The Role of mRNA m^{6}A in Regulation of Gene Expression

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Contents

Abstract N^6-methyladenosine (m^{6}A) is the most prevalent internal methylation in messenger RNA (mRNA). This biochemically reversible modification is deposited by m^{6}A methyltransferases, removed by m^{6}A demethylases and recognized by different RNA-binding proteins. Depending on the localization of m^{6}A and its reader proteins, an array of cellular processes ranging from RNA maturation and export in nucleus, to degradation and translation in cytoplasm, can be affected and consequently lead to diverse cell fates. The essential role of m^{6}A in normal tissue development as well as tumor progression has been revealed in the past few years, emphasizing an additional layer of gene expression regulation.

Keywords N^6-methyladenosine · m^{6}A methylation · Methyltransferase · Demethylase

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1   Introduction

N^6-methyladenosine (m⁶A) modification (Fig. 1), the most abundant internal methylation on mRNA, has emerged as a key regulatory mark on messenger RNA (mRNA). Despite the demonstration of methylation of ribosomal RNA (rRNA) and transfer RNA (tRNA) in mammalian cells by 1970, mRNA methylation was not ascertained until mRNA could be purified with polyA selection. With the pursuit of cap structure of mRNA (or heterogeneous nuclear RNA, hnRNA), Perry and Rottman laboratories found that the internal regions of mRNA and hnRNA are frequently methylated by m⁶A in mammalian cells, with an estimated occurrence of ∼1–2 m⁶A per 1000 nucleotides (Desrosiers et al. 1974; Perry et al. 1975). Subsequent mutation and enzymatic footprinting assays by a number of groups revealed that m⁶A is deposited within a consensus sequence Pu(G>A)m⁶AC(A/C/U) (where Pu represents purine) (Schibler et al. 1977; Wei and Moss 1977; Kane and Beemon 1987). In mammalian cells, the majority of m⁶A modifications are catalyzed by a multicomponent methylation complex identified by Rottman laboratory (Bokar et al. 1994), which contains the core catalytic component METTL3 (methyltransferase like 3, initially called MT-A70) (Bokar et al. 1997). m⁶A was thought to be irreversible for decades until the groundbreaking discovery by Chuan He laboratory with their exciting report of the first m⁶A demethylase FTO (fat mass and obesity-associated protein) in mammalian cells (Jia et al. 2011). Another member of AlkB subfamily of the Fe(II)/2-oxoglutarate (2OG) dioxygenase superfamily, ALKBH5 (alkB homologue 5), was soon discovered to have a similar m⁶A demethylase activity (Zheng et al. 2013). These remarkable discoveries revived the interest from RNA research community and led to the functional characterization of m⁶A. Meanwhile, transcriptomic analysis afforded by high-throughput sequencing enables global mapping and measurement of m⁶A at levels of cells, tissues and species, with the use of an antibody that specifically recognizes m⁶A (Meyer et al. 2012; Dominissini et al. 2012; Schwartz et al. 2013; Batista et al. 2014; Wang et al. 2014b). These m⁶A-seq studies, ranging from yeast to mammalian cells, confirmed that m⁶A residues within the consensus sequences are scattered in coding sequences (CDS), 3′ untranslated regions (UTRs) and 5′ UTRs, and are more enriched in the last exon around stop codon, suggesting a highly conserved m⁶A deposition

Fig. 1 The structures of mRNA methylation

N^6-methyladenosine (m⁶A) $N^{6,2'}$-O-dimethyladenosine (m⁶A$_m$)
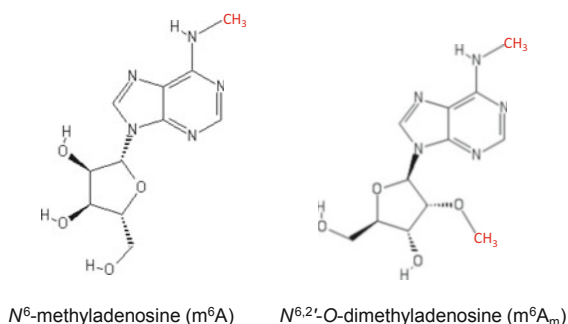
machinery. Pioneer studies by James E. Darnell and others (Sommer et al. 1978; Friderici et al. 1976) suggested a correlation between m^{6}A and mRNA instability, which has been elucidated by He laboratory and others with the demonstration of YTHDF2 (YTH domain-containing family protein 2)-dependent degradation of a subset of mRNAs bearing m^{6}A (Wang et al. 2014a; Du et al. 2016). Shortly after, m^{6}A has been shown to participate in many critical aspects of RNA regulation that include alternative splicing, nuclear export and translation, which are carried out by a variety of RNA-binding proteins and are ultimately essential for many major biological processes, such as embryonic stem cell (ESC) differentiation, circadian clock, and spermatogenesis (Geula et al. 2015; Batista et al. 2014; Wang et al. 2014b; Fustin et al. 2013; Zheng et al. 2013).

2 m^{6}A Sequencing Technologies Enable "Epitranscriptomic" Studies

The transcriptome-wide profile of m^{6}A in eukaryotic cells was first accomplished by two m^{6}A-seq (or MeRIP-seq) studies based on the immunoprecipitation with a specific anti-m^{6}A antibody and high-throughput sequencing (Dominissini et al. 2012; Meyer et al. 2012). m^{6}A-seq starts with purified RNAs that are subjected to polyA-selection or ribosomal RNA depletion, fragmentation, and immunoprecipitation followed by high-throughput sequencing. This straightforward method led to the identification of m^{6}A-marked transcripts corresponding to more than 7000 human genes. When coupled with methyltransferase depletion to reduce the detection background, this approach can achieve a relatively high resolution (Schwartz et al. 2013). Given the limitation of resolution due to the cumulative signals from multiple m^{6}A residues residing within the same ∼100–200 nucleotide fragments, the CLIP-based m^{6}A-CLIP (Cross-Linking and Immunoprecipitation) and PA-m^{6}A-seq (photocrosslinking-assisted m^{6}A sequencing) were introduced to facilitate higher resolution. Both methods use UV irradiation to capture RNA-antibody interactions by creating a covalent crosslink between the antibody and its bound RNA. However, unlike the m^{6}A-CLIP that allows the antibody to be crosslinked directly at m^{6}A sites (Ke et al. 2015; Linder et al. 2015), PA-m^{6}A-seq UV irradiation induces T-to-C transitions at 4-thiouridine residues in m^{6}A nearby regions, which therefore requires the search of consensus sequence to infer the precise m^{6}A sites (Chen et al. 2015). Consistent with the early observations regarding a biased m^{6}A distribution in 3′ end of mRNA (Perry et al. 1975), nearly all m^{6}A-seq studies have m^{6}A peaks mapped enriched in 3′ UTRs and near stop codons. It should be noted that this antibody also binds to m^{6}A$_m$, a frequent modification adjacent to the cap of mRNA, which yields "false" m^{6}A peaks within 500 nt from the transcription start site (TSS). Nonetheless, these peaks can be extracted from m^{6}A-CLIP mapped sites by in silico analysis of their overlap with TSSs and their localization in a sequence context matching the core initiator motif (Mauer et al. 2017).

