Functions and Dynamics of Methylation in Eukaryotic mRNA

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Contents

Abstract Eukaryotic messenger RNA (mRNA) contains non-canonical nucleosides, which are modified mostly by methylation. Although some modifications are known for decades, advances in high-throughput sequencing and mass spectrometric techniques now have allowed to elucidate transcriptome wide methylation patterns. The discovery of methyltransferases that write and demethylases that erase methylations in a sequence-specific manner, as well as reader proteins that recognize these modifications leading to a specific biological response, has triggered wide

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attention converting the research field of mRNA methylation into a current hotspot in molecular biology.

Most research has focussed on N^6-methyladenosine (m^6A), which is the most abundant modification in eukaryotic mRNA. Therefore, this overview has a focus on m^6A summarizing the current knowledge on how specific m^6A patterns are generated and how they are recognized and translated into biological outputs like alternative splicing, altered transcript stability, or modified translational activity of mRNAs. The distribution patterns of other methylations in mRNA, such as N^1-methyladenosine (m^1A), 5-methylcytidine (m^5C) and 5-hydroxymethylcytidine (hm^5C) have also been mapped in recent years. We review the current knowledge regarding these and other minor eukaryotic mRNA methylations and provide an outlook suggesting potential future research directions.

Keywords Methyltransferase · Demethylase · N^6-mAMP · 2′-O-methylation (N_m) · 7-methylguannosine (m^7G) · 3-methylcytidine (m^3C) · N^4-acetylcytidine (ac^4C) · YTH family proteins · N^6-methyl AMP deaminase (MAPDA)

1 Introduction

In addition to the four canonical nucleosides adenosine (A), guanosine (G), cytosine (C) and uridine (U), over 150 chemical nucleoside modifications have been discovered in RNA species of all kingdoms of life. Comparatively few modified nucleosides, mostly altered by methylation, are known in eukaryotic messenger RNA (mRNA), including N^6-methyladenosine (m^6A), 5-methylcytidine (m^5C), N^1-methyladenosine (m^1A), 2′-O-ribose methylation of nucleosides with a canonical base (Nm), N^7-methylguanosine (m^7G), 3-methylcytidine (m^3C), and N^4-acetylcytidine (ac^4C) (Fig. 1). Although the latter is an acetylation, we will also review the very recent discovery of this modification in eukaryotic mRNA. Some of these modifications are known to be reversible and have essential roles in regulating gene expression. In this chapter, we review the function and dynamics of these eukaryotic mRNA methylations, focusing on the most abundant mRNA methylation, m^6A, as well as summarizing the current knowledge concerning other methylations.

2 The Functions and Dynamics of N^6-methyladenosine (m^6A)

N^6-methyladenosine is the most prevalent (0.4–0.6% of adenosine is modified in mammalian and plant mRNA) and currently best studied base modification present in the mRNA of all eukaryotes, including yeast (Bodi et al. 2010), plants (Zhong et al. 2008), flies (Hongay and Orr-Weaver 2011) and mammals (Horowitz et al. 1984; Schibler et al. 1977; Yoon et al. 2017). Although it was already discovered in

