

ADVANCED REVIEW

RNA methylation in nuclear pre-mRNA processing

Helena Covelo-Molares | Marek Bartosovic[†] | Stepanka Vanacova

CEITEC, Masaryk University, Brno, Czech Republic

CorrespondenceStepanka Vanacova, CEITEC, Masaryk University, Brno, Czech Republic.
Email: stepanka.vanacova@ceitec.muni.cz**[†]Present address**

Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Solna, Sweden.

Funding information

Brno City Municipality Scholarship; Ministry of Education, Youth and Sports, Grant/Award Number: LQ1601; Czech Science Foundation, Grant/Award Numbers: 305/11/1095, 14-25884S; Wellcome Trust, Grant/Award Number: 084316/B/07/Z

Eukaryotic RNA can carry more than 100 different types of chemical modifications. Early studies have been focused on modifications of highly abundant RNA, such as ribosomal RNA and transfer RNA, but recent technical advances have made it possible to also study messenger RNA (mRNA). Subsequently, mRNA modifications, namely methylation, have emerged as key players in eukaryotic gene expression regulation. The most abundant and widely studied internal mRNA modification is N⁶-methyladenosine (m⁶A), but the list of mRNA chemical modifications continues to grow as fast as interest in this field. Over the past decade, transcriptome-wide studies combined with advanced biochemistry and the discovery of methylation writers, readers, and erasers revealed roles for mRNA methylation in the regulation of nearly every aspect of the mRNA life cycle and in diverse cellular, developmental, and disease processes. Although large parts of mRNA function are linked to its cytoplasmic stability and regulation of its translation, a number of studies have begun to provide evidence for methylation-regulated nuclear processes. In this review, we summarize the recent advances in RNA methylation research and highlight how these new findings have contributed to our understanding of methylation-dependent RNA processing in the nucleus.

This article is categorized under:

RNA Processing > RNA Editing and Modification

RNA Processing > Splicing Regulation/Alternative Splicing

RNA Interactions with Proteins and Other Molecules > Protein–RNA Interactions: Functional Implications

KEYWORDS

RNA demethylase, RNA methylase, RNA processing

1 | INTRODUCTION

Posttranscriptional processing of messenger RNA (mRNA) is a common feature of eukaryotes. The 5' capping and 3' polyadenylation modifications were discovered almost 50 years ago and have remained as one of the hallmarks of eukaryotic mRNA processing (Edmonds, Vaughan, & Nakazato, 1971; Muthukrishnan, Both, Furuichi, & Shatkin, 1975; C. M. Wei, Gershowitz, & Moss, 1975b). However, the recent discovery of additional internal, functional mRNA modifications has introduced a previously unappreciated level of mRNA metabolism regulation. This review will discuss the latest development in the field of mRNA modifications from the perspective of nuclear mRNA processing.

The pioneering analysis of methylated nucleotides in mammalian mRNA in the 1970s revealed the presence of a 7-methylguanosine (m⁷G) cap at the 5' end. It also unexpectedly detected internal methylated nucleotides (Desrosiers,

Friderici, & Rottman, 1974; Perry & Kelley, 1974); N⁶-methyladenosine (m⁶A) was the most abundant modification (Dubin & Taylor, 1975; Salditt-Georgieff et al., 1976). These studies provided valuable insights into the composition and proportions of various internal mRNA modifications. However, they lacked thorough biochemical and functional analyses due to technical limitations. The big boom in the field came with the development of high-throughput sequencing methods combined with biochemistry and/or specific antibodies, which provided tools to obtain a whole-transcriptome view of RNA modifications. Individual RNA modifications can be detected by specific approaches. Some may be directly detected as mismatch mutations (e.g., A to I or C to U editing) or they must first be chemically modified to induce a mutation (e.g., bisulfite-based sequencing of m⁵C). In other cases, such as N¹-methyladenosine (m¹A) or m⁶A, the detection methods either rely on interference with reverse transcriptase or utilization of specific antibodies (Dominissini et al., 2012; Meyer et al., 2012; Tserovski et al., 2016). The recognition of the biological importance of RNA modifications, along with methodical developments, has led to “epitranscriptomics.” This field, analogous to epigenetics, studies functionally relevant chemical RNA modifications that do not alter the genomic sequence.

Thanks to methodological and technical developments, several major discoveries have changed our view on the importance of RNA modifications. In addition to reversible chemical modifications of DNA and proteins, RNA marks (mainly adenosine methylations) constitute yet another layer of gene expression regulation. In diverse eukaryotic lineages, dynamic RNA methylation plays a key role in such elementary processes as germline maturation (Batista et al., 2014; Geula et al., 2015; Y. Wang et al., 2014), early embryogenesis (Ivanova et al., 2017; Zhao et al., 2017), host defense against pathogens (Martinez-Perez et al., 2017; Tirumuru et al., 2016), and cancer self-renewal and tumorigenesis (Cui et al., 2017; Z. Li et al., 2017; Zhang et al., 2017) (Box 1). On the cellular level, methylation regulates a wide range of RNA-related processes: RNA processing, stability, and translation. The aim of this review is to discuss the function of RNA methylation in nuclear pre-mRNA processing. Most of the recent discoveries have been made in mammalian systems, but there is also new data from other eukaryotes, such as yeast, flies, and plants (Schwartz et al., 2013; Zhang et al., 2015). The spectrum of newly identified mRNA modifications grows every year; however, only a few have been studied in such detail as to be able to draw conclusions about their function. We will first discuss the growing spectrum of mRNA methylation marks, describe the known machinery involved in writing, erasing, and reading methylated mRNA, and finally we will discuss how these marks are involved in the early steps of mRNA processing in the nucleus.

BOX 1

m⁶A IN DEVELOPMENT AND CANCER

Several studies have indicated that m⁶A is a key regulator during early development and gametogenesis in different species. In mammals, m⁶A controls the stability of pluripotency regulators and thus allows embryonic stem cell (ESC) differentiation into specific cell types (Batista et al., 2014; Geula et al., 2015; Y. Wang, Li, et al., 2014). Deletion of the m⁶A writer, the Methyltransferase-Like 3 (METTL3) in (KO) mouse ESCs is viable, but they failed to differentiate into specific lineages. Mettl3 KO in mouse is embryonically lethal (Geula et al., 2015). Failure of embryonic development was also observed in *Arabidopsis thaliana* upon depletion of the METTL3 ortholog (Zhong et al., 2008). *Drosophila* embryos were viable, but they suffered from severe neurological defects and reduced fertility (Hausmann et al., 2016; Lence et al., 2016). Moreover, the m⁶A eraser AlkB Homolog 5 (ALKBH5) was required for mouse spermatogenesis (Zheng et al., 2013), and the m⁶A readers YTHD Domain Family 2 (YTHDF2) and YTH Domain Containing 2 (YTHDC2) were needed for early zygote development in mammals and zebrafish and successful meiotic program in the mammalian germline, respectively (Hsu et al., 2017; Ivanova et al., 2017; Wojtas et al., 2017; Zhao et al., 2017; Box 2). Emerging evidence also suggests that m⁶A could play a role in cancer. For instance, the m⁶A methylation/demethylation pathway regulated glioblastoma stem cell self-renewal and tumorigenesis (Cui et al., 2017; Zhang et al., 2017), and METTL3, METTL14, and Wilms Tumor 1-Associating Protein (WTAP) were highly expressed in myeloid leukemia (Jaffrey & Kharas, 2017). These pioneering and encouraging discoveries require further investigation to discover the mechanistic details underlying the observed phenotypes.