3   m^{6}A Is Primarily Deposited by the METTL3–METTL14 Methyltransferase

The first cloned mRNA m^{6}A methyltransferase METTL3 was identified from a partially purified Hela cell nuclear fraction referred to as MT-A, which contains the subunit that binds to S-adenosylmethionine (SAM), the catalytic co-factor for methyltransferase (Bokar et al. 1997; Bokar et al. 1994). METTL3 has a co-localization with nuclear speckles and a widespread expression pattern in human tissues (Bokar et al. 1997). A phylogenomic analysis revealed over 40 bacterial and eukaryotic proteins that share sequence similarity with the methyltransferase domain of human METTL3 (Bujnicki et al. 2002). METTL3 orthologs are conserved throughout all eukaryotes that include *Saccharomyces cerevisiae* (IME4), *Drosophila melanogaster* (IME4) and *Arabidopsis Thaliana* (MTA). In *S. cerevisiae*, m^{6}A can be detected in sporulating cells at low levels that requires the catalytic activity of Ime4 (Inducer of Meiosis 4) (Clancy et al. 2002). In *D. melanogaster*, *Ime4* mutants are viable but develop behavioural defects with a sex bias towards maleness due to reduced female-specific splicing of the Sex lethal (Sxl), a master switch gene that controls sex determination and dosage compensation (Haussmann et al. 2016; Lence et al. 2016). The METTL3 orthologs are essential for developing embryo to progress past the globular stage in *A. thaliana* (Zhong et al. 2008) and required for embryonic development in mammalian cells (Geula et al. 2015). During the course of evolution, METTL3-mediated m^{6}A RNA modification turns to be more actively involved in critical biological processes. Noteworthy, m^{6}A is also present in bacterial mRNAs that carry a unique consensus motif of GCCAU. With a distinct modification pattern from those in eukaryotes, these m^{6}A-modified genes are associated with respiration, amino acids metabolism, stress response and small RNAs. Homologs of mammalian m^{6}A methyltransferases have not been reported in bacteria, suggesting a distinct bacterial pathway of m^{6}A modification (Deng et al. 2015).

The MT-A fraction most likely contains METTL14 (methyltransferase-like 14) as well, which partners with METTL3 to form a heterodimer (Liu et al. 2014; Wang et al. 2014b), since monomeric METTL3 is nearly inactive (Wang et al. 2016a; Scholler et al. 2018). Beyond the mammalian cells, METTL14 orthologs are present in *A. thaliana* (MTB/EMB1691) and *D. melanogaster* (CG7818). Structural analyses revealed that METTL14 has no catalytic ability, but instead, it provides an RNA-binding platform and stabilizes METTL3 conformation in the tight asymmetric heterodimer (Wang et al. 2016a, b; Sledz and Jinek 2016). A narrow groove lined with conserved positively charged residues that are contributed by the two methyltransferase domains (MTDs) in the heterodimeric METTL3-METTL14 complex, allows single-stranded RNA to fit in, so that the METTL3-METTL14 complex can methylate target adenosine in the consensus sequence of single-stranded RNAs, rather than long stable duplex structures (Wang et al. 2016a, b; Sledz and Jinek 2016; Narayan et al. 1994). In addition to the MTDs, the methyltransferase activity requires two CCCH-type zinc fingers of METTL3 and a domain of Arginine-Glycine-Glycine (RGG) repeats on the C-terminus of METTL14 (Scholler et al.
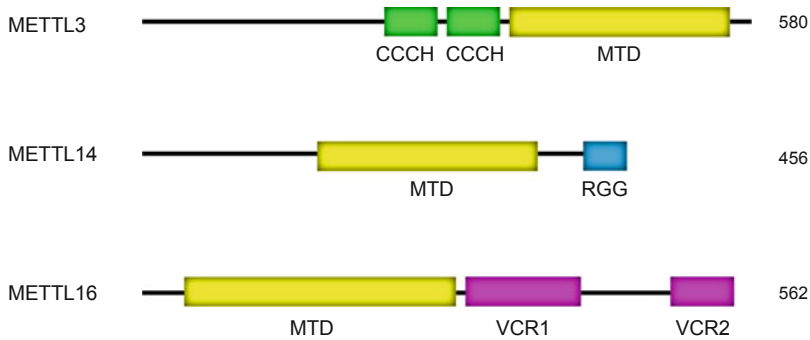
Fig. 2 Schematic domain structures of human mRNA m^{6}A methyltransferases

2018; Wang et al. 2016a) (Fig. 2). Moreover, METTL3-METTL14 can form heterotetramers through the METTL14 homodimer (Ruzicka et al. 2017; Liu et al. 2014), which may affect substrate recognition or catalytic activity.

Interestingly, whereas the METTL3-containing fraction (MT-A) from Hela cell nuclear extract displayed low methyltransferase activity, the addition of an METTL3-absent, methylation-deficient fraction (MT-B) fully restored the activity (Bokar et al. 1994, 1997). There are two possibilities that may be not mutually exclusive: MT-B releases METTL3-METTL14 from an inhibitory state of MT-A; or METTL3-METTL14 interacts with the cofactors in MT-B to create a highly active "Super Methylation Complex" (SMC). Although the exact composition of MT-B fraction (875 kDa) has yet to be defined, some lines of evidence suggest that METTL3-METTL14 may form a SMC through direct or indirect interactions with the cofactors. The evolutionarily conserved SMC is consisted of METTL3-METTL14 core methylation subunits and some cofactors that at least include WTAP (Wilms tumour 1-associated protein), KIAA1429 (VIRMA, vir like m^{6}A methyltransferase associated), RBM15/RBM15B (RNA binding motif protein 15/15B), HAKAI (E3 ubiquitin-protein ligase Hakai) and ZC3H13 (zinc finger CCCH domain-containing protein 13) (Schwartz et al. 2014; Horiuchi et al. 2013; Knuckles et al. 2018; Yue et al. 2018; Zhong et al. 2008). Whereas the core methylation subunits METTL3 and METTL14 tend to have comparable stoichiometry and remain a biochemically stable complex, the cofactors are less associated with the core subunits (Knuckles et al. 2018), which suggests that a highly active SMC may be short-lived to prevent hyperactivity of the methyltransferase, that methylation on different sites is tightly controlled by cofactors, and that heterogenous SMCs may exist in cells.

HAKAI, an E3-ubiquitin ligase that interacts with MTB in *A. thaliana* and WTAP complex through the RING finger domain, has been shown to affect m^{6}A levels to some extent (Ruzicka et al. 2017; Yue et al. 2018; Horiuchi et al. 2013). WTAP has no methyltransferase capacity, but it directly binds to METTL3 and is required for efficient methylation in vivo (Ping et al. 2014; Liu et al. 2014; Zhong et al. 2008). WTAP orthologs that include FIP37 in *A. thaliana,* Fl(2)d in *D. melanogaster* and Mum2 in *S. cerevisiae,* are all METTL3 partners. In addition, GST-WTAP has been

shown to bind to the 3′ UTR of Cyclin A2 mRNA directly in vitro (Horiuchi et al. 2006), suggesting its potential role in selectivity of m⁶A methylation.

