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Where, when and how: context-dependent functions of RNA methylation writers, readers, and erasers

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Abstract

Cellular RNAs are naturally decorated with a variety of chemical modifications. The structural diversity of the modified nucleosides provides regulatory potential to sort groups of RNAs for organized metabolism and functions, thus affecting gene expression. Recent years have witnessed a burst of interest in and understanding of RNA modification biology, thanks to the emerging transcriptome-wide sequencing methods for mapping modified sites, highly-sensitive mass spectrometry for precise modification detection and quantification, and extensive characterization of the modification "effectors", including enzymes ("writers" and "erasers") that alter the modification level and binding proteins ("readers") that recognize the chemical marks. However, challenges remain due to the vast heterogeneity in expression abundance of different RNA species, further complicated by divergent cell-type-specific and tissue-specific expression and localization of the effectors as well as modifications. In this review, we highlight recent progress in understanding the function of N^6-methyladenosine (m^6A), the most abundant internal mark on eukaryotic messenger RNA (mRNA), in light of the specific biological contexts of m^6A effectors. We emphasize the importance of context for RNA modification regulation and function.

eTOC Blurb

RNA N^6-methyladenosine (m^6A) has emerged as a multifaceted controller for gene expression regulation, mediated through its effector proteins—writers, readers, and erasers. *Shi et al.* review recent advances in the mechanistic understandings of m^6A effectors in various biological systems and cellular responses, emphasizing cellular and molecular contexts as important determinants of RNA modification functions.

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Declaration of Interests

C.H. is a scientific founder of Accent Therapeutics, Inc. and a member of its scientific advisory board.

Keywords

RNA modifications; Epitranscriptome; N^6-methyladenosine (m^6A); Gene expression regulation; METTL3/14; YTHDF proteins; FTO; Context-dependent functions

More than 150 distinct chemical marks on cellular RNA have been identified to date (Boccaletto et al., 2018) since the discovery the first structurally modified nucleoside, pseudo-uridine, in the 1950s (Cohn and Volkin, 1951). However, it was only until several years ago that board interest in RNA modification biology resurged, prompted by recognition of the prevalence and functional significance of internal messenger RNA (mRNA) modifications, most prominently N^6-methyladenosine (m^6A, Figure 1). These insights were sparked by the identification of enzymes capable of reversing m^6A (Jia et al., 2011; Zheng et al., 2013) and through the development of detection methods with improved sensitivity and high-throughput sequencing approaches to map modified sites (Dominissini et al., 2012; Meyer et al., 2012).

m^6A has been the best-characterized mRNA modification so far. It was first discovered in 1974 as the major form of internal methylation on mammalian mRNA (Desrosiers et al., 1974; Perry and Kelley, 1974) and early work showed that it occurs in a sequence context as (G/A)(m^6A)C (Schibler et al., 1977; Wei and Moss, 1977). More recent transcriptome-wide m^6A site mapping has given greater details on its localization and prominence, revealing its prevalence in thousands of transcripts and its unique and conserved distribution preferentially centered around stop codons and enriched at 3' untranslated regions (3'UTRs) in the transcriptomes of human and mice (Dominissini et al., 2012; Meyer et al., 2012). Building on the understanding of its widespread prevalence on mRNA, recent work has uncovered that m^6A plays an important role in gene expression regulation (Roundtree et al., 2017a), animal development (Frye et al., 2018), and human diseases (Hsu et al., 2017a).

The effectors in m^6A pathways include "writers" and "erasers" that respectively install and remove the methylation, and "readers" that recognize it (Figure 1). Characterization of these effector proteins in assorted biological systems have underscored multifaceted and tunable features of their functions, emphasizing local contexts as important determinants of their biological roles. Why does context matter in m^6A epitranscriptomics? On one hand, an m^6A effector itself may exhibit different expression levels, post-translational modifications (PTMs), and cellular localization, depending on cell types and/or in response to environmental stimuli. For instance, in most cell lines the m^6A demethylase FTO is largely nucleus localized and mediates ~5–10% of total mRNA m^6A demethylation; however, in certain leukemia cells, FTO is also highly abundant in cell cytoplasm and can mediate up to ~40% m^6A demethylation of total mRNA. On the other hand, the intrinsic heterogeneity of RNA molecules further complicates the local contexts facing m^6A effectors and challenges interpretation of experimental data: (1) diversity in RNA species, including mRNA, tRNA, rRNA, and other abundant non-coding/regulatory RNA; (2) a wide range of cellular abundances for RNA species including individual mRNA sequences—1 to 50 copies for most protein-coding genes, as estimated from single-cell RNA-seq with spike-in quantification (Marinov et al., 2014); (3) complex RNA secondary structures (stem, loop,

and bulge, *etc*); (4) varying compaction and therefore accessibility of individual RNA sequences depending on RNA cellular localization and translation state (Adivarahan et al., 2018); as well as other variable factors. Furthermore, *cis* elements in RNAs and *trans* elements including different RNA binding proteins (RBPs) could regulate the interactions between m^{6}A effectors and RNA substrates. Therefore, the occurrence and biological outcome of m^{6}A methylation at a given site on an mRNA could be highly context-dependent, providing a unique potential to tune gene expression in different biological processes.

In this review, we summarize recent progresses in mechanistic studies of m^{6}A effectors in the light of specific cellular and molecular contexts such as cell types, external stimuli, subcellular localization of effectors, and locations of m^{6}A sites on an mRNA. It is important to take into consideration these context complexities to refine and advance our understanding of RNA modification functions.

Writers for m^{6}A Methylation

m^{6}A is installed on mRNA co-transcriptionally by a complex composed of multiple subunits (Figure 1) with a stable core complex formed between methyltransferase-like 3 (METTL3) and methyltransferase-like 14 (METTL14) (Bokar et al., 1997; Liu et al., 2013)— the former as the catalytic subunit and the latter as an essential component to facilitate RNA binding (Wang et al., 2016a; Wang et al., 2016b). Further studies characterized a handful of additional subunits and revealed how they contribute to the activity and specificity of the writer complex. Wilms tumor 1-associating protein (WTAP) binds to METTL3/14 and is required for optimal substrate recruitment and METTL3/14 localization (Ping et al., 2014; Zhong et al., 2008); Vir like m^{6}A methyltransferase associated (VIRMA) is critical for deposition of m^{6}A specifically to the 3'UTR (Yue et al., 2018); Zinc finger CCCH-type containing 13 (ZC3H13) facilitates nuclear localization of the writer complex (Wen et al., 2018); and RNA binding motif protein 15/15B (RBM15/15B) is reported to bind U-riched regions and may facilitate methylation of certain RNAs (Patil et al., 2016). In fruit flies, Zc3h13/Flacc is shown to stabilize the interaction between Wtap/Fl(2)d and Rbm15/Nito (Knuckles et al., 2018).