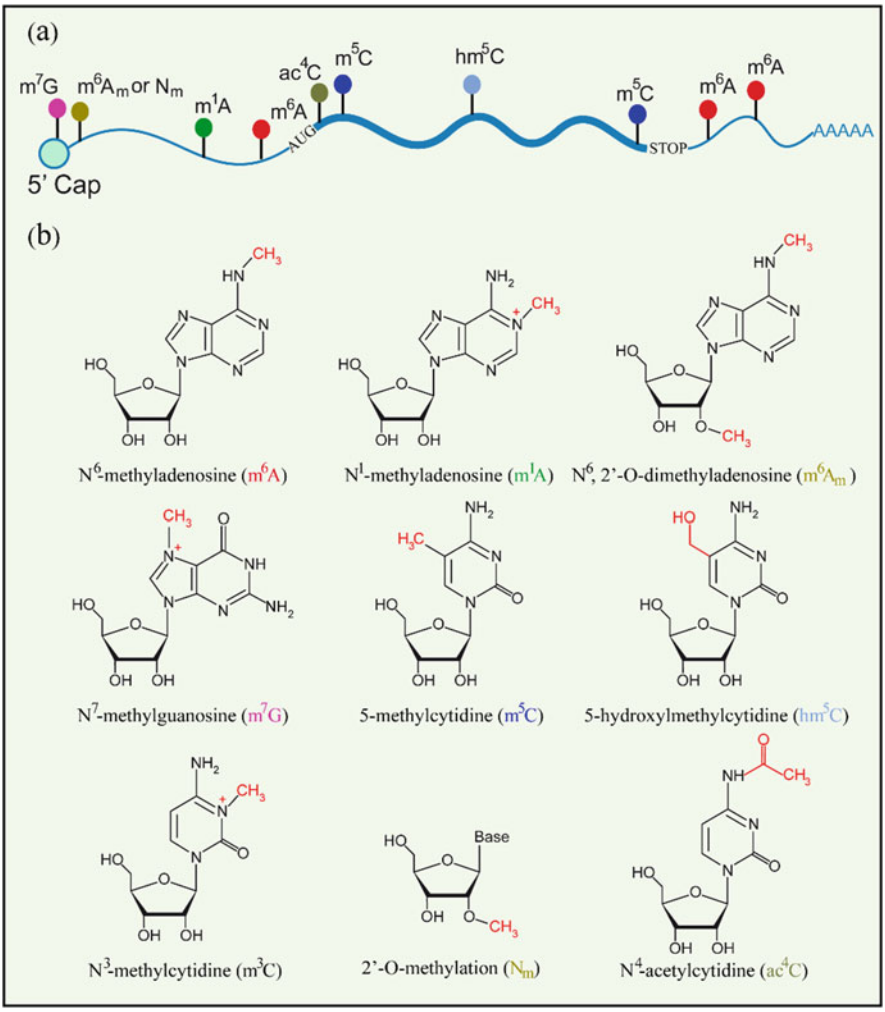

Fig. 1 Currently known nucleoside methylations, hydroxymethylations, and acetylations in eukaryotic mRNA. (**a**) The typical positions of the respective modified nucleosides in eukaryotic mRNA. Note that the typical distribution of m^3C is not yet known. (**b**) The chemical structures of methylated, hydroxymethylated, and acetylated nucleosides found in mRNA

mammalian cells in 1974 (Desrosiers et al. 1974; Perry and Kelley 1974), transcriptome-wide distribution of m^6A was poorly characterized until 2012, when it was mapped on the whole transcriptome for the first time by antibody-mediated immunoprecipitation coupled with high-throughput sequencing (Dominissini et al. 2012). In human, over 12,000 m^6A sites were unveiled in more than 7000 transcripts, clustered around stop codons, 3′ untranslated regions (3′ UTRs), and within long internal exons (Fig. 1). The modification occurs at the consensus RNA motif RRm^6ACH (R = G or A; H = U or A or C) (Dominissini et al. 2012; Meyer et al. 2012). The dynamics and molecular functions of m^6A as a novel post-transcriptional

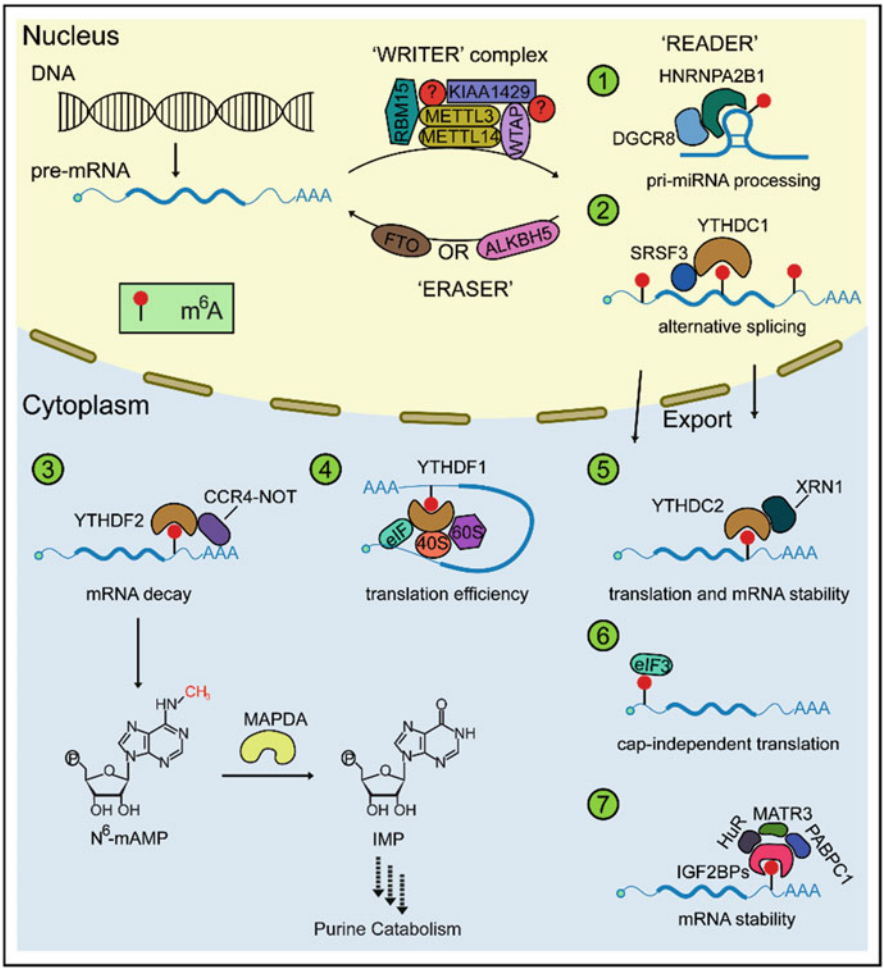

Fig. 2 The dynamics and functions of m^{6}A in eukaryotic mRNA. m^{6}A formation is catalyzed by the writer protein complex at specific sites in pre-mRNA. Marks can be removed by easer proteins. Reader proteins recognize m^{6}A sites and affect various processes including pri-miRNA processing, alternative splicing, translation, and mRNA stability (①–⑦). The N^6-mAMP released from m^{6}A-RNA turnover is catabolized by MAPDA to the canonical nucleotide inosine monophosphate (IMP). All abbreviations for proteins are explained in the text

regulator of gene expression have been established since 2011, when the first m^{6}A demethylase FTO (fat mass and obesity-associated protein) was identified in mammalian cells (Jia et al. 2011). In the nucleus, m^{6}A is installed and removed by so called 'writer' and 'eraser' proteins, respectively. The mature m^{6}A mRNAs are decoded by 'reader' proteins to mediate various biological processes, such as alternative splicing, mRNA translation, and mRNA stability. N^6-mAMP is released from these methylated mRNAs upon their degradation, and is catabolized by N^6-methyl AMP deaminase (MAPDA) to inosine monophosphate (IMP), which is a metabolite of the canonical purine nucleotide metabolism (Fig. 2).

