulation to coordinate gene expression changes. For example, RNA-binding proteins (RBPs) and microRNAs (miRNAs) exploit

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Contributions

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Competing Interests

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sequence elements shared between target mRNAs to enable co-regulation of several functionally related transcripts (~10-500 transcripts may be impacted). At the same time, ubiquitous and crucial modifications such as the 5' cap and poly(A) tail have a vast influence on gene expression through recruitment of proteins such as eIF4E, and poly(A) binding protein to virtually all RNA polymerase II transcripts. Internal m^6A methylation resides between these two levels of regulation allowing regulation of thousands of transcripts in response to developmental and other cues while leaving unmethylated transcripts unaffected.

Importantly, while methylation can dramatically influence RNA metabolism, it is not required for any aspect of the RNA life cycle. This means that otherwise identical transcripts may experience different levels of modification and, as a result, may behave as distinct populations in response to stimuli (Figure 1).

Virtually all RNA species including messenger RNAs (mRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNA), miRNAs and long non-coding RNAs (lncRNAs) can experience methylation at various positions (m^1A, m^5C, 2'-O-methyl etc). Of these modifications, N^6-methyladenosine (m^6A) has the best characterized effects on gene expression and was the first shown to modulate mRNA abundance[7,8], m^6A modification can modulate the fate of an mRNA at the level of splicing[9], cleavage/polyadenylation[10], subcellular localization[11], decay[12] and translation[13](Figure 2). Moreover, m^6A is the most prevalent internal mRNA modification in many eukaryotic species [7,8,14–16]. Although m^6A was discovered over 40 years ago[17–19], its cellular function was only characterized through recent advancements in antibody-based precipitation and high-throughput sequencing. Since 2012, ~10,000 m^6A sites have been documented in over a quarter of human transcripts[7,8,20]. The precise impact m^6A marks have on RNA metabolism and gene expression is dictated by the actions of methyltransferases ('writers'), demethylases ('erasers') and m^6A binding-proteins ('readers'), many of which are now known to play roles in pluripotency and development.

The goal of this review is to provide an up-to-date overview of the factors and mechanisms within the m^6A methylation pathway and then to focus on the influence m^6A methylation has on stem cell maintenance/differentiation and cell state transitions.

2. The How, Where, When and Why of m^6A Writing

The N^6-position of adenosine in RNAs can be methylated by four separate methyltransferase activities: CAPAM (also known as PCIF1) targets 2'-O-methyladenosine found adjacent to the 5' cap to produce m^6A_m[21,22], DIMT1L catalyzes dimethylation two adjacent adenosines in18S rRNA[23,24], whereas METTL16[25–30] (both in Box 1) and the METTL3/METTL14[31] heterodimer are responsible for internal m^6A modifications. For brevity, we will be focusing on the METTL3/METTL14 methyltransferase which installs the vast majority of internal m^6A marks on mRNAs and lncRNAs. The remaining methyltransferase activities are described in more detail in Box 1. METTL3/METTL14 is part of the surprisingly large m^6A writer complex which can be divided into two sub-complexes: the m^6A-METTL complex (MAC), which comprises METTL3 and

METTL14[31], and the m^{6}A-METTL-associated complex (MACOM), comprising WTAP, ZC3H13, RBM15/15B, VIRMA and HAKAI[32–36](Figure 3).

In order to perform catalysis, the m^{6}A writer must be localized appropriately within the cell and directed to appropriate sites on each RNA. Furthermore, activity must be regulated such that methylation is restricted to the appropriate developmental stage and/or RNA region. We discuss how these functions are achieved below.

2.1 Methyltransferase Activity (How is m^{6}A added?)

The MAC is responsible for the methyltransferase activity of the writer complex[31,37]. Briefly, this reaction entails the transfer of a methyl group, from an S-Adenosyl Methionine (SAM) molecule, onto the sixth nitrogen of the adenosine base. METTL3 is the sole catalytic subunit and the only one able to bind the SAM methyl donor[38–41]. While METTL3 possesses weak methyltransferase activity *in vitro,* the METTL3/METTL14 heterodimer exhibits substantially higher catalytic activity[31]. Although METTL14 does not perform catalysis, it is essential for methyltransferase activity, providing structural stability, and allosterically enhancing the methyltransferase activity of METTL3[37–39]. Interactions between METTL3 and METTL14 form a cavity where the RNA substrate is bound[38–40]. The methyltransferase domains of METTL3 and METTL14 are highly conserved among eukaryotes, with functional homologs in *Drosophila* (Ime4 and Cg7818, respectively)[16,42], *Arabidopsis* (Mta and Mtb, respectively)[36,43] and *S.cerevisiae* (IME4 and KAR4; although the role of KAR4 remains uncharacterized)[44,45]. Consistent with their high level of conservation, these proteins are essential for viability in mammals and plants, and play vital roles in embryonic development, sex determination and gametogenesis[46–54]. Depletion of either protein in human cell lines results in a marked global decrease in m^{6}A levels[31,32].

2.2 Methyltransferase Specificity (Where on the mRNA?)

When it comes to methylation, not all adenosines are created equal. The context of the adenosine is critical in determining where and whether a transcript is modified. MAC target sites are enriched for the short and redundant consensus m^{6}A motif, RRACH[31,32,55]. However, the expected occurrence of the RRACH motif in RNAs (~1 modification per 85 nucleotides) is much higher than the observed one m^{6}A modification per ~1000 nucleotides[7,8,20,56]. Only a fraction of adenosines found within this motif are actually modified, supporting the existence of additional layers of regulation. Such regulation likely includes selective recruitment of the MAC to specific RNA substrates and is influenced by the surrounding sequence context (such as structure, and availability of binding sites for competing factors).

Recent studies are starting to reveal how MAC is recruited to specific RNAs. There is evidence for interactions between MAC and chromatin/transcription factors including CEBPZ[57], SMAD2/3[6] and ZFP217[49]. These interactions facilitate (CEBPZ, SMAD2/3) or repress (ZFP217) m^{6}A modification of transcripts derived from promoters dependent on these transcription factors. For example, in stem cells SMAD2/3 is activated by TGFβ signaling and binds promoters of genes involved in early cell fate, including

NANOG. It then recruits WTAP and MAC to allow RNA methylation. Marking these transcripts with m^{6}A helps prime stem cells for a timely exit from pluripotency[6]. More broadly, an interaction between METTL14 and trimethylated histone H3 (H3K36me3) may help recruit MAC to nascent RNAs/RNA polymerase II (RNAP II)[58] and thereby promote m^{6}A deposition. These observations suggest that transcripts can be selected for modification as they are transcribed and also supports that the epigenome can directly influence the epitranscriptome.

Once MAC is recruited to an mRNA, there is further regulation to determine which sites within that transcript will be modified. Specificity is conferred by METTL3 via its two CCCH-type zinc finger domains [41] and METTL14 through a C-terminal domain containing RGG repeats[37]. Recent observations imply that the sequence context, and its match to the preferred consensus may be a driving factor determining the distribution and extent of m^{6}A modification (i.e. the m^{6}A signal is ‘hard-coded’ into the mRNA sequence). In further support of this hypothesis, a remarkable number of m^{6}A sites, and the flanking sequences, are conserved across vertebrates[7,59], and in some cases even in organisms as distant as yeast[60].

It is important to note that m^{6}A is not uniformly distributed across the length of the transcript (Figure 4). There are distinct preferences for the 5’ and 3’ ends[7,8,61,62]. Interestingly, the consensus RRACH motif is also enriched in these areas, particularly near the stop codon[59], and H3K36me3 chromatin marks also show a skewed enrichment around stop codons[58], which may contribute to enhanced MAC recruitment. In addition, the FTO demethylase may prune the methyl group from sites at the 5’ end[63– 65] resulting in an overall enrichment of m^{6}A at the 3’ end of RNAs. In exons, the vast majority of m^{6}A marks are located internally, residing at least 50 nucleotides away from splice junctions[66]. Moreover, there is an enrichment of m^{6}A in long (>200 nucleotides) internal exons[7,59,66,67]. The purpose and mechanism by which this is achieved is unclear. Interestingly, introns are rarely methylated when compared to exons, despite containing nearly three times the amount of RNA[20,66]. One explanation for this observation could be that the splicing machinery outcompetes or has priority over the m^{6}A writer complex for access to nascent RNA.

As well as being concentrated in specific regions of the RNA, m^{6}A sites are often found in clusters, similar to DNA methylation sites in CpG islands[8,20,56,59,66]. Clustering may be achieved in part through an association between the METTL3 and RNAP II that facilitates methylation when transcription slows[68], but also inherently requires that the available consensus sites be clustered.