On the other hand, the association between RBM15/RBM15B proteins and METTL3-METTL14 is WTAP-dependent. An RBM15/RBM15B-containing SMC is likely to be recruited to RBM15/RBM15B binding sites to methylate adjacent consensus motifs (Patil et al. 2016; Kan et al. 2017). Depletion of VIRMA, another WTAP-associated protein, reduces 3′ UTR methylation and seems to increase some 5′ UTR methylation as well (Yue et al. 2018). Likewise, ZC3H13 mainly affects 3′ UTR methylation and appears to bridge RBM15/RBM15B and WTAP through its C-terminal region (Wen et al. 2018; Knuckles et al. 2018). Interestingly, loss of Zc3h13 induces translocation of a great majority of WTAP, Virma, Hakai, Mettl3 and Mettl4 from nucleus to cytoplasm, where WTAP remains to be associated with Virma, Hakai, Mettl3 and Mettl4 (Wen et al. 2018). Since m⁶A modification in polyadenylated RNA has only been found to occur in the nucleus (Wen et al. 2018; Ke et al. 2017; Sommer et al. 1978), ZC3H13 with a related nuclear localization of the m⁶A processing machinery is essential for m⁶A methylation in vivo. Orthologs of VIRMA, RBM15/RBM15B, and ZC3H13 have also been found in *D. melanogaster*, suggesting an evolutionarily conserved mechanism for m⁶A regulation. Then, the question is if there is any SMC component that can stimulate the intrinsic METTL3-METTL14 activity. Thus far, it is not clear whether these proteins influence m⁶A methylation directly or indirectly since these cofactors do not exist excusively in SMC—they exert cellular functions in other complexes as well. As revealed by genetic studies in *Drosophila, ime4*, *mettl14*, *YT521-B* mutants are homozygous viable, whereas *fl(2)d* (WTAP)*, Virilizer* (VIRMA) and *Nito* (RBM15/RBM15B) mutants are homozygous lethal (Kan et al. 2017), which suggests that the participation of methyltransferase in a WTAP complex is not essentially required for the function of the latter, whose defects have broad effects on the gene expression beyond m⁶A.

Since METTL3-METTL14 complexes prepared from *E. coli*, insect cells and mammalian cells display similar activities in vitro, post-translational modifications are not expected to have a substantial impact on the methylation activity (Wang et al. 2016a). Indeed, although multiple phosphorylation sites have been identified in METTL3 and METTL14, none of them seems to affect methylation (Scholler et al. 2018). However, unlike phosphorylation, SUMOylation of METTL3 in the N-terminal region near the two CCCH motifs is suggested to repress its methyltransferase activity with an unknown mechanism (Du et al. 2018).

4 METTL16 Is an m⁶A Methyltransferase That Prefers Structured RNAs

METTL16, the mammalian ortholog of *Escherichia coli* methyltransferase for A1618 in the 23S ribosomal RNA, was reported to be an m⁶A methyltransferase for U6 snRNA A43 methylation and for ~20% m⁶As in either total RNA or

polyadenylated RNA in human HEK293 cells (Pendleton et al. 2017; Warda et al. 2017). It is found that 82% of the METTL16-dependent m⁶A sites are in introns or spanned intron-exon boundaries (Pendleton et al. 2017), and 87% of the METTL16-bound pre-mRNA introns are constitutively spliced (Warda et al. 2017). Despite a far less methylation scope relative to the METTL3-METTL14 complex, homozygous Mettl16⁻/⁻ knockout only allows mouse embryonic development until blastocyst stage (E3.5) and causes developmental arrest around the time of implantation (Mendel et al. 2018). Loss of Mettl16 leads to massive transcriptome dysregulation that includes reduced mRNA levels of the SAM synthetase Mat2a (Mendel et al. 2018), which encodes a catalytic subunit (α2) in the methionine adenosyltransferase isoenzyme MAT II. METTL16 introduces m⁶A at six vertebrate-conserved hairpin structures in the MAT2A 3′ UTR, wherein the binding of METTL16 at hp1 regulates the expression of a nuclear retained-intron isoform fated for nuclear decay. Different from hp1, m⁶As in the hp2–6 cluster promote degradation of MAT2A mRNA without affecting intron retention (Pendleton et al. 2017; Shima et al. 2017; Pendleton et al. 2018). When SAM level is low, METTL16 dwells longer on hp1, which promotes the splicing of an otherwise retained proximal upstream intron to facilitate MAT2A expression and to maintain SAM homeostasis. Of note, it is the binding of METTL16 rather than its methyltransferase activity that induces MAT2A splicing through the METTL16-VCR domains (Pendleton et al. 2017) (Fig. 2). Interestingly, MAT2A protein also associates with METTL16 in mammalian cells (Shima et al. 2017), suggesting an intimate involvement of METTL16 in the SAM-sensing feedback mechanism.

Apart from its interaction with U6 snRNA, METTL16 also associates with other non-coding RNAs, such as XIST (X inactive specific transcript) and MALAT1 (metastasis associated lung adenocarcinoma transcript 1) (Pendleton et al. 2017; Warda et al. 2017). MALAT1 contains a unique triple helix structure that is specifically recognized by METTL16 (Brown et al. 2016), suggesting that METTL16 may prefer structured RNAs. Structural analyses of METTL16 show that the RNA-binding groove comprising positively charged residues from the N-terminal module and the MTD allows RNA binding for subsequent methylation of the target adenosine, which is captured by a hydrophobic pocket (Ruszkowska et al. 2018; Doxtader et al. 2018; Mendel et al. 2018). The MTD contains a polypeptide loop that adopts an autoinhibitory conformation, characterized by a key lysine residue near the SAM binding site. Destabilizing mutations within this K-loop lead to a hyperactivity of METTL16 (Doxtader et al. 2018). Although a consensus sequence for METTL16 methylation is absent, the target adenosine must be unpaired and surrounded by stems. The transition region of the RNA stem-loop is a key region affecting catalysis, which possibly involves a disordered loop within the MTD that is not required for RNA binding (Doxtader et al. 2018; Mendel et al. 2018). Interestingly, the C-terminal region of METTL16 is required for an METTL16/ MALAT1 RNA triple helix interaction with a stoichiometry of 2:1 (Ruszkowska et al. 2018), but it is unclear whether the two METTL16 molecules exist as monomers and homodimer in the complex.