2.1 The Writer Complex: m^6A Methyltransferase Complex

The installation of m^6A occurs in the nucleus by a methyltransferase complex conserved in eukaryotes (Fig. 1) with an approximate molecular weight of 875 kDa (Bokar et al. 1994). The complex contains two core components, Methyltransferase Like 3 (METTL3) (Bokar et al. 1997), and METTL14 (Liu et al. 2014; Wang et al. 2016b), and several accessory proteins like Wilms Tumor 1 Associated Protein (WTAP) (Ping et al. 2014), and RNA Binding Motif Protein 15 (RBM15) (Patil et al. 2016), and KIAA1429 (Schwartz et al. 2014).

In the search for proteins responsible for the m^6A formation, a 70-kDa fraction from partial protein purification was characterized, which exhibited S-adenosylmethionine (SAM)-binding activity (Bokar et al. 1994). Purification of the corresponding recombinant protein identified a m^6A methyltransferase, originally termed MT-A70 but later renamed METTL3. Mutation of the *METTL3* ortholog in Arabidopsis (here called adenosine methylase A, MTA) leads to the complete loss of m^6A in mRNA resulting in failure of the developing embryo to progress past the globular stage (Zhong et al. 2008). Also in other organisms METTL3 has critical functions. In *Saccharomyces cerevisiae*, it is involved in the regulation of cell fate decision during sporulation and meiosis (Agarwala et al. 2012; Bodi et al. 2010; Clancy et al. 2002), while in *Drosophila melanogaster* it modulates neuronal functions and sex determination through alternative splicing of the *Sxl* gene (Haussmann et al. 2016; Lence et al. 2016). METTL3 localizes in the nuclear speckles in plant and mammalian cells.

METTL3 belongs to a larger family of SAM-dependent methyltransferases that is highly conserved in mammals (Schapira 2016). METTL14, another family member, shares 43% identity with METTL3 (Liu et al. 2014). Interestingly, three independent crystallization studies demonstrated that METTL14 has no methyltransferase activity as it lacks a SAM-binding domain (Śledź and Jinek 2016; Wang et al. 2016a, b). It is now known, that METTL3 and METTL14 form a stable heterodimer in the m^6A methyltransferase complex. Crystal structures of the METTL3 and METTL14 complex showed that only METTL3 binds SAM while METTL14 appears to assist in RNA binding in concert with METTL3 and enhances the enzymatic activity of METTL3 (Wang et al. 2016b). Abrogation of *METTL14* in mouse decreases m^6A abundance in mRNAs of embryonic stem cells, which blocks stem cell self-renewal and differentiation leading to early embryonic lethality (Geula et al. 2015; Wang et al. 2014a). The Arabidopsis ortholog of METTL14, MTB (adenosine methylase B), is also a core member of the methyltransferase complex. Its absence results in an arrest of embryonic development at the globular stage and reduces the m^6A/A ratio from 1.6% to 0.75% in mRNA (Růžička et al. 2017).

While characterizing the Arabidopsis m^6A methyltransferase MTA, a yeast two-hybrid screen for MTA-binding proteins was performed, which resulted in the identification of FIP37 (FKBP12-interacting protein of 37 kDa), the plant otholog of human WTAP (Zhong et al. 2008). Subsequent studies highlighted the functional importance of WTAP. It interacts with METTL3 and METTL14, and is required for

their localization into nuclear speckles, which are enriched with pre-mRNA processing factors and are the place for m^6A formation in vivo (Ping et al. 2014). WTAP depletion causes a strong reduction of the RNA-binding capability of METTL3 and loss of METTL3 and METTL14 localization from nuclear speckles (Ping et al. 2014). Therefore, WTAP is considered to be an adaptor protein connecting the m^6A methyltransferase complex to nuclear speckles.

Recently, RBM15 and its paralogue RBM15B were identified in mammalian cells as accessory components of the m^6A methylation complex (Patil et al. 2016). They were shown to recruit the m^6A methylation core complex to specific sites in mRNA. Co-immunoprecipitation analyses revealed that RBM15 and RBM15B interact with METTL3 in the presence of WTAP. Importantly, knockdown of *RBM15* and *RBM15B* led to a significant reduction of m^6A in mRNA, indicating that they are indeed functional components of the m^6A methyltransferase complex. These results are also supported by data from Drosophila where Spenito, the RBM15 ortholog in fly, is required for m^6A formation (Lence et al. 2016). Interestingly, RBM15 and RBM15B bind to uridine-rich regions in mRNAs, which are in proximity to sites that are methylated to m^6A (Patil et al. 2016), suggesting that these proteins guide the m^6A methyltransferase complex to appropriate sites on the mRNA. Additional guide proteins may exist, because not all m^6A methylation sites are found near uridine-rich regions, and RBM15 and RBM15B-mediated methylations represent only a subset of m^6A marks in mRNA.

Two independent proteomic screens revealed that KIAA1429, also known as VIRMA (vir like m^6A methyltransferase associated), associates with the core components of the m^6A methyltransferase complex in mammalian cells (Schwartz et al. 2014; Yue et al. 2018). Similarly, Virilizer, the Drosophola ortholog of VIRMA, interacts with Fl(2)d (female lethal2), the ortholog of WTAP, and regulates alternative splicing of pre-mRNAs involved in sex determination in flies (Ortega et al. 2003). The depletion of VIRMA leads to about four-fold reduction of m^6A, demonstrating that it is required for the full methylation pattern in mammals (Schwartz et al. 2014). Very recently it was reported that VIRMA guides the core components of the m^6A methyltransferase complex to specific sites on mRNAs (Yue et al. 2018).

