RNA modifications modulate gene expression during development

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Abstract:

RNA modifications have recently emerged as critical post-transcriptional regulators of gene expression programs. They impact diverse eukaryotic biological processes and the correct deposition of many of these modifications is required for normal development. Messenger RNA modifications regulate various aspects of mRNA metabolism. For example, N^6-methyladenosine (m^6A) affects the translation and stability of the modified transcripts, thus providing a mechanism to coordinate the regulation of groups of transcripts during cell state maintenance and transition. Similarly, some modifications in transfer RNAs are essential for RNA structure and function. Others are deposited in response to external cues and adapt global protein synthesis and gene-specific translational accordingly, and thereby facilitate proper development.

Main Text:

Understanding normal tissue development and disease susceptibility requires knowledge of the various cellular mechanisms controlling gene expression in multicellular organisms. Much work has focused on investigating lineage-specific transcriptional networks governing stem cell differentiation [ref to other review articles in the special issue]. Yet, gene expression programs are dynamically regulated during development and require the coordination of both mRNA metabolism and protein synthesis. The deposition of chemical modifications onto RNA has emerged as a basic mechanism to modulate cellular transcriptomes and proteomes during lineage fate decision in development.

Many of the over 170 modifications present in RNA have been known for decades, but only in the past several years have sufficiently sensitive tools and high-resolution genome-wide techniques been developed to identify and quantify these modifications in low abundance RNA species such as mRNA (*1, 2*). Some RNA modifications have been shown to affect normal development as certain modifications can control the turnover and translation of transcripts during cell state transitions and therefore, play important roles during tissue development and homeostasis. In particular, the N^6-methyladenosine (m^6A) modification of mRNA is an essential regulator of mammalian gene expression (*3, 4*). Other modifications such as 5-methylcytosine (m^5C) and N^1-methyladenosine (m^1A) are currently best-described for their functional roles in non-coding RNAs but have also been studied in mRNA (*3, 5*).

We summarize here recent studies that elucidate the roles of RNA modifications in modulating gene expression throughout cell differentiation and animal development. Due to space limitations, we will focus on m^6A in mRNA and m^5C in tRNA as notable examples. RNA editing and RNA tail modifications, which have been comprehensively reviewed previously, will not be included.

Types of RNA modifications

Modifications in mRNA. In addition to the 5' cap and 3' polyadenylation, mRNAs contain numerous modified nucleosides, including base isomerization to produce pseudouridine (Ψ), methylation of the bases to produce m^6A, m^1A, and m^5C, methylation of the ribose sugar to install 2'O-methylation ($N_{m,}$ m^6A_m), and oxidation of m^5C to 5-hydroxymethylcytosine (hm^5C) (*3*). Of these, one of the most abundant and well-studied mRNA modifications is m^6A. 20-40% of all transcripts encoded by mammalian cells are m^6A methylated, and methylated mRNAs tend to contain multiple m^6A per transcript (*1, 2*). m^6A and other RNA modifications are also present in lncRNAs and miRNAs.

The biological functions of m^6A are mediated by writer, eraser, and reader proteins (Fig. 1A) (*3*). m^6A is installed by a multi-protein writer complex consisting of the METTL3 catalytic subunit, and many other accessory subunits (*3*). Two demethylases, FTO and ALKBH5, act as erasers (*6, 7*). m^6A can both directly and indirectly affect the binding of reader proteins on methylated mRNAs (*3*). For example, YTHDF2 binds to m^6A in mRNA and targets the transcripts for degradation (*3, 8*), and YTHDF1, YTHDF3 and eIF3a promote translation of m^6A containing transcripts (*3, 9, 10*). The list of m^6A readers regulating mRNA homeostasis is still growing (*11, 12*), and the functions of m^6A could depend on recognition by cell type-specific reader proteins. Reader and eraser proteins for other modifications are less well described.

