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Post-transcriptional gene regulation by mRNA modifications

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Abstract

The recent discovery of reversible mRNA methylation has opened a new realm of post-transcriptional gene regulation in eukaryotes. The identification and functional characterization of proteins that specifically recognize RNA N^6-methyladenosine (m^6A) unveiled it as a modification that cells utilize to accelerate mRNA metabolism and translation. N^6-adenosine methylation directs mRNAs to distinct fates by grouping them for differential processing, translation and decay in processes such as cell differentiation, embryonic development and stress responses. Other mRNA modifications, including N^1-methyladenosine (m^1A), 5-methylcytosine (m^5C) and pseudouridine, together with m^6A form the epitranscriptome and collectively code a new layer of information that controls protein synthesis.

RNA from all living organisms can be post-transcriptionally modified by a collection of more than 100 distinct chemical modifications[1]. Among these modifications, N^6-methyladenosine (m^6A) has been identified as the most abundant internal modification in eukaryotic mRNA since its discovery in the 1970s[2–7]. In early experiments, radioactive labelling of methylation sites in mRNAs revealed that internal regions harboured significant radioactivity[2,3] in addition to 5′ caps. These internal regions were later characterized as primarily m^6A; the remaining fraction included 5-methylcytosine (m^5C)[7,8]. RNA m^6A is widely conserved across plants[9–12] and vertebrates[2,3,13–18], and is also found in viruses[19–23] as well as in single-cell organisms such as archaea[24,25], bacteria[26] and yeast[27] (Supplementary information S1 (box)). The amount of m^6A in isolated RNA is estimated to constitute 0.1–0.4% of all adenosine nucleotides in mammals[18,28], 0.7–0.9% of adenine nucleotides (all within GA dinucleotides) in meiotic *Saccharomyces cerevisiae*[29], and 1–15 sites per virion RNA molecule in various viruses[30]. Mutation and *in vitro* enzymatic studies have identified a consensus motif of RRm^6ACH ([G/A/U][G>A]m^6AC[U>A>C])[31–34]. However, owing to the low abundance of m^6A in mRNA and the lack of effective techniques, functional characterizations of m^6A have been largely absent over the past few decades.

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Competing interests statement

The authors declare no competing interests.

SUPPLEMENTARY INFORMATION

See online article: S1 (box) | S2 (box)

The deposition of m^{6}A is carried out by a multicomponent methyltransferase complex that was first reported in 1994 (REF. [35]). A key protein, methyltransferase-like 3 (METTL3), was subsequently identified as an *S*-adenosyl methionine-binding protein with methyltransferase capacity[36]. Recent studies have identified other components of the m^{6}A methyltransferase complex in mammals, including METTL14 (REFS [37,38]), Wilms tumour 1-associated protein (WTAP)[37,39] and KIAA1429 (REF. [40]) (FIG. 1). Homologues of human WTAP have been identified in yeast (MUM2)[41] and in plants (FKBP12-interacting protein 37 (FIP37))[12]. In 2010, we speculated that RNA modifications such as m^{6}A in mRNA could be dynamic and reversible[42]. This hypothesis was confirmed in 2011 with the discovery of the first m^{6}A demethylase[43], which rekindled interest in the biological relevance of m^{6}A. The removal of m^{6}A is facilitated by fat mass and obesity-associated protein (FTO) and alkB homologue 5 (ALKBH5)[43,44] (FIG. 1), with each possessing distinct subcellular and tissue distributions[44–48] and potentially affecting different subsets of target mRNAs. The demonstration that both these enzymes can catalyse the demethylation of m^{6}A in mRNA[43,44] provided the first evidence of reversible post-transcriptional modification in RNA transcribed by RNA polymerase II, including mRNAs and certain non-coding RNAs.

Following the use of a m^{6}A-specific antibody to identify m^{6}A sites in *S. cerevisiae*[29], m^{6}A-specific antibodies were used for immunoprecipitation followed by high-throughput sequencing to generate transcriptome-wide maps of m^{6}A, charting the m^{6}A epitranscriptome[49,50]. These studies have uncovered the presence of more than 10,000 m^{6}A sites in over 25% of human transcripts, with enrichment in long exons, near stop codons and in 3′ untranslated regions (3′ UTRs). 5′ UTRs and regions surrounding the start codon have also been shown to harbour varied levels of m^{6}A in different species or cell types, or in certain growth conditions[49,51,52]. These observations confirmed that m^{6}A is a prevalent modification in mRNA and confirmed its presence in the consensus sequence RRm^{6}ACH. Many other m^{6}A detection methods have since been developed, including advances in single-nucleotide resolution mapping and high-resolution characterization[53–56] (reviewed in REFS [57,58]).

Proteins that mediate the effects of m^{6}A have also been uncovered (see below), together establishing a complex interplay among m^{6}A deposition, removal and recognition factors (‘writers’, ‘erasers’ and ‘readers’, respectively)[59]. Writers and erasers determine the prevalence and distribution of m^{6}A, whereas readers mediate m^{6}A-dependent functions. The YT521-B homology (YTH) domain family of proteins (YTHDF1, YTHDF2, YTHDF3 and YTHDC1) are direct readers of m^{6}A and have a conserved m^{6}A-binding pocket[49,60–65]. In addition, the heterogeneous nuclear ribonucleoprotein (HNRNP) proteins HNRNPA2B1 and HNRNPC selectively bind m^{6}A-containing mRNAs[66,67]. HNRNPC recognizes m^{6}A-induced changes in mRNA secondary structures[67], whereas the exact mechanism for selective m^{6}A binding by HNRNPA2B1 remains to be elucidated.

The writers, erasers and readers impart the biological functions of m^{6}A (TABLE 1). Recent work has uncovered molecular mechanisms of m^{6}A-dependent control of mRNA fate, along with its biological consequences. In this Review, we discuss the advances in our understanding of the biological effects of this prevalent mRNA modification. We propose a role for m^{6}A in sorting groups of mRNAs for accelerated metabolism through the activity of

reader proteins, which then affects numerous biological processes such as cell differentiation and development. Furthermore, we discuss additional mRNA chemical modifications that have potential roles in post-transcriptional gene regulation.

m^{6}A modulates mRNA metabolism

N^6-adenosine methylation affects almost every stage of mRNA metabolism, from processing in the nucleus to translation and decay in the cytoplasm. Two distinct modes of function have been identified for m^{6}A readers: indirect reading and direct reading. Indirect reading involves m^{6}A alterations to RNA secondary structures, thereby rendering the RNA accessible to a unique set of RNA-binding proteins (RBPs). Direct reading involves m^{6}A selectively binding to RBPs with diverse cellular functions (TABLE 1).

m^{6}A alters RNA folding and structure

The methyl group at the $N6$ position of m^{6}A does not change Watson–Crick A•U base pairing but weakens duplex RNA by up to 1.4 kcal per mol[68]. In unpaired positions, m^{6}A stacks better than an unmodified base, thereby stabilizing surrounding RNA structures[69,70] or promoting the folding of adjacent RNA sequences[71]. Transcriptome-wide RNA structure mapping has confirmed that methylated RNA regions prefer single-stranded structures to double-stranded structures[70,72–74]. In addition, a recent report revealed that m^{6}A within coding regions could induce steric constraints that destabilize pairing between codons and tRNA anticodons, thus affecting translation dynamics[75].