2 | THE SPECTRUM OF MRNA METHYL MARKS

The spectrum of mRNA methyl marks includes, so far, six different modifications. RNA methylation may occur at the N1 and N6 atoms in adenosine, N3 and C5 in cytidine, N7 in guanosine, and at the 2'-OH of ribose (Figure 1).

m⁷G, at the 5' end of mRNA, marks the beginning of nearly all cellular mRNA transcribed by RNA polymerase II (RNAPII) (C. M. Wei et al., 1975b). In higher eukaryotes, the two nucleotides immediately adjacent to the cap show a

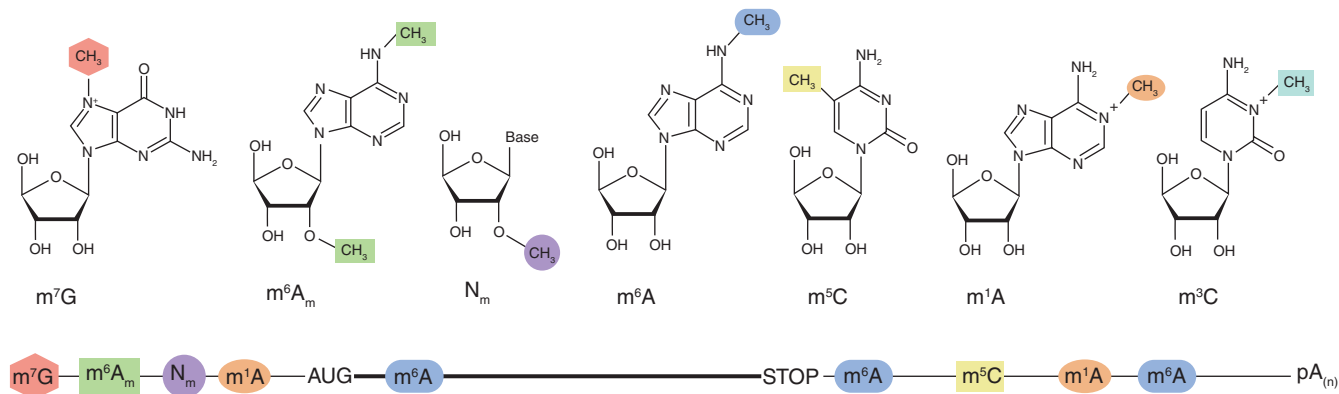


FIGURE 1 A schematic view of the structure and localization of methylated nucleosides in eukaryotic mRNA. The bold line represents the coding sequence, and the thin lines are 5' and 3' untranslated regions (UTRs). Abbreviations: m^7G , 7-methylguanosine; m^6A_m , $N^6,2'$ -O-methyladenosine; N_m , 2'-O-ribose methylation; m^1A , N^1 -methyladenosine; m^6A , N^6 -methyladenosine; m^5C , 5-methylcytosine; m^3C , 3-methylcytosine

complex methylation pattern (Figure 2). The characteristics and roles of the 2'-O-methylation (N_m) and $N^6,2'$ -O-methyladenosine (m^6A_m) found next to the cap will be discussed later in this review. Most modifications found in mRNA, such as m^1A , N^3 -methylcytosine (m^3C), N^5 -methylcytosine (m^5C), and m^6A , were first observed in noncoding RNA (ncRNA), mainly in the abundant and stable transfer RNA (tRNA) and ribosomal RNA (rRNA).

Both adenosine modifications, m^1A and m^6A , were identified by the 1970s; however, more detailed molecular and biochemical studies began only recently. Both modifications are reversible, meaning that they can be added and subsequently erased from mRNA by specific enzymes.

m^1A was first identified on rRNA and tRNA, where it is important for the formation of tertiary structure conformations (Helm, Giege, & Florentz, 1999), and in 2016 it was also reported in mRNA (Dominissini et al., 2016). It is written by TRMT6/TRM61A tRNA methyltransferase (X. Li et al., 2017; Safra et al., 2017), and it can be erased from tRNA and mRNA by the DNA/RNA demethylase ALKBH3 (Aas et al., 2003; Li, Xiong, Wang, et al., 2016; Ougland et al., 2004; Sundheim et al., 2006). In 2016 and 2017, several groups employed a method for site-specific detection of m^1A in mRNA, but different methodological approaches and data analyses led to controversial findings and conclusions. By using a combination of Dimroth rearrangement, antibody enrichment, and stalled reverse transcription, m^1A was located at the 5' untranslated region (UTR) and around start codons of several hundreds to several thousands of different mRNA transcripts (Dominissini et al., 2016; Li, Xiong, Wang, et al., 2016). These studies also revealed that m^1A was dynamically regulated by diverse stress conditions, such as starvation or heat shock (Li, Xiong, Wang, et al., 2016), and its 5' location was proposed to positively regulate

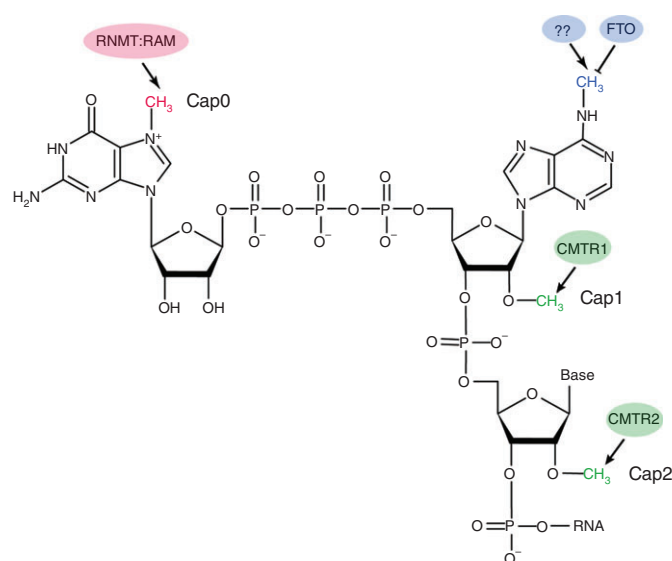


FIGURE 2 The chemical formula of the eukaryotic mRNA 5'- m^7G cap structure with the two downstream nucleotides included. Red: stable methyl group that forms the "Cap 0" structure (m^7GpppN). Green: stable methyl groups that form the "Cap 1" ($m^7GpppNm$) and "Cap 2" ($m^7GpppNmNm$) structures. Blue: if the first nucleotide of the mRNA is adenosine, it can be further methylated at the N^6 position of the base ($m^7Gpppm^6A_m$). The enzymes responsible for methylation/demethylation of the specific groups are depicted

translation initiation (Dominissini et al., 2016). However, by using reverse transcription-based misincorporation and truncation at modified sites as well as more strict bioinformatics analysis, Safra et al. (2017) found m^1A only in a handful of cytosolic mRNAs and a few mitochondrial mRNAs (in strong stem-loop structures) and linked m^1A to translational repression and developmental regulation. Since this field is very young, it needs further biochemical and chemical validations to accompany the high-throughput sequencing studies. For instance, it is important to test the relevance of 5' terminal m^1A s as well as to study the molecular mechanism that underlies the role of m^1A at structured elements. Notably, m^1A has been detected on *MALAT1*-associated small cytoplasmic RNA (mascRNA), which is a small tRNA-like RNA processed from *MALAT1* long noncoding RNA (lncRNA) by an unusual mechanism that involves RNase P and RNase Z (Wilusz, Freier, & Spector, 2008). It is possible that m^1A , like in some tRNA, stabilizes the tertiary conformation of mascRNA and thus promotes its yet unknown function in the cytoplasm.

The additional methylation modifications m^3C and m^5C , which are also typically found in ncRNA (such as tRNA and rRNA) were recently also reported in mRNA (Clark, Evans, Dominissini, Zheng, & Pan, 2016; Cozen et al., 2015; Iwanami & Brown, 1968a, 1968b; Squires et al., 2012; Xu et al., 2017).

Two different enzymes are responsible for m^3C deposition in tRNA, METTL2, and METTL6 (Xu et al., 2017), whereas METTL8 modifies mRNA (Xu et al., 2017). The presence of m^3C on mRNA was indicated by high performance liquid chromatography coupled to mass spectrometry, but the specific locations are not yet known (Xu et al., 2017). Neither erasers nor readers have been identified for m^3C so far.