Modifications in tRNA and rRNA. In addition to mRNA, the faithful translation of the genetic code is orchestrated by at least two more types of RNAs, tRNA and rRNA. Human rRNAs contain a set of chemical modifications which often cluster at functionally important sites of the ribosome, such as the peptidyltransferase center and the decoding site (*13*). Modification in tRNAs are the most diverse, with cytoplasmic and mitochondrial tRNAs carrying over 100 different modifications (Fig. 1B). A human tRNA can contain between 11 to 13 different modifications that are deposited at different steps during its maturation that could directly affect translation (*14*). The modifications range from simple methylation and isomerization events including m^5C, m^1A, Ψ, 5-methyluridine (m^5U), 1- and 7-methylguanosine (m^1G, m^7G), and inosine to complex multiple step chemical modifications (Fig. 1B) (*14*). The function of a modification depends both on its location in the tRNA and its chemical nature. For example, m^5C is site-specifically deposited by at least three enzymes NSUN2, NSUN3 and DNMT2 (Fig. 1B) and all three enzymes influence tRNA metabolism differently. Modifications at the wobble position are the most diverse and often optimize codon usage during gene-specific translation (Fig. 1B) (*15, 16*).

RNA modifications in development

mRNA modifications in development. A wealth of recent studies identified an essential role for m^6A during development, and many of them highlighted a role for m^6A in regulating transcriptome switching during embryonic and adult stem cell differentiation (*3*). An early clue that m^6A is essential for development was the observation that removal of the m^6A writer enzyme Mettl3 is embryonic lethal in mice (*4*). *Mettl3*$^{-/-}$ embryos appear normal pre-implantation, but begin to show defects post-implantation and are absorbed by embryonic day 8.5. Examination of gene expression from these embryos and depletion of *Mettl3* in embryonic stem cells (ESCs) further suggested impaired exit from pluripotency as, for example, expression of the pluripotency factor Nanog was sustained (*4, 17*). Transcripts encoding certain pluripotency factors are methylated (*4, 17, 18*) which affects the turnover of these transcripts during differentiation. At least some of these transcripts are co-transcriptionally methylated through the recruitment of the m^6A writer complex by cell-state specific transcription factors like Smad2 and Smad3 (*19*). Therefore, m^6A marks transcripts encoding important developmental regulators to facilitate their turnover during cell fate transitions and thereby enables cells to properly switch their transcriptomes from one cellular state to another (Fig. 2A).

This paradigm has also been used to explain the differentiation of other cell types. Conditional knockout of *Mettl3* in CD4+ T-cells prevents the proliferation and differentiation of naïve T-cells through stabilization of Socs family genes (*20*). Loss of *Mettl14* (an essential component of the METTL3/14 methyltransferase complex) in the brain delays cortical neurogenesis and is associated with slower cell cycle progression and impaired decay of transcripts involved in lineage specification of cortical neural stem cells (*21*). Similarly, deletion of *Ythdf2* delays mouse neuronal development through impaired proliferation and differentiation of neural stem/progenitor cells (*22*). m^6A-mediated RNA decay also regulates various stages of zebrafish development. For example, during the maternal-to-zygotic transition, embryos lacking *Ythdf2* exhibit impaired clearance of maternal transcripts, delaying embryonic development (*23*). Loss of *Mettl3* blocks the endothelial-to-hematopoietic transition in zebrafish due to loss of the Ythdf2-mediated decay of genes that specify endothelial cell fate, such as *Notch1a* and *Rhoca* (*24*).

While these studies highlight the functional roles of the YTHDF2-mediated clearance of mRNAs, loss of *Ythdf2* only partially accounts for phenotypes associated with loss of *Mettl3*. For example, loss of *Mettl3* impairs priming of mammalian ESCs, yet *Ythdf2* knockout embryos are able to exit pluripotency (*22, 25*). Similarly, *Mettl3* deletion in zebrafish is lethal due to severe hematopoietic defects, but adult *Ythdf2* knockout fish seem to be normal (*23, 24*). Work on gametogenesis highlights the importance of other m^{6}A eraser and reader proteins in development, as loss of *Mettl3, Mettl14*, *Alkbh5*, *Ythdf2*, and *Ythdc2* are all associated with impaired fertility and defects in spermatogenesis and/or oogenesis (*7, 25-31*). These defects were associated with the altered abundance, translation efficiency and splicing of methylated transcripts encoding regulators of gametogenesis. Work in *Drosophila* suggests important roles for m^{6}A in mediating splicing as deletion of *Ime4*, the *Mettl3* homolog, and other m^{6}A writer complex subunits reduces viability of females due to inappropriate splicing of *Sex lethal* (Sxl), an important regulator of dosage compensation and sex determination (*32, 33*).