Multifaceted METTL3: Cellular Localization, Post-translational Modification, and Functions

The cellular distribution of METTL3 varies among cell lines, and there are cases where cellular stress induces its redistribution (Knuckles et al., 2017; Xiang et al., 2017). When interacting with WTAP in the form of a stable dimer with METTL14, METTL3 localizes to the nuclear speckle in HeLa cells (Bokar et al., 1997; Ping et al., 2014). Functional nuclear localization signals (NLS) have been identified in both METTL3 and WTAP, of which key residue mutations abolished preferential nuclear localization of ectopic METTL3 and WTAP in HeLa cells (Scholler et al., 2018). A fraction of METTL3 protein is also detected in the cytoplasm in multiple human cancer cell lines at various percentages, including HeLa cells (Chen et al., 2015; Choe et al., 2018; Lin et al., 2016), breast cancer cells (MDA-MB-231 cells) (Alarcon et al., 2015b), and acute myeloid leukemia cells (MOLM13 cells) (Barbieri et al., 2017). In a rare instance, in mouse cortical neurons METTL14 rather than METTL3

localizes to both cytoplasm and nuclei (Merkurjev et al., 2018). It is not yet clear why METTL3 localization varies. The protein abundance ratios between METTL3 and METTL14 as well as other adaptor subunits of the writer complex could vary among cell lines, which could affect localization of METTL3. It is also possible that post-translational modifications (PTMs) alter interactions between METTL3 and its partner proteins, leading to cytoplasmic presence.

m^6A-containing genes are enriched in important cellular processes, and a subset of m^6A sites appear dynamic in response to stimuli and stress (Dominissini et al., 2012; Meyer et al., 2012). The transcript-specificity of m^6A methylation could be due to writer recruitment at desired chromatin loci, likely through transcription factors (TFs) and/or epigenetic marks (Figure 2A). After heat shock, METTL3 was observed to localize to heat-shock genes in the chromatin, and m^6A installation on those heat-shock transcripts was suggested to ensure their timely clearance after the stress (Knuckles et al., 2017). Upon DNA UV damage METTL3/14 localizes within 2 min to UV-induced damage sites, co-occurring with increased m^6A intensity (Xiang et al., 2017). In the TGFβ signaling in human pluripotent stem cells, activated transcription factors SMAD family member 2/3 (SMAD2/3) interact with METTL3/14-WTAP, facilitating co-transcriptional m^6A installation on selective transcripts (Bertero et al., 2018). A recent study in acute myeloid leukemia cells showed that a fraction of METTL3 associates with the promoter regions of ~80 active genes specified by a transcription factor CCAAT enhancer binding protein zeta (CEBPZ) independent of METTL14 (Barbieri et al., 2017). Their results suggest that specialized “adaptor” proteins might exist in different cells to target the writer to distinct sets of genes in the chromatin, resulting in transcript-specific m^6A methylation. Moreover, a gene-body enriched histone modification Histone H3 trimethylation at lysine 36 (H3K36me3) is suggested to recruit the writer complex through interaction with METTL14 in HepG2 cells, favoring m^6A installation on mRNA coding sequences (CDS) and 3’UTRs (Huang et al., 2019).

In contrast to these nuclear roles, cytoplasmic-localized METTL3 is suggested not to function as an m^6A writer, but instead as a potential m^6A reader (Figure 2B). Independent of its catalytic activity, METTL3 in the cytoplasm in lung cancer cells promotes translation of a reporter mRNA when tethered to its 3’UTR (Lin et al., 2016). Further studies showed that this translation promotion effect depends on interaction between METTL3 and eIF3h, which are coordinately overexpressed in many types of cancers (Choe et al., 2018).

METTL3 could be modulated through PTMs or those of its interacting proteins, which could affect protein stability, localization, writer complex formation, and writer catalytic activity. Human METTL14 has been reported to be phosphorylated at residue Serine399 which lies on the protein-protein interface with METTL3, suggesting regulatory functions (Wang et al., 2016b). SUMOylation sites have also been detected on four lysine residues of human METTL3 and were shown to reduce activity of METTL3/14 in *in vitro* methylation assays (Du et al., 2018). This repressive effect was mediated by a currently unknown mechanism but not through affecting METTL3’s stability, localization, nor interaction with METTL14/WTAP. Further investigations on protein partners of METTL3 in either the nucleus or the cytosol is likely to give more clues to deciphering PTM-mediated regulation of METTL3’s

functions, especially in a biological system where phosphorylation or SUMOylation pathways are misregulated.

Methylation Specificity Driven by Both Sequence and Structure

While the METTL3/14 complex preferentially installs m^{6}A methylation in a sequence motif RRACH (R = A or G; H = A, C, or U) (Liu et al., 2013), another m^{6}A writer, methyltransferase-like 16 (METTL16) installs m^{6}A in a different sequence and structure context (Pendleton et al., 2017). Two validated substrates of METTL16 are U6 small nuclear RNA (snRNA) and a hairpin (hp1) in the 3'UTR of human methionine adenosyltransferase 2A (*MAT2A*) mRNA that encodes the *S*-adenosylmethionine (SAM) synthetase (Pendleton et al., 2017). Truncation/mutation tests, *in vitro* methylation selection assays, and a crystal structure of METTL16 with its RNA substrates together suggest that METTL16 prefers a UAC(m^{6}A)GAGAA sequence in the bulge of a stem-loop structured RNA (Doxtader et al., 2018; Mendel et al., 2018; Pendleton et al., 2017). An important functional implication for the METTL16-mediated *MAT2A* methylation is to establish a negative feedback loop for SAM homeostasis. One working model proposed was that when SAM is limiting, prolonged interaction between METTL16 and *MAT2A* hp1 promotes proper intron splicing in HEK293 cells (Pendleton et al., 2017). A different study in HeLa cells suggested that an increased stability of *MAT2A* mRNA under SAM-limiting conditions also contributes to its elevated abundance.

More recently, a new m^{6}A writer ZCCHC4 was identified to mediate methylation of A4220 on 28S rRNA within an AAC motif and also interact with a subset of mRNAs (Ma et al., 2019).

m^{6}A Readers

Characterization of m^{6}A readers have provided valuable insights into the molecular mechanisms of the m^{6}A-mediated post-transcriptional gene regulation. Methylated probe pull-down and quantitative protein mass spectrometry assays have identified multiple RBPs that favor m^{6}A probes (Dominissini et al., 2012; Edupuganti et al., 2017) through divergent binding modes (Figure 1). Diverse regulatory or functional machineries can be recruited to m^{6}A-containing mRNA through m^{6}A readers, and therefore impact the fate of the target mRNA.