Regarding m^5C , its presence in mRNA remains debated. Several methods based on indirect m^5C detection have been used to localize m^5C in coding and ncRNA. These methods include m^5C -RNA immunoprecipitation (RIP), 5-azacytidine-mediated RIP (Aza-IP), and methylation at individual nucleotide resolution crosslinking and immunoprecipitation (miCLIP) (Hussain, Aleksic, Blanco, Dietmann, & Frye, 2013; Li, Xiong, & Yi, 2016). For direct mapping of m^5C in native RNA, bisulfite sequencing, a technique widely used to study DNA methylation, was adopted (Amort et al., 2017; David et al., 2017; Khoddami & Cairns, 2013; Legrand et al., 2017; Squires et al., 2012). Several of these studies reported thousands of m^5C positions in poly(A) + RNA, with even higher m^5C levels in nuclear fractions (Amort et al., 2017; Khoddami & Cairns, 2013; Squires et al., 2012). Thousands of m^5C marks have been reported in plants. In *Arabidopsis*, the tRNA m^5C methyltransferase TRM4B was linked to the methylation of thousands of sites in mRNA coding sequences, and m^5C was suggested to play a role in mRNA stability and root development (David et al., 2017). The presence of m^5C in eukaryotic mRNA has, however, been strongly questioned by the work of Legrand et al. (2017), who developed a stringent and statistically robust pipeline for whole-transcriptome bisulfite sequencing data analysis and performed a comprehensive methylation analysis of mouse coding and ncRNA. Whereas their results revealed highly reproducible and robust detection of m^5C in tRNA and rRNA, they did not show any significant m^5C in mRNA (Legrand et al., 2017). However, some studies have linked m^5C in mRNA and ncRNA with specific functions in mammals. For instance, the human mRNA export adaptor ALYREF was proposed to act as an m^5C mRNA reader to regulate mRNA export from the nucleus (X. Yang et al., 2017), and loss of m^5C methylation on vault ncRNAs caused aberrant processing into Argonaute-associated small fragments that can function as microRNAs (miRNAs) (Hussain et al., 2013). Together, the presence and function of m^5C in mammalian mRNA remains controversial.

Apart from simple methyl groups, more complex modifications can be found on RNA. Recently, the same family of enzymes that oxidize 5-methylcytosine in DNA was reported to catalyze the formation of 5-hydroxymethylcytosine in mammalian total RNA (5hmC) (Fu et al., 2014; Kohli & Zhang, 2013). In flies, a transcriptome wide study using an adapted version of methylation RNA immunoprecipitation sequencing (MeRIP-seq) with 5hmrC antibodies, reported the presence of 5hmrC in many mRNA coding sequences, with particularly high levels in the brain (Delatte et al., 2016). They found that active translation is associated with high 5hmrC levels, and flies lacking the ten-eleven translocation (TET) enzyme responsible for 5hmrC deposition have impaired brain development. In addition, the removal of m^6A by the demethylase fat mass and obesity-associated protein (FTO) is a multistep process that generates two intermediates, N^6 -hydroxymethyladenosine (hm^6A) and N^6 -formyladenosine (f^6A), with expected lifetimes of approximately 3 hr (Fu et al., 2013). It would be interesting to test whether these intermediates could also be recognized by different factors and change the fate of the modified transcripts.

3 | THE MAMMALIAN m^6A EPITRANSCRIPTOME

The most abundant and extensively studied internal modification of mammalian mRNA is m^6A . A growing body of work provides evidence about its role in nuclear pre-mRNA processing. m^6A was initially identified in the 1970s in both mammalian and viral RNA as a part of the cap structure at the first nucleotide downstream of m^7G (Adams & Cory, 1975; Desrosiers et al., 1974; Dubin & Taylor, 1975). Thirty years later, two groups independently developed MeRIP-Seq

(or m⁶A-Seq), a technique that provided the first data on m⁶A distribution within (pre-)mRNA molecules (Dominissini et al., 2012; Meyer et al., 2012). This approach combines immunoprecipitation of fragmented RNA using an m⁶A-specific antibody with high-throughput sequencing and has been previously reviewed (Helm & Motorin, 2017; Li, Xiong, & Yi, 2016). These transcriptome-wide m⁶A maps revealed that, in mammals, approximately 7,000 transcripts contain at least one m⁶A site. m⁶A marks were enriched in the proximity of the transcription start sites (TSS), around the stop codons, and at the beginning of the last exons (Dominissini et al., 2012; Meyer et al., 2012). Notably, the high-throughput profiling results were highly consistent with previous biochemical analyses (Dubin & Taylor, 1975; Perry & Kelley, 1974; Wei & Moss, 1975). Apart from mRNA, m⁶A sites were also identified in various ncRNA, such as lncRNA (N. Liu et al., 2013; Meyer et al., 2012; Patil et al., 2016; Warda et al., 2017), primary-microRNA (pri-miRNA) (Alarcon, Lee, Goodarzi, Halberg, & Tavazoie, 2015), rRNA (Iwanami & Brown, 1968b), small nuclear RNA (snRNA) (Bringmann & Luhrmann, 1987), and tRNA (Saneyoshi, Harada, & Nishimura, 1969). The majority, but not all, of the internal mRNA m⁶A is found within a semi-defined motif, namely RRACH (R = A/G, H = A/C/T) (Dominissini et al., 2012; Harper, Miceli, Roberts, & Manley, 1990; Meyer et al., 2012; Wei & Moss, 1977). Interestingly, at the 5' UTR, the most prevalent motif is BCA (B = C/U/G), rather than RRACH (Linder et al., 2015). These methylated positions near the TSS are probably m⁶A_m sites, which contain an additional methyl group on the ribose (Mauer et al., 2017; C. Wei, Gershowitz, & Moss, 1975a). Another unique feature of these TSS-proximal sites is that they are independent of WTAP, one of the main auxiliary proteins in the METTL3/14 complex (Schwartz et al., 2014). Overall, the RRACH motif appears to be specifically preferred by the methyltransferase METTL3 and the m⁶A_m sites at the TSS might be deposited by a different, as yet unknown methyltransferase.

3.1 | Regulatory factors of the mammalian m⁶A epitranscriptome

3.1.1 | Enzymes that catalyze m⁶A modification: m⁶A writers

The N⁶-methyl residues are deposited on adenosines by S-adenosyl-methionine (SAM)-dependent methyltransferases. Mammals express at least two enzymes that have the potential to introduce m⁶A in mRNA, the heterodimer METTL3/METTL14 (METTL3/14) and METTL16. METTL3/14 (Liu et al., 2014) can associate with several adaptor proteins: WTAP (Liu et al., 2014; Ping et al., 2014; Schwartz et al., 2014), RBM15/15b (Patil et al., 2016), KIAA1429/VIRMA (Schwartz et al., 2014; Yue et al., 2018), HAKAI (Ruzicka et al., 2017; Yue et al., 2018) and ZC3H13 (Knuckles et al., 2018; Yue et al., 2018) (Table 1). However, the core of the active complex is formed by METTL3 and METTL14, homologous proteins that share 43% amino acid identity. They form a stable heterodimer, but only METTL3 contains catalytic methyltransferase activity, whereas METTL14 acts as a scaffold protein that promotes RNA interaction (Sledz & Jinek, 2016; Wang, Doxtader, & Nam, 2016; X. Wang, Feng, et al., 2016). This methylase complex is conserved across diverse eukaryotes, and homologs are found in all blasted metazoan genomes and many protozoa (our unpublished analyses).