Together, these studies demonstrate that the functional network coordinating mRNA methylation is highly complex and highlight the requirement of m^{6}A for the proper execution of stem cell differentiation programs (Fig. 2A). Transcripts maintaining a cell state are most likely co-transcriptionally decorated with m^{6}A through the recruitment of the writer complex by cell-state specific transcription factors. While m^{6}A promotes the decay of these transcripts, active transcription may maintain them at steady state levels with other readers potentially aiding in mediating their processing and translation. Upon receiving the signal(s) for cells to differentiate and repress transcription of these factors, m^{6}A coordinates the timely decay of these transcripts, allowing cells to differentiate. While other posttranscriptional mechanisms aid in promoting cell state switching, the fact that m^{6}A writers and readers are required for many of these transitions suggests that m^{6}A regulates gene expression in ways that cannot be substituted by other similar mechanisms.

tRNA modification in development. While RNA modifications are highly diverse and found in all RNA species, the recent discoveries underpin an emerging common theme: RNA modifications coordinate translation of transcripts encoding functionally related proteins when cells respond to differentiation or other cellular and environmental cues. Indeed, loss of tRNA modifying enzymes can delay stem cell differentiation, often only in distinct tissues. For instance, knockout of *Nsun2* delays stem cell differentiation in brain and skin (*34, 35*). Depletion of *Dnmt2* or the pseudouridine synthase, *Pus7,* impairs hematopoietic stem cell commitment and delays endochondral ossification respectively (*36, 37*). Knockout of *Elp3*, a core component of Elongator that modifies the tRNA wobble position, is embryonic lethal (*38*).

Several recent studies reveal that the dynamic deposition of tRNA modifications is a fast and efficient way for cells to adapt the protein translation machinery to external stimuli (*37, 39-41*). For example, self-renewing stem cells must be resilient to external differentiation cues and maintain protein synthesis at a low rate, yet their differentiation requires high levels of protein synthesis to produce committed progenitors. (*39, 42, 43*). The deposition of RNA modifications into tRNAs represents an efficient way to adapt energy requirements to specific cell states.

Recent studies discovered that tRNA modifications regulate protein translation rates during development via tRNA-derived small non-coding RNA fragments (tRFs) (*5, 37*). Loss of NSUN2-mediated methylation at the variable loop increases the affinity to the endonuclease angiogenin, promotes cleavage of tRNAs into tRFs and inhibits global protein synthesis (*34, 39*). Similarly, the Ψ writer PUS7 modifies tRNAs and thereby influences the formation of tRFs which then target the translation initiation complex (*37*). Loss of DNMT2-mediated methylation at the anti-codon loop (C38) causes both tRNA-specific fragmentation and codon-specific mistranslation (*36*). Thus, altered tRNA modification patterns shape tRFs biogenesis and determine their intracellular abundances (Fig. 2B). tRFs could act on global and gene-specific protein translation by displacing distinct RNA binding proteins and are therefore important players in stem cell differentiation (*37, 39*), sperm maturation (*44*), retrotransposon silencing (*45*), intergenerational transmission of paternally acquired metabolic disorders (*46*), and breast cancer metastasis (*47*).

Wobble tRNA modifications enhance the versatility of tRNA anti-codons to recognize mRNA to optimize codon usage and translation of cytoplasmic and mitochondrial mRNAs (Fig. 2B) (*15, 48-50*). Mitochondria are crucial players in stem cell activation, fate decisions, tissue regeneration, aging and diseases (*51*). Mitochondrial translation can be affected by mitochondrial tRNA and mRNA modifications. Mammalian mitochondria use folate-bound one-carbon (1C) units to methylate tRNA through the serine hydroxymethyltransferase 2 (SHMT2). SHMT2 provides methyl donors to produce the taurinomethyluridine base at the wobble position of distinct mitochondrial tRNAs. Loss of the catalytic activity of SHMT2 impairs OXPHOS and mitochondrial translation (*52*).