An Expanding List of m^{6}A Reader Proteins Adds Complexity

One class of direct and robust m^{6}A readers are proteins containing the YT521-B homology (YTH) domain, including YTH domain family 1–3 (YTHDF1–3) and YTH domain containing 1–2 (YTHDC1–2) in humans (Figure 1). Cytoplasmic YTHDF2 promotes degradation of its target transcripts partially through recruiting the CCR4-NOT deadenylase complex (Du et al., 2016; Wang et al., 2014). By contrast, the other two cytoplasmic m^{6}A readers, YTHDF1 and YTHDF3, are suggested to promote translation of target transcripts in HeLa cells by recruiting translation initiation factors (Shi et al., 2017; Wang et al., 2015). Nuclear reader YTHDC1 has been suggested to play multiple roles including regulating mRNA splicing by preferably recruiting a certain splicing factor (Xiao et al., 2016),

expediting mRNA export (Roundtree et al., 2017b), and accelerating decay of certain transcripts (Shima et al., 2017). YTHDC2 mediates both mRNA stability and translation and regulates spermatogenesis (Hsu et al., 2017b), while its additional RNA binding domains and helicase domains render detailed mechanistic studies enigmatic.

A different group of m^{6}A readers utilize common RNA binding domains (RBDs) such as K homology (KH) domains, RNA recognition motif (RRM) domains, and arginine/glycine-rich (RGG) domains, to preferentially bind m^{6}A-containing RNAs; in some of these cases RNA structure may play a role (Figure 1). The presence of m^{6}A can remodel local RNA structure and consequently modulates RNA-protein interactions around or nearby, termed an "m^{6}A-switch" (Liu et al., 2015). Several heterogeneous nuclear ribonucleoproteins (HNRNPs) fall into this category, including HNRNPC, HNRNPG, and HNRNPA2B1, which regulate alternative splicing or processing of target transcripts (Alarcon et al., 2015a; Liu et al., 2015; Liu et al., 2017; Wu et al., 2018). Fragile X mental retardation 1 (FMR1) contains three KH domains and one RGG domain, and has been shown to prefer m^{6}A-containing RNA, impacting both RNA translation and RNA stability likely through interplay with YTHDF1 and YTHDF2 (Edupuganti et al., 2017; Zhang et al., 2018). An additional class of readers, insulin-like growth factor 2 mRNA-binding proteins 1–3 (IGF2BP1–3), was reported to stabilize target mRNA in an m^{6}A-dependent manner (Huang et al., 2018), in contrast to the role of YTHDF2 in decreasing mRNA stability. Mutation and truncation assays identified domains KH3–4 of the IGF2BP proteins critical for *in vitro* binding to an m^{6}A-modified RNA probe. A recent study added proline rich coiled-coil 2A (Prrc2a) to the list of m^{6}A readers (Wu et al., 2019), showing that recombinant Prrc2a prefers to bind a methylated probe and Prrc2a stabilizes a critical m^{6}A-modified transcript required for myelination. The exact domains of Prrc2a responsible for m^{6}A recognition remain to be explored.

We currently do not know how readers achieve selectivity towards certain m^{6}A sites or certain m^{6}A-modified transcripts. One likely scenario is that readers may be localized to different regions of mRNA through interacting with other RBPs that recognize distinct features of the RNA. IGF2BP1–3 and YTHDF2, which regulate mRNA stability in opposite directions, were shown to bind distinct sites on mRNA: the former favors 3'UTR in HEK293T cells examined while the latter shows more binding to CDS (Huang et al., 2018). RNA recognition modes of RBPs can depend on multiple variables including binding site sequences, flanking sequences, and RNA secondary structures (Dominguez et al., 2018). Therefore, RBPs that interact with reader proteins might convey specificity towards certain m^{6}A sites or m^{6}A-modified transcripts. Secondly, the density and sequence contexts of m^{6}A sites are likely to matter. Densely populated m^{6}A regions could be more frequently occupied by m^{6}A readers. In the case of FMR1, it binds consensus RNA sequences of GGA and ACU similar to the m^{6}A-containing sequence (Ascano et al., 2012; Edupuganti et al., 2017). Perhaps the protein has evolved to further recognize the methyl group in these particular sequence contexts in order to add an additional regulation layer. Thirdly, reader proteins might enrich at specific cellular compartments, and therefore preferentially interact with local RNA species. For instance, reader proteins YTHDF1–3, FMR1, and HNRNPA2B1, were identified in mammalian stress granule cores (Jain et al., 2016). YTHDF1–3, HNRNPK, and IGF2BP2–3 were found enriched in the cell protrusion compared to the cell body in breast cancer cells (Mardakheh et al., 2015). The methylation could promote

translation or affect stability of a certain group of transcripts depending on which reader is present or dominates under the specific cellular contexts (Figure 3).

Readers Acting in Response to Stimuli

It has been observed that predominately cytoplasmic YTHDF proteins could redistribute to the nuclei of cells under certain stimuli including heat shock stress and viral infection. YTHDF2 was reported to be greatly upregulated at both transcript and protein levels within hours after heat shock and the majority of YTHDF2 translocated to the nuclei (Zhou et al., 2015). The authors proposed that the nuclear YTHDF2 would compete with the demethylase fat mass and obesity-associated protein (FTO, discussed below) to prevent demethylation of 5'UTR-m^{6}A in heat shock response genes, thereby enhancing their cap-independent translation in the cytosol. *In vitro* demethylation activity of FTO was inhibited by the presence of YTHDF2, supporting the competition hypothesis. In a similar scenario where monkey kidney cells (Vero cells) were infected with enterovirus type 71, YTHDF1 and YTHDF2 were upregulated and distributed into both cytosol and the nucleus 12 and 24 hours post infection (Hao et al., 2019). Although its molecular basis and outcome remain to be elucidated, this reader translocation event emphasizes the dynamic nature and versatility in m^{6}A-mediated regulation pathways.