5 m⁶A Methylation Occurs Before Splicing and Depends on Transcription

The early radioactive labeling experiments by J. E. Darnell laboratory suggested that (1) m^6A deposition mainly occurs in exon regions of mRNA co-transcriptionally or shortly after transcription completion (Lavi et al. 1977; Sommer et al. 1978) and (2) methylation can occur before splicing (Chen-Kiang et al. 1979). These pioneer studies defined the m^6A-formation step in the course of mRNA biogenesis and processing in steady-state cells, although it is unknown when m^6A occurs in cells under stress conditions, such as heat shock and ultraviolet-induced DNA damage response (Zhou et al. 2015; Xiang et al. 2017). In agreement with these concepts, METTL3 can localize to transcription active sites in an RNA-dependent manner as marked by RNAP II (RNA polymerase II) co-localization (Haussmann et al. 2016). An association between METTL3 and RNAP II was observed when cells were treated with camptothecin, an topoisomerase I inhibitor (Slobodin et al. 2017). Moreover, upon transcription inhibition by actinomycin D, WTAP nulear localization is altered, which may partially explain the m^6A dependence on transcription (Little et al. 2000). On the other hand, the intronic existence of m^6A in eukaryotic cells (Carroll et al. 1990; Dominissini et al. 2012), albeit at a very low abundance, suggests that at least these mRNAs are methylated before spliced. The m^6A-CLIP study from Robert B. Darnell laboratory showed that a majority of m^6As are added to exons of nascent pre-mRNA (Ke et al. 2017). However, ~43–49% and ~29–34% of binding sites for METTL3/METTL14/WTAP identified by PAR-CLIP largely fall into intergenic regions and introns, respectively (Liu et al. 2014). Consistent with the PAR-CLIP study, ~57% of early m^6A peaks around consensus motif or motifs sharing an SAG core arose within introns by bromouridine (BrU) pulse-chase of nascent RNAs (Louloupi et al. 2018). The discrepancies could be caused by different factors that include microRNAs (Alarcon et al. 2015) in these regions, distinct kinetics of transcription elongation, RNA processing, methylation and demethylation, among others.

Despite the establishment of m^6A as a primarily co-transcriptional process, the mechanisms underlying the interplay of m^6A deposition and transcription are unclear. Several intriguing questions of m^6A formation are begging answers: (1) only selected consensus sequences are methylated; (2) methylation is nonstoichiometric, with most genes displaying less than 50% methylation levels; (3) methylation of a gene transcript may be changed in different conditions. While the selection of primary sequences may reflect a context effect of the RNA substrates which emphasizes the interaction with SMC components, the latter two facts are likely to be contributed by different transcription activators, co-factors or regulators. This conceptual model can be supported by several lines of evidence: (1) the recruitment or displacment of METTL3 at genomic regions can be induced by transcription activators in response to stimulus or development conditions; (2) an METTL3 accumulation in nuclear speckles where most proteins are not active in RNA processing suggests an inducible interaction between METTL3 and nascent RNAs that are synthesized in the

chromatin-associated regions. This model also implies that histone modifications and DNA methylation may play indirect role in m^6A formation (Zhang 2018).

For the METTL3-METTL14 complex, it is tempting to hypothesize that the sequential recruitment of m^6A methylation cofactors to nascent transcripts following transcription activation nucleates a pre-methylation complex (PMC), which further assembles to an active SMC upon interaction with the METTL3-METTL14 core subunits (Zhang 2018). In this transcription → PMC (without core subunits) → SMC → m^6A methylation pathway, RNA-binding proteins (RBPs) with different target sequence preferences, such as RBM15 (and possibly WTAP), first bind to nascent transcripts and seed the PMC. If the methylation core subunits join, a SMC will be formed, stabilized and able to modify nearby consensus sequences around RBP binding sites. In this methylation pathway, the PMC formation on a near consensus sequence and its transition to a SMC would be rate-limiting after transcription activation. It would appear therefore that various associations between RBPs and SMC components, or potential heterogenous SMCs under different conditions, cooperatively define the precise temporal and spatial m^6A landscapes.

For METTL16 methyltransferase, the relationship between m^6A decoration and transcription is currently unknown. The METTL16 substrate U6 snRNA is transcribed by RNA polymerase III. An early study showed that U6 A43 can be methylated by HeLa whole cell S-100 extract with a high efficiency (Shimba et al. 1995), suggesting transcription might not be essential to this type of methylation. Whether m^6A methylation of RNA pol II transcribed genes by METTL16 depends on transcription warrants further study.

6 m^6A Demethylation Occurs on Nascent Transcripts in Mammalian Cells

The first identified m^6A demethylase FTO was initially shown to utilize α-ketoglutarate (α-KG)-and Fe(II) to demethylate 3-methylthymine (3-meT) and 3-methyluracil (3-meU) in single-stranded DNA and RNA, respectively, with low in vitro activities (Jia et al. 2008; Gerken et al. 2007). Later, FTO was found to demethylate ~8–20% cellular m^6As in mammalian cells (Wei et al. 2018a; Jia et al. 2011), with N^6-hydroxymethyladenosine (hm^6A) and N^6-formyladenosine (f^6A) as intermediate products during the demethylation (Fu et al. 2013). Soon after, the target spectrum of FTO in vivo was further extended to include the N^6, 2′-O-dimethyladenosine (m^6A_m) adjacent to the N^7-methylguanosine (m^7G) cap in mRNA (Mauer et al. 2017) (Fig. 1), N^1-methyladenosine (m^1A) in tRNA, m^6A in U6 RNA and m^6A_m in several snRNAs (Wei et al. 2018a). Notably, whereas the demethylase activity of FTO on cap m^6A_m is higher relative to internal m^6A, (Wei et al. 2018a; Mauer et al. 2017), the cellular levels of m^6A_m in polyadenylated RNA are only ~1/10–1/25 of that of m^6A (Wei et al. 2018a). Upon demethylation, the m^6A_m of $m^7Gpppm^6A_m$ is converted to 2′-O-methyladenosine (A_m) via an N^6-

hydroxymethyl intermediate ($m^7Gppphm^6A_m$). The consequence of demethylation was thought to reduce RNA stability (Mauer et al. 2017). However, a more careful estimation of demethylation effect on the transcripts containing m^6A_m without any m^6A suggests no statistically noticeable increase of RNA levels upon FTO knockdown in HEK293T cells (Wei et al. 2018a). Considering the lack of m^6A_m in *Arabidopsis* and *Drosophila* polyadenylated RNA, whether the reversible modification near cap plays any important role in gene expression remains an open question. The other demethylase ALKBH5 has been reported to demethylate an estimated ~9–30% of total m^6As in human cells (Zheng et al. 2013; Zhang et al. 2017). Both FTO and ALKBH5 belong to the mammalian AlkB family of Fe(II)- and α-ketoglutarate-dependent dioxygenases that is consisted of nine AlkB homologs (ALKBH1-8, FTO). FTO and ALKBH5 are not essential for embryonic development, but each possesses non-redundant functions in specific tissues. Fto is widely expressed in adult and fetal tissues, with expression highest in the brain. Fto$^{-/-}$ mice show postnatal growth retardation with a significant reduction in adipose tissue and lean body mass (Fischer et al. 2009). Alkbh5 is also widely expressed, with most abundant expression in the testes where it is essential for spermatogenesis and male fertility (Zheng et al. 2013). The characterization of ALKBH10B and ALKBH9B, two *Arabidopsis* ALKBH5 orthologs with an m^6A demethylase activity on single-stranded RNA, indicates that demethylation occurs not solely in mammalian cells. Inactivation of ALKBH10B demethylase activity destabilizes mRNA transcripts of *FT, SPL3*, and *SPL9*, delays flowering, and represses vegetative growth (Duan et al. 2017). ALKBH9B was identified as an interactive protein with coat protein (CP) of alfalfa mosaic virus (AMV) by an yeast two-hybrid screen. ALKBH9B positively regulates AMV infection and influences m^6A levels in the AMV genome. In contrast, cucumber mosaic virus (CMV) CP fails to interact with ALKBH9B. Moreover, m^6A-modified viral RNAs of CMV differ from those of AMV in that neither the methylation levels nor viral infection is affected by ALKBH9B, suggesting a selectivity for substrate demethylation (Martinez-Perez et al. 2017). Interestingly, ALKBH9B exhibits a partial colocalization with siRNA bodies and P bodies in cytoplasm, thus raising the possibility of cytoplasmic demethylation in *A. thaliana*.