Finally, the MAC can be directed to a specific location on an RNA through interaction with components of the MACOM, some of which are RNA-binding proteins. Specifically, VIRMA recruits the m^{6}A writer complex to sites near the stop codon and within the 3’ UTR[34], while RBM15 and RBM15B binding sites are enriched in the 5’ UTR of transcripts[61]. RBM15/15B both bind U-rich sequences through canonical RRMs, whereas VIRMA lacks a characterized RNA binding domain, but may be recruited to mRNAs through interaction with the cleavage and polyadenylation machinery[34]. Interestingly,

depletion of VIRMA in human cells resulted not only in a decrease in m^{6}A marks near the stop codon and within the 3' UTR, but also a significant increase in methylation at 5' UTRs[34]. This suggests that RBM15/15B and VIRMA may compete for recruitment of the writer complex to different regions of a transcript. HAKAI, an E3 ubiquitin ligase, may also guide MACOM to specific sites or modulate activity of the complex, but more work is needed to uncover its exact role[36]. Changes in expression or activity of proteins like these may facilitate developmental changes in the distribution of m^{6}A, such as the switch from 3' UTR to 5' UTR m^{6}A seen during cerebellum development in mice[69].

To sum up, the MAC is recruited to specific RNAs/RNAP II through interactions with histones and transcription factors. m^{6}A is then deposited on RNAP II transcripts in positions that are selected in large part on the basis of how close they match the preferred motif for METTL3/METTL14. However, the final distribution of m^{6}A is also influenced through recruitment and repression of the methyltransferase by RNA-binding proteins, and histone marks, as well as through removal of the modification at the 5' end by the demethylase, FTO[63,70]. As a result, m^{6}A is concentrated towards the 3' end of RNAs.

2.3 Methyltransferase Localization

It is clear that m^{6}A modification occurs early in the life of an mRNA[66,71] and this is consistent with the observation that the writer complex accumulates in nuclear speckles. These membraneless organelles congregate near sites of active transcription and serve as a reservoir for factors that participate in transcription and pre-mRNA processing[72]. WTAP, the third subunit of the core methyltransferase, plays no characterized role in catalysis or substrate selection but is essential for proper MAC localization to nuclear speckles[31], as is ZC3H13[73]. Importantly, if the MAC is not appropriately localized, it does not function: depletion of WTAP or ZC3H13 results in a profound global reduction in m^{6}A methylation[31,32,73].

2.4 Regulating the Writer

Cells modulate m^{6}A profiles in response to differentiation cues and external signals such as heat shock, DNA damage or stress[65,74–77]. The complexity of the m^{6}A writer allows for many levels of regulation; changes in abundance of individual subunits, and post-translational modifications likely influence activity at different stages of development and differentiation. However, to date we have only scratched the surface in identifying how such regulation is achieved. METTL3, being the catalytically active subunit, is an obvious target for regulation, and is targeted by miRNAs[78] and SUMOylation[79]. Other subunits appear to be dependent on METTL3 for their stability or expression[80] and can also be impacted by post-translational modifications (PTMs)[37]. However, there are still many knowledge gaps with respect to how these events regulate methyltransferase activity and the m^{6}A landscape overall.

3. m^{6}A erasure

Modifications of nucleic acids, including the 5' cap and poly(A) tail on RNA and methylation of cytosine in DNA, are generally reversible; their removal is catalyzed by

dedicated enzymes (decapping enzymes, deadenylases, and T5-methylcytosine hydroxylases, respectively) with well characterized consequences such as decay/inactivation of RNAs and de-repression of gene expression. The m^6A modification is also reversible, through the action of two enzymes in the α-ketoglutarate-dependent dioxygenase (AlkB) family (ALKBH5 and FTO/ALKBH9). Both ALKBH5 and FTO remove internal m^6A modifications, and thus can directly oppose the action of METTL3. However, as discussed below, ALKBH5 activity is restricted both temporally and spatially, suggesting that it is not a global regulator of all methylated mRNAs. A major impact of FTO may arise from its ability to target m^6A_m adjacent to the 5' cap structure, especially in snRNAs[81,82](see Box 1).

Like other AlkB proteins, ALKBH5 and FTO utilize an α-ketoglutarate (αKG) domain to recognize the m^6A-modified RNA, and also bind Fe(II) and αKG cofactors[70,83,84]. In addition, they possess a nucleotide recognition lid that interacts with the RNA substrate. Upon substrate recognition the demethylases catalyze oxidative dealkylation of $m^6A$to regenerate an unmodified adenosine[84–88]. The ALKBH5 protein is localized to nuclear speckles and is thought to act upon nascent mRNAs[84], whereas FTO shuttles between nucleus and cytoplasm[65,89] and can affect both nascent and mature transcripts[63,66,70,81,89,90]. Importantly, although the AlkB protein family is conserved across eukaryotic and bacterial domains, ALKBH5 and FTO are found only in vertebrates[84,91], although Arabidopsis have two alternative demethylases, ALKBH9B[92] and ALKBH10B[93]. The fact that eraser proteins are absent from lower eukaryotic organisms, which have both writers and readers of m^6A, suggests that demethylation is unlikely to play a general role in reversing all m^6A modifications but rather may have evolved to act on specific substrates and/or in response to external signals.

In support of this idea, knockout of ALKBH5 has no discernable effect on overall health in mice but results in profound defects in spermatogenesis, leading to male infertility[84]. Although ALKBH5 has the potential to broadly counteract the action of METTL3, it appears this is not its primary role in most cell types. Outside of the testes, the impact of ALKBH5-mediated demethylation on overall levels of m^6A is modest[84] and m^6A status of mRNAs does not vary greatly as they mature and enter the cytoplasm[66]. These two observations again support that only a subset of m^6A modifications are targeted for removal by ALKBH5. However, it is not clear how selectivity may be achieved as the evidence for any sequence preference is weak[84,94]. Although the overall influence of demethylation by ALKBH5 in most normal cells is modest based on the robust health of knockout mice[84,95], the protein is upregulated in hypoxia[96] and in certain cancer cells[97] and modulates expression of number of transcripts such as TFEB, FOXM1, and NANOG[97–99]. Thus, for some transcripts and responses ALKBH5 may be important.

In contrast to ALKBH5, FTO knockout has quite dramatic effects in mice with links to obesity and neurogenesis[69,91,100,101] although some phenotypes may be due to indirect effects on neighboring genes[102,103]. Since the recent discovery that FTO specifically targets m^6A_m to reverse methylation adjacent to the cap[81], it is not so clear what proportion of FTO's impact on gene expression is due to removal of m^6A_m (see Box) versus m^6A or even m^1A[89]. It should also be noted that MeRIP-seq experiments are, at least in

part, at the mercy of m^6A antibody specificity which currently have difficulties distinguishing between m^6A and m^6A_m[81,104]. There is evidence to suggest FTO may prune m^6A from the 5' UTR of specific mRNAs in the nucleus and that this activity is blocked during stress[63–65,75](see below). This is consistent with m^6A being more abundant at the 3' end and around the stop codon in mammals, while being prevalent around both the start and stop codons in *Arabidopsis*[15], which lack FTO[15,91]. Conversely, there is also evidence that FTO acts on m^6A sites outside of the 5' UTR [105,106], suggesting that the demethylase activity of FTO is not confined to any one region of transcripts. In addition, FTO associates with intronic sequences which may partially explain why introns exhibit significantly lower levels of modification than exons[107](Figure 4). However, it is hard to distinguish whether effects of FTO on splicing are due to altered function of snRNAs (which have m^6A_m cap structures and are essential components of the splicing machinery) versus changes in binding of RBPs that are recruited or repressed by m^6A (see below for more details).

Overall, demethylation may have a significant effect on expression and/or processing of specific transcripts, or during certain cellular responses, but more research is required to tease out when in the RNA life cycle demethylation occurs and how widespread its influence really is. This topic has been debated recently[64,66,81,108,109].

4. How m^6A affects RNA fate: Functions of the m^6A readers

m^6A alone is of little consequence to an RNA transcript; deposition of m^6A does not immediately trigger degradation or activate translation. Rather, m^6A influences RNA secondary structure and association with proteins and other RNAs to direct altered processing[9,10], export[11], translation[13,110,111] or decay[12,110,112,113](See Figure 2). The presence of m^6A favors a single stranded conformation and can therefore disrupt stem-loop structures[114] in a process termed 'm^6A switching'[115]. In some cases, this can allow enhanced binding of proteins like HNRNPC, which, in turn, alters abundance and alternative splicing of target mRNAs[116,117]. Alternatively, m^6A can block proteins from binding[62,118,119]. For example, inhibition of Human antigen R (HuR or ELAVL1) binding, due to the presence of m^6A, leaves mRNAs susceptible to miRNA-mediated degradation[120]. Although indirect influences on protein and miRNA binding have a significant impact, for brevity, this section will focus on the recruitment of proteins that directly recognize m^6A (a.k.a. m^6A readers) and their effects on RNA metabolism.