Consistent with MeRIP-seq experiments, RNA binding studies by photoactivatable ribonucleoside-enhanced CLIP (PAR-CLIP) revealed that METTL3 and METTL14 preferentially occupy regions that contain the RRACH motif (Liu et al., 2014; Ping et al., 2014). METTL3 and METTL14 colocalize in the nucleus, specifically in nuclear speckles (T. Chen et al., 2015; Liu et al., 2014; Ping et al., 2014); however, association with other regulatory proteins influences their cellular localization and RNA substrates (Patil et al., 2016; Ping et al., 2014; Schwartz et al., 2014). For example, interaction of METTL3 with *XIST* lncRNA is regulated by the RNA binding protein RBM15/15b (Patil et al., 2016). WTAP appears to mediate modification of the non-TSS m⁶A sites (Schwartz et al., 2014), and it is essential for the nuclear localization of the complex. Downregulation of WTAP promotes METTL3/14 relocalization from nuclear speckles (Ping et al., 2014). The nuclear speckle localization of METTL3 was also disrupted upon knockdown of the miRNA processing factor Dicer (T. Chen, Hao, et al., 2015). Studies of polysome fractions from METTL3-depleted cells suggested that METTL3 associates with polysomes and enhanced translation by recruiting eIF3 to the translation initiation complex (Lin, Choe, Du, Triboulet, & Gregory, 2016). However, the cytoplasmic role of METTL3 remains elusive and requires further clarification. Most of the studies indicate that METTL3/14 has primarily a nuclear function (Ke et al., 2017; Knuckles et al., 2017; Liu et al., 2014; Ping et al., 2014; Xiang et al., 2017).

The RRACH motif statistically occurs approximately once every 86 nucleotides, which is more than the experimentally observed methylation frequency. Despite intensive research in this field, it remains unclear how enrichment of m⁶A at specific locations is achieved. Taking into consideration the complexity of known interactors of METTL3 and METTL14 (Schwartz et al., 2014), it will be intriguing to see how other unknown mRNA binding factors influence m⁶A mRNA methylation.

The other m⁶A methyltransferase, METTL16, targets both ncRNA and protein coding mRNA (Pendleton et al., 2017; Warda et al., 2017). In contrast to METTL3/14, it does not associate with the RRACH motif. Therefore, some of the m⁶A

TABLE 1 A summary of the known features of m⁶A protein factors, their molecular function, cellular localization, and a brief description of the m⁶A-mediated role or activity of the mammalian m⁶A writers, erasers, and readers

Protein	Description	Localization	Molecular function	References
METTL3	Catalytic core of the METTL3/14 methyltransferase complex	N, C	Methyltransferase, transferase, binding RNA/protein/SAM	Liu et al. (2014), Sledz and Jinek (2016), X. Wang, Feng, et al. (2016)
MFTTL14	RNA-binding scaffold of the METTL3/14 complex	N	Methyltransferase, transferase, binding RNA/protein/SAM	Liu et al. (2014), Sledz and Jinek (2016), X. Wang, Feng, et al. (2016)
WTAP	Adaptor protein of the MFTTL3/14 complex essential for localization to nuclear speckles	N	Binding protein	Ping et al. (2014), Schwartz et al. (2014)
VIRMA/KIAA1429	Adaptor protein of the METTL3/14 complex involved in guiding it to specific RNAs	N	Binding RNA	Schwartz et al. (2014), Yue et al. (2018)
RBM15/15B	Adaptor protein of the METTL3/14 complex involved in guiding it to specific RNAs	N	Binding protein/RNA/nucleic acid	Patil et al. (2016)
HAKAI	Adaptor protein of the MFTTL3/14 complex	N	Ubiquitin-protein transferase/ligase activity, binding protein/metal ion	Ruzicka et al. (2017), Yue et al. (2018)
ZC3H13	Adaptor protein of the METTL3/14 complex required for protein protein interactions within the complex	N	Binding protein/RNA/metal ion	Yue et al. (2018), Knuckles et al. (2018)
METTL16	N6-methyltransferase that methylates snRNAs and a subset of mRNAs	N	Methyltransferase, transferase, binding RNA/U6 snRNA 3' end/RNA stem-loop	Pendleton et al. (2017), Warda et al. (2017)
FTO	m ⁶ A/m ⁶ A _m RNA demethylase. Influences splicing and regulates cap-independent translation after heat shock stress	N, C	Oxidoreductase activity, dioxygenase activity, oxidative RNA demethylase activity, oxidative DNA demethylase activity, binding metal ion	Jia et al. (2011), Bartosovic et al. (2017), Zhao et al. (2014)
ALKBH5	m ⁶ A RNA demethylase. Influences mRNA export	N	Oxidoreductase activity, dioxygenase activity, oxidative RNA demethylase activity, binding RNA/metal ion	Zheng et al. (2013)
YTHDF1	Direct m ⁶ A reader. Promotes translation of target m ⁶ A-modified mRNAs	C	Binding RNA/m ⁶ A-containing RNA/protein/ribosome	Wang et al. (2015)
YTHDF2	Direct m ⁶ A reader. Mediates decay and promotes cap-independent translation after heat shock. YTHDF2-mediated decay regulates mammalian oocyte maturation and zebrafish maternal mRNA clearance and haematopoietic stem cell specification	N, C	Binding RNA/m ⁶ A-containing RNA/protein	X. Wang et al. (2014), Ivanova et al. (2017), Zhao et al. (2017), Zhou et al. (2015)
YTHDF3	Direct m ⁶ A reader. Promotes translation of target m ⁶ A-modified mRNAs and circular RNAs	C	Binding RNA/m ⁶ A-containing RNA/protein/ribosome	Y. Yang et al. (2017), A. Li et al. (2017)
YTHDC1	Direct m ⁶ A reader. Regulates splicing modulating the binding of SRSF3 and SRSF10. Mediates export of m ⁶ A-modified mRNAs	N	Binding RNA/m ⁶ A containing RNA/protein	Xiao et al. (2016), Roundtree et al. (2017)
YTHDC2	Direct m ⁶ A reader. Regulates the meiotic program in mammalian germline	C	Binding RNA/m ⁶ A-containing RNA/protein/ATP/RNA polymerase, helicase activity, ATP-dependent RNA helicase activity, RNA-dependent ATPase activity	Hsu et al. (2017), Wojtas et al. (2017)
HNRNPC	Indirect m ⁶ A reader. Mediates splicing events dependent on "m ⁶ A switch"	N	Binding RNA/m ⁶ A-containing RNA/mRNA 3'UTR/ poly(U) RNA/telomerase RNA/nucleosomal DNA/RNA polymerase II proximal promoter and distal enhancer sequence-specific DNA binding/protein	Liu et al. (2015)
HNRNPG	Indirect m ⁶ A reader. Mediates splicing events dependent on "m ⁶ A switch"	N	Binding RNA/protein	Liu et al. (2017)
HNRNPA2B1	Direct m ⁶ A reader. Promotes processing of m ⁶ A-modified pri-miRNA precursors. Regulates splicing of m ⁶ A-modified transcripts	N, C	Binding RNA/ m ⁶ A containing RNA/mRNA 3'-UTR/miRNA/pre-mRNA intronic binding/single-stranded telomeric DNA binding/G-rich strand telomeric DNA binding/protein	Alarcon, Goodarzi, et al., 2015, Alarcon, Lee, et al., 2015
eIF3	Direct m ⁶ A reader. Promotes translation of target mRNAs with m ⁶ A within the 5' UTR	C	Binding RNA/protein, translation initiation factor activity	Meyer et al. (2015)

Note. N, nuclear; C, cytoplasmic localization.

localization studies based on this sequence motif may have underestimated the total number of sites (Warda et al., 2017). It is yet to be investigated whether METTL16 associates with any adaptor proteins. Additionally, thorough biochemical characterizations need to be performed to clarify its substrate specificity and enzymatic activities.

3.1.2 | The dynamics of m⁶A: m⁶A/m⁶A_m erasers

Similar to m¹A (see above), m⁶A is a reversible mark. It can be readily removed in mammals by two currently known demethylases, FTO and ALKBH5 (Jia et al., 2011; Zheng et al., 2013) (Table 1). FTO and ALKBH5 are members of a protein family that harbors an ALKB domain. ALKB proteins are Fe²⁺/α-ketoglutarate-dependent dioxygenases that catalyze the conversion of methylated RNA and DNA nucleotides (Fedele, Singh, Delaney, Li, & Essigmann, 2015). Similar to the methylase complex, both FTO and ALKBH5 are localized in nuclear speckles (Jia et al., 2011; Zheng et al., 2013). Although FTO can also be localized to the cytoplasm (Gulati et al., 2014), final m⁶A decoration, and thus the majority of demethylation, occurs on chromatin-associated pre-mRNA (Ke et al., 2017) to influence pre-mRNA processing (Bartosovic et al., 2017; Zhao et al., 2014) (see below).