In summary, stem cell differentiation requires the constant and dynamic adaption of energy supply to fuel protein synthesis. A highly efficient and fast trigger to adapt global and gene-specifc protein translation rates to external stimuli is the dynamic deposition, removal or oxidation of m^5C to other forms of C in tRNAs.

RNA modifications in disease

tRNA modifications in disease. Complex human pathologies that are directly linked to tRNA modifications include cancer, type 2 diabetes, neurological disorders, and mitochondrial-linked disorders (*53*). The human brain is particular sensitive to defects in tRNA modifications (*54*), and the cellular defects are commonly caused by impaired translational efficiency and misfolded proteins leading to a deleterious activation of the cellular stress response.

Similar to normal tissues, tumor cells are challenged by a changing micro-environment, for example through hypoxia, inflammatory cell infiltration and exposure to cytotoxic drug treatments (*39*). Thus, tumor cell populations rely on the correct deposition of tRNA modifications to switch their transcriptional and translation programs dynamically in response to external stimuli. For instance, skin tumors lacking the NSUN2-mediated m^5C modification repress global protein synthesis leading to an enlarged undifferentiated tumour-initiating cell population (*39*). However, the up-regulation of NSUN2 and methylation of tRNAs is strictly required for cell survival in response to chemotherapeutic drug treatment, and NSUN2-negative tumors fail to regenerate after exposure to cytotoxic drug treatments (*39*). Thus, tumor-initiating cell populations require the tight control of protein synthesis for accurate cell responses and to maintain the bulk tumor.

Similarly, modifications found in other non-coding RNAs are likely to play important roles in their biogenesis and function. For instance, the biogenesis of rRNA is known to be significantly affected by various modifications, the defect of which could contribute to human ribosomopathies (*55*).

mRNA modifications in disease. mRNA modifications also contribute to the survival and growth of tumor cells, further highlighting the importance of mRNA modifications in regulating cell fate decisions. Both subunits of the m^6A writer complex, METTL3 and METTL14 are highly expressed in human hematopoietic stem and progenitor cells (HSPCs) and the expression of these two subunits declines during differentiation of HSPCs along the myeloid lineage (*56, 57*). Over-expression of METTL3 inhibits cell differentiation and increases cell growth (*56, 57*). Consistent with a role in maintaining self-renewal programs, METTL3 and METTL14 are overexpressed in acute myeloiod leukemia (AML) and AML cells are sensitive to depletion of METTL3 and METTL14 (*56-58*). These effects could be mediated by changes in the methylation of cell-state specific transcripts such as *MYC*, *MYB*, *BCL2*, *PTEN*, and *SP1* that help to maintain self-renewal and prevent differentiation (*56-58*). Interestingly, the stabilization of certain m^6A methylated transcripts in AML cells may be mediated by the IGF2BP1-3 family of m^6A reader proteins rather than the YTHDF1-3 family (*12, 57*).

An opposite role for m^6A in leukemogenesis was found in certain subtypes of AML with increased expression of the demethylase FTO, resulting in decreased m^6A and elevated levels of oncogene transcripts (*59*). Inhibition of FTO reduces AML cell proliferation and viability in these cell types (*59, 60*). The mechanisms and pathways for the writers and eraser to impact AML are likely distinct. While elevated writers block differentiation of HPSCs contributing to AML initiation and cell survival, elevated FTO mostly impacts AML proliferation. This distinction is exemplified by the dual role of the oncometabolite R-2HG; its inhibition of TET2 contributes to AML initiation but also inhibits FTO in a subset of AML leading to repressed proliferation (*60*). Decreased m^6A is also associated with some solid tumors, likely promoting their proliferation. For example, in breast cancer, hypoxia was shown to induce the overexpression of ALKBH5, an m^6A eraser, and ZNF217, a transcription factor that can inhibit METTL3, resulting in reduction of the m^6A methylation and decay of transcripts like *Nanog* (*61, 62*). Similarly, overexpression of ALKBH5 or downregulation of METTL3 or METTL14 promotes the tumorigenicity of glioblastoma cells through stabilization of pro-proliferative transcripts like *FOXM1* (*63, 64*). Additional mechanisms for how m^6A alters gene expression to help drive cancer progression are likely to be discovered in the future.