Because the mammalian demethylases were found to mainly localize in nucleus, demethylation in mammalian cells was thought to be a nuclear event in normal conditions (Jia et al. 2011; Zheng et al. 2013; Zhang et al. 2017). However, FTO is also found in the cytoplasm of some cell lines (Wei et al. 2018a). HEK293T cells expressing a cytoplasm-retained FTO mutant for lack of a nuclear localization signal at N-terminal region show reduced m^6A_m but not m^6A in polyadenylated RNA. A more detailed analysis on nuclear and cytoplasmic fractions suggested that the FTO-mediated demethylation of m^6A is prominent in the nucleus, whereas m^6A_m is more affected in the cytoplasm of HEK293T cells. This observation contrasts with the finding that FTO knockdown in some acute myeloid leukemia (AML) cell lines leads to increased m^6A levels in both nucleus and cytoplasm, although the m^6A_m levels only increase in the cytoplasm (Wei et al. 2018a). These findings unambiguously favor nucleus as the primary site for FTO-mediated m^6A demethylation and also point out that FTO-mediated demethylation of m^6A_m mainly occurs in

cytoplasm. The concomitant m^{6}A change in cytoplasm could be caused by RNA export and turnover of the m^{6}A-methylated fraction.

Since m^{6}A of mammalian cell mRNA is added and removed in nucleus, an interesting question is whether demethylation takes place on mature mRNA or nascent transcripts. Based on the subcellular fractionation experiments, Zhang et al. (2017) measured the levels of ALKBH5 protein and FOXM1 (Forkhead box M1) transcripts in three subcellular fractions: a cytoplasmic fraction, a soluble nuclear fraction (nucleoplasmic) and an insoluble fraction that contains chromatin and associated ternary transcription complexes (RNAPII with attached nascent transcripts). It is found that, in glioblastoma cells, FOXM1 nascent transcripts are exclusively present in the insoluble fraction, where there is also a large amount of ALKBH5 proteins. The depletion of ALKBH5 increases m^{6}A levels on FOXM1 nascent transcripts and mature mRNAs equally. The native RNA immunoprecipitation with nuclear ALKBH5 confirmed its interaction with FOXM1 nascent rather than mature transcripts even though the latter is readily detected in nucleus, indicating that nascent transcripts attached to the chromatin are the nuclear substrates for ALKBH5 (Zhang et al. 2017). Similar subcellular fractionation experiments carried out by R.B. Darnell laboratory were further coupled with m^{6}A-CLIP to map and quantify each m^{6}A in RNAs from the three fractions (Ke et al. 2017). In HeLa cells, about 10% of the m^{6}As in pre-mRNA exons disappear when these RNAs are released from chromatin, whereas >99% of nucleoplasmic m^{6}As remain unchanged when they become cytoplasmic (Ke et al. 2017). These two studies pointed out that, in steady state, m^{6}A demethylation takes place in the nascent transcripts associated with chromatin. However, where demethylation occurs in response to stress is not known (Xiang et al. 2017; Zhou et al. 2018). Since the demethylase FTO is active in cytoplasm where m^{6}A demethylation rarely happens, it is tempting to speculate an unidentified factor that directs demethylases to nascent transcripts and perhaps stimulates their activities as well. The possible candidates include protein modifications, long non-coding RNAs and other trans and cis factors, depending on what is achieved from certain contexts. A relevant example that has been illustrated above, is the specific interaction of ALKBH9B and AMV CP. Similarly, viral infection induces a critical acetylation of the nuclear DEAD-box helicase member DDX46 at Lys470 that allows ALKBH5 recruitment via DDX46's DEAD helicase domain to demethylate m^{6}A-modified antiviral transcripts, such as MAVS, TRAF3 and TRAF6, and therefore to enforce their retention in the macrophage nucleus and to reduce their translation (Zheng et al. 2017) (the molecular mechanism of nuclear retention will be discussed later). Long non-coding RNAs with sequence complementary to pre-mRNA have also been suggested to enhance ALKBH5-mediated demethylation on specific nascent transcripts (Zhang et al. 2017).

In contrast to the preferential exonic deposition of m^{6}A as reported by R. B. Darnell laboratory, two studies addressing FLAG-FTO targets by CLIP showed the preferential binding of FTO in introns relative to exons (Bartosovic et al. 2017; Wei et al. 2018a). It should be mentioned that the m^{6}A consensus motif is not enriched in FTO binding sequences and that the FTO binding sites appear not to overlap with m^{6}A sites very well (Bartosovic et al. 2017). Nevertheless, it agrees

with the reports that show a large fraction of early m⁶As and METTL3-METTL14 binding sites in introns (Liu et al. 2014; Louloupi et al. 2018). An explanation would be these intronic m⁶As are extremely short-lived due to frequent demethylation. In fact, this is not entirely impossible because upon UV stress, m⁶A can be efficiently added by METTL3 and removed by FTO both within a few minutes (Xiang et al. 2017). Future studies should provide direct evidence to prove the emergence of a large group of intronic m⁶As in cells depleted of any m⁶A demethylase activity, if the undetectable intronic m⁶As are indeed because of rapid demethylation.

7 Nuclear m⁶A Regulates Alternative Splicing and Export

Since m⁶A methylation occurs before splicing completion, m⁶A was conceived to participate in the regulation of RNA processing a few decades ago (Stoltzfus and Dane 1982; Camper et al. 1984). However, embryonic stem cells depleted of Mettl3 have all m⁶A-containing constitutive exons spliced normally, with only a minor change of alternative splicing events, which suggests no common obligatory role of m⁶A in splicing (Ke et al. 2017). It is of importance to be noted that any change of alternative splicing by depletion of methyltransferase or demethylase cannot be simply interpreted as a direct effect of m⁶A, because some small non-coding RNAs involved in splicing are possibly regulated by m⁶A or m⁶Aₘ and, as exemplified by METTL16, methylation activity is not required for its regulation of MAT2A intron retention. A direct contribution of m⁶A to alternative splicing is that m⁶A destabilizes the hairpin structure to increase the accessibility of its surrounding single-stranded RNA binding motifs to RNA binding proteins, such as heterogeneous nuclear ribonucleoprotein C (HNRNPC) and HNRNPG, which modulate the alternative splicing of some gene transcripts (Liu et al. 2015, 2017). In the case of HNRNPC, the presence of methylation in an m⁶A-containing stem loop enhances the HNRNPC interaction with the opposing poly-U strand that is otherwise buried within their local RNA structures. The majority of these m⁶A-switches are found in introns and depend on METTL3-METTL14 mediated methylation. Knockdown of HNRNPC or METTL3-METTL14 resulted in differentially expressed splice variants associated with 221 m⁶A-switch-containing genes in HEK293T cells (Liu et al. 2015). Similarly, m⁶A-switches also modulate the binding affinity of HNRNPG to RNA (Liu et al. 2017). On the other hand, the cofactors of methyltransferase with additional roles in splicing may provide a connection with m⁶A, such as WTAP, which facilitates alternative splicing (Ortega et al. 2003). Likewise, YT521-B, the ortholog of human m⁶A binding protein YTHDC1, regulates alternative splicing of Sxl in *D. melanogaster* (Haussmann et al. 2016; Lence et al. 2016). Moreover, *Alkbh5* knockout in the mouse testes leads to several hundred of shorter 3′ UTR transcripts during spermatogenesis (Tang et al. 2018), likely due to alternative splicing or alternative polyA (APA) usage in the last exon (Ke et al. 2015). APA could be partially mediated by VIRMA, an SMC component that is associated with polyadenylation cleavage factors CPSF5 (cleavage and