In post-mitotic cells, the translation-promoting effect of YTHDF1 becomes especially apparent when active translation of functional proteins is desired (Figure 3). In the dorsal root ganglion (DRG) model of injury-induced axon regeneration, regeneration-associated genes are heavily m^{6}A methylated, and YTHDF1 is required for robust global *de novo* protein synthesis during this recovery process; in comparison, the basal translation enhancing effect of YTHDF1 before the injury is minor (Weng et al., 2018). This observation resembles the initial study on YTHDF1 in HeLa cells, showing that reporter transcripts tethered by YTHDF1 demonstrated a faster recovery from the translation arrest after removal of sodium arsenite stress (Wang et al., 2015). More recently, we studied in detail how the m^{6}A- and YTHDF1-mediated translation regulation contributes to learning and memory process (Shi et al., 2018), in which the formation of long-term memory relies on production of new proteins in synapses. In the mouse model, YTHDF1 deficiency impaired hippocampus-dependent neuronal functions such as spatial learning and memory as well as contextual fear memory, which could be rescued by re-introducing YTHDF1 protein specifically in the hippocampus. At the molecular level, transcripts that are m^{6}A-modified and also bound by YTHDF1 enrich genes functioning in synaptic transmission and long-term potentiation. Interestingly, nascent protein labelling and luciferase reporter assays revealed a stimulus-dependent feature for YTHDF1: cultured neurons stimulated by potassium chloride depolarization showed YTHDF1-dependent translation enhancement in 2 to 4 hours but not before the stimulation. These studies showed that while the YTHDF1-dependent upregulation of translation could be constitutively activated in certain cells such as cancer cells (Liu et al., 2018; Wang et al., 2015), in other cells this process could be stimulation-induced. How stimuli trigger YTHDF1 to promote translation remains to be studied.

Functions of Readers: Redundant or Convoluted?

YTHDF1–3 are similar in their domain structures: they all contain an *N*-terminal low-complexity sequence and a *C*-terminal conserved YTH domain. There have been arguments that YTHDF1–3 target almost identical m^6A sites in HEK cells, supporting a model that YTHDF1–3 are redundant in function in mammals (Meyer and Jaffrey, 2017). However, in the case of endometrial cancer cells, YTHDF1 and YTHDF2 clearly target unique transcripts and respectively regulate mRNA translation and decay (Liu et al., 2018). Transcripts in the AKT pathway are highly m^6A methylated. YTHDF1 upregulates protein production from the phosphatase coding mRNA *PHLPP2* while YTHDF2 downregulates the transcript level of kinase coding mRNAs *PRR5*, *PRR5L*, and *mTOR*. Therefore, YTHDF1 and YTHDF2 collectively inhibit the downstream AKT phosphorylation, preventing the AKT pathway from being hyper-activated. Moreover, an RNA-targeting platform has been developed to study the precise functions of readers on specific transcripts by fusing the catalytically inactive Cas13b to the *N*-terminal domain of YTHDF1 or YTHDF2, respectively (Rauch et al., 2018). In this context, YTHDF2-dCas13b delivery resulted in roughly an equal decrease in mRNA and protein level, while YTHDF1-dCas13b enhanced translation with minor mRNA destabilization effect, supporting different functions of the two proteins. Taken together, the notion that YTHDF1–3 function redundantly is oversimplified.

There have been multiple examples showing a crosstalk or competition between m^6A reader proteins (Edupuganti et al., 2017; Wu et al., 2019; Zhang et al., 2018) and even between the eraser FTO and readers during certain cellular responses (Zhou et al., 2015). These proteins form a network of physical or functional interactions. Appreciating their context-dependent regulation will be important for deciphering the roles of m^6A in future studies.

m^6A Erasers

m^6A methylation can be reversed *via* active demethylation by m^6A demethylases FTO or AlkB homolog 5 (ALKBH5), thereby the methylation-dependent processes can be reversed and controlled.

The RNA Demethylation by FTO is Context Dependent

FTO, the first RNA demethylase identified, was reported to remove the methyl group of N^6-methyladenosine (m^6A) in mRNA both *in vitro* and inside cells (Fu et al., 2013; Jia et al., 2011). FTO has also been reported to demethylate N^6, 2-*O*-dimethyladenosine (m^6A_m), a modification that has an identical chemical structure in the base moiety to m^6A and is found on the second base adjacent to the 5’ cap (cap-m^6A_m) in a portion of mRNAs (Adams and Cory, 1975; Wei et al., 1975), both *in vitro* (Fu, 2012) and inside cells (Mauer et al., 2017) (Figure 4). We have comprehensively characterized substrate spectrum of FTO by carefully validating FTO binding targets from cross-linking immunoprecipitation followed by high-throughput sequencing (CLIP-Seq) results, confirming that FTO possesses effective demethylation activity towards m^1A in specific tRNAs, m^6A_m in some snRNAs, and internal m^6A and cap-m^6A_m in mRNA (Wei et al., 2018).

A recent crystal structure of human FTO bound to 6mA-modified ssDNA revealed the molecular basis of its function towards internal m^6A as well as cap m^6A_m (Zhang et al., 2019). The superimposition of FTO with NOP2/Sun RNA methyltransferase family member 6 (NSUN6), a known tRNA m^5C methyltransferase (Haag et al., 2015), revealed similarities in their overall structures, supporting that FTO can also acts as a tRNA demethylase on stem-loop substrates (Wei et al., 2018).

The spatial distribution of FTO also modulates its accessibility towards physiologically relevant substrate(s). FTO was originally reported to be a nuclear protein containing an NLS in the *N*-terminus (Sanchez-Pulido and Andrade-Navarro, 2007) and partially colocalizes with nuclear speckles (Jia et al., 2011), but it was later shown to localize in both the nucleus and the cytoplasm in certain cell lines (Aas et al., 2017; Gulati et al., 2014). Cellular localization of FTO varies among several mammalian cell lines. In certain AML cell lines, a large portion of FTO proteins localize in cell cytoplasm (Wei et al., 2018); the spatial regulation of FTO results in the distinct substrate preference in the nucleus *versus* the cytoplasm. FTO mediates mRNA m^6A and cap m^6A_m demethylation in cytoplasm but mostly mRNA m^6A demethylation in cell nucleus (Wei et al., 2018), likely because the cap moiety is bound by cap-binding proteins and not accessible for demethylation. While it is true that 5–10% of mRNA m^6A are subjected to the FTO-mediated demethylation in common cell lines such as HeLa and HEK (Jia et al., 2011; Wei et al., 2018), perhaps because nuclear mRNA accounts for a small portion of total cellular mRNA and they may not be adequately accessed by FTO, in some AML cells FTO is highly elevated, and mostly localizes in the cytoplasm. In these cells up to ~40% of all mRNA m^6A are subjected to demethylation by FTO (Li et al., 2017; Su et al., 2018; Wei et al., 2018). The highly expressed FTO and its prevalent m^6A demethylation play an oncogenic role in these AML cells; inhibition of FTO by an oncometabolite 2-hydroxyglutarate (2HG) suppresses leukemia progression (Li et al., 2017; Su et al., 2018). This effect is again context dependent, as FTO high AML cells are more sensitive to 2HG inhibition.