4.1 Reader Recognition of m^6A

The best characterized m^6A readers are members of the YTH domain-containing family of proteins, namely YTHDF1-3, YTHDC1 and 2 in mammals. The YTH domain was originally identified in the splicing factor YT521-B/YTHDC1[121,122], and is now known to confer m^6A-dependent RNA-binding activity[123,124]. In all cases, the preference for m^6A-modified RNA is specified by an aromatic cage that forms a hydrophobic pocket around the modified nucleotide[125–128]. Beyond this, it is not clear whether each YTH protein recognizes a distinct motif that allows it to act on a unique population of mRNAs, or if binding is dictated primarily by relative availability and affinity of each family member.

Furthermore, whether YTH domains can accommodate m^6A_m adjacent to the 5' cap has not been explored. Although there is some evidence for sequence preference outside of the clear requirement for m^6A, this must be evaluated critically, as experiments involving capture and sequencing of RNAs bound by YTH proteins inevitably give a motif that overlaps with that of the MAC (i.e. RRACH)[9,12,13,111,129]. For YTHDC1, *in vitro* and structural analyses support that there is a specific interaction with the G at the −1 position that does not occur in other YTH proteins[125,126]. Other interactions between the RNA and YTH domain[113,127,128], and possibly regions outside of the YTH domain[12,126,130], could confer additional sequence specificity. Such interactions must be important at some level, as RNAs less than 5 nucleotides long bind poorly to YTH proteins compared to longer transcripts[126]. Additional specificity may also be derived from interactions of the YTH proteins with factors associating elsewhere on the RNA. For example, YTHDC1 interacts with 3' end formation factors which could favor binding towards the 3' end of mRNAs[10]. The subcellular location of the RNA also influences its accessibility to different YTH proteins. In the nucleus, YTHDC1 has a much higher concentration[9] whereas the other YTH proteins are more abundant in the cytoplasm[12,13,110,129]. At this point, the extent to which the sequence context of an m^6A site dictates reader binding potential and the subsequent fate of a modified RNA is an area in need of further investigation. Moreover, while the YTH domain accommodates m^6A, there is evidence that these proteins also associate with N^1-methyladenosine modified substrates[131] and that binding can occur independent of METTL3-mediated methylation[132]. It is also worth considering that multiple YTH proteins are programmed to recognize the same or adjacent methylated residues, as YTHDF1-3 have numerous shared targets[61,110,111]. These proteins appear to cooperate to modulate translation and decay, but it is not clear whether multiple YTH proteins bind simultaneously to the RNA substrate at adjacent m^6A residues, or if the first YTH protein to bind engages the others through protein-protein interactions (Figure 5).

Although the majority of research on m^6A readers and their biological functions focuses on YTH proteins, other proteins with different RNA binding domains have been implicated as m^6A readers. The best supported of these are the insulin-like growth factor 2 mRNA-binding proteins (IGF2BP1-3)[133,134], the eIF3 complex[135] and PRRC2A[136]. In these cases, there is compelling evidence for preferential binding of each factor to m^6A modified RNAs, but the structural basis of these interactions is unknown as all available crystal structures were derived using unmodified substrates. FMR1, or FMRP, has also been implicated as a potential m^6A reader[119,137], but binding of FMR1 to m^6A sites may be influenced more by the sequence context rather than the modification itself [138].

4.2 m^6A effects on RNA splicing

The timing of m^6A deposition and how it interfaces with other nuclear RNA processing events like splicing and polyadenylation is the subject of intense investigation and also has fueled debate[64,66,108]. There is an apparent disconnect between the distribution of m^6A (primarily in exons, and away from exon junctions) and its ability to influence splicing. One reason for the discrepancy may be that conclusions about the distribution of m^6A are drawn from analysis of m^6A across the whole transcriptome, whereas effects on splicing are uncovered by scrutinizing splicing on a gene by gene basis. It is also possible that our maps

of m^{6}A are not accurate due to bias and limitations of the approaches used to identify m^{6}A sites as well as variation between different cell types[109]. And finally, it is important to remember that most experiments only assess the abundance of different mRNA isoforms at steady state, and cannot distinguish whether variation is caused by differential synthesis (i.e. changes in splice site choice) or differential stability. Although deposition patterns are not consistent with splicing regulation being the primary function for m^{6}A, and METTL3 depletion has only modest effects on splicing, for certain transcripts methylation occurs near sequence elements important for splicing and has a dramatic influence on splice site selection. A significant portion of this regulation is achieved through recruitment of a methyl-reader that was first identified as a splicing factor, YTHDC1/YT521B[9]. Splicing may also be influenced by m^{6}A-mediated changes in RNA structure that can favor or inhibit association of other RNA binding proteins like hnRNPA2B1[139].

Among the YTH domain-containing family of proteins, YTHDC1 is the only one that is constitutively enriched in the nucleus under physiological conditions[9]; it accumulates in foci termed YT bodies that are found near active transcription sites and adjacent to splicing speckles[121,140]. YTHDC1 directly interacts with splicing factors SRSF3 and SRFS10 to modulate exon inclusion events. One model asserts binding of SRSF3 to YTHDC1 facilitates its recruitment to splicing substrates and simultaneously prevents association of SRSF10 via steric hindrance[9]. SRSF3 is more abundant and favors exon inclusion, thus SRSF10 is only able to promote exon skipping when methylation or YTHDC1 function are disrupted. Indeed, YTHDC1 deficiency in mouse oocytes affected splicing of almost 2000 genes[10]. Overall, YTHDC1 may not directly influence splice site choice, but by binding to m^{6}A-modified RNAs and interacting with splicing regulators it can have a profound effect on processing events.

4.3 Cleavage and Polyadenylation

Given that m^{6}A modification is concentrated in the 3’ UTR and around the stop codon, it is well positioned to influence 3’ end processing. YTHDC1 interacts with CPSF6, a subunit of the Cleavage Factor I_m complex that is responsible for proper polyadenylation[141]. Furthermore, loss of YTHDC1 results in extensive alteration of polyadenylation sites in mouse oocytes[10]. Depletion of METTL3 and VIRMA also induces changes in polyadenylation site[34], but further study is required to determine whether the effects of reduced methylation are mediated entirely through loss of YTHDC1 binding or if other factors are involved.

4.4 Nuclear Export

One of the most dramatic effects of m^{6}A is on RNA export. Inhibition of methylation delays export of mature mRNAs[142] and knockdown of the ALKBFI5 demethylase accelerates export[84]. Recent studies have discovered that in HeLa cells YTHDC1 mediates this facet of m^{6}A function by recruiting SRSF3, which in turn interacts with the nuclear RNA export factor 1 (NXF1) to facilitate export of methylated mRNAs[11]. Interestingly, SRSF3 has been identified as a key regulator of pluripotency in murine iPSCs, where it binds to NANOG mRNA and facilitates its export, as well as modulating splicing and expression of

numerous pluripotency genes[143]. In the future, it will be interesting to assess how much of this regulation relies on recruitment by YTHDC1.

4.5 Regulation of YTHDC1

As outlined above, YTHDC1 is crucial for the efficient and accurate processing and export of methylated mRNAs and is the major direct reader of m^{6}A in the nucleus. It is therefore worth highlighting that YTHDC1 is a substrate for a number of tyrosine kinases including FYN, c-Src and c-Abl and phosphorylation of YTHDC1 influences both its localization and function. The phosphorylated form of YTHDC1 is dispersed throughout the nucleoplasm, insoluble, and unable to influence splice site selection[144]. At this time it is not known when or where phosphorylation naturally occurs but, based on the observation that YT bodies disperse during mitosis, it seems likely that activity of YTHDC1 is modulated in a cell-cycle dependent manner[144].

4.6 m^{6}A effects on translation

Once a methylated mRNA reaches the cytoplasm, it becomes available for binding by other YTH proteins although whether, and how, exchange of readers occurs is unknown. In the cytoplasmic compartment, m^{6}A modulates translation and decay of modified transcripts. YTHDF1[13,145–147], YTHDF3[110,111], YTHDC2[129,148,149] and even the methyl-writer, METTL3[150], have all been implicated as translation regulators, but are recruited to different regions of the mRNA and interact with different components of the translation machinery.