polyadenylation specificity factor subunit 5) and CPSF6 in an RNA-dependent manner (Yue et al. 2018). Depletion of VIRMA or METTL3 leads to 3′ UTR lengthening of a few hundred of genes in HeLa cells, indicating a potential role of m[6]A in the selection of proximal polyadenylation sites (Yue et al. 2018). Again, despite the correlation, unresolved is if the m[6]A within 3′ UTR *per se* regulates APA, or vice versa.

After splicing is completed, RNA export may also be influenced by m[6]A. A delayed appearance of mRNA in the HeLa cell cytoplasm caused by methyltransferase inhibitors was reported by Rottman laboratory based on radioactive labeling (Camper et al. 1984). Subsequent studies showed that a delayed cytoplasmic appearance of bPRL mRNA was accompanied with nuclear accumulation of precursor RNA (Carroll et al. 1990). Similarly, the application of methylation inhibitors leads to a prolonged nuclear retention of two key clock genes, *PER2* (period circadian clock 2) and *ARNTL* (aryl hydrocarbon receptor nuclear translocator like), and thereby an altered schedule of gene expression that results in an elongated circadian period (Fustin et al. 2013). On the other hand, RNA export is enhanced by ALKBH5 knockdown (Zheng et al. 2013), which may be mediated by YTHDC1, the only nuclear exclusive YTH domain family member. Despite its role in *D. melanogaster*, YTHDC1 mainly binds to exons (Xu et al. 2014) and has very minor effect on splicing in Hela cells (Roundtree et al. 2017b). Depletion of YTHDC1 induces a nuclear accumulation of mature m[6]A-marked mRNAs and a decrease in their cytoplasmic appearance. The nuclear export function of YTHDC1 is mediated by an RNA-independent interaction between the C-terminal YTHDC1 and SRSF3 (serine/arginine-rich splicing factor 3), which interacts with the canonical mRNA export receptor NXF1 (nuclear RNA export factor 1) (Roundtree et al. 2017b).

8 Cytoplasmic m[6]A Regulates mRNA Stability and Translation Efficiency

Before the advance of the "omics" era, biochemical studies detected a positive correlation between m[6]A decoration and mRNA decay (Friderici et al. 1976; Sommer et al. 1978). These radioactive labeling experiments provided the first evidence of the functional relevance of m[6]A. The principle of m[6]A-mediated post-transcriptional gene regulation is that RBPs recruited or repelled by m[6]As modify the fate of mRNA, such as decay and translation, depending on the function of RBP *per se* or through the association with a traditional pathway. Among these RBPs, the proteins harboring a YTH domain are described as the m[6]A "readers" that specifically recognize and regulate m[6]A-decorated transcripts (Dominissini et al. 2012; Wang et al. 2014a). The mammalian YTH domain proteins that have been largely characterized by He laboratory include the cytoplasmic YTHDF1, YTHDF2, YTHDF3, YTHDC2 and the nuclear YTHDC1, all of which employ their conserved

aromatic cage to recognize m^{6}A (Xu et al. 2014; Zhu et al. 2014). With the list of "reader" proteins extended by other RBPs that are absent of a YTH domain, a critical regulatory network has emerged that impacts multiple aspects of mRNA homeostasis, which cannot be achieved by the primary sequence alone.

8.1 m^{6}A and mRNA Decay

The first confirmed m^{6}A reader-mediated effect on gene expression arose from the mechanistic studies of YTHDF2 (Wang et al. 2014a). YTHDF2 accelerates mRNA deadenylation and degradation, with the C-terminal YTH domain specifically recognizing m^{6}A-methylated RNA and the N-terminal region interacting with the CCR4– NOT deadenylase complex through CNOT1 (CCR4–NOT transcription complex subunit 1) (Wang et al. 2014a; Du et al. 2016). In early life of zebrafish embryos, the depletion of Ythdf2 stabilizes m^{6}A-modified maternal mRNAs and delays maternal-to-zygotic transition (Zhao et al. 2017). Likewise, Ythdf2 is maternally required for oocyte maturation (Ivanova et al. 2017). Moreover, Ythdf2 depletion leads to a disturbed m^{6}A-dependent degradation of neural development-related mRNA targets, and impaired proliferation and differentiation capabilities of neural stem/progenitor cells, consequently leading to defective neurogenesis. Homozygous *Ythdf2*$^{-/-}$ mice in C57BL/6 background are embryonic lethal, with the majority of *Ythdf2*$^{-/-}$ embryos lost between E14.5 and E18.5. Semi-lethality was observed for the *Ythdf2*$^{+/-}$ mice, with one third of the surviving mice having malfunctioning eyes (Li et al. 2018). It is not surprising that depletion of m^{6}A methyltransferase, accompanied with a failure of m^{6}A-induced decay of methylated transcripts, would give rise to similar or more severe phenotypes. Indeed, a loss of m^{6}A methyltransferase exhibits significant impacts on mouse ESC differentiation and preimplantation development (Batista et al. 2014; Wang et al. 2014b; Geula et al. 2015). *Mettl3* knockout mice display more severe early embryo lethality phenotypes than *Ythdf2* knockout, with E5.5-E7.5 *Mettl3*$^{-/-}$ embryos having abnormal characteristics (Geula et al. 2015), indicating additional mechanisms for m^{6}A-mediated effects as discussed later. Interestingly, depending on the dominance of already-expressed genes in different cell states to be stabilized, the loss of methyltransferase in murine ESCs can lead to opposite cell fates, i.e., an improved self-renewal in naïve state but a defective cell regeneration in primed state (Wang et al. 2014b; Geula et al. 2015; Batista et al. 2014). In general, mRNAs that encode key proteins with regulatory functions tend to have more m^{6}As with faster turnover (Wang et al. 2014b; Ke et al. 2017). A proposed model describes that m^{6}A induces rapid clearance of a group of mRNAs encoding key regulators, such as transcription factors, in a coordinated manner during the transition of cell states (Roundtree et al. 2017a). Additionally, m^{6}A may destabilize some transcripts by repelling RBPs without a YTH domain, such as G3BP1 (G3BP stress granule assembly factor 1) and HuR (ELAV-like protein 1) (Edupuganti et al. 2017; Wang et al. 2014b).