In addition to the above components, other members of m^6A methyltransferase complex, such as the E3 ubiquitin ligase HAKAI (Horiuchi et al. 2013; Růžička et al. 2017), may also guide the methyltransferase complex to specific methylation sites.

2.2 *The Erasers: m^6A Demethylases*

An important advance in the m^6A field was the identification of the first m^6A demethylation enzyme, fat mass and obesity-associated protein (FTO) (Jia et al. 2011). Soon later, another m^6A demethylase, α-ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5), was identified (Zheng et al. 2013). The discovery of enzymatic activities that remove m^6A marks indicated that these RNA modifications are dynamically regulated (Fig. 2).

FTO belongs to the non-heme Fe(II)- and α-ketoglutarate-dependent dioxygenase AlkB family of proteins, and was initially characterized as an enzyme demethylating m^3T and m^3U in single-stranded DNA (ssDNA) and single-stranded RNA (ssRNA) in vitro (Gerken et al. 2007; Jia et al. 2008), although both observed activities were much lower than those of the other AlkB-family proteins (Lee et al. 2005). In 2011, Jia et al. found that FTO could efficiently demethylate m^6A in mRNA both in vitro and in vivo (Jia et al. 2011). During the oxidation of m^6A to A, FTO generates N^6-hydroxymethyladenosine and N^6-formyladenosine as intermediate products. The depletion of *FTO* in mammalian cells induced a significant increase of m^6A content in mRNA. Interestingly, another study demonstrated that FTO also exhibited demethylation activity towards N^6, 2′-O-dimethyladenosine (m^6A_m), which is exclusively located at the +1 position downstream of the 7-methylguanosine cap (Fig. 1) in many mRNAs (Mauer et al. 2017). By selectively demethylating m^6A_m, FTO reduces the stability of m^6A_m-containing mRNAs. A very recent study comprehensively investigated the cellular distribution and the substrates of FTO in vivo (Wei et al. 2018a). The authors demonstrated that FTO preferentially demethylates internal m^6A of pre-mRNAs in the nucleus as well as the cap m^6A_m and m^6A in mRNA in the cytosol. Additionally, internal m^6A in U6 RNA, internal and cap m^6A_m in small nuclear RNAs (snRNAs) and N^1-methyladenosine (m^1A) in tRNA can be demethylated by FTO.

Another m^6A demethylase, ALKBH5, was initially identified in a biochemical screen testing the demethylation activity of recombinant human AlkB homologs towards m^6A-labelled ssRNA substrates (Zheng et al. 2013). As FTO, it is a demethylase that can reverse m^6A methylation by oxidation. The depletion of *ALKBH5* in mammalian cells resulted in approximately 9% increase of the m^6A level in mRNA, whereas a 29% decrease was observed by its overexpression. Similar to the m^6A methyltransferase METTL3, ALKBH5 is located in nuclear speckles, suggesting that it is involved in pre-mRNA processing. In addition, the knockdown of *ALKBH5* leads to a dramatic increase of cytoplasmic mRNA amounts as a result of accelerated nuclear mRNA export (Zheng et al. 2013).

In plants, *ALKBH10B*, a homolog of mammalian *ALKBH5*, influences floral transition. *ALKBH10B* mutation leads to global m^6A hypermethylation and reduces the stability of its target transcripts. Mutant plants exhibit late flowering, producing approximately seven extra leaves before the first bud bloomes (Duan et al. 2017). Furthermore, Arabidopsis ALKBH9B has been reported to be an additional plant m^6A demethylase in vitro. It co-localizes with siRNA bodies and associates with processing bodies (P-bodies) in the cytoplasm, implying its involvement in mRNA silencing and mRNA decay (Martínez-Pérez et al. 2017).

2.3 *The Readers: m^6A Recognition Proteins*

N^6-methyladenosine in mRNA can directly affect the secondary structure by altering RNA-RNA base pairing, as well as RNA-protein interactions of the transcripts. It can

influence every step of an mRNA's lifetime, including splicing, export, translation and degradation (Roundtree et al. 2017). The major mechanism by which m^6A exerts its function is by recruiting specific RNA-binding proteins, the so-called reader proteins. To date, two types of reader proteins for m^6A recognition were identified. The first type binds directly to the m^6A base and includes the YTH family proteins (Hsu et al. 2017; Li et al. 2017b; Shi et al. 2017; Wang et al. 2014b, 2015; Xiao et al. 2016), the eIF3 (Meyer et al. 2015), and the IGF2BPs (Huang et al. 2018). The second type, for example the reader protein HNRNPA2B1 (Alarcón et al. 2015), selectively recognizes an m^6A-mediated secondary structure in the mRNA (Fig. 2).

The definition of the YTH family proteins began when an approximately 140 amino acid domain of the human splicing factor YT521-B was shown to have RNA binding capacity (Hartmann et al. 1999). Basic Local Alignment Search Tool (BLAST) analyses revealed that this domain is highly conserved in YT521-B homologs as well as in other proteins of eukaryotes. Accordingly, the YT521-B homologs were called YTH domain-containing family proteins (YTHDFs) while other proteins, which otherwise are not homologous to YT521-B, were called YTH domain-containing proteins (YTHDCs). Together, these proteins are named YTH (for YT521-B homology) family proteins (Stoilov et al. 2002).