4.6.1 5’ UTR-mediated regulation: m^{6}A methylation in the 5’ UTR promotes cap-independent translation[135] and influences selection of the translation start site[106]. Cap-independent/m^{6}A-dependent translation is achieved by recruiting the translation initiation factor, eIF3 which in turn engages the 40S ribosomal subunit[135]. There is evidence that the ATP binding protein, ABCF1, and YTHDF3 also play a role[151]. During stress, cap-dependent translation is often blocked, thus this m^{6}A-dependent mechanism facilitates recovery by directing translation of chaperones and other required factors. As m^{6}A is deposited on capped RNAs, it also has the capacity to compete or collaborate with canonical cap-dependent translation under physiological/non-stress conditions[151]. Importantly, 5’ UTR m^{6}A is specifically increased during heat shock with no effect observed in the body or 3’ UTR of mRNAs[135]. This is achieved through relocalization of the normally cytoplasmic YTHDF2 to the nucleus where it specifically protects 5’ m^{6}A sites from demethylation by FTO[65,75]. Increased m^{6}A methylation in the 5’ UTR is also observed during oxidative stress which induces accumulation of many translation-arrested transcripts in cytoplasmic bodies known as stress granules (SGs). Under these conditions, 5’ UTR m^{6}A facilitates translation silencing and SG localization through binding of YTHDF3[152]. There is obviously still more to be learned about these mechanisms: How are 5’ UTR m^{6}A sites are selectively protected or modified? How do oxidative and heat stress both induce 5’ UTR methylation but have opposing effects on translation of m^{6}A modified mRNAs? It will also be interesting to investigate whether these novel translation mechanisms are important during development and differentiation.

4.6.2 3' UTR/Coding Region methylation: As noted above, the majority of m^{6}A marks are clustered around the stop codon and in the 3' UTR, where they are ideally situated to influence both translation and decay[7,8,56]. Both METTL3 and YTHDF1 have been independently implicated as readers of m^{6}A located at the 3' end of mRNAs, and both were reported to act by recruiting eIF3[13,150]. Tethering experiments suggest that YTHDF1 and METTL3 proteins function in separate pathways rather than one enlisting the other. However, it is not clear why eIF3 requires participation of other factors to recognize m^{6}A near the stop codon when it appears to bind m^{6}A in the 5' UTR directly[135]. In addition, the precise mechanism by which METTL3 interacts with an mRNA to promote translation requires further investigation. There is evidence from tethering assays that the translation-promoting activity of METTL3 does not require methyltransferase activity or METTL14/WTAP[150], but it is not clear whether METTL14/WTAP are required for association of METTL3 with RNAs in the cytoplasm. It is also not known whether the MAC is removed from the mRNA after m^{6}A deposition, with METTL3 reuniting with the transcript at a later time to promote translation. Alternatively, METTL3/MAC may be retained on some sites after it catalyzes the methylation reaction, while being ejected from other sites to allow binding of other factors.

YTHDF1 and YTHDF3 share many substrates and appear to cooperate to promote translation. However YTHDF3 does not influence translation on its own, although it does exhibit interactions with ribosomal proteins[110,111]. In contrast, when bound to circular RNAs, YTHDF3 promotes translation by acting in concert with elF4G2[153].

A third member of the YTH family, YTHDC2, also directly interacts with m^{6}A-modified transcripts to facilitate translation. YTHDC2 is extremely important for germline development and specifically for the switch from mitosis to meiosis in male germ cells[113,149,154]. Tethered YTHDC2 enhances translation and loss of YTHDC2 results in a marked decrease in translational efficiency of its targets[129]. Moreover, YTHDC2 interacts directly with the small ribosomal subunit[148]. It is hypothesized that the RNA helicase activity of YTHDC2 might facilitate access for translation initiation factors and/or the ribosome[113,148,155].

Beyond the YTH family of proteins, IGF2BPs also co-localize with SG markers[134] and sequester target transcripts to stress granules[156], suggesting they could perform a similar triaging role to that of YTHDF3. IGF2BP1 specifically regulates the translation of *MYC* mRNA in an m^{6}A-dependent manner through a coding region element that contains m^{6}A sites[134]. The IGF2BPs have previously been linked to several mechanisms of translation regulation[156], including of viral transcripts[157]. However, the precise mechanism for m^{6}A dependent regulation by IGF2BPs remains elusive.

4.7 m^{6}A effects on decay

The final effect of m^{6}A on the RNA life cycle is its impact on RNA decay rates. Multiple studies have shown that m^{6}A methylated transcripts are less stable than their unmodified counterparts[12,20,50,74,110,112], and YTHDF2 is the primary culprit causing this destabilization[12]. Under physiological conditions, a majority of YTHDF2 binding sites are found near the stop codon and within the 3' UTR[12], which likely reflects the natural

distribution of m^{6}A[7,8]. Upon substrate binding YTHDF2 accelerates the degradation of target transcripts through direct recruitment of the CCR4-NOT deadenylase complex[112] via an interaction between YTHDF2 and the CNOT1 subunit of the deadenylase. As deadenylation is the first and rate-limiting step in decay of most mRNAs, modulating this step has a significant effect on overall mRNA decay rates[158]. At the same time, YTHDF2 can also work with the HRSP12 RBP to recruit RNAse P/MRP endonuclease[159]. YTHDF2-mediated decay represses transcripts encoding cell cycle regulators such as Cyclin A2 (CCNA2) and CDK2[101], and also clears populations of unwanted mRNAs such as maternal transcripts during oocyte maturation[2] and transcripts favoring self-renewal in differentiating hematopoietic stem cells (HSCs)[160]. YTHDF2 plays an important role in clearing RNAs during cell state transitions such as the maternal-to-zygotic[1] and the epithelial-mesenchymal transitions[161].

While YTHDF2 makes the most obvious contribution to m^{6}A dependent mRNA decay, there is evidence that other YTH proteins also interface with the mRNA decay machinery. YTHDF2 and YTHDF3 both enhance deadenylation somewhat when tethered to a reporter mRNA[112], but this might perhaps be explained by observations that YTH proteins, and particularly YTHDF3, can augment the binding of other family members (such as YTHDF2) to their substrates[110]. YTHDC2 is also able to down-regulate its targets, presumably by destabilizing them[129], and has been implicated as an RNA stability factor in the germline, where it interacts with a meiosis-specific protein known as MEIOC[162,163]. The interaction of MEIOC and YTHDC2 with mitosis-promoting transcripts, including CCNA2 enhances their decay and facilitates a sharp transition into meiosis[149,163]. These experiments indicate a primitive role for m^{6}A in meiosis that may be conserved from yeast to humans, as m^{6}A deposition only occurs during meiosis in *S. cerevisiae*[164–166]. The exact mechanism by which YTHDC2 influences decay in meiotic cells needs more study. While there is strong evidence for direct interaction between YTHDC2 and the 5’−3’ exoribonuclease XRN1[148], XRN1 can only act on mRNAs that have already been committed to destruction by endonucleolytic cleavage or decapping[167]. Furthermore, XRN1 is required for meiosis in *S. cerevisiae,* but its catalytic activity is dispensable[168], suggesting that the interaction with XRN1 may not be directly responsible for the ability of YTHDC2 to enhance mRNA turnover.

The IGF2BP family of proteins also modulate the decay of m^{6}A-modified transcripts but unlike YTHDF2 and YTHDC2, IGF2BPs stabilize methylated transcripts[134]. In some cases, this is achieved through sequestration of methylated mRNAs into stress granules[156], but stabilization can also be caused by impeding binding of destabilizing factors such as miRNAs[133]. It is also worth noting that m^{6}A on its own can also modulate mRNA stability by perturbing binding of factors with overlapping target sites. For example, the presence of m^{6}A impedes HuR binding and thereby favors association miRNAs at neighboring sites to enhance decay of developmental transcripts such as *IGF2BP3* and *FGF5*[120].

Regulation of reader activity can be achieved through standard mechanisms including gene expression, subcellular localization and PTMs. In terms of gene expression, YTHDF2 is repressed by two miRNAs[169,170], while YTHDC2 is inducible by TNFα[171].

Previously mentioned observations of YTHDF2 re-localizing to the nucleus during heat shock[65,75] and PTMs on YTHDC1[144] infer there may be other instances of regulation awaiting discovery. Future studies regarding the mechanisms and situations by which reader activity is regulated will provide useful insight about the impact m^6A has on global gene expression.

5. How does m^6A methylation of mRNAs influence pluripotency and development?

To recap, m^6A is added to a large proportion of mRNAs and non-coding RNAs in many different tissues and cell types and can influence most, if not all, aspects of RNA metabolism. It is not surprising then that m^6A methylation has a great influence on both cell and organism function. However, despite the almost ubiquitous nature of m^6A modification, its power is observed most clearly in stem cells and during development[50,51,74,120,172], when large-scale coordinated changes in gene expression occur to determine cell fate. The effects of reducing m^6A methylation are highly cell-type dependent, suggesting that methyltransferase activity, the RNA targets it modifies, and/or the activity/abundance of methyl-readers are regulated in a cell-specific manner. Ultimately though, a common theme is that cell fate decisions are skewed following loss of methylation[50,52,53,172–176], suggesting that m^6A helps maintain stem cells in a balanced state where they are able to self-renew but also primed to rapidly and efficiently differentiate down one or more pathways. When either methylation itself, or the activity of methyl-readers, is perturbed, the balance tips and cells are committed to a specific fate or permanently detained in the self-renewal program. The effects of m^6A on developmental decisions appear to occur through at least three mechanisms, which are discussed below.

