



REVIEW

N⁶-methyl-adenosine (m⁶A) in RNA: An Old Modification with A Novel Epigenetic Function

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Abstract N⁶-methyl-adenosine (m⁶A) is one of the most common and abundant modifications on RNA molecules present in eukaryotes. However, the biological significance of m⁶A methylation remains largely unknown. Several independent lines of evidence suggest that the dynamic regulation of m⁶A may have a profound impact on gene expression regulation. The m⁶A modification is catalyzed by an unidentified methyltransferase complex containing at least one subunit methyltransferase like 3 (METTL3). m⁶A modification on messenger RNAs (mRNAs) mainly occurs in the exonic regions and 3'-untranslated region (3'-UTR) as revealed by high-throughput m⁶A-seq. One significant advance in m⁶A research is the recent discovery of the first two m⁶A RNA demethylases fat mass and obesity-associated (FTO) gene and ALKBH5, which catalyze m⁶A demethylation in an α -ketoglutarate (α -KG)- and Fe²⁺-dependent manner. Recent studies in model organisms demonstrate that METTL3, FTO and ALKBH5 play important roles in many biological processes, ranging from development and metabolism to fertility. Moreover, perturbation of activities of these enzymes leads to the disturbed expression of thousands of genes at the cellular level, implicating a regulatory role of m⁶A in RNA metabolism. Given the vital roles of DNA and histone methylations in epigenetic regulation of basic life processes in mammals, the dynamic and reversible chemical m⁶A modification on RNA may also serve as a novel epigenetic marker of profound biological significances.

Introduction

More than 100 modifications have been identified in native cellular RNAs including mRNAs, tRNAs, rRNAs, small nuclear RNA (snRNAs) and small nucleolar RNAs (snoRNAs) [1,2]. As one of the most common modifications, methylation occurs on either nitrogen or oxygen atoms at the post-transcriptional level with S-adenosylmethionine (SAM, Adomet) serving as the methyl donor for m⁶A formation [3–6]. The most prevalent methylated nucleoside in eukaryotic mRNA is N⁶-methyl

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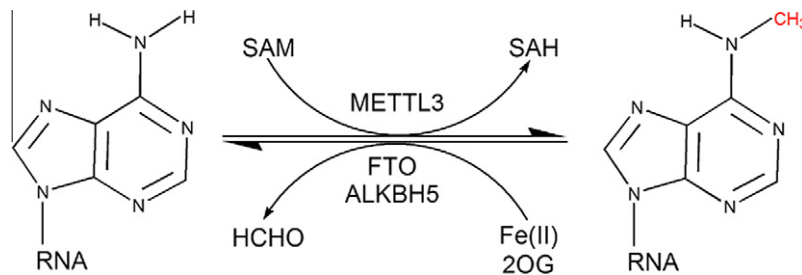


Figure 1 Reversible m⁶A methylation in mRNA

METTL3-containing methyltransferase complex catalyzes m⁶A methylation with SAM as a methyl donor. FTO and ALKBH5 demethylate m⁶A in an iron and α -ketoglutarate-dependent manner.

adenosine (m⁶A) (**Figure 1**), which accounts for more than 80% of all RNA base methylations and exists in various species [7–15]. m⁶A modification is not susceptible to chemical modifications like bisulfate treatment for 5-mC detection [16]. It cannot affect the base pairing ability either [17,18]. Therefore, although having been identified for 5 decades, the biological functions of m⁶A modification remain elusive due to limited detection strategies [19–22]. Recently, an antibody-based affinity approach was developed to identify the transcriptome profile of m⁶A sites in combination with next-generation sequencing (NGS) techniques. Around 7000 mRNAs have been found to possess m⁶A sites in both human and mouse cells [23,24]. Furthermore, two novel mRNA m⁶A demethylases have been identified recently confirming the dynamic regulation of m⁶A [25,26]. Given that the recent major progress of m⁶A research is related to mRNA metabolism, we hereby discuss m⁶A modification in mRNAs in the hope of elucidating its novel epigenetic regulatory functions.

Distribution of m⁶A along mRNAs

To understand the fundamental roles of m⁶A in mRNAs, it is necessary to determine the positions of m⁶A in the gene transcripts. The m⁶A-containing consensus sequence is different from other RNA regulatory elements such as AU-rich elements or poly(A) signal, suggesting that it may have different regulatory functions from those known elements. Traditional strategies such as thin layer chromatography (TLC) and HPLC have been utilized to study the distribution of m⁶A in several well-known m⁶A-containing mRNAs [12,27,28]. Furthermore two lines of independent transcriptome analysis have revealed that m⁶A modification occurs in mRNAs with a frequency of 1 m⁶A per 2000 ribonucleotides on average [23,24]. m⁶A modification was found to occur in highly-conserved regions with a consensus sequence identified as: RRACH (R = G or A; H = A, C or U) [10,29–33]. Mutation from GAC to GAU in Rous sarcoma virus mRNA effectively abolished the m⁶A modification [34]. In addition, motif search from m⁶A peaks enriched in both human and mouse cells also pointed to the tendency for m⁶A to occur in this consensus sequence [23,24]. However the frequency of this consensus sequence in the genome is much higher than the frequency of m⁶A occurrence, therefore additional sequences or RNA structures may also play a role in determining the methylation sites.