Not only m[6]A methyltransferase and its cofactors are evolutionarily conserved, the "readers" coevolve from yeast to mammals as well (Schwartz et al. 2013). A phylogenetic analysis of 297 YTH-containing proteins from 32 representative species suggests that 57 of them belong to the DC group and 240 to the DF group (Scutenaire et al. 2018). Eleven of the 13 members of the *Arabidopsis* YTH protein family, namely ECT1-11 for Evolutionarily Conserved C Terminus, fall into the YTHDF clade. One of the two YTHDC-type proteins possesses three highly conserved N-terminal zinc fingers in addition to the YTH domain and is a member of the plant polyadenylation complex (CPSF30; AT1G30460). ECT2 is required for trichome branching and involved in the redundant control of timing of leaf formation with ECT3 (Arribas-Hernandez et al. 2018). Surprisingly, by formaldehyde-assisted crosslinking of ECT2 and associated RNA in *A. thaliana*, G. Jia laboratory found that the majority of ECT2-binding sites are located within the 3′ UTR of mRNA containing a unique URUAY motif (R=G>A, Y=U>A, where the majority [over 90%] UGUAY)(Wei et al. 2018b). ECT2 appears to bind methylated UGUAA and methylated GGACU with similar affinities (Wei et al. 2018b), suggesting the sequence bias may reflect the relative abundance of methylated motifs in the cells, and in line with this, *Arabidopsis* nuclear extracts exhibit higher methylation activity for UGUA relative to GGACU. However, the RRACH is more significantly enriched than UGUAY in overall m[6]A peaks, and interestingly, UGUAY is identical to a specific UGUAACA methylation signature near the start codon (Luo et al. 2014). The ECT2 binding motifs also share sequence similarity with far upstream elements that regulate polyadenylation, likely lending evidence of a potential regulatory role in RNA processing (Wei et al. 2018b). However, ECT2 mostly accumulates in the cytoplasm with a diffuse pattern or aggregates in stress granules upon heat stress (Scutenaire et al. 2018; Arribas-Hernandez et al. 2018), which suggests its function on cytoplasmic RNAs. This is partially evidenced by ECT2-dependent stabilization of some RNA transcripts associated with trichome morphogenesis (Wei et al. 2018b).

ECT2 is not the first reader that was found to stabilize m[6]A-marked transcripts. In mammalian cells, insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs; including IGF2BP1/2/3) were reported to enhance RNA stability and translation under normal and stress conditions through the K homology domains that recognize m[6]A residues (Huang et al. 2018). Moreover, depletion of FTO appears to increase the levels of some m[6]A-methylated transcripts (Wei et al. 2018a). Therefore, despite the m[6]A-mediated destabilization of a majority of transcripts, a subgroup of transcripts become more stable, which diverts the fates of mRNAs for dynamic physiological roles.

8.2   m⁶A and mRNA Translation

In bacterial translation systems, m[6]A within coding regions acts as a barrier to tRNA accommodation during translation elongation (Choi et al. 2016). However, m[6]A was found to associate with enhanced translation efficiency of dihydrofolate reductase

transcripts (Tuck et al. 1999). Recent studies support the positive role of m⁶A in translation through several mechanisms. First, the cap-dependent translation can be enhanced by the m⁶A-bound YTHDF1 through recruitment of eIF3 (translation initiation factor complex 3) (Wang et al. 2015). Interestingly, YTHDF1 was found to associate with G3BP1 in an RNA-dependent manner (Wang et al. 2015). Although genetic deletion of YTHDF1 allows mice to develop normally at least to four months of age, these mice show learning and memory defects, impaired hippocampal synaptic transmission and long-term potentiation due to the loss of YTHDF1-dependent expedited translation of m⁶A-methylated neuronal mRNAs in response to neuronal stimulation in the hippocampus (Shi et al. 2018). Moreover, YTHDF3 also promotes translation through interaction with YTHDF1 (Shi et al. 2017). Surprisingly, cytoplasmic METTL3 enhances translation of mRNAs bearing m⁶As that are close to stop codon independently of its methyltransferase activity or YTHDF1. In cytoplasm, in addition to a direct METTL3-m⁶A interaction, the N-terminal region of METTL3 binds eIF3h (translation initiation factor 3 subunit h) to facilitate mRNA looping, thus promoting translation of a large subset of oncogenic mRNAs, such as BRD4 (bromodomain-containing protein 4) in human primary lung tumors (Lin et al. 2016; Choe et al. 2018). Second, m⁶As within 5′ UTR can be recognized by eIF3, which recruits the 43S complex and initiates translation in a cap- and internal ribosome entry site (IRES)-independent manner (Meyer et al. 2015; Zhou et al. 2015). ABCF1 (ATP-binding cassette sub-family F member 1) and YTHDF3 appear to contribute to this type of m⁶A-facilitated mRNA translation to some extent (Coots et al. 2017). Other RBPs implicated in translation include YTHDC2 that increases translation efficiency (Hsu et al. 2017), and FMR1 (fragile X mental retardation 1) (Edupuganti et al. 2017), a polyribosome-associated neuronal RNA-binding protein associated with Fragile X syndrome that represses translation by stalling ribosome translocation.

Elucidating m⁶A function at different conditions is central for unraveling the role of m⁶A in more complex processes. Upon heat shock, YTHDF2 translocates into nucleus and binds to the m⁶A within 5′ UTR, which blocks FTO-mediated demethylation and hence enhances translation of stress-induced transcripts (Zhou et al. 2015). During integrated stress response, the 5′ UTR-located m⁶A controls ribosome scanning and start codon selection, while demethylation promotes the reinitiation of ATF4 (activating transcription factor 4) translation (Zhou et al. 2018). These complex m⁶A-mediated pathways of translational regulation undoubtedly add new dimensions to the dynamic effects of m⁶A in relation to gene expression.

9 m⁶A Methyltransferases and Demethylases Play Important Roles in Cancer

The comprehensive functional analyses for m⁶A regulators in cancer were initiated by an FTO study in leukemia and an ALKBH5 study in glioblastoma (Li et al. 2017; Zhang et al. 2017), and have been extended by a plethora of studies focused on the

dysregulation of m^{6}A methylation and associated proteins. FTO is highly expressed in several subtypes of AMLs, including those that carry t(11q23)/*MLL*-rearrangements, t(15;17)/*PML-RARA* translocation, *FLT3* gene internal tandem duplication (ITD) mutations and/or *NPM1* mutation. FTO demethylates 3′ or/and 5′ UTR-located m^{6}As on the transcripts of *ASB2* (ankyrin repeat and SOCS box containing 2) and *RARA* (retinoic acid receptor alpha), which encode key regulators in all-trans-retinoic acid (ATRA)-induced differentiation of leukemia cells. It therefore enhances ASB2 and RARA mRNA decay and induces downregulation of these two genes. The oncogenic role of FTO in leukemic cell transformation and leukemogenes was confirmed by a series of in vitro and in vivo assays (Li et al. 2017). Interestingly, the oncogenic activity of FTO is inhibited by 2-hydroxyglutarate (R-2HG), an oncometabolite competitive inhibitor of α-KG-dependent dioxygenases that is produced by the cancer-associated mutant *IDH1/2* (isocitrate dehydrogenase 1/2) (Su et al. 2018). In leukemic cells that highly express FTO with a "medium" level of MYC, FTO affects the stability of MYC transcripts by changing m^{6}A levels at MYC 5′ UTR and CDS regions, which confers R-2HG sensitivity in these cells. However, hyperactivation of MYC-associated pathways renders leukemic cells resistant to R-2HG. On the other hand, in glioblastoma multiforme (GBM), ALKBH5 is highly expressed in the patient-derived glioma stem cells (GSCs) and informs poor survival for GBM patients (Zhang et al. 2017). ALKBH5 depletion impaires GSC growth in vitro and in vivo due to the loss expression of FOXM1, an essential transcription factor for GSC self-renewal and proliferation. ALKBH5 binds to and demethylates the 3′ UTR of *FOXM1* nascent transcripts, thereby enhancing FOXM1 mRNA expression through interaction with HuR, an abundant nuclear RBP that promotes pre-mRNA expression. The demethylation seems to be facilitated by a long noncoding RNA transcribed antisense to the *FOXM1*, which harbors an overlapping region with FOXM1 3′ UTR.