Future perspectives

While these studies demonstrate the roles of RNA modifications in various developmental processes, our understanding of how RNA modifications contribute to these processes remains incomplete, especially at the mechanistic level (Fig. 3). The development of new tools that can determine the transcriptome-wide distribution of RNA modifications at nucleotide resolution with quantitative information about the modification fraction would greatly help in these endeavors. Further, it will be essential to understand the intrinsic and extrinsic factors determining the specificity of the RNA modification writers, readers and erasers, and how these proteins are regulated in different cell types across development. For many RNA modifications, there is only very little information available on how other RNA modifications recruit or repel RNA-binding proteins, yet this information is essential for understanding how RNA modifications modulate the RNA processing or protein translation machineries. In addition, how cells adjust RNA modifications and adapt the protein synthesis machinery in response to metabolic requirements remains largely unclear, in particular, how these changes in translation could have cell-type specific effects. Finally, recent studies have suggested that m^6A could directly or indirectly influence chromatin state and transcription through regulation of chromatin regulatory complexes and lncRNAs (*65, 66*). The potential roles of m^6A and other RNA modifications in shaping chromatin states may provide additional mechanisms for explaining how these modifications contribute to gene regulation in development.

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Figures:

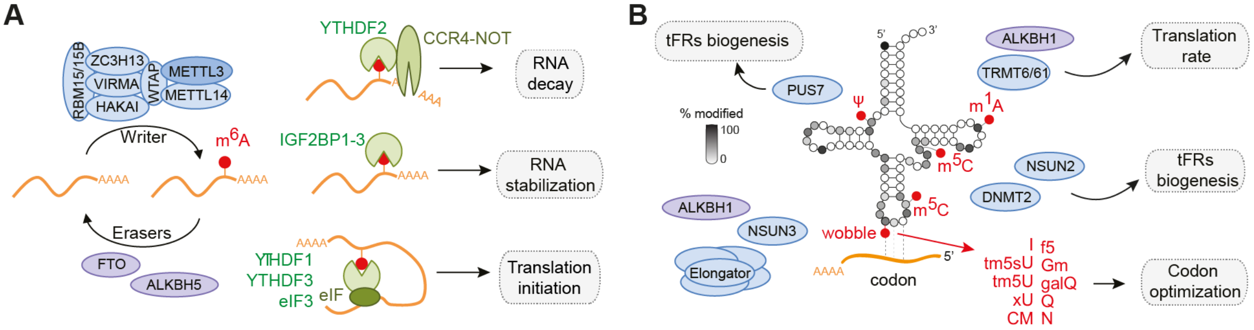

Figure 1. Regulation of gene expression by RNA modifications. (a) m^6A is installed by a multicomponent writer complex with the catalytic subunit METTL3 and removed by the demethylase enzymes FTO and ALKBH5. m^6A reader proteins can specifically bind m^6A transcripts and effect different outcomes for methylated mRNAs. **(b)** RNA modifications in human eukaryotic tRNAs according to Modomics (http://genesilico.pl/trnamodviz/jit_viz/select_tRNA/). xU: other modified uracil (U). N: unknown modified. How often a base is modified is shown by the greyscale. Only examples of writers (TRM6/61, DNMT2, NSUN2, NSUN3, PUS7 and Elongator) and erasers (ALKBH1) are shown and how they affect translation. Modifications at the wobble base are most diverse.