Consistent with this substrate preference, the transcriptome-wide distribution pattern of FTO binding sites identified in FTO CLIP-Seq also varies among cell lines (Yixing Li et al., 2019). CLIP-Seq data analysis showed that GAC- and/or GGAC-containing motifs are enriched in FTO binding sites in certain cells. Especially in HeLa cells where FTO localizes mostly inside nucleus, the binding to GAC-containing and RRACH motifs is enhanced by FTO overexpression.

What is the molecular basis for differential cellular localization of FTO? A recent study suggested that localization and expression of FTO can be regulated by its post-translational ubiquitination on Lys-216 (Zhu et al., 2018). Knocking in the ubiquitin-deficient K216R mutation reduces the nuclear expression of FTO and prohibits the nuclear translocation of FTO in response to amino acid starvation. It is also likely that FTO is recruited to different subcellular fractions by its interacting partners. It will be interesting to comprehensively investigate how the posttranslational modifications and the protein partners of FTO contribute to the spatial regulation of FTO.

m^6A versus m^6A_m Demethylation by FTO

Cap m^6A_m, residing at the +1 ribose from the 5'cap in mRNA, was discovered around almost the same time as internal m^6A (Adams and Cory, 1975; Wei et al., 1975). Unlike m^6A, which is estimated to be 0.1%−0.4% of that of adenosines (that is, present at ~3 sites per mRNA), m^6A_m mainly occurs next to the cap with the second nucleoside 2'-*O*-methylated (m^7GpppN_m). This m^7GpppN_m contains less than 30% m^6A_m (Wei et al., 1976), as measured by a labeling method. Thus, the level of m^6A_m is at or below one tenth of that of m^6A in mRNA, with the ratio m^6A_m/m^6A ratios varying between 1/10 to 1/15 in HeLa, HEK293T, 3T3-L1, MEL624 and brain tissues, as quantified in several recent studies (Engel et al., 2018; Sendinc et al., 2018; Sun et al., 2019; Wei et al., 2018) using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-QqQ-MS/MS). This ratio further decreases to ½0 to 1/30 in certain acute myeloid leukemia (AML) cells (Su et al., 2018; Wei et al., 2018).

Because of the much higher abundance of m^6A than m^6A_m in mRNA, FTO mediates predominant demethylation of m^6A *versus* m^6A_m inside cells despite m^6A_m being a preferred substrate *in vitro* (Wei et al., 2018). Both can be demethylated by cytoplasmic FTO. Since almost all mRNA caps are bound by cap-binding proteins inside the cell nucleus (Marcotrigiano et al., 1997), the lack of m^6A_m demethylation in most cell nucleus may reflect restricted cap-m^6A_m accessibility by FTO (Figure 4). In addition, recent studies (Bartosovic et al., 2017; Louloupi et al., 2018) indicated the considerable intronic binding of FTO, suggesting additional roles of FTO on pre-mRNAs or chromosome associated RNAs.

So far, all functionally relevant reports of FTO have been consistent with internal m^6A but not cap m^6A_m in mRNA being the relevant substrate, including but not limited to facilitating the 3T3-L1 cell differentiation (Zhao et al., 2014); responding to heat shock (Zhou et al., 2015) and UV damage (Xiang et al., 2017); regulating the hepatitis C virus infection (Gokhale et al., 2016); promoting the growth and self-renewal of glioblastoma stem cells (Cui et al., 2017), and playing key oncogenic roles in leukemogenesis (Li et al., 2017; Su et al., 2018), with numerous other examples in recent years (Engel et al., 2018; Yan et al., 2018; Yu et al., 2018; Zhou et al., 2018).

The mRNA cap m^6A_m Methylation Does Not Affect Transcript Stability

A main evidence to assign the FTO-mediated cap m^6A_m demethylation as functionally relevant was the reported role of cap m^6A_m in protecting mRNA from DCP2-mediated decapping and microRNA-mediated mRNA degradation (Mauer et al., 2017). When FTO was knocked down, substantial changes in the levels of FTO target mRNAs were observed; these effects were assigned to cap m^6A_m demethylation as to show its functional effect (Mauer et al., 2017). This conclusion was not supported by a more careful analysis of the correlations between changes of expression levels of transcripts containing m^6A only or m^6A_m only upon FTO knockdown (Wei et al., 2018). Only internal m^6A-modified transcripts showed correlation with the transcript level changes induced by FTO knockdown.

The confusion was recently resolved when three groups independently identified PCIF1 as the mRNA cap m^6A_m methyltransferase (Akichika et al., 2019; Boulias et al., 2018; Sendinc

et al., 2018). PCIF1 possess only cap m^6A_m but not internal methylation activity. Two research groups independently showed that the PCIF1-mediated deposition of m^6A_m does not alter gene expression or transcript stability (Akichika et al., 2019; Sendinc et al., 2018), which refutes the results and conclusion that the mRNA cap m^6A_m demethylation by FTO alters mRNA levels (Mauer et al., 2017). Thus, the transcript stabilizing effect previously observed for cap m^6A_m upon FTO knockdown is mostly derived from the stabilizing role of internal m^6As but not cap m^6A_m.

Cap m^6A_m was also proposed to promote translation efficiency of m^6A_m-containing mRNA with the FTO knockdown leading to decreased translation of several target genes (Mauer et al., 2017). This result was recapitulated in one of the recent cap-m^6A_m methyltransferase studies (Akichika et al., 2019). However, two recent studies reported that cap m^6A_m has negligible effects on translation under basal conditions (Boulias et al., 2018), or even suppresses translation based on *in vitro* translation assays and *in vivo* reporter assays (Sendinc et al., 2018). The effect of cap m^6A_m on translation could also be context dependent and remains to be clarified.

Substrate and Roles of ALKBH5

ALKBH5 is the second RNA demethylase discovered with m^6A as the only known substrate so far (Zheng et al., 2013). ALKBH5 plays an important role in mouse spermatogenesis. Its crystal structures revealed mechanism for m^6A recognition and demethylation (Xu et al., 2014). The expression patterns of ALKBH5 and FTO are distinct among tissues. For instance, in mouse, while *Alkbh5* is most highly expressed in testis, *Fto* has the highest expression in the brain. Differential expression of ALKBH5 and FTO among tissues could be one reason that these two m^6A demethylases participate in different biological pathways. They also interact with different protein partners, leading to different substrate repertoire.