Owing to these thermodynamic effects, m^{6}A-triggered structural remodelling may change the accessibility of RBP interaction motifs to RNA, a phenomenon termed the m^{6}A switch[67]. The methylation of an RRACH motif in a stem structure in several genes, for example, in metastasis associated lung adenocarcinoma transcript 1 (*MALAT1*) and CDP-diacylglycerol synthase 2 (*CDS2*), causes its destabilization and the opening of the duplex. A single-stranded U-tract is then exposed, which can be recognized by HNRNPC to regulate the splicing of these transcripts[67,76] (FIG. 2a). m^{6}A switches may be widespread across the transcriptome and therefore may have profound roles in mediating interactions between RNA and RBPs[67,76].

m^{6}A affects mRNA maturation

Processing of pre-mRNA to mature mRNA consists of three main steps: 5′ capping, 3′ polyadenylation and splicing. m^{6}A was initially proposed to function as a splicing regulator, as early studies found it to be more abundant in pre-mRNA than in mature mRNA[77], with many m^{6}A sites concentrated in introns[78,79]. mRNAs that undergo alternative splicing also have more METTL3-binding sites and more N^6-adenosine methylation sites[39,49]. Writers and erasers of m^{6}A localize predominantly in nuclear speckles[39,43,44,63], which are sites of mRNA splicing and storage. PAR-CLIP (photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation) data showed that most METTL3-binding sites reside in introns[39], and the depletion of *Mettl3* in mouse embryonic stem cells (mouse ES cells) generally favours exon skipping and intron retention[80]. These results indicate that the recruitment of METTL3 to pre-mRNA is a co-transcriptional event, with methylation

potentially preceding and influencing splicing. FTO also modulates alternative splicing by removing m^{6}A around splice sites and by preventing binding of serine- and arginine-rich splicing factor 2 (SRSF2)[81]. m^{6}A readers also affect splicing. YTHDC1 recruits SRSF3 while blocking binding by SRSF10, leading to exon inclusion[63] (FIG. 2a). HNRNPA2B1 and HNRNPC are also active splicing regulators[67,82,83]. HNRNPA2B1 regulates alternative splicing events in a similar manner to METTL3 (REF. [66]), as well as microRNA (miRNA) biogenesis from intronic sequences, which is a process closely coupled with splicing[66,84] (Supplementary information S2 (box)).

`Alternative polyadenylation` (APA) is coupled to splicing of the last intron[85] and associated with mRNA N^6-adenosine methylation. Two-thirds of m^{6}A sites in the last exon are found at the 3′ UTR, where APA sites reside[56], and the knockdown of m^{6}A writers can cause APA[56]. Recent studies of APA mRNA isoforms have revealed that isoforms with m^{6}A tend to utilize proximal APA sites and have shorter 3′ UTRs compared to non-methylated isoforms[86]. Collectively, these results demonstrate that m^{6}A methylation is intimately linked to early mRNA processing.

m^{6}A enhances nuclear processing and export of mRNAs

mRNA nuclear export is a key process that connects transcription and processing in the nucleus to translation in the cytosol, and can selectively modulate gene expression[87]. N^6-adenosine methylation was suggested to promote mRNA export: depletion of *METTL3* inhibited mRNA export[88], whereas depletion of *ALKBH5* enhanced mRNA export to the cytoplasm[44]. Mechanistic details are yet to be reported, but it is conceivable that nuclear readers have an active role in this process. Facilitating mRNA export to the cytoplasm could be a major mechanism by which m^{6}A regulates gene expression.

m^{6}A promotes mRNA translation

Translation is promoted by N^6-adenosine methylation through several mechanisms. YTHDF1 globally promotes translation of m^{6}A-methylated mRNAs by binding m^{6}A-modified mRNA and recruiting translation initiation factors, thereby significantly improving the efficiency of cap-dependent translation[61]. YTHDF1 not only couples methylated transcripts with ribosomes but also recruits the translation initiation factor complex eukaryotic initiation factor 3 (eIF3) (FIG. 2a) to promote the rate-limiting step of translation. METTL3 was recently shown to function also as an m^{6}A reader by enhancing eIF4E-dependent translation in a specific subset of mRNAs by recruiting eIF3 during translation initiation[89]. This effect was independent of its methyltransferase activity or the YTHDF1–eIF3 pathway[89]. Two recent studies have further indicated that the presence of m^{6}A at the 5′ UTR improves cap-independent translation[52,90], and eIF3 was proposed to interact with m^{6}A and facilitate ribosome loading[90]. Collectively, these findings suggest several distinct mechanisms by which m^{6}A promotes mRNA translation.

m^{6}A marks mRNA for decay

Decay is the final step in mRNA metabolism, during which mRNA is destabilized and degraded. m^{6}A has been linked to reduced mRNA stability, as knockdown of *METTL3* and *METTL14* in human and mouse cells has been shown to lead to increases in the expression

of their respective target mRNAs[37,38]. Although most m^{6}A sites appear to accelerate mRNA decay, the impact on the transcriptome, including both methylated and unmethylated transcripts, can be more complex. This reasoning is because many mRNAs encoding transcription repressors are target substrates of the methyltransferase complex; reduced methylation of these transcripts could cause transcription repression.

Mechanistic studies of the cytoplasmic m^{6}A reader YTHDF2 (REFS [49,60]) provided the first direct evidence of an m^{6}A-dependent mRNA decay pathway[60]. Similar to YTHDF1, YTHDF2 is a two-domain protein: the carboxy-terminal YTH domain selectively binds methylated transcripts, whereas the amino-terminal functional domain delivers the YTHDF2-bound transcripts to cytoplasmic RNA decay machinery for dedicated degradation[60]. Knockdown of YTHDF2 prolonged the stability of its target mRNAs, indicating that it promotes mRNA decay of *N*^{6}-adenosine methylation. YTHDF2 was shown to associate with CCR4–NOT transcription complex subunit 1 (CNOT1)[60], which facilitates the recruitment of the CCR4–NOT complex and induces accelerated deadenylation of YTHDF2-bound mRNA[91]. The YTHDF2-dependent degradation of *N*^{6}-adenosine-methylated mRNAs represents a crucial role for m^{6}A, which is in accordance with increased gene expression observed with the knockdown of m^{6}A writers[37,38].