m⁶A also occurs in rRNA [35], tRNA [36] and snRNA [19], but not within the same consensus motifs as it does in mRNA. An *in vitro* analysis using U6 snRNA as substrate showed that

a 3'-stem loop structure was necessary for m⁶A formation [37], suggesting that different from other RNA species, m⁶A in mRNA may perform specific functions.

m⁶A is mainly enriched in mature mRNAs as immunoblotting analysis revealed that m⁶A was only detected in polyadenylated RNAs. However, m⁶A does not exist in the poly(A) tail since depletion of the poly(A) tail from mRNA does not reduce the level of m⁶A in mRNA [23]. Previous studies at the single gene level revealed that there is a high abundance of m⁶A in the 3'-terminal region of bovine prolactin (bPRL) mRNA [32,33]. Later on, m⁶A-immunoprecipitation combined with high-throughput analysis confirmed that, for most genes, the m⁶A modification tends to mainly occur in intragenic regions including coding sequences (CDS), stop codon flanking regions and 3'-UTR, especially the 3'-end of CDS and the first quarter of the 3'-UTR [23,24]. Besides, m⁶A is also enriched near transcription starting sites (TSS) [24]. m⁶A sites do not spread along the transcript randomly, but is always clustered in adjacent regions along the transcript. Collectively, these data suggest a potential functional significance for m⁶A in RNA. The molecular mechanisms involved in what determines the m⁶A methylation sites along these consensus motifs in mRNAs remain to be further elucidated.

m⁶A methyltransferase

To understand this important biological modification, unveiling the methyltransferase complex responsible for catalyzing m⁶A formation in RNA remains one of the top priorities. An *in vitro* methylation system has been established to investigate the mRNA m⁶A modification as it occurs *in vivo* [38]. Since HeLa cell nuclear extracts are capable of catalyzing the formation of m⁶A *in vitro* using an *in vitro* transcribed or synthetic mRNA substrate, the extracts were further fractionated to identify individual components responsible for m⁶A methylation activity. Three fractions termed MT-A1, MT-A2 and MT-B were initially screened and demonstrated to be parts of a methyltransferase complex [39,40]. Later on MT-A1 was shown not to be essential, therefore MT-A (200 kDa, previously designated as MT-A2) and MT-B (800 kDa) are generally considered as the two key multi-component factors of the whole methyltransferase complex responsible for complete m⁶A methylation activity. Subsequently a 70-kDa Adomet-binding subunit termed methyltransferase like 3 (METTL3) was identified from MT-A [41]. Two functional domains responsible for methylation activity have been identified in human METTL3 including consensus methylation motif I (CM I), which is the Adomet-binding