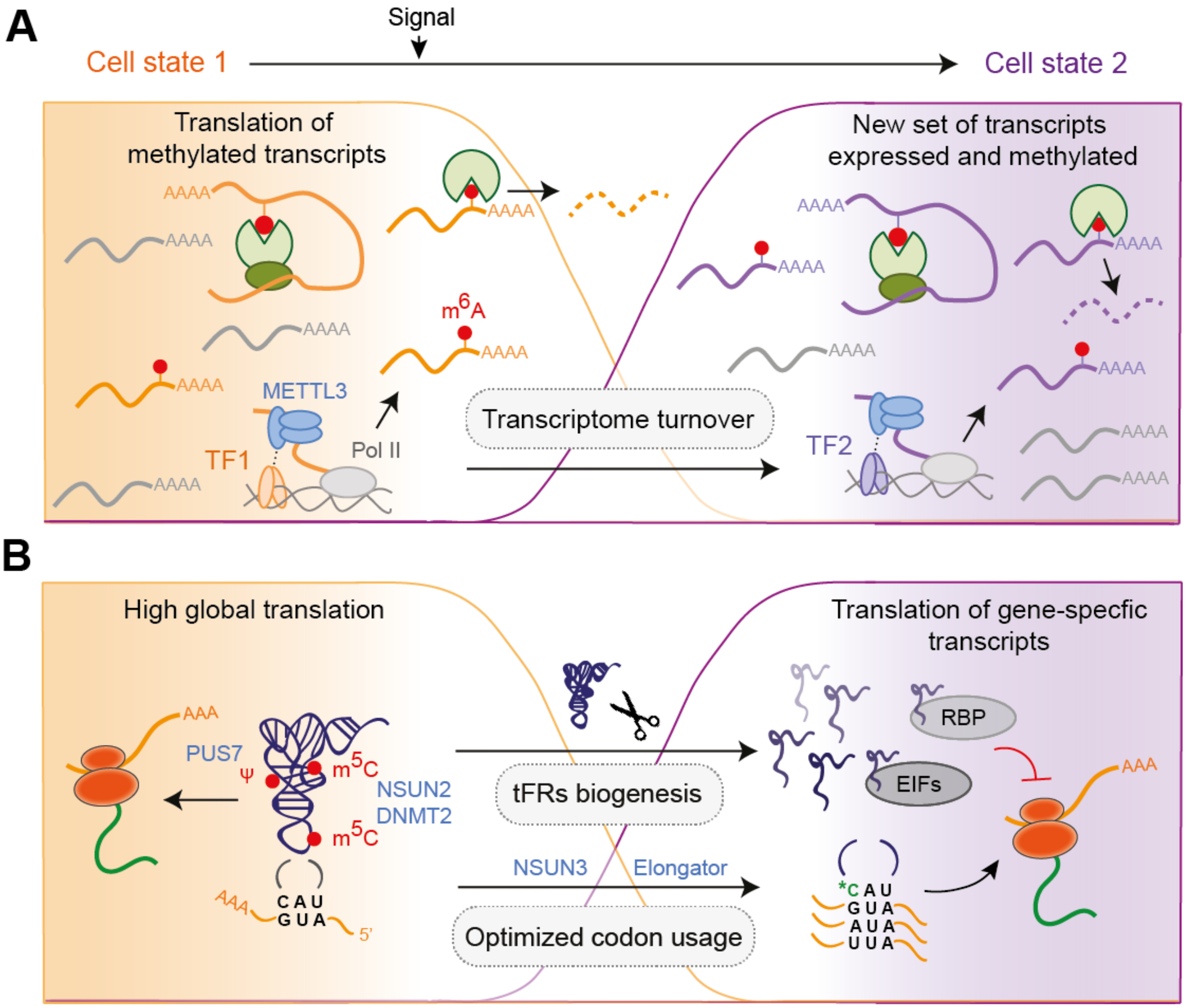

Fig. 2. RNA modifications regulate cell differentiation and development. (a) Model for the roles of m^6A in cell differentiation. In the naïve, undifferentiated state, cell-state specific master transcription factors recruit the METTL3 complex to methylate transcripts encoding cell fate factors. Translation of these methylated factors may aid in the maintenance of cell state and prevent differentiation. When cells initiate differentiation and switch their transcriptional program, reader proteins mediate the turnover of the methylated transcripts to facilitate transcriptome switching. **(b)** Modification by NSUN2, DNMT2 and PUS7 protects tRNAs from cleavage and production of tRFs, which enables high global translation. In a different cell state, tRFs can affect global and gene-specific protein translation by displacing distinct RNA binding proteins (RBP) and are therefore important players in stem cell differentiation. Wobble tRNA modifications, for example by NSUN3 and Elongator, enhance the versatility of tRNA anti-codon to recognize mRNA to optimize codon usage and translation of cytoplasmic and mitochondrial mRNAs during differentiation.