Additionally, the YTHDF2-bound m^{6}A sites may also be recognized by other effectors of mRNA stability, such as ELAV-like RNA binding protein 1 (ELAV1; also known as HuR)[38,92], miRNAs[93] and the Toll-like receptor (TLR) family protein members TLR3 and TLR7 (REF. [94]). Considering the proposed role of m^{6}A in miRNA biogenesis (Supplementary information S2 (box)), these pathways could intersect to cooperatively control the stability of target mRNAs. The decay of methylated transcripts appears to be a major factor in promoting mouse ES cell differentiation and facilitating mouse embryogenesis[80]. In summary, m^{6}A generally functions as a destabilizer of mRNAs and facilitates the degradation of methylated transcripts in various biological contexts.

m^{6}A sorts transcripts into a fast track for mRNA metabolism

The life cycle of mRNAs is regulated by transcriptional and post-transcriptional regulatory processes, including processing, export, translation and decay. Recent studies have revealed that m^{6}A and its related factors influence each of these steps. As these processes are generally coupled, we propose that mediators of *N*^{6}-adenosine methylation may work in concert to shape the methylation pattern and protein binding of specific transcripts, thereby affecting their metabolism. One example of such cooperation is the co-regulation of translation and decay by YTHDF1 and YTHDF2 of their shared targets[61]. Both the translation efficiency and the degradation of these mRNAs are reduced by double knockdown of YTHDF1 and YTHDF2. The combined function of the YTHDF1-dependent translation promotion and YTHDF2-dependent decay may result in a spike in protein production[61] (FIG. 2b). This effect, along with other m^{6}A-mediated effects such as accelerated export of certain methylated mRNA, suggests a critical function for m^{6}A-based gene regulation: writers and erasers dictate the levels of target-specific m^{6}A. In turn, readers decode these messages and may functionally sort methylated mRNAs into distinct functional groups. During cell differentiation and development, when the translation of groups of

transcripts is accomplished within a short time span, methylation could sort these transcript groups into a fast track for processing, translation and decay. Methylation could be particularly beneficial in grouping and synchronizing the expression of hundreds to thousands of mRNAs that otherwise may possess markedly different properties with varied stabilities and translation efficiencies. Such a mechanism may also help in generating translation 'pulses' to satisfy the need for bursts of protein synthesis as well as rapid decay to regulate cell differentiation during early development (FIG. 2b).

m^{6}A shapes cell function and identity

The molecular functions of m^{6}A collectively translate into the control of complex cellular functions. Such controls may be required during the cellular transition between distinct states during differentiation and development, when cells rapidly replace their stage-specific transcriptomes to re-establish a new identity. m^{6}A could be important in shaping the levels of mRNAs of various transcription factors and therefore may serve as barriers to or act as facilitators of these transitions.

m^{6}A functions in circadian rhythm maintenance and cell cycle regulation

One of the earliest identified effects of m^{6}A on cell function was discovered during a study of the mammalian circadian rhythm[88]. Maintenance of the circadian rhythm (clock) involves a negative feedback loop of gene expression, in which clock proteins downregulate the transcription of clock genes. However, only one-fifth of these rhythmic genes are driven by *de novo* transcription[95], indicating that post-transcriptional regulation has prominent roles in circadian rhythm control. Transcripts of numerous clock genes and clock output genes are modified by N^6-adenosine methylation[88]. *METTL3* knockdown leads to reduced N^6-adenosine methylation of two key clock genes, period circadian clock 2 (*PER2*) and aryl hydrocarbon receptor nuclear translocator like (*ARNTL*), which prolongs their nuclear retention and thereby the circadian period[88]. These results demonstrate how changes in mRNA metabolism can have prolonged effects. Similarly, the cell cycle is an oscillating process that is functionally coupled with the circadian clock[96]. A notable shift in cell cycle duration following perturbation of m^{6}A in mRNAs of transcription factors was also reported in mouse ES cells[97]. More detailed studies are needed to fully elucidate the mechanism of cell cycle regulation by m^{6}A.

m^{6}A functions in cell differentiation and reprogramming

Cell differentiation is an essential process during which a cell changes its identity and specialization. N^6-adenosine methylation in mRNAs affects cell differentiation and the expression of numerous transcription factors. For instance, m^{6}A affects the differentiation of pre-adipocytes during adipogenesis[81,98]. FTO controls m^{6}A levels, which in turn affects SRSF2 binding, thereby affecting the alternative splicing of numerous key genes that are required for adipogenesis[81,98]. ALKBH5 affects cell differentiation in human breast cancer stem cells (BCSCs)[99]. Exposure of BCSCs to hypoxia induces m^{6}A demethylation by ALKBH5 of the key pluripotency factor NANOG, which increases transcript stability and promotes BCSC proliferation.

In-depth mechanistic studies carried out in mouse ES cells have demonstrated that proper differentiation requires m^{6}A. Decreased m^{6}A levels and reduced self-renewal of mouse ES cells were observed upon partial short hairpin RNA-mediated knockdown of *Mettl3* and *Mettl14* (REF. [38]). By contrast, a CRISPR–Cas9 knockout of *Mettl3* resulted in improved self-renewal and impaired differentiation[69]. Using homologous recombination to completely inactivate *Mettl3*, a subsequent study confirmed the crucial role of METTL3 in stem cell differentiation and detailed how m^{6}A may drive mouse ES cells away from the pluripotent state[80]. *Mettl3* knockout is embryonic lethal, which is probably due to the retention of pervasive NANOG expression and severely limited `embryonic priming`. Specifically, m^{6}A supports the timely transition from naive pluripotency to lineage commitment, potentially by facilitating the decay of naive pluripotency-promoting transcripts[80]. Cell reprogramming, the reverse process of cell differentiation, was shown to be affected by METTL3 in the same study, in which the reprogramming of differentiated mouse epiblast stem cells to mouse ES cells was blocked by inactivation of *Mettl3* early during development but facilitated by inactivation late during development. Zinc finger protein 217 (ZFP217) is partially responsible for stabilizing key pluripotency and reprogramming transcripts by inhibiting their METTL3-mediated methylation, thus promoting self-renewal of mouse ES cell and reprogramming of somatic cells[97].