In vertebrates, there are three YTHDFs called YTHDF1, YTHDF2, and YTHDF3. YTHDF2 is the first m^6A reader protein identified from mammalian cells (Wang et al. 2014b). Over 3000 cellular RNAs with a conserved core motif of G(m^6A)C were identified as the targets of YTHDF2. Upon YTHDF2 binding such mRNAs are transferred from the translatable pool to P-bodies, where mRNA decay occurs. Further research revealed that YTHDF2 recruited the CCR4-NOT deadenylase complex (Fig. 2 ③) through a direct interaction and then mediated the degradation of m^6A-containing RNAs (Du et al. 2016). The knockdown of *YTHDF2* results in a prolonged lifetime and increased expression of its target transcripts (Wang et al. 2014b). A homolog of YTHDF2, YTHDF1 can also recognize m^6A on mRNA. In contrast to YTHDF2, YTHDF1 actively promotes protein synthesis by interacting with the translation machinery, including the 40S ribosome, the 60S ribosome, and the translation initiation factor eIF3 (Fig. 2 ④). The knockdown of *YTHDF1* leads to reduced translation of its target transcripts (Wang et al. 2014b). YTHDF3, interacts with both YTHDF1 and YTHDF2 and affects the RNA binding specificity of each of them (Li et al. 2017b; Shi et al. 2017). YTHDF3 promotes translation in synergy with YTHDF1 and accelerates m^6A-containing mRNA decay through the interaction with YTHDF2. All three YTHDFs are located in the cytoplasm, where they together regulate the cytoplasmic metabolism of m^6A mRNA.

YTHDCs have two members, named YTHDC1 and YTHDC2. Among the YTH family proteins, YTHDC1 is the only member located in the nucleus (Fig. 2 ②) where it binds the m^6A-containing pre-mRNAs and regulates their splicing (Xiao et al. 2016). Protein-protein interaction and PAR-CLIP (photoactivatable ribonucleoside crosslinking and immunoprecipitation) sequencing revealed that YTHDC1 interacts with the serine/arginine-rich splicing factors SRSF3 and SRSF10. SRSF3 binds maturing mRNA to promote exon inclusion whereas SRSF10 facilitates exon

skipping. YTHDC1 targets m^6A in exonic regions, where it recruits SRSF3 but blocks SRSF10-mRNA binding to modulate alternative splicing of pre-mRNA (Xiao et al. 2016). Another study showed that YTHDC1 also binds the m^6A residues on lncRNA *XIST*, which in total contains more than 78 m^6A sites, to trigger transcriptional repression on the X chromosome (Patil et al. 2016).

The cytoplasmic YTHDC2 has the highest molecular weight of all YTH family proteins (~160 kDa vs ~60 kDa for other members). Biochemical analyses and CLIP-seq (crosslink immunoprecipitation sequencing) indicated that YTHDC2 selectively binds m^6A mRNA at its consensus motif, GGm^6ACU, which is frequently found around the stop codon (Fig. 2 ⑤). The knockout of *YTHDC2* in mouse leads to an increase in target mRNA abundance and a decrease in translation efficiency, which in turn affects spermatogenesis and results in both male and female infertility (Hsu et al. 2017). YTHDC2 contains not only a YTH domain but also several other domains, including an R3H domain (a RNA binding domain with an R-(X_3)-H motif), a DEAH-box helicase core domain, two ankyrin repeat (ANK) domains, and an HELICc helicase domain (Kretschmer et al. 2018; Meyer and Jaffrey 2017). The presence of many domains in YTHDC2 implies that this protein may have multiple functions. Subsequent studies demonstrated that YTHDC2 is also an RNA-induced ATPase with 3′→5′ RNA helicase activity, and it interacts with the 5′→3′ exoribonuclease XRN1 via the ankyrin repeats, which are strategically located between the two helicase domains of YTHDC2 (Wojtas et al. 2017). In addition, the R3H domain contributes together with the YTH domain to the selective binding of YTHDC2 to m^6A-containing RNA (Kretschmer et al. 2018).

Thirteen YTH domain-containing proteins (ECT1-12 and CPSF30) were identified in *A. thaliana* (Li et al. 2014). Among them, *ECT2* is the most highly and most widely expressed member. It binds the m^6A residues, which are enriched in the 3′ UTRs of target mRNAs (Scutenaire et al. 2018; Wei et al. 2018b). Subcellular localization and mRNA-seq analyses suggested that ECT2 regulates 3′UTR processing in the nucleus and its binding to the mRNA promotes transcript stability in the cytoplasm (Wei et al. 2018b). Depletion of *ECT2* leads to increased trichome branching (Arribas-Hernández et al. 2018; Scutenaire et al. 2018; Wei et al. 2018b), which is due to the accelerated degradation of three ECT2-binding trichome morphogenesis related transcripts (Wei et al. 2018b). Furthermore, disruption of *ECT2* and *ECT3* together results in a delayed emergence of the first true leaves (Arribas-Hernández et al. 2018).

Eukaryotic initiation factor 3 (eIF3) is an m^6A reader without a YTH domain (Meyer et al. 2015). Normally, the cap-binding protein eIF4 is necessary for translation initiation (Jackson et al. 2010). However, transcripts containing m^6A in the 5′UTR do not require eIF4, but recruit eIF3 (Fig. 2 ⑥) and other initiation factors for their translation. Therefore, 5′UTR m^6A promotes the translation of certain mRNAs (Meyer et al. 2015). It appears that eIF3 is involved in two distinct types of translational enhancement via m^6A. Either eIF3 binds directly to the m^6A site in the 5′UTR (Fig. 2 ⑥) (Meyer et al. 2015) or YTHDF1 recognizes m^6A residues close to the stop codon and then interacts and transfers eIF3 to the 5′UTR of the transcript (Fig. 2 ④) In this latter scenario eIF3 itself is technically not a reader protein, because the reader function is exerted by YTHDF1 (Wang et al. 2015).