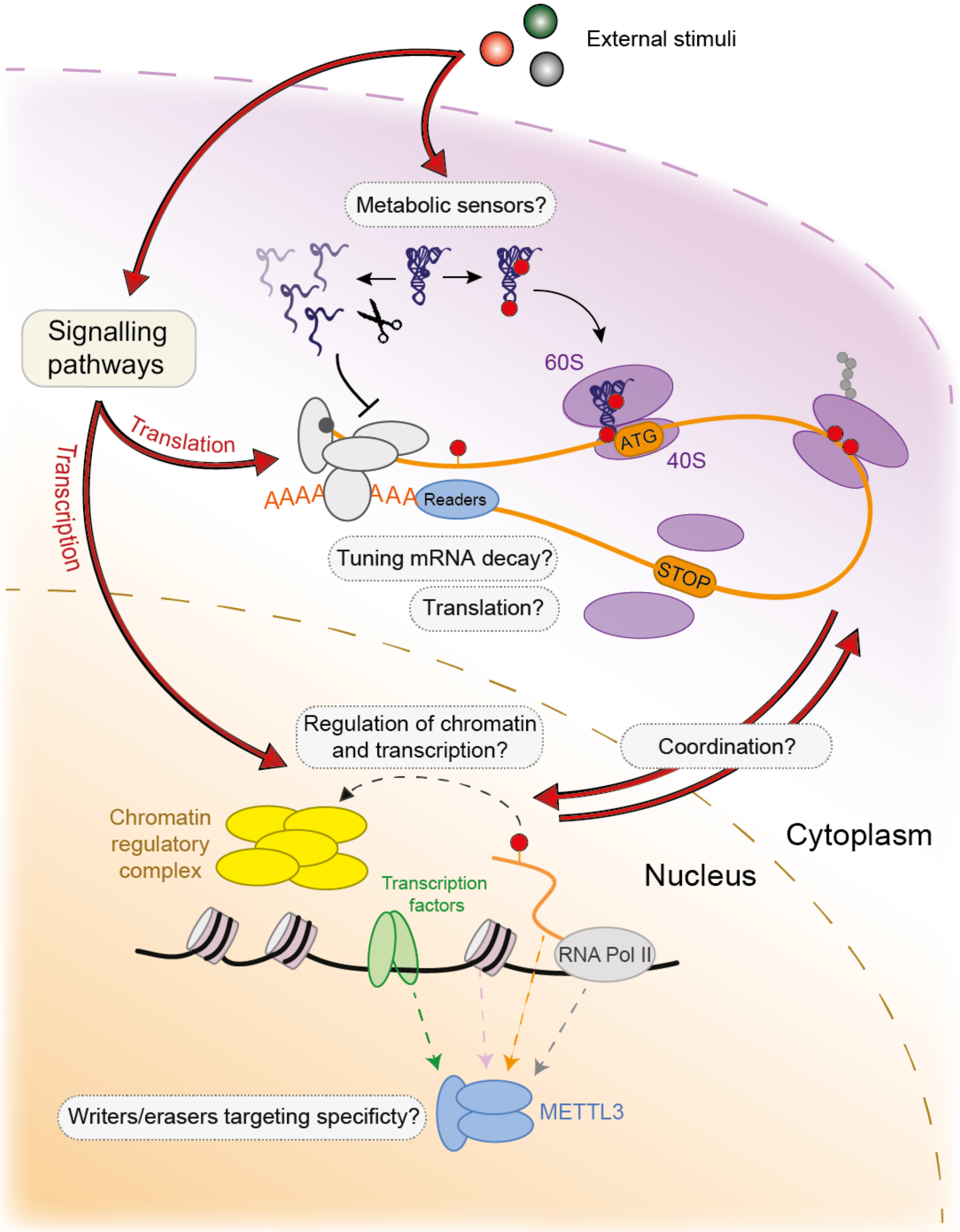

Fig. 3. Future directions for research into gene regulation by RNA modifications. Unresolved questions in the field include: Mechanistically, how do external stimuli regulate RNA modification to affect protein translation rates and transcription? How do RNA modifying enzymes act as metabolic sensors? How do RNA modifications directly or indirectly regulate chromatin regulatory complexes to affect chromatin state or transcription? What factors, such as transcription factors, chromatin, RNA, RNA binding proteins or components of the RNA polymerase II complex, recruit m^{6}A writer and eraser enzymes to their targets? How are the protein synthesis and transcription machineries coordinated by RNA modifications?