m^{6}A facilitates cell state transitions

The collective influence of m^{6}A affects cell function most probably through the regulation of a subset of key transcription factors that determine cell fate. *N*^{6}-adenosine methylation has been found on transcripts of numerous transcription factors that control cellular state and lineage commitment. Such transcription factors include the core pluripotency factors NANOG and the so-called Yamanaka reprogramming factors[100] — the transcription factors POU domain, class 5, transcription factor 1 (POU5F1; also known as OCT4), SOX2, MYC and Krueppel-like factor 4 (KLF4) — that are necessary and sufficient to induce the formation of pluripotent stem cells[69,97,99]. The cellular composition of expressed transcription factors can either maintain cell state or promote cell differentiation. By facilitating the downregulation of transcripts encoding such dominant transcription factors, the barrier to cell-state transition could be tuned by changes in mRNA m^{6}A levels (FIG. 3). Thus, in cells in which *N*^{6}-adenosine-methylated transcripts maintain pluripotency (or any other cell state), reducing m^{6}A levels may increase the barrier for differentiation by reducing the decay and prolonging the lifetime of these mRNAs. Conversely, in cells in which methylated transcripts drive a transition to a new cell state, reducing m^{6}A levels could promote cell differentiation, whereas increased overall methylation could induce stemness and suppress cell differentiation. Experimental results so far suggest that m^{6}A is crucial for shaping cell states during cell differentiation and development. The effects of m^{6}A on mammalian development and human diseases such as cancer progression and metastasis are only beginning to emerge (BOX 1).

m^{6}A functions in stress responses

Owing to rapid response kinetics and potential for both short-term and long-term effects, the regulation of mRNA metabolism is particularly crucial under stress conditions such as extreme temperatures, deprivation of oxygen or nutrients, and exposure to toxins. Early

work suggested that N^6-adenosine methylation on key transcripts in budding yeast is crucial for meiosis initiation in response to nitrogen starvation[74]. The m^{6}A reader protein YTHDF1 was shown to localize to stress granules along with stalled translation machinery following arsenite-induced oxidative stress, and to facilitate a post-stress recovery response by promoting translation restoration[61]. During the heat shock response, YTHDF2 localizes to the nucleus and promotes 5′ UTR methylation by inhibiting FTO binding. This process enables selective cap-independent translation under stress conditions despite global translation suppression[52]. In addition, hypoxic stress induces hypoxia-inducible factor (HIF)-dependent ALKBH5 expression and subsequent removal of m^{6}A from the transcripts encoding the pluripotency factor NANOG, leading to increased NANOG expression and BCSC proliferation[99]. These results indicate that the proposed m^{6}A-mediated fast-tracking mechanism of mRNA processing could also be employed in response to various cellular stresses in addition to affecting cell state transitions.

Other mRNA modifications

Several distinct chemical modifications are abundant in many RNA species, in particular on tRNAs, for which modifications are known to affect translation[101–104]. Apart from the 5′ cap modifications and the 3′ poly(A) tail found on eukaryotic mRNA, coding transcripts feature several chemical modifications with emerging regulatory functions. The availability of high-throughput sequencing techniques, combined with highly sensitive mass spectrometry technology, has aided the discovery of new modifications that have potential regulatory functions.

N^1-methyladenosine

First discovered in total RNA samples decades ago[105], N^1-methyladenosine (m^1A) is a modification that was previously known to regulate the structure and stability of tRNA and rRNA[106,107]. Recent studies have also revealed the presence of m^1A in eukaryo tic mRNAs[108,109]. The positive charge associated with this modification could potentially augment its biological impact by strengthening RNA–protein interactions or by altering RNA secondary structures. A recent study showed that m^1A disrupts RNA base-pairing and induces local RNA duplex melting[110]. In addition to the structural modifications induced by m^1A, two recent reports have described the transcriptome-wide distributions of m^1A in human and mouse cells and tissues[108,109]. Methylated RNA immunoprecipitation followed by high-throughput sequencing was used to map more than 7,000 m^1A locations in coding and long non-coding RNAs, and a combination of immunoprecipitation followed by sequencing with the `Dimroth rearrangement` of m^1A to m^{6}A was used to obtain high-resolution information of RNA N^1-adenosine methylation[108]. The other study exploited the inherent ability of m^1A to stall reverse transcription and identified more than 900 m^1A sites in 600 genes[109]. Both studies suggested that each modified transcript contains on average one m^1A, in contrast to m^{6}A-containing transcripts, which tend to be methylated in multiple sites. The distribution of m^1A in mRNAs is unique in its proximity to translation starting sites and the first splice site — a pattern distinct from the 3′ UTR enrichment of m^{6}A (FIG. 4a). The function of m^1A remains unclear, although it probably promotes translation[108].

Future work to identify key mediators of N^1-adenosine methylation as well as its biological functions will be essential to understanding this new component of the epitranscriptome.

5-Methylcytosine

m^5C (FIG. 4b) has long been studied as an epigenetic modification in DNA. m^5C is known to exist in eukaryotic mRNA at levels well below that of m^6A levels[8], but, until recently, its distribution and function in mRNAs have not been characterized. Adopting the `bisulfite treatment` that was originally developed for m^5C detection in DNA, several m^5C sites in tRNA and rRNA have been characterized[111–113], followed by the report of high-resolution maps of m^5C in mRNAs[114]. Following the report in yeast that tRNA methyltransferase 4 (Trm4) is a tRNA 5-cytosine methyltransferase[115], tRNA aspartic acid methyltransferase 1 (also known as Dnmt2) was reported to be a tRNA m^5C writer in several eukaryotic species and shown to have protective functions against stress-induced tRNA cleavage[111,116–118]. Another tRNA methyltransferase, NOP2/Sun RNA methyltransferase family member 2 (NSUN2, a homologue of yeast Trm4)[119], was reported to also methylate 5-cytosine in mRNAs and in various non-coding RNAs[114,120–122]. Nevertheless, biological functions of m^5C in eukaryotic mRNAs remain largely elusive, although the oxidative derivatives of m^5C, 5-hydroxymethylcytosine and 5-formylcytosine, have been detected in RNA from *Drosophila* spp. to mammalian cells and brain tissues[123–126], suggesting that it is a dynamic modification with potential regulatory roles.

Pseudouridine

The most abundant modification in cellular RNA is pseudouridine (ψ)[127] (FIG. 4b). Generated by the isomerization of uridine, this modified base is relatively highly abundant in rRNA and tRNA. Pseudouridine mapping has been recently accomplished at single-base resolution, identifying hundreds of sites in yeast mRNAs[128,129] as well as 96 sites in mammalian mRNAs[129]. A chemical biology approach was used to effectively enrich for pseudouridine-containing transcripts, revealing thousands of modified sites in mammalian mRNAs[130]. The ability of pseudouridine to alter base-pairing interactions allows it to affect not only RNA structures but also mRNA coding[131], underscoring its potential as a regulatory element. Known pseudouridine synthase enzymes have been shown to mediate isomerization in mRNA as well as tRNA, suggesting that they may serve as mRNA writer proteins[132].