FTO substrate specificity has been debated numerous times. It was initially reported as a DNA demethylase that preferentially targeted 3-methylthymine (3-meT) in single-stranded DNA (ssDNA) (Gerken et al., 2007). However, only a year later, *in vitro* studies demonstrated that recombinant human and mouse FTO displayed higher activity on 3-methyluracil in ssRNA rather than 3-meT in ssDNA (Jia et al., 2008). This view was again altered a few years later, when m⁶A RNA was identified as the main FTO substrate both *in vitro* and *in vivo* (Jia et al., 2011). Most recently, biochemical analyses combined with thin layer chromatography revealed dimethylated adenosine m⁶A_m as the major substrate of FTO in the cytoplasm (Mauer et al., 2017). The current view is that FTO preferentially targets m⁶A and m⁶A_m; its activity on other modified RNA and DNA nucleotides needs to be clarified. Currently, ALKBH5 and FTO have only been found in vertebrates. Plants contain another m⁶A demethylase ALKBH9B (Martinez-Perez et al., 2017).

3.1.3 | The recognition of m⁶A by specific reader proteins

m⁶A mRNA modification has emerged as a regulator of RNA structure (Liu et al., 2015; Spitale et al., 2015) and protein–RNA interactions. m⁶A functions are mediated by distinct RNA binding proteins termed m⁶A readers (Table 1). The m⁶A readers recognize modified RNAs either by direct recognition of m⁶A-containing sequence or through affinity to particular RNA conformations that are sensitive to m⁶A.

m⁶A-modified RNA pulldown combined with mass spectrometry (MS) led to the identification of several m⁶A RNA binders (Dominissini et al., 2012). These proteins include several members of the YTH family (YT521, the founding member of the family), namely YTHDF1, YTHDF2, and YTHDF3 (Dominissini et al., 2012; Theler, Dominguez, Blatter, Boudet, & Allain, 2014; X. Wang, Lu, et al., 2014; Zhu et al., 2014). Although the YTH domain is found in several m⁶A reader proteins, direct m⁶A recognition does not fully rely on this domain. For instance, the translation initiation factor eIF3, which does not contain this domain, binds m⁶A in the 5′ UTR (Meyer et al., 2015). m⁶A can have both positive and negative effects on interactions with particular proteins. For instance, the binding affinity of ELAV-1/HuR to some mRNA is reduced when they are m⁶A-modified, although this reduction is largely dependent on the spatial distribution of m⁶A and HuR binding sites (X. Wang, Lu, et al., 2014).

Besides direct recognition, m⁶A can also indirectly affect protein binding by modulating adjacent binding sites that do not overlap with m⁶A. *in vivo* measurements of RNA secondary structures by *in vivo* click selective 2-hydroxyl acylation and profiling experiment (icSHAPE) revealed decreased folding of methylated RNA sequences comparing to unmethylated; the finding underscored the role of m⁶A in regulating mRNA folding (Spitale et al., 2015). Disruption of dsRNA hairpins by m⁶A exposes ssRNA regions that facilitate their indirect recognition by ssRNA binding proteins. This m⁶A-dependent RNA remodeling was termed “m⁶A switch” (Liu et al., 2015), and it can influence the binding of different RNA binding proteins (Liu et al., 2015, 2017). The versatility of m⁶A writer adaptors, the possibility for removal by erasers and a plethora of recognition mechanisms by readers (summarized in Table 1), confers m⁶A modification a regulatory potential that needs to be further studied.

4 | THE ROLE OF METHYLATION IN PRE-mRNA PROCESSING

Initial primary transcripts produced by RNAPII undergo several processing steps to become mature mRNA. For most pre-mRNA, these steps include three main processes: addition and modification of a 5′ cap, removal of introns by RNA splicing, and formation of mature 3′ ends by cleavage and polyadenylation. Transcription and processing are not independent processes; they occur simultaneously and there is a crosstalk between the factors involved, largely through the carboxy-terminal domain (CTD) of RNAPII (Lenasi & Barboric, 2013). Several lines of evidence support the notion that m⁶A deposition and removal occurs co-transcriptionally (Baltz et al., 2012; Bartosovic et al., 2017; Ke et al., 2017; Knuckles et al., 2017; Slobodin et al., 2017). METTL3 can be co-precipitated with slowed-down RNAPII upon camptothecin (CPT) treatment (Slobodin et al., 2017), and METTL3 chromatin immunoprecipitation (ChIP) experiments demonstrated its direct association with chromatin (Knuckles et al., 2017). Furthermore, using a very different approach, Ke et al. (2017) reported that m⁶A deposition

occurs during transcription while RNA is still attached to chromatin. The comparison of m⁶A-CLIP profiles among chromatin-associated RNA (pre-mRNA), nucleoplasmic, and cytoplasmic mRNA did not reveal any significant differences; this finding implied that final m⁶A decoration is completed while the mRNA is still associated with the transcription locus. All the other m⁶A players, METTL16, FTO, and ALKBH5 target pre-mRNA (Baltz et al., 2012; Bartosovic et al., 2017; Warda et al., 2017). However, their direct association with chromatin and transcription units remains unknown. Altogether, these studies suggest that m⁶A methylation/demethylation has the potential to regulate the very early steps of nuclear pre-mRNA processing. Therefore, we will review the current knowledge on the role of m⁶A in pre-mRNA processing and describe the open questions and discrepancies in the field.

4.1 | The complex methyl code at the 5' end of mRNA

Most eukaryotic mRNA is co-transcriptionally modified at the 5' end by a cap structure (Figure 2). The 5' cap protects mRNA from 5'→3' exoribonucleases, helps to recruit the molecular machinery necessary for translation initiation and splicing, and acts as an export signal for snRNA (Hocine, Singer, & Grunwald, 2010; Topisirovic, Svitkin, Sonenberg, & Shatkin, 2011).

The 5' cap is formed by an unusual 5'-5' triphosphate linkage of a guanosine methylated at the seventh position of the guanosine ring (m⁷G) to the first 5' terminal nucleotide of the mRNA (Wei, Gershowitz, & Moss, 1976; Wei & Moss, 1975). Guanosine m⁷G is the best characterized nucleotide methylation site of the cap (Ramanathan, Robb, & Chan, 2016; Topisirovic et al., 2011). However, 5' mRNA termini have a more complex array of modifications. The two nucleotides immediately downstream of m⁷G can also be methylated at the second carbon of the ribose (2'-OH position) to create 2'-O-methylation (Nm). If just the first nucleotide after m⁷G is methylated, it forms the so-called cap 1 structure (m⁷GpppNm). In mammals, this methylation is performed by the enzyme CMTR1 (Belanger, Stepinski, Darzynkiewicz, & Pelletier, 2010). Additional 2'-O-methylation of the second position can occur in the cytoplasm, mediated by CMTR2 (Werner et al., 2011), to form cap 2 (m⁷GpppNmNm). These two additional methylations serve as a mark to distinguish self and nonself mRNA and to avoid the recognition of cellular mRNA by the innate immune system (Daffis et al., 2010; Züst et al., 2011). However, many viruses that replicate in the cytoplasm have evolved their own RNA-modifying enzymes to mimic cellular mRNA in order to evade the host immune system (Daffis et al., 2010; Züst et al., 2011). 2'-O-methylation of viral mRNA avoids recognition by the cytoplasmic RNA sensor MDA5 and, consequently, the production of type I interferon, and thus subverts the host antiviral response (Züst et al., 2011).