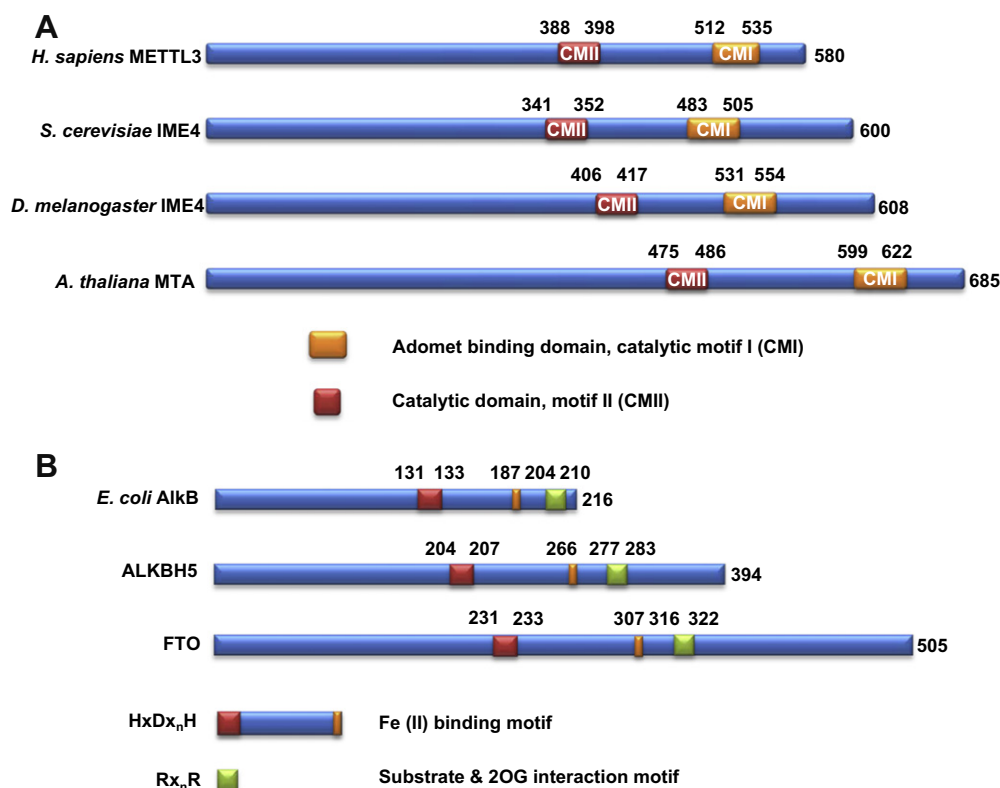


Figure 2 Schematic functional domains of m⁶A methyltransferases and demethylases

A. Structural alignment of human METTL3 and its homologues in *S. cerevisiae*, *D. melanogaster*, and *A. thaliana*. Orange box represents motif I, which is a potential Adomet binding domain. Red box represents motif II, which is a potential catalytic domain. Individual protein size (aa) and position of functional domains are shown as indicated. **B.** Structural alignment of m⁶A demethylases. Both FTO and ALKBH5 belong to the AlkB family. Individual protein size (aa) and positions of conserved iron-binding domain, substrates and α -ketoglutarate interaction motifs are shown as indicated.

domain, and CM II that contains catalytic residues for methylation activity (Figure 1; Figure 2A). Two isoforms of METTL3 have been discovered and the ratio of their expression varies greatly among tissues or cell types, suggesting distinct biological functions for each of them [42].

Northern blot analysis revealed that METTL3 is ubiquitously expressed in human tissues but at a much higher level in testis [41]. METTL3 knockdown in HepG2 cells resulted in increased apoptosis. Meanwhile gene ontology analysis of differentially-expressed genes before and after METTL3 knockdown in HepG2 cells also indicated an enrichment of the p53 signaling pathway [24]. Immunofluorescence analysis showed that METTL3 is localized in nucleoplasm with strong staining in nuclear speckles that are enriched with mRNA processing factors [41]. These results are consistent with the recent finding that m⁶A may regulate spermatogenesis by interfering with RNA metabolism [26].

Besides mammalian cells, METTL3 homologues have also been identified in *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Arabidopsis thaliana* [43]. All these proteins possess the two consensus methyltransferase motifs CM I and CM II (Figure 2A). Inducer of Meiosis 4 (IME4, also termed as SPO8) is the m⁶A methyltransferase found in *S. cerevisiae* [44], which is a key player involved in regulating meiosis and sporulation in yeast [15,45]. Inactivation of the IME4-encoding gene leads to the loss of m⁶A in the mRNA of yeast

mutants as well as sporulation defects. Similarly, the homolog of METTL3 in *A. thaliana*, MT-A, is mainly expressed in dividing tissues, particularly in reproductive organs, shoot meristems and emerging lateral roots [7]. Inactivation of MT-A led to deficiency of m⁶A modification and subsequent failure of the developing embryo to progress beyond the globular stage. *Dm ime4* is another homolog of METTL3 in *D. melanogaster*, which is mainly expressed in ovaries and testes [46]. These data indicate an evolutionarily conserved function for this enzyme in gametogenesis that is dependent on Notch signaling pathway [46].

Since the methylation activity exists only when both MT-A and MT-B complexes are present, it has been speculated that METTL3 exert its methylation activity with the assistance of additional factors. The complexity of m⁶A methyltransferase is unique compared with other methyltransferases identified so far. Nucleolar 2'-O-methyltransferase [47], N⁷-methyltransferase [48] and N²-guanine tRNA methyltransferase [49] are all purified as single active components and are able to exploit their enzymatic activity independently. Components besides METTL3 in the methyltransferase complexes still remain to be discovered. The fact that neither nuclease treatment nor anti-methylguanosine depletion could affect the methylation activity of HeLa cell nuclear extract indicates that RNA is not an essential component of the enzyme [31,39]. MT-A in *A. thaliana* was shown to interact *in vivo* with At FIP37, which

is a plant homolog of the gene female-lethal(2)d (FL(2)(D)) in *D. melanogaster* and Wilms' tumor-associated protein (WTAP) in human [7]. Disruption of At FIP37 in *A. thaliana* also results in an embryo-lethal phenotype with developmental arrest at the globular stage [7]. Together with the evidence that METTL3 mRNA expression level changes during the embryo development [50], m⁶A may play a role in regulating development.

m⁶A demethylases

m⁶A methylation is a dynamic RNA modification as observed by monitoring m⁶A levels throughout brain development [23]. Similar to the reversible methylations of DNA and histones catalyzed by methyltransferases and demethylases, it has been speculated that RNA demethylases should also exist to remove the methyl group from the m⁶A base. Consistent with this speculation, FTO gene and ALKBH5 have been demonstrated to function as the first two RNA m⁶A demethylases both *in vitro* and *in vivo* [25,26].