2′-O-methylnucleosides

2′-*O*-methylation (2′OMe) is a common RNA modification that resides on the 2′ hydroxyl ribose moiety of all four ribonucleosides (FIG. 4b). 2′OMe has been found in all major classes of eukaryotic RNA[133–135], and its existence in human mRNA was reported at the same time that m^6A was discovered[2]. 2′OMe can inhibit A to I RNA editing *in vitro*[136]. Small nucleolar RNAs (snoRNAs) are known to guide 2′OMe on eukaryotic rRNA[137], and recent studies have suggested that certain snoRNAs may also target other RNA species such as mRNA[137,138]. Several methods exist for 2′OMe detection[139], including PCR-based quantitative methods[140,141] and a high-throughput sequencing strategy (RiboMeth-Seq)[142],

which have the potential to be applied to study mRNA modifications. However, the precise sites of 2′OMe in eukaryotic mRNA or its function are currently unclear.

Concluding remarks and perspectives

The main features of m^{6}A on mRNA are its prevalence, unique distribution patterns along transcripts and dynamic nature. The biologically relevant reversibility of m^{6}A distinguishes it from irreversible RNA *cis* elements. Additionally, although more than 7,000 human genes contain m^{6}A sites (making m^{6}A the most widespread mRNA functional element), the majority of potential m^{6}A sites (RRACH motifs) are unmethylated, and most methylated sites are only partially (6–80%) modified[67]. Such sub-stoichiometry suggests that a large dynamic margin exists for m^{6}A-based regulation across the transcriptome and that *N*^{6}-adenosine methylation provides greater capacity (compared to binary regulatory elements) for fine-tuning regulatory mechanisms. The notion of incompleteness is further supported by the highly uneven distribution of m^{6}A along transcripts and the tissue-specific m^{6}A patterns observed in many organisms. These features indicate that for each m^{6}A site on a given transcript the ratio between methylated and unmethylated forms can be dynamic, potentially allowing for a rapidly tuned response to various internal and external stimuli.

The effects of incomplete *N*^{6}-adenosine methylation may be accomplished by numerous mechanisms, and methylation patterns of individual transcripts may function as molecular markers decoded by relevant readers. mRNA transcripts could therefore be sorted into different groups with differential downstream metabolism. This hypothesis is supported by the recent report that methylated transcript isoforms have shorter 3′ UTRs and lower stability than non-methylated transcripts[86]. We propose that the ‘fast-track’ group of methylated transcripts enjoys accelerated nuclear export, translation and degradation, which are facilitated by the concerted binding of m^{6}A readers. This process means that methylated mRNAs, in particular those encoding transcription factors and regulatory proteins, can be synchronized in response to differentiation, development and other stimuli. The stimuli-triggered changes of writer and/or eraser activities may themselves be induced by transcription factors (for example, HIF-dependent ALKBH5 activity[99] and ZFP217-dependent METTL3 activity[97]) or miRNAs (such as miRNA-dependent METTL3 activity[143]), suggesting a complex interplay between m^{6}A and other regulatory pathways.

We propose two hallmarks to mRNA *N*^{6}-adenosine methylation: that it serves as a marker to group and synchronize cohorts of transcripts for fast-tracking mRNA processing and metabolism; and that it considerably affects cell-state transition during cell differentiation. How selectivity and transcript grouping are achieved and how writers, erasers and readers are coordinated in response to different signalling pathways are unknown. We propose that the same stimuli and regulatory processes that tune transcription and translation may also affect these writers, erasers and readers through different forms of post-translational modifications. For example, when certain transcription factors are activated they may directly affect the accessibility and recruitment of writers. The same signalling pathway may coordinately activate or inactivate erasers and readers through direct recruitment or post-translational modifications (FIG. 5). This process could be a fundamental mechanism that mammalian cells exploit to coordinate gene expression during development. Defects in such

processes may cause or contribute to human diseases. For example, various human cancers are known to have perturbations in the expression of m^{6}A writers, erasers and readers (BOX 1). Thus, the epitranscriptome may contribute to tumorigenesis.

Finally, m^{6}A is but one of many post-transcriptional modifications in mRNA with regulatory roles that have been discovered or re-discovered in recent years. In addition to m^{6}A, m^1A, m^5C, pseudouridine and 2′OMe, we suggest that additional regulatory chemical modifications may be discovered in mRNA. Each of these modifications may have a dedicated set of writers, erasers and readers, although some of them might be shared. The potential to decorate distinct parts of the pre-mRNA (5′ UTR, coding sequence, 3′ UTR, splice sites) could be used to modify different groups of transcripts in response to various stimuli. These mRNA chemical modifications could be regulated individually or combinatorially to affect the fate of individual mRNA species. More quantitative technologies will need to be developed to precisely map locations of these mRNA chemical modifications. Future work will also need to test and confirm the hallmarks of mRNA N^6-adenosine methylation we have proposed above and to explore the effects of various mRNA modifications in biological processes such as cell differentiation and development. Further understanding is also needed in how transcript selectivity and site selectivity are achieved, how these mRNA chemical modification processes and their connections with different signalling pathways are regulated, the interplay and potential synergy between modification regulators and other cellular components, and their roles in human physiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

_S_-Adenosyl methionine
A biochemical cofactor and methyl donor for mRNA N^6-methyladenosine methylation and other methyl group transfer processes

Epitranscriptome
The biochemical features of the transcriptome that are not genetically encoded in the ribonucleotide sequence

m^{6}A switch
mRNA sequence that adopts a secondary structure in dependence on N^6-adenosine methylation

Alternative polyadenylation
(APA). The alternative use of different polyadenylation sites at 3′ ends of transcripts

CCR4–NOT complex
The complex multi-subunit carbon catabolite repression 4 (CCR4)–negative on TATA-less (NOT) is one of the major deadenylases in eukaryotic cells

Clock output genes
A set of genes that are regulated transcriptionally by clock genes usually they control metabolic processes.

Embryonic priming
The molecular transition of mouse embryonic stem cells from a naive cell state to a more differentiated or primed cell state, resembling transitions that occur during embryonic development *in vivo*

Dimroth rearrangement
A rearrangement of 1,2,3-triazoles in which the endocyclic and exocyclic nitrogen atoms change place (here, allowing conversion of N^6-methyladenosine to N^1-methyladenosine in basic conditions)

Bisulfite treatment
Treatment of nucleic acid with bisulfite to convert cytosine to uracil, leaving 5-methylcytosine unchanged and distinguishable by reverse transcription or PCR

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Box 1

m^{6}A-related phenotypes in various organisms

In this Box, we summarize the reported phenotypic effects associated with N^6-methyladenosine (m^{6}A) and its regulatory factors in multicellular eukaryotes.