Recently, the insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs, including IGF2BP1-3) were identified as an additional family of m^6A readers in mammals (Huang et al. 2018). All of them contain four tandem KH (K homology) domains, which are conserved ssRNA-binding domains required for m^6A recognition. IGF2BPs target thousands of cytoplasmic m^6A-bearing mRNAs through the consensus GG(m^6A)C motif and promote their stability. Further investigation revealed that several mRNA-stabilizing proteins, including Hu antigen R (HuR), matrin 3 (MATR3), and poly(A)-binding protein cytoplasmic 1 (PABPC1), interact with IGF2BPs. Co-localization of HuR and IGF2BPs (Fig. 2 ⑦) was observed in P-bodies, suggesting that they work together to protect their targets from degradation. Upon heat shock, IGF2BPs are recruited to stress granules possibly to protect mRNAs from harmful conditions (Huang et al. 2018).

Heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1), containing an RNA recognition motif (RRM) domain, has been reported to be a nuclear m^6A reader in the regulation of microRNA processing (Alarcón et al. 2015). Depletion of *HNRNPA2B1* leads to an accumulation of pri-miRNA transcripts and to a reduction of mature microRNAs, implying that this reader protein operates in the processing of pri-miRNAs to pre-miRNAs in the nucleus. Further biochemical analyses demonstrated that HNRNPA2B1 influences the microRNA processing via recruiting the microprocessor machinery protein DGCR8 (Fig. 2 ①). In addition, disruption of *HNRNPA2B1* affects alternative splicing patterns similarly to *METTL3* mutation. This suggests that the absence of m^6A marks or the inability to read them has similar effects on splicing (Alarcón et al. 2015). Intriguingly, a structure-based study indicated that HNRNPA2B1 does not directly bind m^6A (Wu et al. 2018), unlike the other reader proteins. In structural studies of the other reader proteins, aromatic cage-like surfaces were identified as key m^6A-specificity elements. However, such an aromatic pocket is not found in the RRM domain of HNRNPA2B1. By contrast, HNRNPA2B1 exhibits higher binding affinity towards non-methylated RNA substrates (Wu et al. 2018). Another study suggested that HNRNPA2B1 may selectively bind m^6A-containing RNAs through an m^6A-switch mode (Liu et al. 2015; Zhou and Pan 2018), in which m^6A decreases the stability of Watson-Crick base-pairing in RNA loops altering the local structure of the transcripts thereby facilitating the interaction of HNRNPA2B1 with the RNA (Fig. 2 ①).

2.4 The Metabolic Fate of the m^6A Mark

Quantitatively little alterations of m^6A patterns are observed after mRNAs are exported to the cytoplasm (Ke et al. 2017). Hence, N^6-methyl-AMP (N^6-mAMP) will be released with other nucleotides from m^6A-containing mRNA turnover at the end of its lifetime. Metabolite analysis showed that N^6-mAMP does not accumulate in aging Arabidopsis leaf tissue, suggesting that a catabolic pathway for N^6-mAMP exists. We recently identified an N^6-mAMP deaminase (MAPDA), which is widely conserved in eukaryotes. This enzyme catalyzes the hydrolysis of N^6-mAMP to

inosine monophosphate (IMP) and methylamine in the cytoplasm (Chen et al. 2018) and thus represents the endpoint of m^6A modification (Fig. 2). Mutation of *MAPDA* in *A. thaliana* or depletion in human HeLa cells both lead to a significant increase of N^6-mAMP content confirming the function of MAPDA in vivo. Also some N^6-mATP accumulates in *mapda* plants, which in vitro can be incorporated into newly synthesized RNAs. Therefore, we investigated whether m^6A abundance is altered in *mapda* plants but observed that possible changes of m^6A content in vivo were too small to be reliably quantified above the natural background of m^6A in RNA (Chen et al. 2018). We speculated that the RNA is protected not only by MAPDA but also by additional molecular filters from random incorporation of N^6-mATP. Cytoplasmic adenylate kinases, which exhibit a strong substrate preference for AMP over N^6-mAMP were tentatively identified as such additional filters (Chen et al. 2018).

3 Other mRNA Methylations and Acetylations in Eukaryotes

3.1 5-methylcytidine (m^5C)

DNA methylation at the 5′ position of cytosine has been known as an epigenetic maker for decades (Zhang et al. 2018). Interestingly, 5-methylcytidine (m^5C) also occurs in mRNA (Cui et al. 2017; David et al. 2017) and contributes to the epigenetic regulation of various biological processes (Motorin et al. 2009; Yang et al. 2017). Over 10000 m^5C sites were mapped in human mRNAs and other non-coding RNAs, and most of them were identified in untranslated regions, particularly at the binding sites for Argonaute proteins (Squires et al. 2012) and immediately downstream of translation initiation sites (Yang et al. 2017). In plants, 6045 m^5C peaks were detected in 4465 transcripts, and these marks are mainly located in the coding sequences of mRNAs with low translation activity immediately after the start codon and before the stop codon (Fig. 1) (Cui et al. 2017). The m^5C modification in mRNA is mainly introduced by the tRNA m^5C methyltransferase NOL1/NOP2/Sun domain family member 2 (NSUN2) and specifically bound by the mRNA export factor ALYREF in mammals (Yang et al. 2017). ALYREF, together with NSUN2, regulates the nuclear-cytoplasmic shuttling of the m^5C-containing mRNAs. In plants, abrogation of the m^5C methyltransferase reduces cell division in the root apical meristem, causing shorter primary roots and enhanced sensitivity to oxidative stress (David et al. 2017). Disruption of all four *ALY* genes in Arabidopsis (ALYs are homologs of ALYREF in mammals), results in nuclear mRNA accumulation. Phenotypically, vegetative and reproductive defects are observed, including severely reduced leave and root growth, altered flower morphology, as well as reduced seed production (Pfaff et al. 2018).