FTO and ALKBH5 belong to the AlkB family owing to the conserved iron binding motif and α -ketoglutarate interaction domain [51–54] (Figure 1; Figure 2B). Although FTO has been known for its critical function in human obesity and energy homeostasis, its physiological substrates as a demethylase have been obscure [55–58]. The discovery of its m⁶A demethylation activity will pave the way to understanding molecular mechanisms and pathways mediated by FTO.

In contrast to FTO, very little information was available for the biological functions of ALKBH5. A photocrosslinking-based mRNA-bound proteomics analysis has identified ALKBH5 to be an mRNA-binding protein [59], which is consistent with our finding of ALKBH5 as demethylase of m⁶A in mRNA [26]. ALKBH5-deficiency has been shown to increase m⁶A modification levels in mouse tubular mRNA, resulting in testis atrophy and reduction of sperm numbers and motility [26]. This indicates that ALKBH5 regulates spermatogenesis via demethylation of m⁶A in mRNAs. Interestingly, ALKBH5 gene expression can be regulated by either protein arginine methyltransferase 7 (PRMT7) upon genotoxic stresses or hypoxia stimulation [53,60], suggesting a broad role of ALKBH5 in distinct biological processes.

Both FTO and ALKBH5 are ubiquitously expressed proteins with m⁶A demethylase activities. Nonetheless, their biological functions are distinct from each other. This might be attributed to their differential expression profiles in different tissues. For instance, FTO is highly expressed in brain and muscle [58], while ALKBH5 is highly expressed in testis and lung [26]. In addition, to constrain their potential redundancy, FTO and ALKBH5 and additional RNA demethylases may catalyze different mRNA substrates in a cell-/tissue-specific context.

Effect of m⁶A on RNA metabolism

The relative abundance of m⁶A mRNA transcripts varies a lot in different types of mRNA. For instance, prolactin mRNA has only one m⁶A site [33,61]; Rous sarcoma virus mRNA has seven methylation sites [62]; the dihydrofolate reductase (DHFR) transcript has three m⁶A sites [40], SV40 mRNA may have more than 10 m⁶A sites [10], whilst histone and globin mRNA do not have m⁶A modifications [61,63]. A detailed

analysis at transcriptome level shows that 46% of mRNAs contain only one m⁶A peak, 37.3% have two peaks and the remaining have more than two peaks [23]. This indicates that m⁶A itself may have a regulatory role in RNA metabolism.

The primary knowledge about m⁶A function is mainly obtained from chemical methylation inhibitors. RNA metabolism, including mRNA transcription, splicing, nuclear export, translation ability and stability, has been examined by using at least one of the three classical methylation inhibitors: cycloleucine [64], neplanocin A (NPC) [65] and S-tubercidinylhomocysteine (STH) [66]. SAM is a primary biological methyl donor that extensively contributes to DNA/RNA/protein methylation [67]. Cycloleucine is a competitive and reversible inhibitor of methionine adenosyltransferase (MAT), which catalyzes SAM synthesis during methionine metabolism [68]. Cycloleucine can inhibit both 5'-terminal 2'-O-methyl ribose and internal m⁶A modifications, but not the 5'-terminal m⁷G modifications of mRNA. NPC is a cyclopentenyl adenosine analogue which exhibits antiviral activity against a wide variety of viruses, including vaccinia virus, vesicular stomatitis virus, reovirus and measles virus [69,70]. NPC can inhibit cellular S-adenosylhomocysteine (SAH) hydrolase. Intracellular accumulation of SAH inhibits methyltransferases which use SAM as a methyl donor. Therefore, NPC treatment can block the formation of methyl-6-adenosine and 2'-O-methylation. STH is another such inhibitor of m⁶A and 2'-O-methylation due to its structural similarity to SAH. It mainly affects methylation of mRNA but not rRNA [66].

From the analysis of cellular phenotypes observed after treatment with methylation inhibitors, it is found that m⁶A modification is involved in almost all aspects of RNA metabolism. Nuclear speckle staining of FTO and ALKBH5 also suggests a close relation between mRNA demethylation and mRNA splicing [25,26]. In addition, gene ontology analysis of m⁶A-containing genes also revealed that the most correlated functional pathways are related to RNA metabolism [24]. The role of m⁶A in RNA metabolism is summarized and discussed below.

mRNA transcription

As described before, m⁶A modification also exists in the mRNAs of various kinds of viruses, such as influenza virus, Rous sarcoma virus and simian virus [10,11,28]. Occurrence of m⁶A in viral mRNA was shown to enhance the priming efficiency of mRNA. Consistently, when influenza virus-infected CHO cells were treated with NPC, viral mRNA accumulation was greatly reduced [71]. Additionally, in an attempt to identify the biological function of ALKBH5, the effect of ALKBH5-deficiency on gene expression was evaluated using RNA-seq. As a result, several thousand genes showed either up- or down-regulated expression, similar to that seen in FTO knockdown cells via a cDNA expression microarray analysis [72]. In addition, when METTL3 was knocked down in HepG2 cells, the expression levels of nearly 2000 genes were affected, especially those having m⁶A modification [24]. These results suggest that m⁶A modification may be potentially involved in gene expression regulation.