Plants

m^{6}A was first found in monocot plants[9–11] and subsequently in *Arabidopsis thaliana*, in which the writer protein MT-A70-like protein (homologue of the mammalian methyltransferase-like 3 (METLL3)) is required for embryogenesis[12]. Depletion of m^{6}A during development affects normal growth patterns and apical dominance[144]. In *A. thaliana*, m^{6}A is enriched in 3′ untranslated regions (3′ UTRs)[144] near stop codons and, uniquely, around start codons[51]. Genes with these unique m^{6}A sites are enriched in plant-specific pathways involving chloroplast components[51]. m^{6}A deposition is also positively correlated with the abundance of a large fraction of *A. thaliana* transcripts, suggesting that it has regulatory roles in plant gene expression[51]. Another study found that m^{6}A patterns differ between plant organs, suggesting that m^{6}A affects organogenesis and has cell-type specific functions[145]. Numerous homologues of mammalian m^{6}A demethylases and the YT521-B homology (YTH) domain reader proteins exist in plant genomes. At least one *A. thaliana* alkB homologue (ALKBH) protein can catalyse m^{6}A demethylation, which regulates floral transition and vegetative growth (G. Jia, personal communication, 2016).

Insects

Drosophila melanogaster was among the first organisms in which internal mRNA m^{6}A was detected[146]. Inducer of meiosis 4 (homologue of mammalian METTL3) affects Notch signalling during egg chamber development[147], and another potential component of the m^{6}A writer complex, female lethal d (homologue of the mammalian Wilms tumour 1 associated protein (WTAP)), affects sexual determination via splicing regulation of two key genes[148,149].

Fish

In zebrafish, the knockdown of *mettl3* or *wtap* leads to several developmental defects, whereas a combined knockdown leads to increased apoptosis[39]. As zebrafish development requires rapid mRNA clearance during the maternal-to-zygotic transition[150], an m^{6}A-dependent mRNA degradation mechanism was proposed and shown to be crucial for zebrafish embryogenesis based on the function of Ythdf2 (B.S.Z. and C.H., unpublished observations).

Mouse

In mice, in addition to the regulation of embryonic development by METTL3 discussed in the main text, *Alkbh5* knockout mice suffer from impaired spermatogenesis and male infertility[44], whereas fat mass and obesity-associated protein (*Fto*)-deficient mice exhibit reduced body mass and early mortality[45,151].

Human

m^{6}A is linked to numerous human diseases. Several cancer types have been linked to m^{6}A, although many of these connections are indirect[152–163]. More direct evidence emerged from the depletion of METTL3, which caused apoptosis and reduced invasiveness of cancer cells[14,89], and from the activation of ALKBH5 by hypoxia, which caused cancer stem cell enrichment[99]. m^{6}A has also been implicated in the regulation of metabolism and obesity: FTO was suggested to influence pre-adipocyte differentiation[81,98,164], and SNPs in *FTO* associate with body mass index in human populations and the occurrence of obesity and diabetes[165–169], despite a recent work arguing that the functional target of these obesity-associated SNPs is not *FTO*[170]. The connection between m^{6}A and neuronal disorders has also been documented. For instance, dopamine signalling is dependent on FTO and on N^6-adenosine methylation of key signalling transcripts[46], and mutations in the prion-like domain of the reader protein heterogeneous nuclear ribonucleoprotein A2/B1 (*HNRNPA2B1*) are known to cause neurodegeneration through dysregulated protein polymerization[171]. FTO and ALKBH5 have been associated with the developments of depressive disorders[172–175], and addiction, epilepsy, attention deficit disorder and other neurological disorders have also been associated with m^{6}A regulators[176–178]. Reproductive disorders, viral infection, inflammation are also among the diseases influenced by m^{6}A[158,179–183]. Viral RNAs carry internal m^{6}A (Supplementary information S1 (box)), which is deposited by host enzymes and could be utilized by viruses to enhance their survival in mammalian host cells[183].

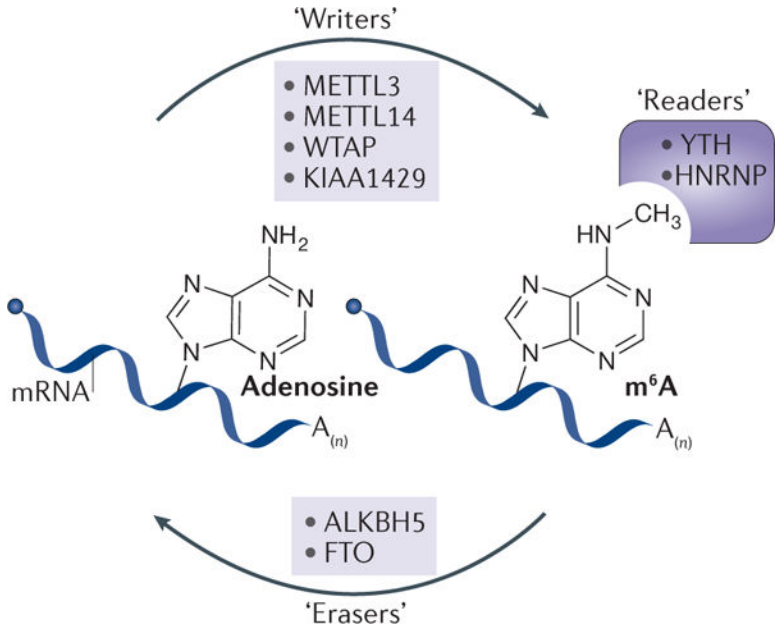

Figure 1. The writer, eraser and reader proteins of m^{6}A

The deposition, removal and recognition of *N*^{6}-methyladenosine (m^{6}A) are carried out by cognate factors termed writers, erasers and readers, respectively. Mammalian m^{6}A writers function as a protein complex with four identified components so far: methyltransferase-like 3 (METTL3), METTL14, Wilms tumour 1-associated protein (WTAP) and KIAA1429. Two m^{6}A erasers have been reported: fat mass and obesity-associated protein (FTO) and alkB homologue 5 (ALKBH5). The function of m^{6}A is mediated partly by reader proteins, which have been identified in members of the YT521-B homology (YTH) domain-containing protein and the heterogeneous nuclear ribonucleoprotein (HNRNP) protein families.