5.1 Tipping the balance of dosage-sensitive master regulators

In many cases, methylation of mRNAs encoding one or two dosage sensitive master regulators (generally signaling or transcription factors) is pivotal. If expression of these key targets rises above, or falls below a threshold it pushes the cell to differentiate, or alternatively into a permanent state of self-renewal or quiescence.

Some recently characterized examples are shown in Figure 6. In bone marrow mesenchymal stem cells (BMSCs) knockout of METTL3 strongly favors differentiation down the adipocyte lineage over osteoblast production, leading to osteoporosis. The Parathyroid Hormone Receptor (PTH1R) mRNA is a key target of METTL3-mediated methylation in BMSCs; its translation is compromised following METTL3 knockdown and this disrupts the PTH signaling axis[175](Figure 6A). The importance of m^6A modification of this single mRNA for osteogenesis is highlighted by the fact that restoration of PTH1R expression partially rescues osteogenic gene expression in METTL3-deficient cells[175].

Methylation is also important in myeloid differentiation (Figure 6B). Loss of METTL3 results in activation of quiescent HSCs to a proliferative state[177], while METTL14[178] and YTHDF2[160,179] are required for self-renewal of active HSCs - silencing either gene favors myeloid differentiation. Two key targets of METTL14 in this process are the MYB

and MYC transcription factor mRNAs whose expression is down-regulated following METTL14 depletion[178]. Moreover, upstream of this, m^{6}A deposition on the *NOTCH1* mRNA can promote its destabilization via YTHDF2 and repress Notch signaling. This favors the endothelial-hematopoietic transition[172,180].

The above examples highlight that some genes are uniquely sensitive to methylation of their transcripts. Further study is required to determine what makes these genes so responsive. It could be simply that the proteins encoded by these genes are very sensitive to small changes in expression. Alternatively, the extent of methylation and/or context of the methylation site(s) could directly influence recruitment of readers and effector proteins to these transcripts.

5.2 Clearing unwanted mRNAs from the transcriptome

During differentiation and development, massive changes in gene expression occur which require that existing transcripts are cleared to make way for those being induced to specify the new cell type. Recent studies have found that m^{6}A methylation, through recruitment of the decay promoting factor YTHDF2, plays vital roles in clearing unwanted transcripts. During the maternal-zygotic transition in zebrafish, YTHDF2 associates with over one third of maternally loaded mRNAs and induces their decay[1]. Similarly, maternal mRNA clearance during mouse oocyte maturation is disrupted following loss of YTHDF2[2]. Interestingly, newly transcribed zygotic mRNAs are m^{6}A methylated, but their abundance is not generally affected by removal of YTHDF2[1]. Further investigation will be needed to determine what distinguishes the maternally supplied methylated mRNAs from those derived from the zygotic genome. It is possible that both populations are targeted for decay but that zygotic transcription is down-regulated to compensate for loss of YTHDF2. Alternatively, the proteins deposited on mRNAs during zygotic transcription are likely to be different than those found on maternal transcripts and could influence m^{6}A recognition and impact. It is important to note that although m^{6}A clearly plays a role in clearance of some maternal mRNAs, it is not an essential one, as *Ythdf2*$^{-/-}$ embryos are generally viable, although they do exhibit delayed development[1,2]. Evidence suggests that there are multiple redundant mechanisms for maternal mRNA clearance which ensures robustness and reduces the influence of random variation[181].

In the examples above, m^{6}A induces clearance of a population of mRNAs at a specific developmental transition. In other scenarios, m^{6}A facilitates a phenomenon known as transcriptional pre-patterning, where mRNAs specifying an alternative cell fate are actively transcribed but constantly targeted for m^{6}A-mediated decay by YTHDF2 before being translated[182]. This can create a balanced state where stem cells are poised for rapid differentiation while still maintaining a stem cell phenotype. Strong evidence for this role of m^{6}A comes from studies of neural development, where destabilization of neuron-specific mRNAs by m^{6}A tagging and recruitment of YTHDF2 may both clear these transcripts in neural stem cells and facilitate their coordinated upregulation following induction of neuronal differentiation[174]. Although differentiation can still occur in the absence of methylation, it is less robust, at least in part due to prolonged cell cycle progression[174,182].

5.3 Potentiating transcriptional changes for sharp transitions

It is clear that m^{6}A is deposited on many mRNAs, and thus has a widespread impact on overall gene expression. In this respect, m^{6}A modification is reminiscent of miRNA-mediated regulation. miRNAs fine tune gene expression, buffer against noise and confer robustness on biological systems[183]; all roles that can also be attributed to methylation. Specifically, while transcriptional changes are central to initiating cell fate transitions during development, methylation and its downstream effects are key to ensuring these transitions are carried through in a sharp and coordinated fashion.

An excellent example is seen in a recent study evaluating the impact of m^{6}A during nerve regeneration following injury. Robust axon regeneration requires transcription and translation of regeneration-associated genes (RAGs), many of which are enriched among mRNAs tagged with m^{6}A following nerve injury. Both the site and level of methylation on RAG mRNAs is altered following injury. Importantly, upregulation of RAG proteins is delayed following deletion of METTL14. This delay can be attributed, in part, to reduced recruitment of the translation regulator, YTHDF1, to the RAG mRNAs, but reduced expression of translation factors may also be an influence[145]. Overall, axon regeneration is more robust in the presence of a functional m^{6}A pathway; recovery of damaged axons is impaired following loss of METTL14 or YTHDF1 (figure 6C).

Methylation is also used to facilitate neuroectoderm differentiation through a mechanism activated by TGFβ signaling[6]. TGFβ signaling is required to maintain a pluripotent state and functions by activating SMAD2/3 transcription factors. The MAC is recruited by activated SMAD2/3 to its target genes, resulting in enhanced methylation and destabilization of several mRNAs whose transcription is induced by TGFβ signaling, including that encoding the pluripotency factor NANOG[6]. It is well known that unstable transcripts respond more rapidly to external cues[184,185], thus by destabilizing its target mRNAs, methylation facilitates sharper down-regulation of pluripotency factors when TGFβ signaling is inhibited. This in turn allows timely exit from pluripotency and efficient entry into the neuroectoderm differentiation program (Figure 6D).

6. Where does the m^{6}A field go from here?

The ability to study m^{6}A and its impact on biological processes became readily available relatively recently, through advances in next generation sequencing technologies that allow isolation of methylated RNAs (MeRIP-Seq)[7,8]. Since then, adaptations have been implemented to increase the resolution[20,56,186,187], but the method remains at the mercy of antibody specificity, leaving it vulnerable to nonspecific signals or sequencing artifacts[104]. The reliance on antibodies also prevents quantitative assessment of m^{6}A levels on a global scale. Other methods, like SCARLET[188], are able to accurately quantify m^{6}A at a specific site, but the method is laborious, works only on relatively abundant transcripts and can only characterize one site at a time. The very recent introduction of sequencing approaches relying on m^{6}A-sensitive endonucleases[59,60] and nanopore-based direct RNA sequencing[189] will undoubtedly reveal more information regarding the stoichiometry of m^{6}A modification at individual sites. As these techniques are adopted, new insights into how m^{6}A stoichiometry changes during development and in response to stimuli

will be revealed, as well as a better understanding of relative roles of the writers and erasers in defining the m^{6}A landscape under different conditions.

There are many avenues for future research connected to the m^{6}A epitranscriptome and its impacts on development and pluripotency. We have much to learn about how m^{6}A deposition integrates with other events that are dependent on the same SAM methyl-donor such as histone and DNA methylation. Through the SAM donor and methyltransferase activities, methylation is used to connect stem cell metabolism to gene expression at multiple levels. The effects of SAM depletion on pluripotent cells include impaired self-renewal, with cells entering a state poised for differentiation and, if deprivation is prolonged, eventually apoptosis[190]. The naïve-to-primed hESC transition is also dependent on SAM levels[191]. Methionine deprivation (which leads to SAM depletion) is known to influence the modification of DNA and histones, but the effects of reducing this metabolite on METTL3/14 and the major pathway of m^{6}A deposition are virtually unstudied. Given the compelling evidence that RNA methylation is integral to self-renewal and differentiation of stem cells, it seems likely that it contributes to the effects of SAM depletion on these processes and may be tightly coordinated with similar modifications occurring at the chromatin level.