However, whether m⁶A does have an effect on mRNA transcription remains controversial. For example, when SV40-infected BSC-1 cells were treated with NPC, no change was

detected with the transcription of SV40 mRNA [73]. In HeLa cells treated with STH, nascent mRNA synthesis did not alter significantly when compared to untreated samples [66]. Cycloleucine treatment of CHO cells did not affect the biosynthesis of mRNA either [68]. It has to be pointed out that since these inhibitors also target other methylation besides m⁶A, further evidence is required to clarify the specific role of m⁶A in mRNA transcription.

On the other hand, besides transcription efficiency, transcription kinetics are also likely affected by m⁶A modification. Consistent with this speculation, although efficiency of nascent RNA synthesis is not greatly affected, the speed of synthesis is accelerated in m⁶A demethylase ALKBH5 deficient cells [26], indicating that methylation levels of m⁶A-containing mRNA transcripts may have a regulatory role in the control of RNA synthesis kinetics.

mRNA splicing

Pre-mRNA splicing is an essential step in gene expression. It involves precise excision of introns and joining of exons from primary transcripts in the nucleus to generate mature mRNA [74]. Emerging evidence supports the correlation of m⁶A with RNA splicing, although the precise mechanism remains unclear. For instance, there is a significant accumulation of premature mRNA but decrease of mature mRNA in cycloleucine-treated avian sarcoma virus-affected cells [75]. In addition to its impact on global mRNA splicing, impact of m⁶A on specific mRNA splicing has also been determined. Quantitative S1 nuclease mapping of nuclear bPRL revealed that NPC-treated CHO cells contained 4–6-fold more bPRL precursor in the nucleus than control cells [61]. In cycloleucine-treated CHO cells, the average size of heterogeneous nuclear RNA (hnRNA) also shifted significantly from low to higher molecular weight [68]. Similar results have also been observed with SV40 RNA after NPC treatment [73], indicating that m⁶A modification favors the progression of RNA splicing.

Interestingly, numerous genes, especially the methylated genes, showed differential expression at isoform levels but not gene levels as shown from m⁶A-IP/RNA-seq data analysis of METTL3-knockdown HepG2 cells. Meanwhile, spliced exons and introns were significantly enriched with m⁶A peaks, indicating an intrinsic connection between m⁶A and alternative splicing of mRNAs [24].

The mechanism of m⁶A regulation on splicing remains poorly understood. We speculate that occurrence of methylation may interfere with the interaction between splicing factors and mRNA. m⁶A clusters may serve as docking sites for certain kinds of RNA-binding proteins; alternatively, since A-U base pairing may be destabilized by m⁶A [76,77], RNA secondary structure might be affected as well. As aforementioned, MT-A interacts with At FIP37 [7], which is an RNA binding protein involved in regulating RNA splicing [78]. Therefore another possible mechanism might be through the interacting partners of methyltransferases or demethylases. A recent study of ALKBH5 raised another possibility that m⁶A levels may affect gene expression or nuclear speckle assembly of splicing factors [26]. Immunofluorescence analysis showed that ALKBH5-deficiency significantly affected nuclear speckle staining of several splicing factors including SC35, ASF/SF2 and SRPK1. Since expression and phosphorylation levels of

these factors are closely related with their activities in the regulation of pre-mRNA splicing, alternative splicing would be affected as a consequence. Taken together, m⁶A may play a regulatory role in pre-mRNA splicing, but further experimental evidence is required to consolidate this connection.

mRNA nuclear export

After splicing, mature mRNA has to be exported out of nucleus to be either translated or degraded in the cytoplasm. In STH-treated HeLa cells, retention time of nuclear mRNA increased by 40%, but there was no change to non-polyadenylated RNA [66]. This suggests that m⁶A modification itself is not utilized for nuclear export, but plays a regulatory role in the nuclear export machinery. In ALKBH5-deficient cells that have enhanced m⁶A levels, nascent synthesized RNA was found mainly distributed in cytoplasm as observed via 5-bromouridine (BrU) incorporation analysis [26]. Furthermore, to clarify whether only mRNA was affected, poly(A) RNA and rRNA specific FISH experiments have been performed to monitor the dynamics of nuclear export of mRNA and rRNA, respectively. The results confirmed that nuclear retention time of mRNA only was decreased when the m⁶A level was enhanced as a result of ALKBH5 deficiency.