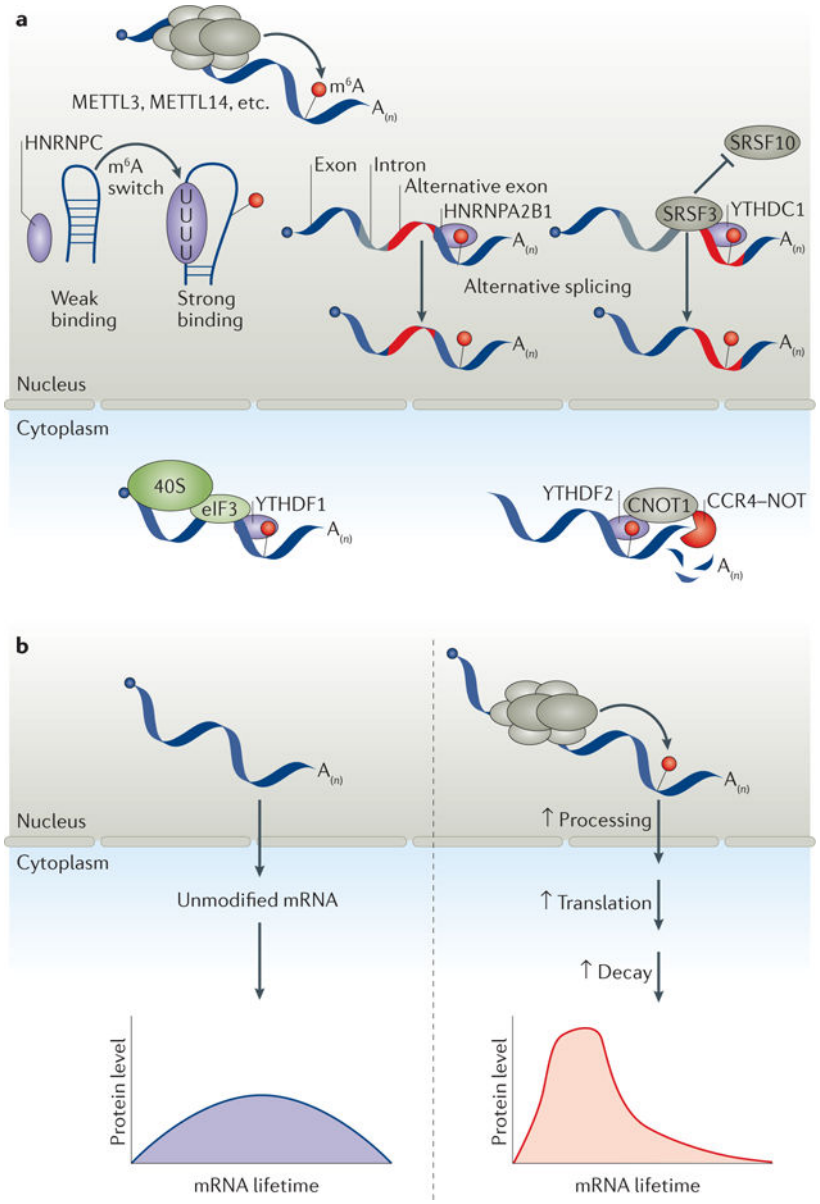

Figure 2. m^{6}A-dependent mRNA processing promotes translation and decay, and affects splicing

a| After being deposited by the methyltransferase core catalytic components methyltransferase-like 3 (METTL3) and METTL14, *N*^{6}-methyladenosine (m^{6}A) is recognized by various reader proteins. In the nucleus, heterogeneous nuclear ribonucleoprotein C (HNRNPC) functions as an indirect m^{6}A reader by binding unstructured m^{6}A switch regions and regulating splicing, whereas YT521-B homology (YTH) domain-containing 1 (YTHDC1) regulates alternative splicing by binding m^{6}A directly and recruiting the splicing factors serine and arginine-rich splicing factor 3 (SRSF3) while blocking binding by SRSF10. HNRNPA2B1 also mediates alternative splicing in a manner similar to YTHDC1. In the cytoplasm, YTHDF1 mediates translation initiation of m^{6}A-containing transcripts by binding directly to m^{6}A and recruiting eukaryotic initiation factor 3 (eIF3), thereby facilitating the loading of the eukaryotic small ribosomal subunit

(40S). YTHDF2 promotes mRNA decay by binding to CCR4–NOT transcription complex subunit 1 (CNOT1), thereby facilitating the recruitment of the CCR4–NOT complex and inducing accelerated deadenylation. **b** | Methylated transcripts may be sorted by reader proteins into a fast track (right) for processing, translation and decay. This fast-tracking effectively groups transcripts with otherwise markedly different properties to ensure their timely and coordinated translation and degradation, possibly generating a sharp ‘pulse’ of gene expression to satisfy a need for translational bursts and subsequent clearance of these transcripts.

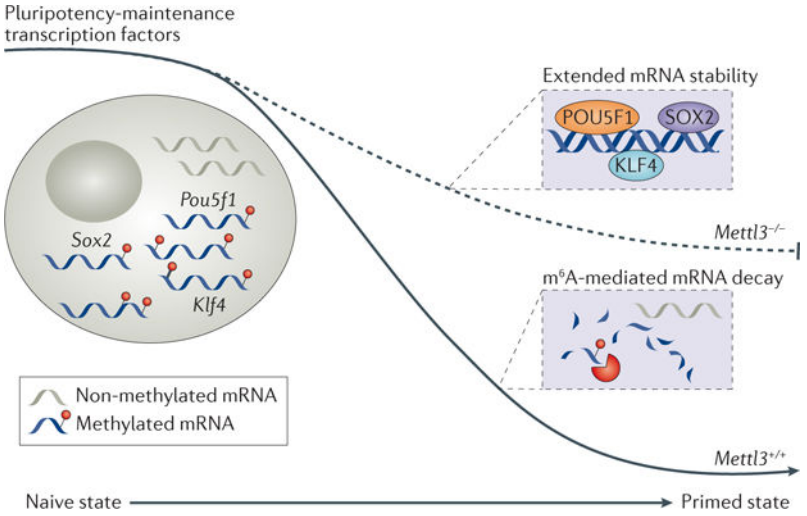

Figure 3. m^{6}A affects mouse embryonic stem cell differentiation

The N^6-methyl-adenosine (m^{6}A) methyltransferase METTL3 (methyltransferase-like 3) is required for the transition of mouse embryonic stem cells (mouse ES cells) from a naive to the more differentiated primed state. During this process, the key pluripotency factor transcripts POU domain, class 5, transcription factor 1(*Pou5f1*), Krueppel-like factor 4 (*Klf4*) and *Sox2* must be cleared. In mouse ES cells lacking *Mettl3*, this clearance is defective because non-methylated mRNAs are less subjected to decay, which prevents the establishment of a differentiated transcriptome required to achieve a primed mouse ES cell state.