ALKBH5 knockdown in human cell lines can accelerate the export of target RNAs from the nucleus to the cytoplasm and this function is affected by its demethylation activity (Zheng et al., 2013). Almost all reported ALKBH5 functional studies revealed a similar molecular pathway—ALKBH5 mediates the demethylation of 3’UTR m^6A in certain transcripts—including facilitating hypoxia induced HIF-dependent breast cancer stem cell phenotype (Zhang et al., 2016), regulating glioblastoma proliferation and tumorigenesis through the ALKBH5-FOXM1 pathway (Zhang et al., 2017), and modulating splicing and stability of long 3’ UTR mRNAs in male germ cells (Tang et al., 2018).

Conclusions and Future Perspectives

Recent years have witnessed a rapid advance in the understanding of the complexity of gene expression regulation at the level of RNA modifications. The regulatory roles of m^6A could be modulated by multiple layers of contexts: (1) cell differentiation and developmental status. The mRNA decay function of m^6A mediated by YTHDF2 is critical for cell state transitions where a transcriptome switch is typically needed (Ivanova et al., 2017; Li et al., 2018; Zhao et al., 2017). In cultured cell lines or post-mitotic cells, the role of YTHDF2 might not be as significant; (2) effects from environmental stimuli or cellular signaling. The functions of m^6A may not be activated in the absence of relevant cellular cues. Thus, simply

investigating cell line systems under standard growth conditions may not adequately capture the real biology of RNA methylation; (3) subcellular localization of m^6A effectors. Changes in protein PTMs or the presence or absence of protein partners might account for variable distribution of writer, reader, and erasers in different biological systems. The writer METTL3 shows multifaceted functions likely depending on its cellular localization and PTMs. The eraser FTO exhibits differential substrate preferences in the cytoplasm *versus* in the nucleus, and can mediate substantial mRNA demethylation if the level is elevated in cell cytoplasm. Certain RNA modifications may enrich and exhibit functions in sub-cellular compartments; (4) along the same transcript, the exact locations of m^6A or other modifications may matter and have different regulatory functions. The diversity in the binding modes of and crosstalk between different classes of m^6A readers and their protein partners may confer specificity in the fate regulation of m^6A-modified mRNA.

Moving forward, some of the key questions remaining to be addressed in the field are most likely to be context dependent. The writers and erasers likely target different groups of transcripts in different cell types and in different biological processes. The erasers and different readers may also modulate or recognize m^6A at specific regions of a transcript, leading to different functional outcomes. How are the selectivity, both transcript selectivity and site selectivity, achieved? Different readers may affect distinct sets of transcripts in different cell types or tissues. How are these readers recognizing their target transcripts? How are writers, readers and erasers regulated and integrated into diverse biological signaling and regulation? RNA and RNA modifications effector proteins can exist in different cellular compartments. The correct cellular context should be an essential element of future functional investigations of RNA modifications.

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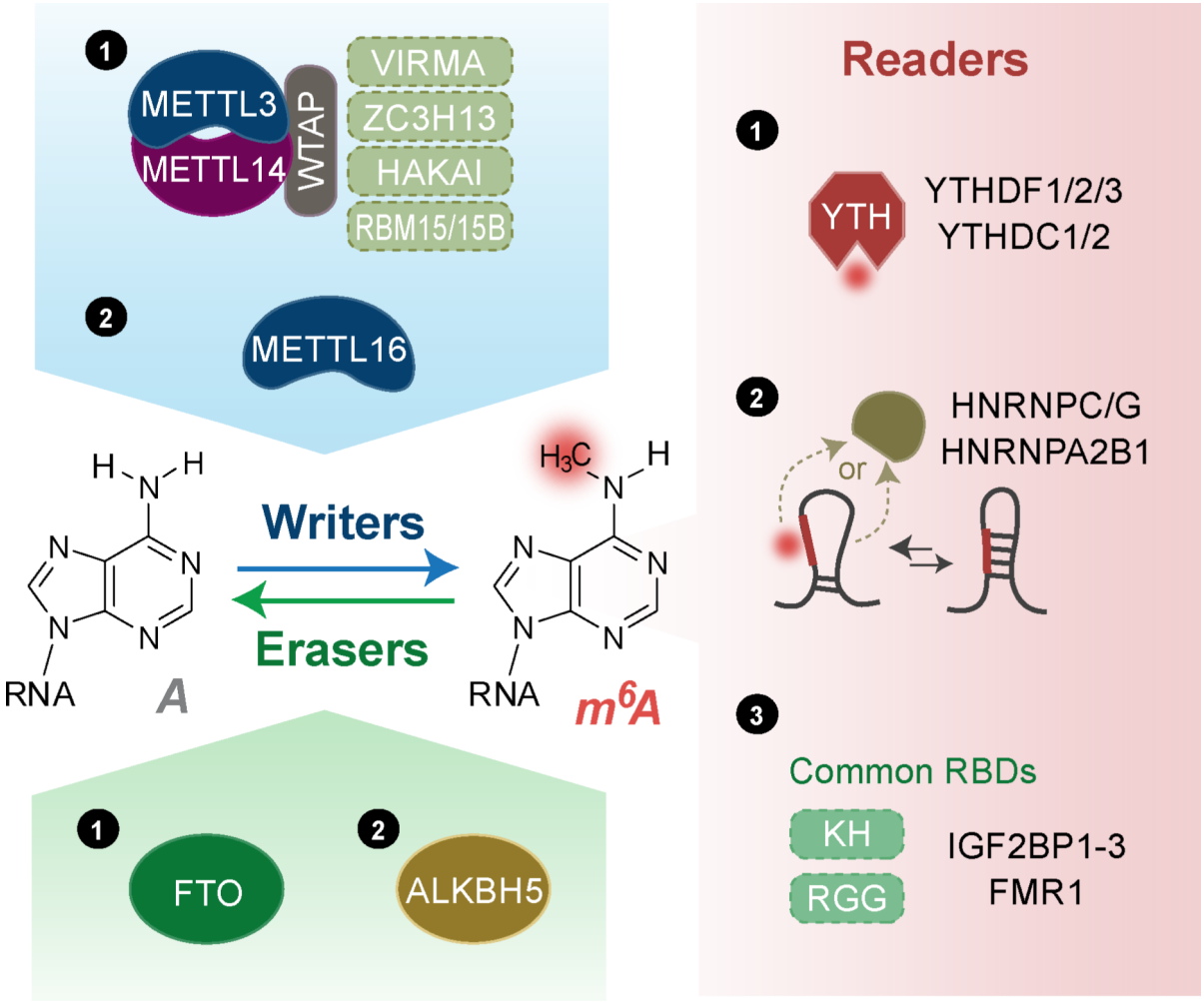

Figure 1. m^{6}A effectors: writers, erasers, and readers.