Furthermore, a small portion of m^5C in RNA (~0.02% m^5C) can be oxidized to 5-hydroxymethylcytosine (hm^5C) by the ten-eleven translocation (Tet)-family

enzymes in Drosophila and mammalian cells (Delatte et al. 2016; Fu et al. 2014). Tet-deficient flies have decreased RNA hydroxymethylation and suffer from impaired brain development. Notably, hm^5C was also present in RNA of *Caenorhabditis elegans* and *A. thaliana*, which do not contain hm^5dC in their DNA and lack *Tet* homologs in their genomes, implying a non-Tet mediated mechanism for hm^5C formation in RNA (Huber et al. 2015). Transcriptome-wide mapping in Drosophila revealed that hm^5C occurs in the transcripts of many genes at the consensus motif of 'UCCUC' and is more abundant in coding sequences (Delatte et al. 2016). The function of hm^5C in RNA, however, remains largely unknown.

3.2 N^1-methyladenosine (m^1A)

Besides m^6A, also a methylation at the N^1 position of adenosine (m^1A) occurs in eukaryotic mRNA (Dominissini et al. 2016; Li et al. 2016). However, m^1A is less abundant with an m^1A/A ratio of about 0.02% in mRNA of human cells. The modification is enriched in the 5′ untranslated region (Fig. 1) of mRNA transcripts (Li et al. 2016) and around the start codon upstream of the first splice site (Dominissini et al. 2016). m^1A can rearrange to m^6A under alkaline conditions at elevated temperatures (Dimroth rearrangement) (Macon and Wolfenden 1968), therefore, it might be erased in the detection process leading to its underestimation and the introduction of noise in m^6A measurements. In 2016, Dominissini and colleagues identified more than 7000 m^1A modification sites in human mRNA (Dominissini et al. 2016), while Li and colleagues detected 901 m^1A modification sites originating from 887 transcripts, encoded by 600 human genes (Li et al. 2016). Later, a m^1A-seq study reaching single-base resolution reported only 7 m^1A nucleotides at internal sites of cytosolic mRNAs and 5 m^1A modifications in the mitochondrial mRNA (Safra et al. 2017). Interestingly, Li and colleagues, who previously found 901 m^1A modification sites, also developed a single-nucleotide resolution mapping method for m^1A profiling at same time, and they identified 473 sites in cytosolic mRNA and 22 m^1A sites from mitochondrial genes (Li et al. 2017a). It appears that the actual number of m^1A modifications fluctuates either for biological or technical reasons.

The tRNA methyltransferase complex TRMT6/61A is required for a part of the m^1A marks in mRNA (Li et al. 2017a). The RNA demethylase ALKBH3 can remove m^1A. The m^1A profile is dynamic in response to stimuli, such as nutrient starvation and heat shock (Li et al. 2016). A very recent study demonstrated that YTHDF1-3 and YTHDC1, but not YTHDC2, bind directly to m^1A in RNA (Dai et al. 2018). However, the mechanism of m^1A installation in RNA, including writer protein(s) other than TRMT6/61A and their preferred sequence motives, as well as the functional roles of this mRNA methylation have yet to be elucidated.

3.3 2′-O-methylation (N_m)

Ribose methylation in mRNA usually occurs at the 2′O position (2′-O-methylation, N_m) in the second or the third base adjacent to the 5′ cap (Schibler and Perry 1977), and these are installed by the Cap-specific mRNA (nucleoside-2′-O-)-methyltransferase 1 (CMTR1) (Belanger et al. 2010) and CMTR2 (Werner et al. 2011), respectively, in mammals. N_m might be involved in the discrimination of self and non-self mRNAs (Daffis et al. 2010). A portion of N_m modifications also bear m^6A methylations to form m^6A_m (Linder et al. 2015). m^6A_m-containing transcripts are more stable than others, because the modification prevents DCP2-mediated decapping and microRNA-induced mRNA decay (Mauer et al. 2017). In vertebrates, the methylation of the N^6 position generating m^6A_m is introduced by PCIF1 (Phosphorylated C-terminal domain Interacting Factor 1), which interacts with the Ser5-phosphorylated C-terminal domain of RNA polymerase II, as cap-specific adenosine methyltransferase (CAPAM) complex (Akichika et al. 2018). Mutating *PCIF1* by CRISPR-Cas9 in human cells completely abrogates the N^6 methylation on A_m in the cap structure. The m^6A_m modification was restored when knockout cells were rescued by plasmid-encoded PCIF1 (Akichika et al. 2018). Dynamically, the m^6A modification of m^6A_m can be removed by the demethylase FTO (Mauer et al. 2017).