While there has been significant progress in identifying components of the m^{6}A writing machinery, and numerous readers have been characterized, the interactions between these proteins need to be better defined. For example, the MACOM subunits may compete for binding to the MAC which could result in an array of methyltransferase complexes with different preferences and expression patterns. This in turn could account for changes in the m^{6}A landscape that have been observed in response to stimuli. At the level of m^{6}A reading, there is evidence that readers cooperate, but we have not yet determined whether each must bind m^{6}A directly for recruitment, whether different readers have unique site preferences, or whether reader proteins are exchanged as the mRNP matures and progresses to translation and eventually decay.

Finally, while connections between m^{6}A and development are clear, the exact role of m^{6}A in facilitating massive reorganization of the transcriptome must be further dissected to understand how one set of methylated transcripts are targeted for destruction (e.g. maternal RNAs) while others (e.g. zygotic transcripts) are not. It could be that the m^{6}A distribution changes and/or that different readers/effectors are recruited as transcription changes. Sifting through the links between chromatin/transcription and m^{6}A deposition will likely give important insights in this area.

At this point it is clear that m^{6}A methylation is an essential means of coordinating and regulating gene expression during cell state transitions. With advances in technology, and increased attention, it seems inevitable that this avenue of investigation will lead to a much clearer understanding of pluripotency and differentiation and also lead to novel therapeutic approaches for cancer and other diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box:

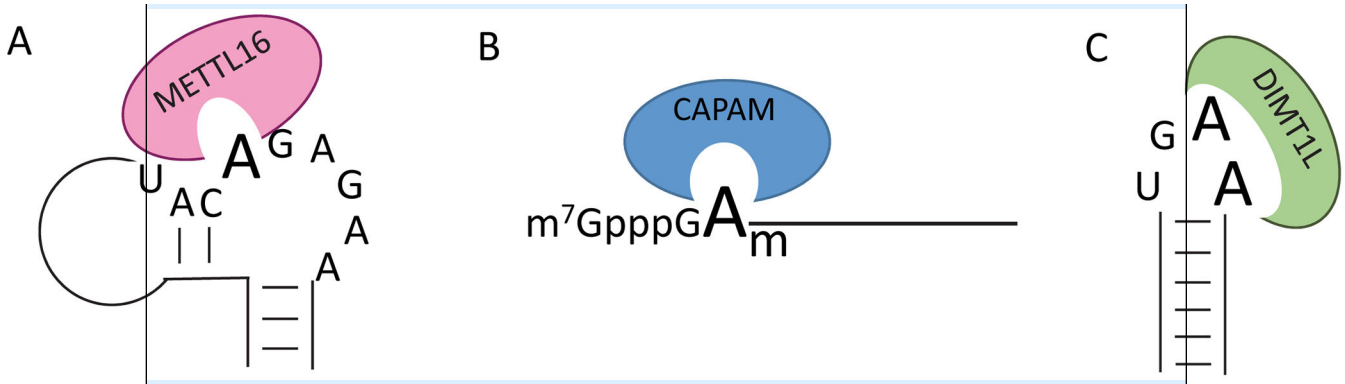
Other ways to deposit m^6A

While we have focused primarily on METTL3-mediated m^6A deposition, it is important to note that there are other enzymes capable of installing methyl groups on the N6 position of adenosine. In each case the substrate/site of modification is distinct from that recognized by the MAC.

METTL16 METTL16 is in the same family as METTL3 and METTL14 but targets a smaller set of transcripts at specific sites containing a UACAGAGAA motif and unique secondary structure where the adenosine is bulged out[27,29,192](Panel A). To date, targets of METTL16 include non-coding RNAs, lncRNAs and pre-mRNAs[25]. METTL16-mediated methylation of U6 snRNA facilitates snRNA-pre-mRNA interactions during splicing[25], while methylation in the 3' UTR of the SAM synthetase, *MAT2A,* regulates alternative splicing to control SAM homeostasis[27]. In the latter example, the methylated adenosine is recognized by YTHDC1 under high SAM conditions to down-regulate MAT2A expression[28], while prolonged association of METTL16 under low SAM conditions causes intron retention and eventually decay of the MAT2A mRNA. Loss of METTL16 leads to massive effects on the transcriptome and embryonic lethality in mice[193], primarily because of dysregulation of SAM homeostasis. It is not known whether m^6A deposited by METTL16 can be removed by ALKBH5 or FTO.

CAPAM/PCIF1 Recently, CAPAM was identified as the enzyme responsible for modifying 2'O-methyladenosine adjacent to the 5' cap of RNAP II transcripts, including mRNAs and snRNAs, to generate m^6A_m[21,194](Panel B). Being adjacent to the cap allows m^6A_m to promote translation of capped mRNAs independent of eIF4E[21]. m^6A_m is also crucial for proper snRNA biogenesis[82], which in turn affects mRNA splicing. Notably, m^6A_m is removed by FTO[82] and therefore effects of FTO on splicing may be largely due to hyper-methylation of snRNAs. As some immunoprecipitation-based approaches fail to distinguish internal 5' UTR m^6A from m^6A_m, existing research on the effects of m^6A at the 5' end of mRNAs must be carefully interpreted.

DIM1/DIMT1L N^6-dimethylation of 18S rRNA is performed by Dimlp in yeast[24] and its homolog DIMT1L in mammals[23] at two adjacent positions in a highly conserved region near the decoding site. Interestingly, association of the methyltransferase with the 18S rRNA is required for rRNA processing and catalysis is delayed until after cleavage occurs. This requirement ensures that all cleaved rRNAs are committed to modification.



Box Figure: Alternative methyltransferases recognize distinct properties of their target RNAs.

A: METTL16 targets a unique structure found in U6 snRNA, MALAT lncRNA, and the 3' UTR of the MAT2A mRNA as well as other RNAs. B: CAPAM targets the 2'O-methyladenozyne found adjacent to the 5' cap in many RNA polymerase II transcripts. C: DIMT1L targets two adjacent adenosine residues in a stem-loop structure of 18S rRNA.

Highlights

- m^6A is an abundant post-transcriptional modification that enables rapid responses to external cues
- The m^6A methylation pathway consists of readers, writers and erasers
- m^6A modifications impact multiple facets of RNA metabolism
- m^6A regulation is crucial for stem cell differentiation and development