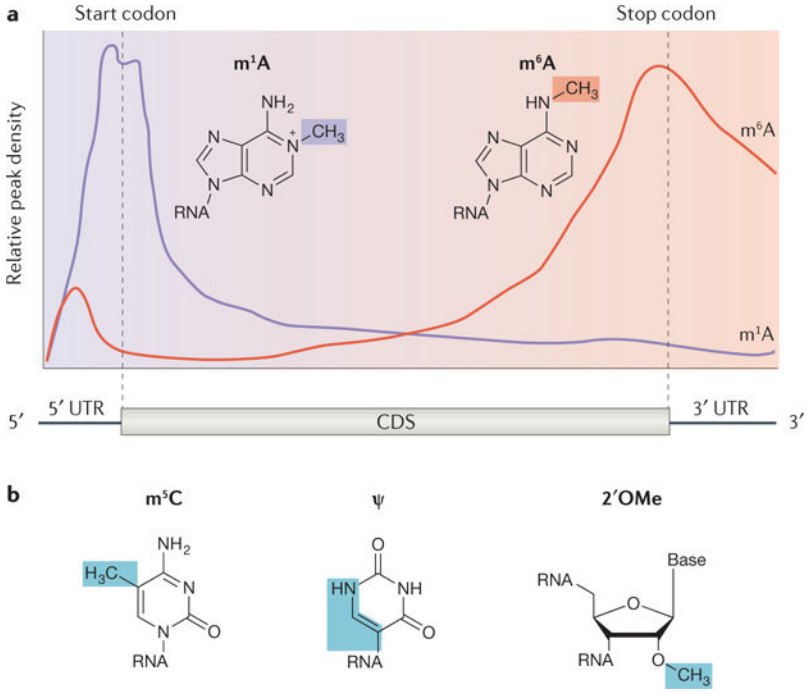

Figure 4. m^{6}A and other mRNA post-transcriptional modifications

a| Qualitative distribution profiles of *N*^{1}-methyladenosine (*m*^{1}A; purple) and *N*^{6}-methyladenosine (m^{6}A; red) in mRNA. *m*^{1}A is found primarily near translation start codons and first splice sites[108], whereas m^{6}A is primarily found in long exons and within 3′ untranslated regions (3′ UTRs)[49]. **b** | In addition to m^{6}A and *m*^{1}A, other chemical modifications found on eukaryotic mRNA with emerging regulatory functions include 5-methylcytosine (*m*^{5}C), pseudouridine (ψ) and 2′-*O*-methylation (2′OMe). CDS, coding DNA sequence.

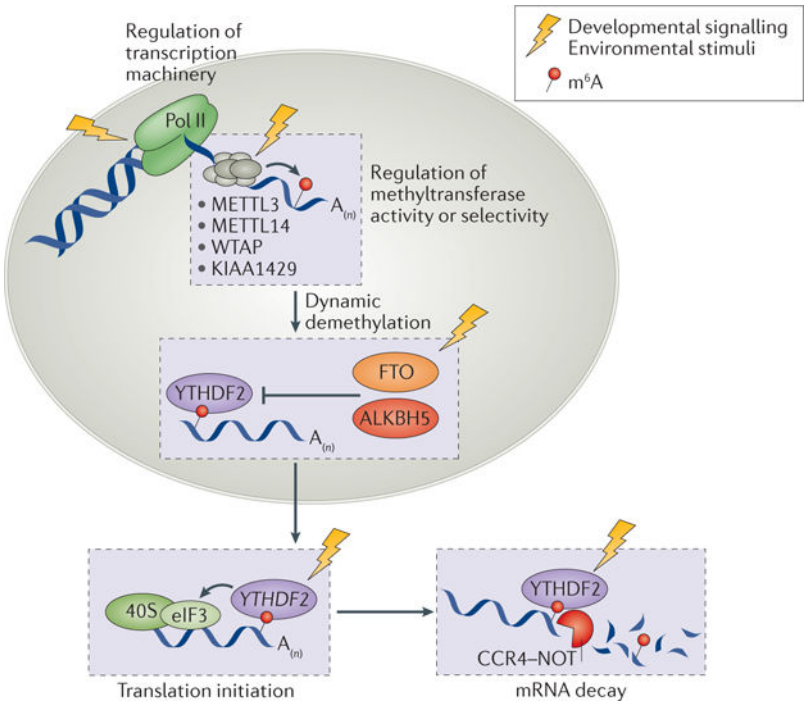

Figure 5. m^{6}A synchronizes mRNA processing in response to various internal and external stimuli

The activities of N^6-methyladenosine (m^{6}A) writers, erasers and readers may be regulated by the same signalling pathways and stimuli that tune transcription and translation, potentially through various post-translational modifications (not shown) on writers, erasers and readers. This process could constitute an additional mechanism to post-transcriptionally coordinate the expression of large groups of genes in response to internal and external stimuli, which may affect many physiological processes that require rapid responses involving multiple genes. ALKBH5, alkB homologue 5; CCR4–NOT, carbon catabolite repression 4–negative on TATA-less; eIF3, eukaryotic initiation factor 3; FTO, fat mass and obesity-associated protein; METTL, methyltransferase-like; Pol II, RNA polymerase II; WTAP, Wilms tumour 1-associated protein; YTHDF2, YT521-B homology domain-containing family protein 2.

Table 1

m^6A regulators in humans

Protein	Functional classification	m^6A-associated biological function(s)	Refs
Methyltransferase-like 3 (METTL3)	Catalytic subunit of m^6A methyltransferase	Installs m^6A; promotes translation independently of its catalytic activity	36, 37, 89
Methyltransferase-like 14 (METTL14)	A core subunit of m^6A methyltransferase	A key component for m^6A installation	37
Wilms tumour 1-associated protein (WTAP)	Regulatory subunit of m^6A methyltransferase	Facilitates m^6A installation	37,39
KIAA1429	Regulatory subunit of m^6A methyltransferase	Facilitates m^6A installation	40
Fat mass and obesity-associated (FTO)	m^6A demethylase	mRNA splicing, translation and adipogenesis	43,52, 81,98
AlkB homologue 5 (ALKBH5)	m^6A demethylase	mRNA nuclear processing, mRNA export, promotes stemness phenotype of breast cancer stem cells	44,52, 99
Heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1)	m^6A reader	mRNA splicing, miRNA biogenesis	66
Heterogeneous nuclear ribonucleoprotein C (HNRNPC)	m^6A reader that recognizes m^6A-induced structural changes	m^6A structural switch, mRNA splicing	67
YTH domain-containing 1 (YTHDC1)	Direct m^6A reader	mRNA splicing, transcriptional silencing	62,63, 184
YTH m^6A-binding protein 1 (YTHDF1)	Direct m^6A reader	Translation initiation	60,61
YTH m^6A-binding protein 2 (YTHDF2)	Direct m^6A reader	mRNA decay	60
YTH m^6A-binding protein 3 (YTHDF3)	Direct m^6A reader	Unknown	60

m^6A, N^6-methyladenosine; YTH, YT521-B homology.