In addition to ribose 2'-O-methylation, if the downstream adjacent nucleotide to m⁷G is A, it can be methylated at the sixth purine position to form m⁶A_m (C. Wei et al., 1975a). This methylation is reversible by FTO (Mauer et al., 2017) to produce 2'-O-methyladenosine; however, it is unknown which enzyme adds the N⁶-methyl group. These TSS-associated m⁶A marks appear to play a major role in the cytoplasm to stabilize mRNA transcripts (Mauer et al., 2017) and activate translation initiation (Meyer et al., 2015; Y. Yang, Fan, et al., 2017; Zhou et al., 2015). The m⁶A marks within the 5' UTR can act as so-called m⁶A-induced ribosome engagement sites (MIREs), and they promote mRNA translation under certain stress conditions (Meyer et al., 2015; Zhou et al., 2015). Based on the Zhou et al. (2015) study, heat shock induces relocalization of the cytoplasmic m⁶A reader YTHDF2 to the nucleus where it protects 5' UTR m⁶As from demethylation by FTO. This event in turn promotes cap-independent translation initiation by the direct recognition of m⁶A by eIF3, which recruits the pre-initiation complex independently of the cap-binding factor eIF4E (Meyer et al., 2015). A similar m⁶A-driven translation initiation, but mediated by the m⁶A reader YTHDF3 and the translation initiation factor eIF4G2, was demonstrated with circular RNA (circRNA) (Y. Yang, Fan, et al., 2017).

In summary, the 5' cap structure of mammalian mRNA can contain up to four methyl groups: the stable m⁷G, two Nm sites, and one reversible methylation site on transcripts that contain adenosine at the TSS (m⁶A_m). It would be interesting to determine whether additional methyl groups are present on the cap and how the cellular machinery interprets this complex modification at the beginning of mRNA transcripts.

4.2 | The role of RNA methylation in the regulation of pre-mRNA splicing

m⁶A has been thought to play a critical role in constitutive splicing (Stoltzfus & Dane, 1982). The regulation of pre-mRNA splicing is not fully understood. The spliceosome machinery is composed of small nuclear ribonucleoproteins (snRNPs), in which snRNA possess a number of nucleotide modifications, including base and 2'-O-ribose methylations. With some exceptions, the role and enzymatic pathways that produce most of these modifications are unknown. METTL16 catalyzes methylation at position A43 of U6 snRNA (Pendleton et al., 2017; Shimba, Bokar, Rottman, & Reddy, 1995; Warda et al., 2017). The exact function of this modification is unknown, but structural studies suggested that it might be important for local structure or base pairing in the U4/U6.U5 tri-snRNP spliceosome (Agafonov et al., 2016).

Internal mRNA adenosine methylations were also linked to pre-mRNA splicing *in cis*. Downregulation of m⁶A by METTL3 knockdown resulted in transcriptome-wide alternative splicing (AS) changes (Dominissini et al., 2012), and a number of follow-up studies have established robust links between both m⁶A and m⁶A factors and pre-mRNA splicing (Bartosovic et al., 2017; Haussmann et al., 2016; Lence et al., 2016; Liu et al., 2015, 2017; Molinie et al., 2016; Patil et al., 2016; Xiao et al., 2016; Zhao et al., 2014). m⁶A modification may affect pre-mRNA splicing by at least three different mechanisms (Figure 3), as described below:

1. m⁶A regulates the binding of specific splicing factors through RNA conformation. Mechanistically, m⁶A impacts the dynamics of RNA conformations that favor the transition from paired to unpaired RNA (Spitale et al., 2015). This mechanism was termed “m⁶A-switch” and has the potential to regulate access of RNA binding proteins. RNA folding occurs co-transcriptionally, and mRNA conformation immediately regulates co-transcriptional processing, including splicing (Liu, Hu, & Zhang, 2016). To date, the presence of m⁶A was shown to facilitate the binding of hnRNPc to its target RNA and in turn influenced their splicing pattern (Liu et al., 2015). hnRNPc preferentially binds RNA on single-stranded U-tracts (>4 Us). Liu et al. (2017) demonstrated that the presence of m⁶A opposite to a U-tract in a hairpin of *MALAT1* made the U-tract more accessible and enhanced its interaction with hnRNPc.
2. Methylated mRNA recruits m⁶A reader proteins that bind in the vicinity of splice sites (SSs) and promote or repress recruitment of trans-acting splicing factors. This mechanism occurred for YTHDC1, which bound m⁶A-modified regions in the vicinity of the 5' and 3' SS and recruited the splicing factor SRSF3 while inhibiting the binding of SRSF10 (Xiao et al., 2016). SRSF3 and SRSF10 have opposing roles in AS; the former promotes exon inclusion and the latter exon skipping. Therefore, at exons with a SS that contains SRSF3 and 10 binding sites, the presence or absence of m⁶A, and in turn whether YTHDC1 is bound to that residue, can regulate AS (Xiao et al., 2016). YTHDC1 also plays a role in dosage compensation in mammals (Patil et al., 2016). m⁶A marks in the *X-inactive specific transcript (XIST)* were required to mediate gene silencing of the X chromosome, although it is unknown how YTHDC1 binding contributes to gene silencing (Patil et al., 2016). Interestingly, the role of m⁶A in dosage compensation is conserved. In *Drosophila*, binding of the m⁶A