However, cycloleucine was found to affect nuclear retention time of rRNA in CHO cells [64,79]. Considering that cycloleucine inhibits both m⁶A and 2'-O-methylation to the same extent, and 2'-O-methylation is abundant in rRNA, it is possible that rRNA nuclear export was suppressed via the pathway mediated by 2-O-methylation instead of m⁶A.

It has been reported that different RNA species utilize different pathways for nuclear export through nuclear pore complexes [80]. The TAP-P15 complex is the main exporter utilized for mRNA export with the assistance of adaptor proteins, such as ALY/REF adaptor, SR proteins and the TREX complex [81,82]. Since phosphorylation levels of ASF/SF2 determine its function in splicing or nuclear export, we speculate that decreased phosphorylation of ASF/SF2 conferred by ALKBH5-deficiency would strengthen its interaction with the TAP/P15 complex, thereby leading to accelerated nuclear mRNA export. In contrast to mRNA, rRNA can recruit several different pathways to make its export more efficient. The Ran system and Crm1 export receptor bridged by Nmd3 adaptor are the dominant export systems [83,84], while the TAP-p15 complex [85] or Arx1 [86] are just auxiliary shuttling export receptors. Therefore, rRNA nuclear export may not be significantly affected by ALKBH5 deficiency. Interestingly ALKBH5-deficiency also led to abnormal aggregation of SRPK1 protein in cytoplasm [26]. SRPK1 is responsible for phosphorylation of splicing factors to facilitate their involvement in splicing of pre-mRNA [87]. Given the importance of the demethylation activity of ALKBH5 on the cellular locations of some of these mRNA processing factors, it is likely that the m⁶A methylation status of mRNA transcripts targeted by ALKBH5 influences the cellular dynamics of their processing factors. The detailed pathways and mechanisms should be further investigated.

mRNA translation

Most m⁶A modification occurs in exons, thus m⁶A remains in the mature mRNA after splicing. Therefore, m⁶A may also

affect the translation of m⁶A-containing mRNA transcripts. Mouse DHFR mRNA was *in vitro* methylated followed by *in vitro* translation using rabbit reticulocyte system. A 1.5-fold increase was detected when comparing the translation level of methylated transcripts to that of unmethylated transcripts [88]. Similarly, when cytoplasmic transcripts purified from cycloleucine-treated cells were *in vitro* translated, the amount of DHFR protein produced from undermethylated mRNA was 20% less than untreated mRNA [89]. Although cycloleucine can inhibit both 2'-O-methylation and m⁶A methylation, 2'-O-methylation normally plays a negative effect on protein translation; therefore the observed enhancement of translation after cycloleucine treatment should be caused solely by m⁶A modification.

mRNA stability

Regulation of mRNA decay is a major factor affecting overall cellular mRNA abundance. Since m⁶A is not detected in the poly (A) tail, we speculate that at least it is not involved in polyadenylation-dependent mRNA decay. Camper SA et al. have checked the effect on mRNA stability by treating of HeLa cells with STH. They found that although m⁶A inhibition was almost complete in the presence of up to 500 μM STH, mRNA stability was not greatly affected [66].

However, as identified by high throughput sequencing analysis, m⁶A is enriched in the 3'-UTR region. 3'-UTR contains several important functional domains required for mRNA decay, such as AU-rich element (ARE), iron-responsive element (IRE) and cytoplasmic polyadenylation element (CPE) [90]. Meanwhile, 3'-UTR is a region targeted by microRNAs (miRNAs) [91], therefore we cannot rule out the possibility that m⁶A may be involved in regulation of mRNA stability. As an example, ELAV1/HuR, a potential m⁶A-binding protein, was able to bind to the ARE region and stabilize corresponding transcripts [92].

The effect of ALKBH5 deficiency on overall RNA stability has been evaluated, but only a very modest decrease of RNA stability was detected [26]. Since 5-ethynyluridine could be labeled into all kinds of RNA, what is observed might be an effect on a mixed RNA population. Thus it is difficult to draw conclusions as to how mRNA stability is affected upon ALKBH5-deficiency. The other possibility could be that the effect of m⁶A on mRNA stability may be gene-specific, therefore a significant change in global RNA stability is unlikely to happen.

m⁶A-binding proteins

Similar to 5-methylcytosine (5-mC), m⁶A itself may serve as a docking site for RNA-binding proteins. Using an RNA affinity chromatography approach, several potential m⁶A-binding proteins have been suggested such as ELAV1, YTHDF2 and YTHDF3 [24], although their individual biochemical features remain to be further elucidated. m⁶A levels should be closely related with the binding affinity of these proteins on mRNA. Alternatively, occurrence of m⁶A may affect secondary structures of RNA and their accessibility by other RNA binding proteins.