Writers: Majority of m^{6}A methylation on mRNA is installed by a writer complex ① composed of core subunits METTL3 and METTL14 and additional adaptors proteins including WTAP, VIRMA, ZC3H13, HAKAI, and RBM15/15B in a sequence context of RRACH (R = A or G; H = A, C, or U). The other known writer METTL16 ② installs m^{6}A in a sequence context of UAC(m^{6}A)GAGAA on top of a hairpin structure in transcript *MAT2A*. Erasers: two erasers have been characterized for m^{6}A methylation on mRNA, including FTO and ALKBH5. Readers: Three classes of reader proteins utilize different mechanisms to prefer binding m^{6}A-containing RNAs. ① YTH-domain containing proteins (YTHDF1–3, YTHDC1–2) use a well-characterized YTH domain to direct recognize m^{6}A methylation. ② A local structure disrupted by the presence of m^{6}A could favor RNA-binding events of HNRNPC/G and HNRNPA2B1. ③ RNA binding proteins including IGF2BP1–3 and FMR1 prefer m^{6}A-containing RNAs through their tandem common RNA binding domains (RBDs) via a mechanism yet to be characterized.

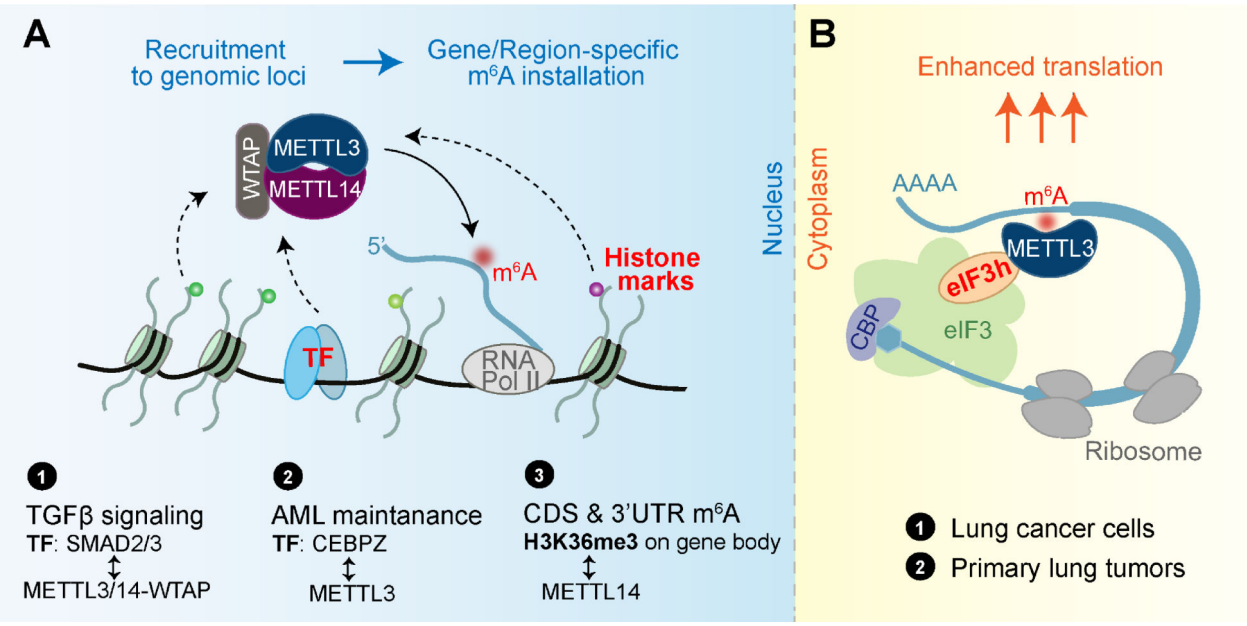

Figure 2. A model of cellular-localization dependent functions of m^{6}A writer proteins.

(A) m^{6}A writer complex installs m^{6}A co-transcriptionally in the nucleus. The recruitment of the writer complex to specific genomic loci by transcription factors (TFs) or histone marks may contribute to the gene- or region-specificity in m^{6}A installation. Examples include ① TF SMAD2/3 interacts with m^{6}A writer complex in response to TGFβ signaling; ② In acute myeloid leukemia (AML) cells, METTL3 is recruited to TSS (transcription start sites) regions with dependence on TF CEBPZ, which subsequently mediates methylation of transcripts important for cancer maintenance; ③ Gene-body enriched histone mark H3K36me3 recruits the m^{6}A writer complex by interacting with METTL14, a process contributing to preferential m^{6}A installation at CDS and 3'UTR of nascent transcripts. (B) In the cytoplasm, METTL3 itself recognizes 3'UTR m^{6}A sites on mRNA and promotes protein translation from the transcript by facilitating translation loop formation through interaction with eIF3h.

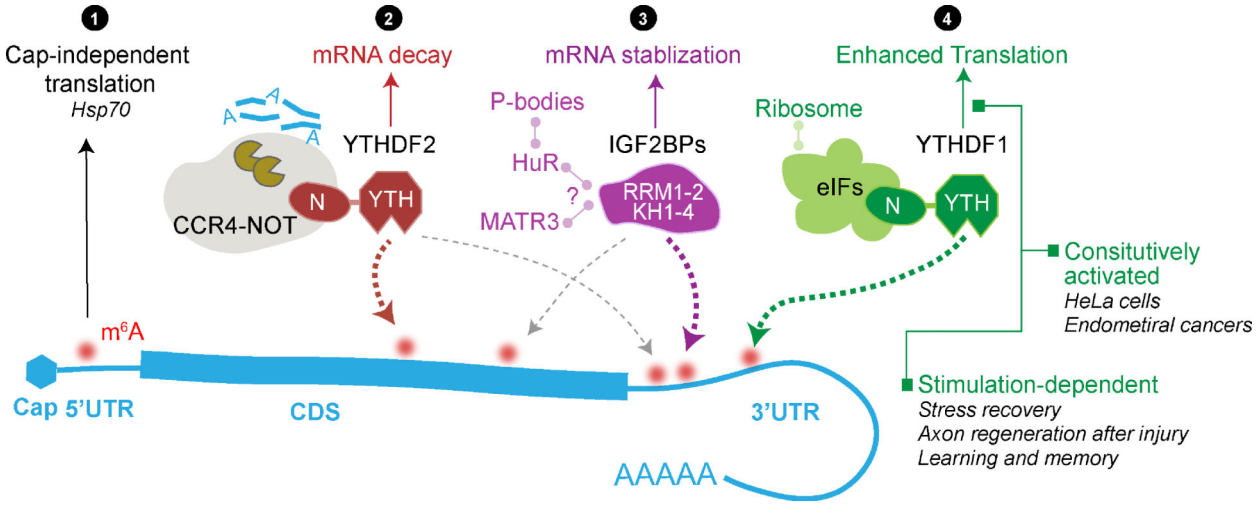

Figure 3. Region-, reader-, and stimulation-dependent roles of m^{6}A methylation on mRNA.