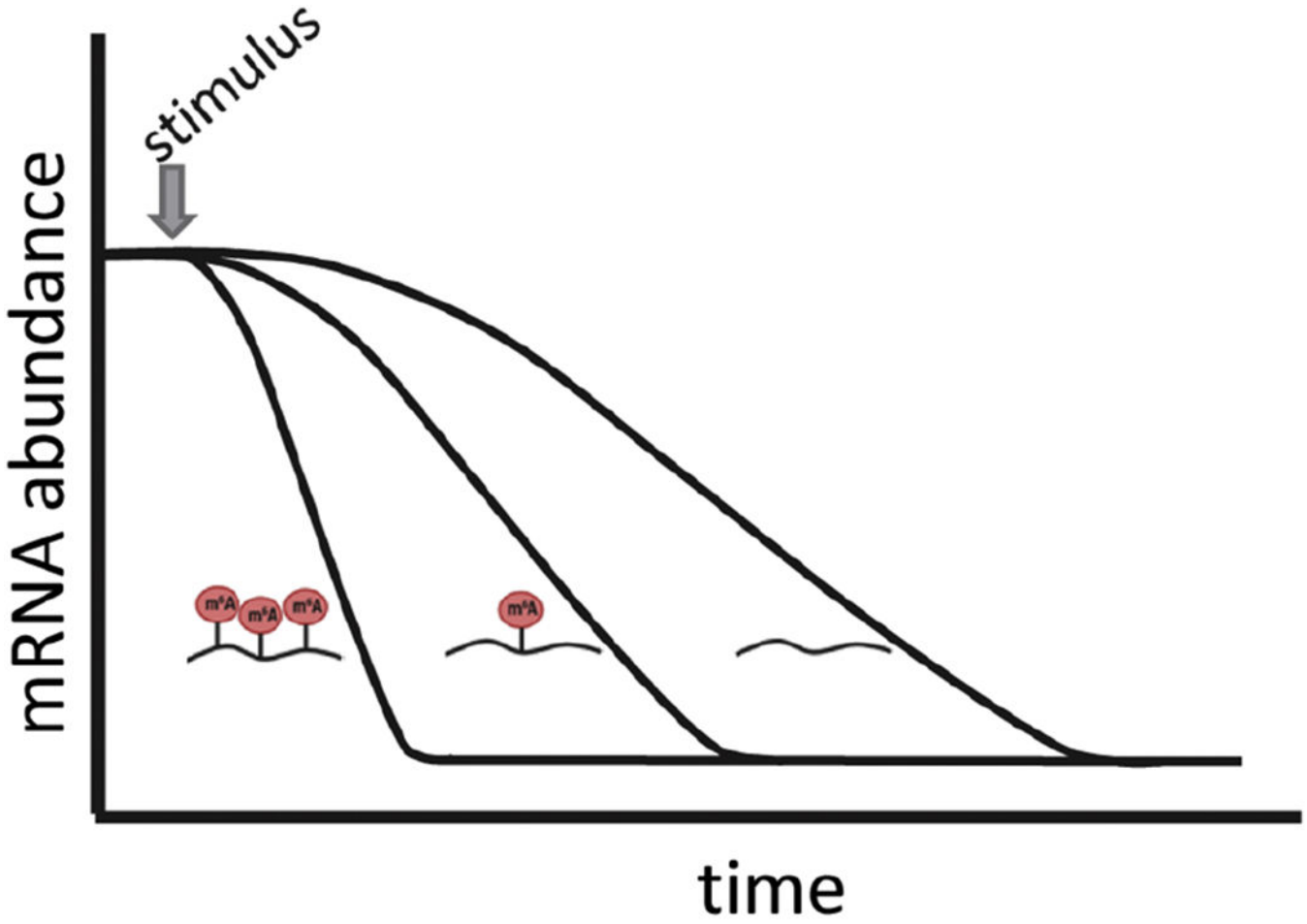


Figure 1: The impact of m^{6}A on mRNA abundance on mRNA stability.

Different subpopulations of transcripts from the same gene can exhibit different responses based on their level of modification. Following stimulation, the sharpest change in mRNA abundance is seen for transcripts with clusters of m^{6}A. Transcripts with single m^{6}A modification may experience a more gradual decrease, while transcripts with no m^{6}A are the slowest to change.

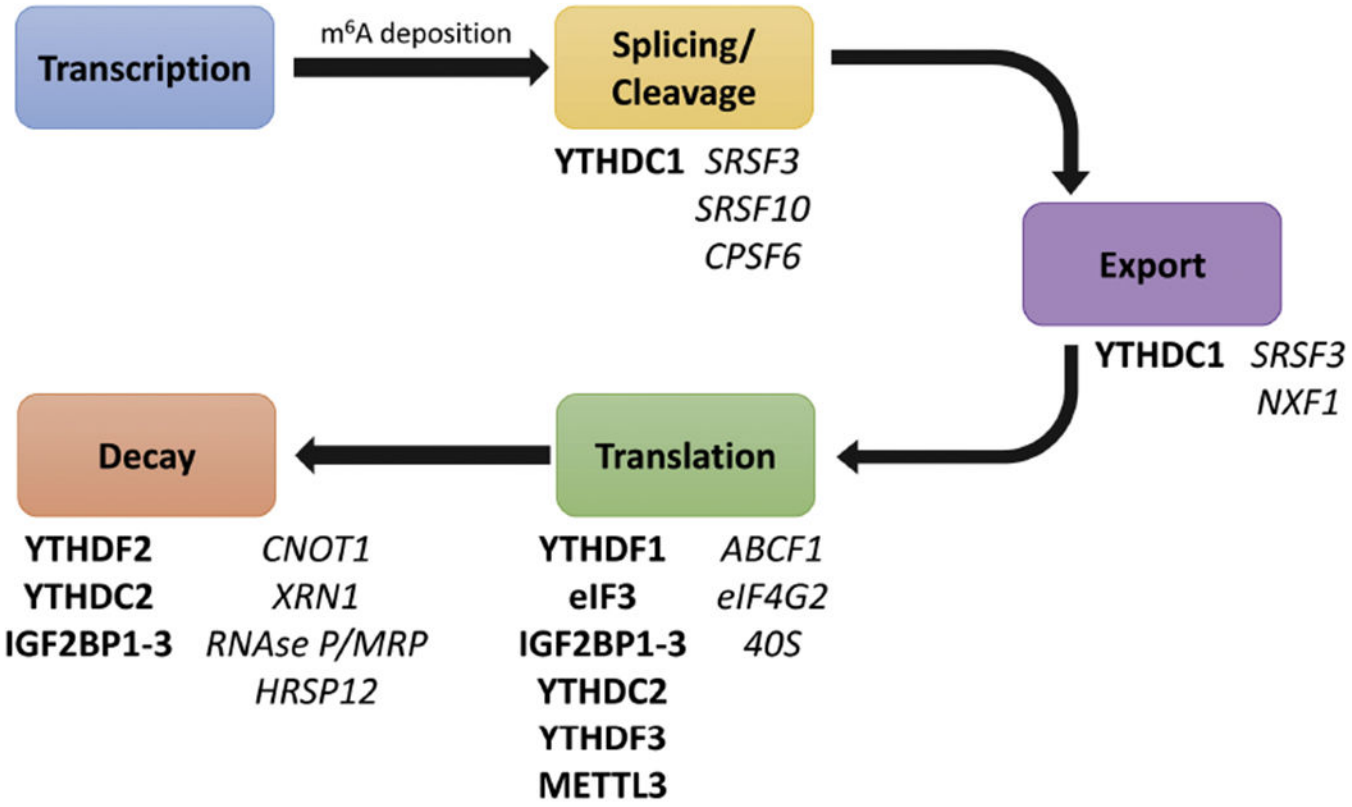


Figure 2: m^{6}A influences mRNA function from cradle to grave.

Recognition of m^{6}A by methyl-reader proteins, bolded, can influence various RNA processes including splicing/cleavage, nuclear export, translation and decay. Often, reader proteins interact with or recruit effector proteins, italicized, to carry out a given function. They can also compete with miRNAs and RBPs.

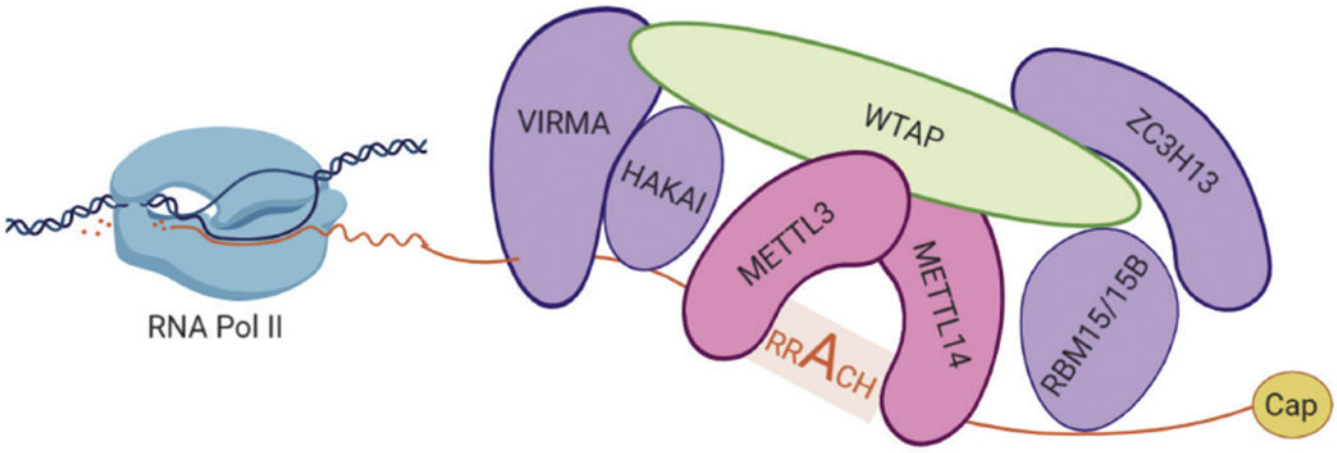


Figure 3: The m^{6}A writer complex.

The m^{6}A writer complex targets RNAs as they are transcribed. The m^{6}A-METTL complex (MAC; shown in pink), consisting of the METTL3/14 heterodimer, is the catalytically active subcomplex which methylates adenosine at sites matching the RRACH consensus. WTAP is the major scaffolding protein that connects the MAC and m^{6}A-METTL-associated complex (MACOM). The composition and/or activity of MACOM subunits can influence the distribution of m^{6}A across a transcript. VIRMA and HAKAI favor 3' methylation, while RBM15/15B facilitates 5' methylation.