Interestingly, although these demethylases behave as oncogenes in these cancers, the methyltransferase complex does not simply play the opposite role, i.e., METTL3-METTL14 being oncogenic in AMLs (Barbieri et al. 2017; Vu et al. 2017; Weng et al. 2018). Studies from different laboratories suggest that METTL3-METTL14 complex may employ multiple mechanisms to activate oncogenic pathways that at least include some key transcription factors, such as MYC (Barbieri et al. 2017; Vu et al. 2017; Weng et al. 2018). Starting with a catalytic activity domain-focused Cas9-induced indel mutation screen in mouse primary leukemia cells, Barbieri *et al.* (Barbieri et al. 2017) confirmed the requirement of *METTL3*, *METTL14* and *METTL16* for cell growth in ten human AML cell lines. They also found that METTL3 and METTL14 can be crosslinked to TSSs of distinct groups of coding genes that are decorated with bimodal trimethylation of lysine 4 on histone H3 (H3K4me3) marks by chromatin immunoprecipitation assays. The promoter-bound METTL3, likely recruited by CEBPZ (CCAAT enhancer binding protein zeta), enhances translation of target genes without affecting mRNA levels, such as the transcription factors *SP1* and *SP2*, which activate the transcription of *MYC* oncogene. Similarly, Vu et al. (2017) reported METTL3 as an essential gene to maintain the undifferentiated state and survival of hematopoietic stem/progenitor

cells (HSPCs) and myeloid leukemia cell lines. METTL3 depletion reduces translational efficiency and causes decreased levels of MYC, BCL2 and PTEN (phosphatase and tensin homolog) protein expression in AML cells. Weng et al. (2017) showed that METTL14 is expressed at high levels in HSPCs and AML cells carrying t(11q23), t(15;17), or t(8;21) and is downregulated during myeloid differentiation. METTL14 sustains the development and maintenance of AMLs and self-renewal of leukemia stem/initiation cells through promoting stability and translation of MYB and MYC mRNAs. METTL14 itself is transcriptionally suppressed by the transcription factor SPI1. In addition, mutations of methyltransferases have also been implicated in tumor development. The hotspot R298P mutation of METTL14 in endometrial tumors is correlated with reductions in m^6A methylation, and promotes proliferation and tumorigenicity of endometrial cancer cells through activation of the AKT pathway (Liu et al. 2018).

The connections of m^6A and tumor development are not limited to aforementioned types of tumor. A growing body of studies have indicated that multiple types of cancer take advantage of the tunable regulatory mechanisms to survive and propagate, through mutations or aberrant expression of components of the m^6A regulatory network (e.g., writers, erasers and readers).

10 Conclusion and Future Perspective

mRNA m^6A methylation was discovered over 40 years ago. However, the attempts from RNA biologists to understand the function and regulation of this abundant modification at molecular, cellular and tissue levels have just started in very recent years, because of the important biological functions of reversible RNA methylation revealed by a few groups (Zheng et al. 2013; Li et al. 2017; Zhang et al. 2017) and the advance of new technologies afforded by high-throughput sequencing. A current view of m^6A on mRNA is that it is deposited, removed, sensed and perhaps regulated by several conserved machineries from fly to human, and that m^6A plays distinct roles in life when incorporated into diverse biological processes. In Fig. 3, a simplified model of METTL3-METTL14-mediated m^6A deposition is hypothesized—the recruitment of a Pre-Methylation Complex (PMC) with subsequent assembly to a Super Methylation Complex (SMC) allows efficient m^6A methylation at consensus sequence adjacent to the RBP binding sites. The intracellular and extracellular signals that trigger transcription allow m^6A methylation for specific genes, while the selection of consensus motif to be modified is decided by the PMC (Fig. 3a). The signals may also regulate PMC component expression, modification, or localization, etc., and as a result, m^6A distribution. On the other hand, the "free" methyltransferase core subunits have intrinsic ability to bind and catalyze single-stranded RNA with low activity, likely giving rise to abortive methylation in the absence of PMC or low methylation "noise" in cells (Fig. 3b). Therefore, the sequential recruitment of SMC components ensures accurate m^6A deposition in a large number of transcripts. After methylation, m^6A
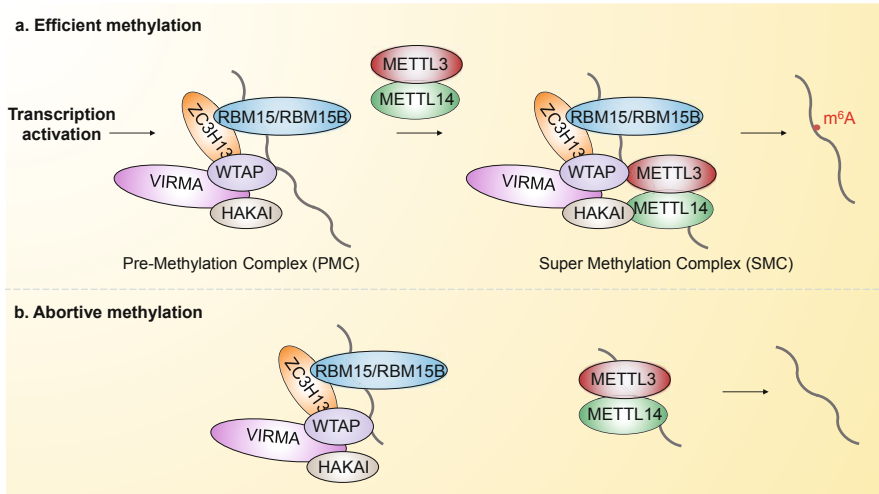
Fig. 3 A hypothetical model of METTL3-METTL14-dependent m⁶A deposition. The overlaps of proteins indicate confirmed or proposed interactions

demethylation occurs on a subset of nascent transcripts in mammalian cells, which is regulated by a poorly understood mechanism. Depending on the functions of the RBPs that display higher or lower binding affinities to m⁶A-marked transcripts, a wide range of events throughout RNA life are influenced by m⁶As that include RNA processing, localization, decay and translation, which altogether modulate gene expression for dynamic physiological functions. Elucidating how m⁶A affects gene expression not only aids our understanding of essential physiology but also enables novel therapeutic approaches for human diseases.

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