Multiple layers of contexts could substantially affect how m^{6}A methylation regulates the fate of modified mRNA. Region-dependent regulation: 5'UTR m^{6}A is linked to cap-independent translation, especially during stress response when cap-dependent translation is repressed (①). Reader-dependent regulation: YTHDF2 and IGF2BPs can affect mRNA stability in opposite directions. YTHDF2 recruits CCR4-NOT complex via its *N*-terminal domain to promote mRNA decay (②) while IGF2BPs stabilizes mRNA likely through co-factors HuR and MATR3 (③). They may recognize distinct m^{6}A sites and exhibit differential binding density in CDS and 3'UTR. Stimulation-dependent regulation exemplified by YTHDF1 (④): YTHDF1 preferentially binds 3'UTR m^{6}A and promotes translation through interaction with translation initiation factors. While it constitutively promotes translation in cancer cells including HeLa cells and endometrial cancer cells, in post-mitotic cells the translation promotion effect only significantly manifests when induced by stimulations such as recovery/repair processes or learning signals.

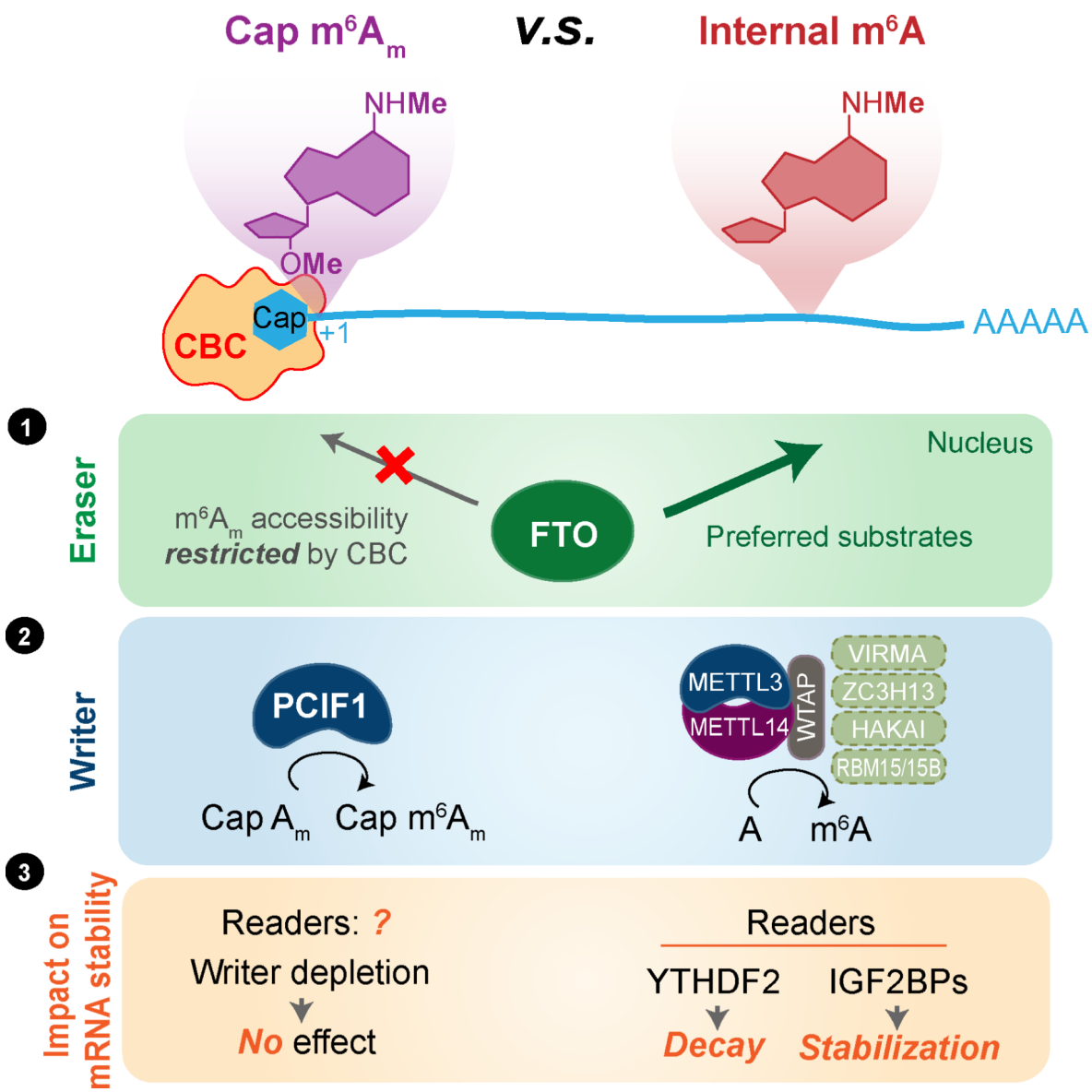

Figure 4. Distinct effectors and functions of cap *N*^{6}, 2-*O*-dimethyladenosine (m^{6}A$_m$) *versus* internal m^{6}A in mRNA.

While FTO exhibits catalytic demethylation activity towards both cap m^{6}A$_m$ and internal m^{6}A in mRNA, in the cell nucleus, m^{6}A is the main substrate of FTO since cap m^{6}A$_m$ is most likely masked by cap-binding complex (CBC) in mRNA (①). The methyl group on the *N*^{6} position of m^{6}A$_m$ is installed by a cap-specific methyltransferase PCIF1 while internal m^{6}A is installed by METTL3-METTL14 in a multi-component writer complex (②). The role of m^{6}A on mRNA stability through the m^{6}A readers have been established. However, recent characterization of the m^{6}A$_m$ writer PCIF1 showed that depletion of the writer had minimum effect on the abundance/stability of the cap-m^{6}A$_m$-modified mRNA in cell lines tested(③), refusing the role of the FTO-mediated mRNA cap m^{6}A$_m$ demethylation in affecting transcript stability.