ELAV1 protein is one of the potential m⁶A-binding proteins, which is highly expressed in cancer cells and also gets involved in inflammation through regulation of mRNA

stability, splicing and translation [93]. ELAV1 regulates mRNA stability by binding to ARE located in the 3'-UTRs of the mRNA [94]. As an example, ELAV1 can specifically bind to the 3'-UTR of c-myc mRNA [95]. Meanwhile, m⁶A-IP-seq analysis reveals that the localization of destabilization element of c-myc also overlaps with m⁶A peaks [24]. Therefore, we speculate that m⁶A methylation levels may have some link with the ELAV1 protein-mRNA interaction. There is also evidence suggesting that regulation of ELAV1-bound transcripts is also dependent on its interplay with miRNAs [96]. ELAV1 and miRNA have both competitive and cooperative interactions since they share the same target mRNAs [97,98]. A transcriptome-wide analysis revealed that ELAV1-binding sites on mRNA are in close proximity to miRNA-targeting sites, but they do not overlap with each other [99]. As described before, m⁶A methylation sites are also proximal but not overlapping with miRNA targeting sites [24]. It may be worthwhile studying the role of m⁶A in the functional interplay between ELAV1 and miRNAs. Another potential candidate protein YTHDF2 is found to be a translocation partner of RUNX1 in acute myeloid leukemia patients [100]. The existence of m⁶A-binding proteins may connect m⁶A to its vital functions in distinct basic life processes.

Remarks

GO analysis of m⁶A-containing mRNA transcripts reveals that m⁶A-containing transcripts are involved in many functional categories, indicating that m⁶A has broad and fundamental functions [24]. However there are numerous challenging issues to be addressed before detailed elucidation of epigenetic regulatory roles of m⁶A in RNA metabolism.

The first issue is to explore a correlation of m⁶A position along mRNA and its function. The 3'-UTR region is important for mRNA regulation through its influence on mRNA stability, cellular localization, and translation efficiency via its interaction with RNA binding proteins. Since transcriptome-wide analysis revealed that m⁶A is enriched in the 3'-UTR region, it is possible that occurrence of m⁶A modification may affect the docking of those RNA binding proteins on the 3'-UTR and their functions subsequently. Indeed it has been reported that there is an overlap between known RNA regulatory elements and m⁶A peaks in certain kinds of mRNAs [24]. On the other hand, since m⁶A sites in the 3'-UTR are proximal to miRNA-targeting sites and there is an inverse localization between miRNA targeting sites and m⁶A sites, it is also possible that m⁶A methylation may affect the targeting of mRNA by specific miRNA, and consequently corresponding RNA functions are negatively affected.

Human methyltransferase consists of MT-A and MT-B complexes, but so far only a 70-kDa subunit METTL3 from MT-A has been identified [41]. Therefore, the second crucial issue is to identify additional methyltransferase components and their biological functions. Transcription levels of METTL3 are dependent upon intracellular levels of Adomet, which is regulated by methionine availability. Interestingly, methionine metabolism is closely associated with cancer [101]. Furthermore, higher m⁶A methyltransferase activity was detected in transformed cells than in non-transformed cells, indicating an intrinsic relationship between m⁶A methylation and cancer [102]. Immunoblotting analysis using m⁶A antibody confirmed