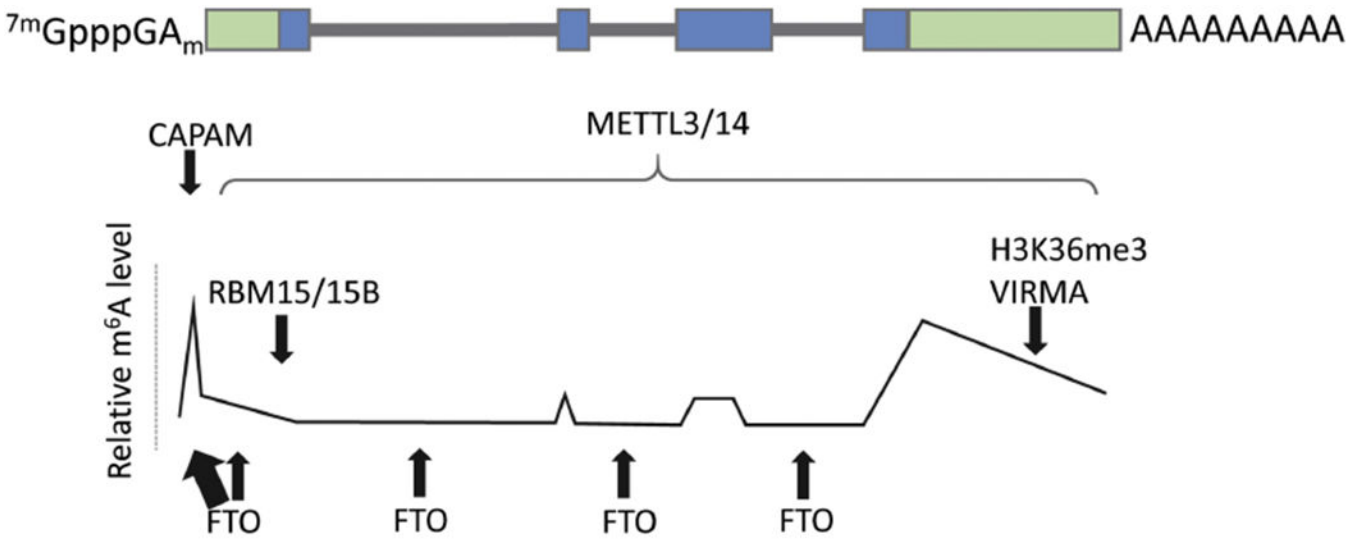


Figure 4: Factors influencing the distribution of m^6A and m^6A_m on mammalian mRNAs.

The overall m^6A landscape in physiological conditions is determined primarily by the distribution of consensus motifs recognized by the methyltransferases (CAPAM, METTL3/14). However, it can be influenced by demethylases such as FTO, which prunes m^6A from introns and across the transcript. Moreover, certain components of the MACOM, namely RBM15/15B and VIRMA, can favor methylation at the 5' and 3' ends respectively. Finally, there is evidence that histone modifications can influence m^6A deposition.

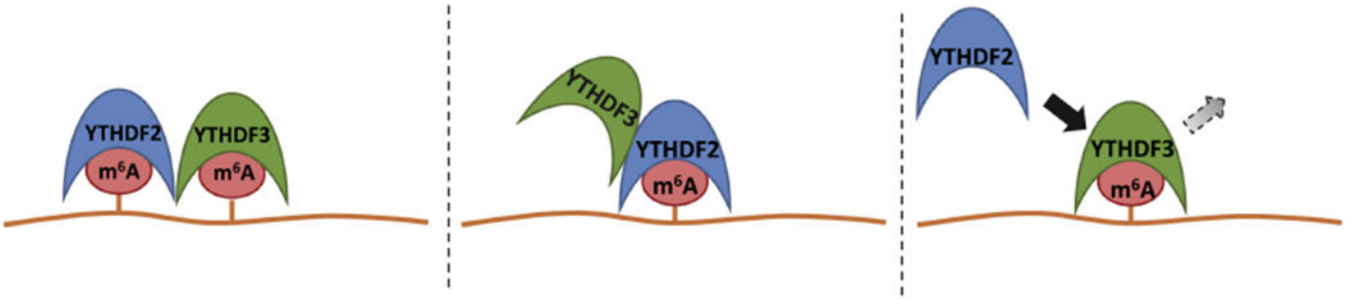


Figure 5: Competition and Collaboration between Reader Proteins.

A: Reader proteins bind adjacent m^{6}A and recruit effectors to simultaneously influence multiple aspects of metabolism. B: Reader protein binds m^{6}A and recruits other readers along with effectors, again allowing simultaneous impact on multiple pathways. C: Reader proteins compete for single m^{6}A and shuttle mRNA down different pathways.

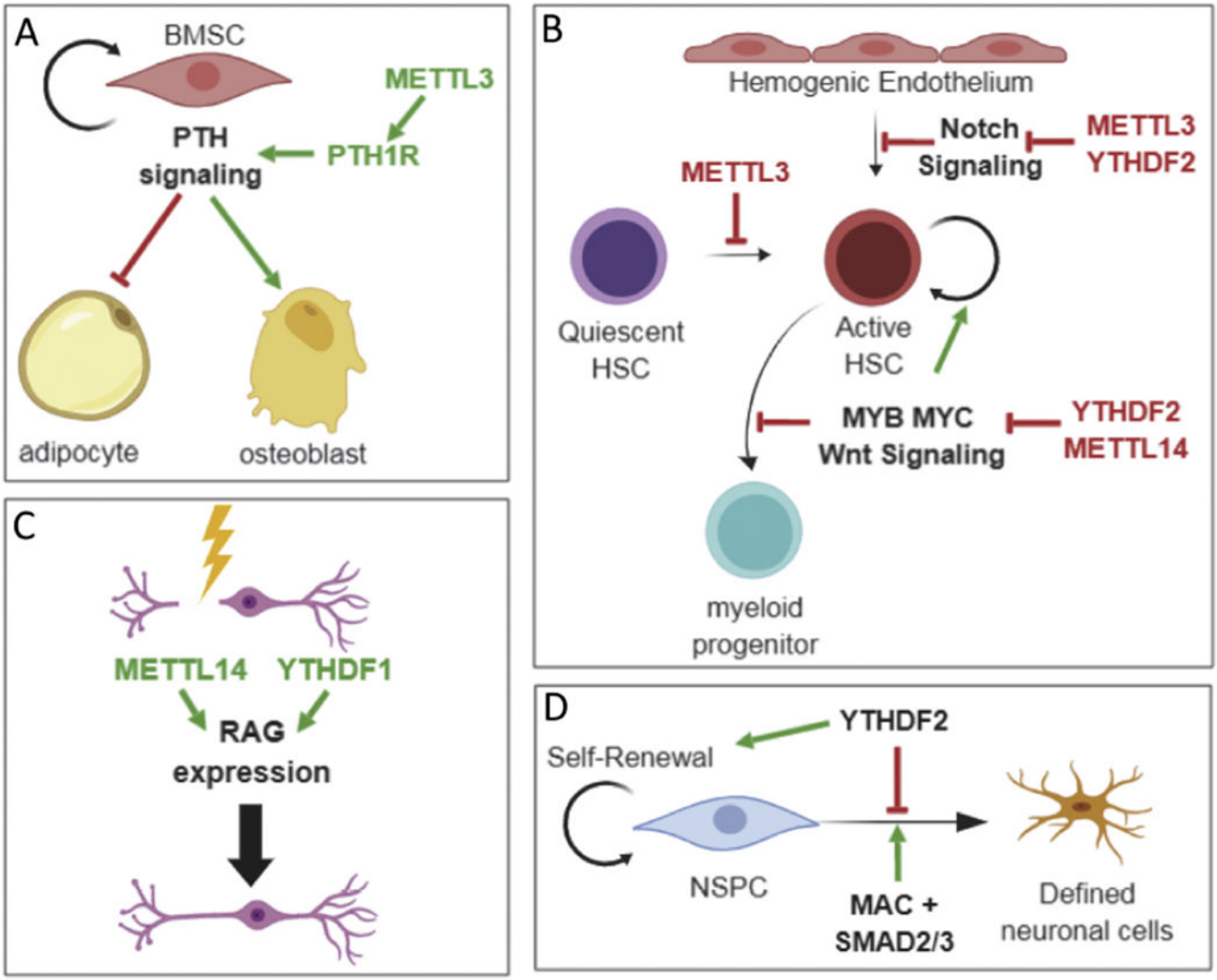


Figure 6: m^{6}A methylation influences key events during development and differentiation.

A: A: In bone marrow stem cells (BMSC), translation of the parathyroid hormone receptor (PTH1R) mRNA is regulated by METTL3-mediated methylation to favor production of osteoblasts. B: B: m^{6}A influences hematopoiesis at multiple steps. C: Following nerve injury, regeneration associated genes are subject to m^{6}A-mediated regulation to promote recovery. D: Neural Progenitor Stem Cells (NPSCs) rely on m^{6}A methylation for self-renewal and for a sharp transition upon receipt of a cue to differentiate. See text for more details.