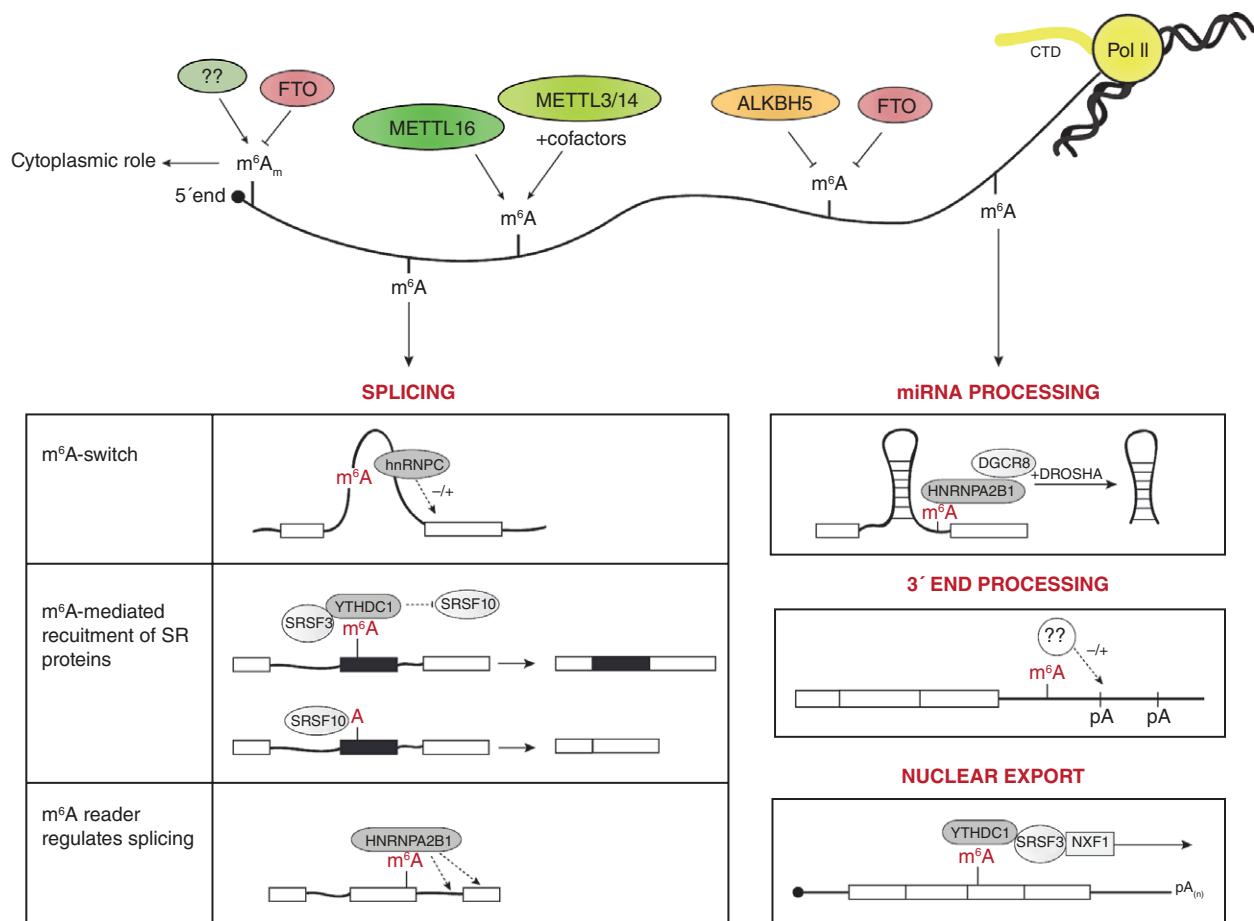


FIGURE 3 A schematic view of the role of the m⁶A methylation/demethylation pathway in nuclear pre-mRNA processing. Writers, erasers, and known readers of m⁶A that play a role in splicing, 3' end processing, and nuclear mRNA export are shown

reader YT521-B to the methylated sex determination factor *Sex lethal* (*Sxl*), regulated the female-specific AS of *sxl*, a key regulator of dosage compensation (Haussmann et al., 2016; Lence et al., 2016).

3. The third mechanism involves direct recognition of m⁶A-modified motifs by splicing factors; only one example has been reported. The splicing factor hnRNPA2B1 was proposed to mediate some of the m⁶A-METTL3-dependent AS events, although the study lacks direct proof (Alarcon, Goodarzi, et al., 2015).

4.2.1 | The role of m⁶A demethylases in AS regulation

The role of m⁶A in AS regulation is supported by studies on m⁶A demethylases. FTO appears to regulate AS of a distinct subset of genes in mice and humans. In mouse adipocytes (3T3 cells), FTO-mediated demethylation was proposed to affect splicing of a group of exons that rely on the splicing factor SRSF2 (Zhao et al., 2014). Among the genes, FTO seems important for exon exclusion in a transcript encoding the adipogenic regulatory factor *RUNX1T1*; this finding links FTO and adipogenesis (Merkestein et al., 2015; Zhao et al., 2014). In human HEK293T cells, FTO bound intronic regions in the proximity to alternatively spliced exons, and its activity affected both exon inclusion and exclusion for several hundred pre-mRNAs (Bartosovic et al., 2017). The detailed mechanisms and splicing factor(s) involved have yet to be uncovered. ALKBH5 plays a key role in splicing regulation during spermatogenesis in mice (Tang et al., 2018). Its activity was required for correct splicing to generate long 3' UTR transcripts in mitotic and meiotic male germ cells. These transcripts are crucial for timely degradation in later steps of spermatogenesis (Tang et al., 2018).

On the other hand, a study from the Darnel group suggested that the major role of m⁶A is mRNA stability rather than processing (Ke et al., 2017). They analyzed constitutive and AS in METTL3 knockout mouse embryonic stem cells (mESCs) and observed only minor changes compared with wild type cells. Nevertheless, alternatively spliced exons that contained m⁶A showed 4% and 1% more frequent inclusion and exclusion, respectively, in METTL3 knockout mESCs. In agreement with the proposed role of m⁶A in promoting mRNA degradation (Du et al., 2016; X. Wang, Lu, et al., 2014), they found that m⁶A correlated with rapid turnover (Ke et al., 2017).

AS is an extremely complex and highly regulated process that results in tissue and developmental stage-specific splicing and proteome patterns (Nilsen & Graveley, 2010). Seemingly, m⁶A is one of the many AS regulators. It is important to further investigate which mechanisms and factors are involved in m⁶A-regulated AS and also what regulates m⁶A deposition on distinct alternatively spliced transcripts.

4.3 | Adenosine methylation in the formation of 3' mRNA ends

Processing at the 3' end of RNAPII transcripts involves a highly complex process and machinery capable of recognizing the precise localization of polyadenylation (poly(A)), endonucleolytic cleavage, and polyadenylation. It is mediated by a large multisubunit complex. Cleavage position is dictated by several sequence elements in the 3' UTR. The best characterized example is the so-called poly(A) site (PA). In humans, it is estimated that approximately 50% of protein coding genes contain more than one PA, so-called alternative polyadenylation (APA) sites (Tian, Hu, Zhang, & Lutz, 2005). The differential usage of specific APAs subsequently determines the 3' UTR length and presence or absence of additional regulatory sequences, such as miRNA binding sites, secondary structures, or motifs for RNA-binding factors. The 3' end quality thus impacts mRNA stability, localization, and translation rate.

With respect to the 3' mRNA end, one of the two most prominent m⁶A sites is in the vicinity of stop codons and the beginning of the 3' UTR (Batista et al., 2014; K. Chen et al., 2015; Dominissini et al., 2012; Linder et al., 2015; Meyer et al., 2012; Schwartz et al., 2014) or at the beginning of the last exon (Ke et al., 2015). Numerous studies established that m⁶A marks at 3' mRNA ends regulate primarily cytoplasmic events such as stability and translation efficiency (X. Wang, Lu, et al., 2014; Wang et al., 2015; Y. Wang, Li, et al., 2014) (Box 2). These mechanisms have been studied in great detail (Fu, Dominissini, Rechavi, & He, 2014; Hoernes & Erlacher, 2017). However, several studies also provided evidence that m⁶A was involved in nuclear pre-mRNA processing of 3' ends (Bartosovic et al., 2017; Ke et al., 2015; Molinie et al., 2016).

Interestingly, the combination of m⁶A-IP with UV-crosslinking and high-throughput sequencing uncovered that 70% of all m⁶A modifications were in the last exons, particularly in long last exons (Ke et al., 2015). It was proposed that long last exons could carry more m⁶A residues, and this increase in modified residues could inhibit proximal APA selection. However, downregulation of METTL3, METTL14, and WTAP resulted in differential APA selection in both proximal and distal sites in a subset of mRNAs (Ke et al., 2015). The study did not provide any further insights on the possible molecular mechanism of the m⁶A-linked APA selection.

Since the development of the first transcriptome-wide methods to study m⁶A location and distribution along mRNA, different approaches have improved several aspects of the technique (K. Chen, Lu, et al., 2015; Linder et al., 2015; Liu et al., 2013; Molinie et al., 2016). One improvement was the development of the so-called “m⁶A-LAIC-Seq” (Molinie et al., 2016)

BOX 2THE CYTOPLASMIC ROLE OF m⁶A

m⁶A has been shown to regulate, in large part positively, mRNA translation and decay in the cytoplasm. The cytoplasmic reader YTHDF1 promoted translation of its target m⁶A-modified mRNA (Wang et al., 2015). One of the most versatile m⁶A readers, YTHDF2, is connected with both translation and decay. Acting as a cytoplasmic reader, YTHDF2 promoted decay of m⁶A-modified transcripts (X. Wang, Lu, et al., 2014) by recruiting the CCR4-NOT complex (Du et al., 2016). This YTHDF2 role has been linked with clearance of specific mRNA transcripts during early development and oocyte maturation (Ivanova et al., 2017; Zhao et al., 2017) (see Box 1). On the other hand, as a nuclear reader, it is linked to translation. During heat shock, YTHDF2 protects m⁶A/m⁶A_m marks at the 5' UTR of specific transcripts, namely *Hsp70*, from the demethylase FTO (Zhou et al., 2015). These methylated transcripts are recognized in the cytoplasm by eIF3 to promote cap-independent translation (Meyer et al., 2015). Interestingly, m⁶A seems to play a dual role during heat shock. Under this condition, the m⁶A marks preferentially deposited by METTL3 in heat-response transcripts also serve as marks for targeted and rapid degradation to ensure an acute response (Knuckles et al., 2017). Another protein of the same family, YTHDF3, promotes translation of target mRNA and circRNA (A. Li, Chen, et al., 2017; Y. Yang, Fan, et al., 2017). Only one study has linked methylation with increased stability. Transcripts with m⁶A_m at the 5' cap seem to be more stable than monomethylated transcripts (m⁷GpppNm) probably due to their resistance to DCP2-mediated decapping (Mauer et al., 2017).