2′-O-methylation is also present at the 3′ end of microRNAs, thus protecting microRNA from 3′→5′ degradation. Such modification is installed in plants by the methyltransferase HUA-ENHANCER-1 (HEN1) (Yu et al. 2005).

3.4 7-methylguanosine (m^7G)

In addition to mRNA methylations listed above, there are a variety of other less abundant modifications in mRNA. Recently, 7-methylguanosine (m^7G), which was previously thought to only exist in the 5′ cap structure of mRNA, was mapped at internal positions of mRNA (Chu et al. 2018). In this research, the authors developed a novel analytical method to differentiate internal m^7G from that present in the 5′ cap. In detail, by comparing the digestion properties of different nucleases, namely S1 nuclease and phosphodiesterase I, they found that phosphodiesterase I can digest both cap and internal m^7G containing RNA while the S1 nuclease is only able to digest RNA with internal m^7G (Chu et al. 2018). High resolution mass spectrometry analyses coupled with differential nuclease treatments of mRNA revealed the existence of internal m^7G in mRNA in human cells, rats, as well as in plants. In plants, m^7G in mRNA occurs at a higher frequency (about 3.5×10^{-5}–5.5×10^{-5} m^7G/G) than in mammals (about 0.5×10^{-5}–1.5×10^{-5} m^7G/G). The abundance of m^7G in mRNA was elevated in rice when plants suffered environmental stress, such exposure to the higher concentrations of cadmium, which is widely distributed in soil (Chu et al. 2018).

3.5 3-methylcytidine (m^3C)

The rare modification 3-methylcytidine (m^3C, 0.004% of cytosine) was detected in mRNA from mouse liver and human cells recently (Xu et al. 2017). Apart from mRNA, m^3C is also present and comparatively abundant in tRNA (about 1.7% m^3C/C) but is absent in rRNA of mammalian cells. Dramatic reduction of m^3C abundance in mRNA was observed in *mettl8* knockout mice, indicating that its formation in mRNA is mediated by METTL8 (Xu et al. 2017). It was also shown that METTL2 and METTL6 contribute m^3C formation in certain tRNAs (Xu et al. 2017). Mice lacking either METTL2 or METTL6 or METTL8, showed no developmental defects, and growth rates of human cells, that were mutated in *METTL2* and *METTL8* as well as knockdown for *METTL6*, were not significantly altered (Xu et al. 2017). Up to date, little is known about the distribution, dynamics, and function of this newly identified mRNA modification.

3.6 N^4-acetylcytidine (ac^4C)

A very recent publication reported the identification of a novel mRNA modification, N^4-acetylcytidine (ac^4C), occurring with an abundance of approximately 0.2% ac^4C/C in the mRNA of mammalian cells (Arango et al. 2018). ac^4C immunoprecipitation coupled with the next-generation sequencing (acRIP-seq) revealed that the majority of acetylated transcripts possess one to two ac^4C modification site(s), and that the distribution of ac^4C generally displays a 5′ positional bias, especially clustering proximal to translation start sites within coding sequences. Acetyltransferase NAT10, previously determined as a protein acetyltransferase for α-tubulin, histones, and p53, is responsible for ac^4C installation in mRNA. Its mutation resulted in approximately 80% to 90% reduction of ac^4C modification in mRNA. Analyses of mRNA half-lives indicated that the NAT10-mediated mRNA acetylation increases the stability of target transcripts. Further biochemical studies showed that ac^4C also promotes the efficiency of translation (Arango et al. 2018).

4 Concluding Remarks and Future Perspectives

The discovery of mRNA methylation revealed a new layer of epigenetic regulation. RNA methylation shares many of the characteristics with the well-known epigenetic DNA and histone modifications, such as the reversibility and the regulatory effects on gene expression. In the nucleus, writer proteins install methylations at specific sites in mRNA according to consensus RNA motives or structures. Eraser proteins can dynamically remove modification marks, and they are hypothesized to fine tune the precise methylation pattern maybe depending on environmental cues. In

accordance, only a sub fraction of the consensus m^6A RNA methylation motif (RRm^6ACH) in mammals is actually methylated (Dominissini et al. 2012), which might be controlled by m^6A demethylases. m^6A marks in some pre-mRNAs can be recognized by nuclear reader proteins to mediate differential splicing. Mature mRNAs are exported to the cytosol, where their methylation pattern is decoded by different cytoplasmic reader proteins. These readers fine tune several biological processes, such as mRNA decay and translation. Upon RNA turnover, methylated nucleotides are released together with canonical ones. Special enzymes for the catabolism of modified nucleotides will often be required to remove the modifications thereby facilitating the entry of the altered nucleotides into the general nucleotide metabolism.

Today, most of our mechanistic understanding of mRNA methylation is derived from studies on m^6A, and little is known about the other methylations, including m^5C, m^1A, N_m, m^7G, m^3C, and ac^4C. Substantial efforts investigating these methylations (acetylations) will still be necessary. It will be required (1) to find enzymes with the respective writer activities and (2) to map their respective binding sites, and (3) to elucidate the biological processes these methylations are involved in, and (4) to describe the metabolic fate of the corresponding methylated nucleotides released upon degradation of the modified mRNA. For all mRNA methylations, including m^6A, the large amount of information gained from high-throughput modification mapping approaches is highly valuable to obtain further insights into the biological function of these modifications. This tool will need to be employed to investigate the spatiotemporal properties of mRNA methylations, for example in different tissues and in response to environmental stimuli. It can be predicted that higher spatial resolution and dissection of the temporal dynamics of methylation will greatly enhance our understanding of the fine tuning of gene expression.

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