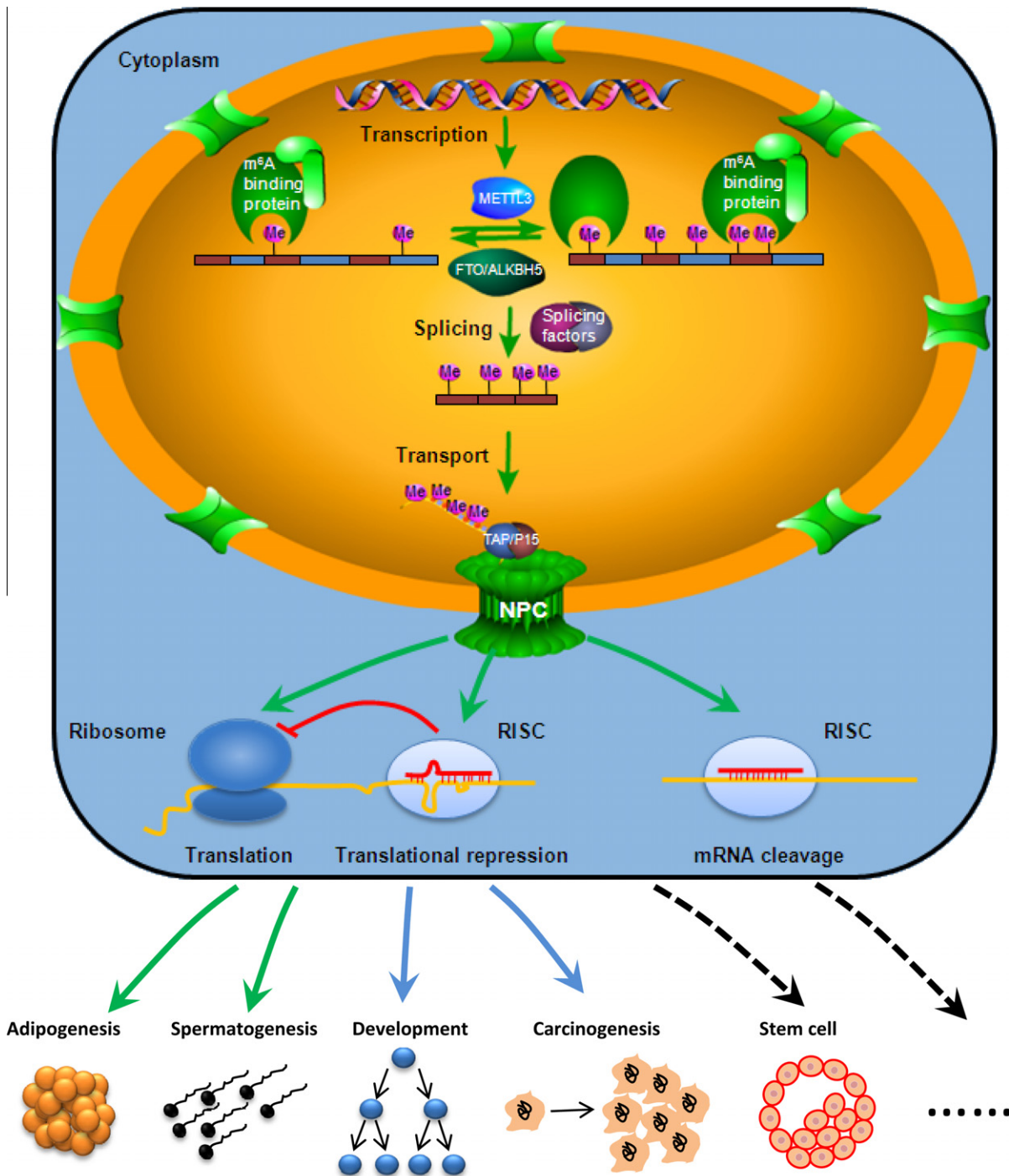


Figure 3 Working model of m⁶A in regulating mRNA metabolism

As a ubiquitous modification in mRNAs, m⁶A methylation occurs immediately after pre-mRNA transcription by METTL3-containing methyltransferases; while FTO and ALKBH5 are responsible for m⁶A demethylation. Through mutual interplay between methyltransferases and demethylases, m⁶A level is kept in balance to form a docking site for m⁶A binding proteins and proper assembly of RNA secondary structure. The mRNA transcripts with adequate m⁶A level can be properly spliced, transported, translated or degraded. Unbalanced m⁶A regulation will cause defects in RNA metabolism at each step shown above. Functional studies on methyltransferase, demethylases and potential m⁶A-binding proteins reveal that m⁶A in RNA may be involved in adipogenesis (FTO), spermatogenesis (ALKBH5), development (METTL3, FTO and ALKBH5), carcinogenesis (YTHDF2), stem cell renewal and other unidentified life processes.

that m⁶A levels in mouse and rat also varied a lot throughout brain development [23]. Coincidentally, METTL3 is differen-

tially expressed during embryo development, suggesting that m⁶A may be correlated with development as well [50].

Another significant issue is to investigate m⁶A-mediated biological functions from the angle of RNA demethylases. Although both belong to the AlkB family, FTO and ALKBH5 have been shown to have distinct biological functions so far. This may be attributed to their different tissue-specific expression patterns and different substrate preferences. We speculate that m⁶A plays a role in FTO-regulated homeostasis, however the mechanism remains unclear. Additionally, the phenotype of ALKBH5-deficient mice and increased m⁶A levels in testis tubular mRNA suggests an essential regulatory role of m⁶A in spermatogenesis. In addition, considering the ubiquitous distribution and fundamental functions of m⁶A modification, we cannot rule out the possibility that additional RNA demethylases may exist and catalyze different RNA substrates including mRNAs, rRNAs, tRNAs in a cell-/tissue-specific context. Similar to 5-mC, m⁶A itself may serve as a docking site for RNA-binding proteins. Furthermore, the identification and characterization of m⁶A-binding proteins is also vital to unveil the functions of m⁶A modification in distinct basic life processes.

In conclusion, m⁶A methylation plays broad and important roles through the functional interplay among m⁶A methylases, demethylases and m⁶A binding proteins [103]. Abnormal m⁶A methylation levels induced by defects in any factor in this network may lead to dysfunction of RNA and cause diseases. The 5-mC modification of DNA is known to play dynamic roles in epigenetic regulation in mammalian cells. Discovery and characterization of the first two RNA demethylases FTO and ALKBH5 indicates that, similar to the epigenetic regulatory role of DNA methylation, the reversible m⁶A modification in mammalian mRNA represents another novel epigenetic marker with broad roles in fundamental biological processes including adipogenesis, spermatogenesis, development, carcinogenesis, stem cell renewal and other as yet uncharacterized processes (Figure 3).

Competing interests

The authors have declared that no competing interests exist.

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