that relies on sequencing intact full-length transcripts from m⁶A enriched and nonenriched samples. Sequencing nonfragmented RNA allows comparison of m⁶A enrichment on transcript variants of individual genes. Using this novel technique, Molinie et al. (2016) noted that m⁶A-enriched transcripts tend to more frequently use proximal APA sites than their corresponding nonmethylated partners. On the other hand, for the total RNA population (input), they observed that m⁶A levels positively correlated with 3' UTR length, in accordance with the findings by Ke et al. that m⁶A accumulated in long last exons (Ke et al., 2015; Molinie et al., 2016). This apparent discrepancy could be explained by m⁶A-mediated degradation. Molinie et al. proposed that these m⁶A-enriched transcripts with short 3' UTRs turn over more rapidly and therefore are underrepresented in the total RNA population. This model would also be supported by recent findings about FTO. Depletion of FTO by CRISPR-Cas9 in HEK293 cells resulted in longer 3' termini for a large subset of genes, which coincided with the position of validated APA sites (Bartosovic et al., 2017; Gruber et al., 2016). However, whether stabilization of FTO-dependent m⁶A marks really promotes degradation of short 3' UTR transcripts remains to be tested.

In summary, there are myriad questions and still very few answers regarding the potential role of m⁶A in 3' end processing. It would be interesting and challenging to explore whether some of the players in the m⁶A pathway are involved with 3' end processing machinery and help in poly(A) site selection (Figure 3).

4.4 | RNA methylation as a regulator of mRNA export to the cytoplasm

Mechanisms that connect efficient mRNA processing and export are critical for proper gene expression. Previous observations suggested that the cytoplasmic appearance of mRNA is dependent on m⁶A methylation (Camper, Albers, Coward, & Rottman, 1984; Finkel & Groner, 1983). Reducing m⁶A levels using the S-adenosylhomocysteine analog S-tubercidinylhomocysteine (STH) in HeLa cells resulted in delayed nuclear export of mature mRNA (Camper et al., 1984), and the same effect was observed upon METTL3 knockdown (Fustin et al., 2013). Correspondingly, ALKBH5 depletion led to rapid cytoplasmic mRNA appearance (Zheng et al., 2013).

Mechanistically, this process is mediated, at least for some mRNA, by YTHDC1. YTHDC1 incorporates the methylated target mRNA into the nuclear export pathway by interaction with SRSF3 (Roundtree et al., 2017). SRSF3 then acts as an adaptor between YTHDC1 and nuclear export factor 1 (NXF1) to facilitate the export of the m⁶A-modified mRNA bound by the reader protein (Roundtree et al., 2017; Figure 3).

These pioneer studies have proposed mechanistic insights on mRNA methylation-driven nuclear export. Future studies will reveal the extent of this regulation and the molecular details which link methylation, pre-mRNA processing, and export.

4.5 | Methylation and small RNA processing

The accumulation of methyl marks, namely m⁶A at 3' UTRs, partially correlated with miRNA binding sites (T. Chen, Hao, et al., 2015; Dominissini et al., 2012; Meyer et al., 2012). In addition, knockdown of Dicer, the endonuclease responsible for

producing mature miRNA, reduced m⁶A levels in mammalian neuronal stem cells and HeLa cells and caused delocalization of METTL3 from nuclear speckles (T. Chen, Hao, et al., 2015). However, METTL3 and Dicer do not interact in vivo, and the changes in m⁶A levels seem to be mediated directly by miRNA; exogenous introduction of miRNA directed against non-methylated transcripts increased m⁶A levels on their target sites (T. Chen, Hao, et al., 2015). It was proposed that miRNA could help to direct METTL3/14 to modify specific sites, although no further mechanistic details were provided (T. Chen, Hao, et al., 2015).

In turn, m⁶A marks were also detected on pri-miRNA. m⁶A promoted pri-miRNA processing by enhancing its recognition by DGCR8 (Alarcon, Lee, et al., 2015). This process was mediated by the nuclear m⁶A reader HNRNPA2B1, which recognized the methyl marks and interacted with the microprocessor complex and DGCR8 (Alarcon, Goodarzi, et al., 2015) (Figure 3). Altogether, these works suggest that METTL3 and miRNA processing pathways are co-regulated and the interplay between them affects both mRNA methylation and miRNA processing.

5 | CONCLUSION

In the early days of RNA research, it was already recognized that this molecule bears variable chemical modifications. However, due to technical limitations, the majority of research on RNA modifications was restricted to the three classes of highly expressed and stable RNA: rRNA, tRNA, and snRNA. Only recently have major technical advances revived interest in RNA biology and its processing, modification, and physiology. This expanding interest, in turn, acts as driving force to keep improving the technological tools. Biochemical approaches, coupled to high-throughput sequencing, have made it possible to study not only whole transcriptomes but also to map individual modifications and reveal the function of RNA-modifying enzymes or protein binding factors. All these tools would be inconceivable without increasing progress in computing power and development of bioinformatics tools. Improved sensitivity of mass spectrometry made it the gold standard for the identification of new chemical modifications and their assignment to specific types of RNA. The revolutionizing step in m⁶A studies was the development and utilization of an anti-m⁶A antibody for immunoprecipitation of methylated RNA fragments followed by RNA sequencing. All of these factors, combined with developments in cell manipulation, including RNAi tools, CRISPR-Cas9 gene editing, and protein tagging, allowed the identification of several writers, readers, and erasers in the field of epitranscriptomics.

A certain degree of controversy and inconsistency currently exists in the field. Careful steps must be taken to clarify the presence of methylation other than m⁶A in the eukaryotic mRNA. The biggest challenge to this task is still how to precisely and reliably detect individual modifications with single nucleotide resolution. Rectification will include improved protocols for pure mRNA isolation (free of contaminating ncRNA) and development of new approaches that allow direct, site-specific detection of methyl marks without the use of indirect tools (e.g., antibody enrichment or relying on reverse transcriptase “errors”). Most importantly, further elaboration of robust and available bioinformatics analyses tools should be a key step in comparative analyses of data obtained from various groups. Currently, attempts are being made to interconnect the published data in databases (Boccaletto et al., 2018).

It will be crucial to understand the functions of methyl marks on mRNA and other ncRNA. mRNA exhibits a complex co- and post-transcriptional life, which in the nucleus involves a number of processing and modification steps, stability, and nucleo-cytoplasmic export. Most of these processes are interconnected. Several studies implied that methylation and demethylation occur co-transcriptionally; therefore, it will be important to reveal to what extent methylation can affect transcription, and *vis-a-vis*, how transcription efficiency may regulate mRNA methylation patterns. The dynamics of m⁶A and m¹A resembles the dynamics of DNA modifications, which play a crucial role in gene expression regulation. Since the modifiers are to a certain extent homologous to DNA-modifying proteins, it will be interesting to address whether there is any crosstalk between epigenetic and epitranscriptomic pathways.

The expanding interest in the field has led to the identification and characterization of the key factors of the m⁶A mRNA pathway. The mammalian methyltransferase core complex has been characterized biochemically, structurally, and to certain extent biologically. However, we still do not know how auxiliary factors, such as Virilizer homolog, WTAP, or RBM15/15b, regulate methylation. One of the big questions that remains is what defines the final m⁶A (or other mark) pattern. m⁶A and its writers have been located to RRACH motifs, but only a very small portion of such motifs at specific mRNA locations are methylated. This phenomenon could be regulated by adaptor factors, including RNA binding proteins, METTL3/14 auxiliary factors, or even other RNAs (e.g., miRNA) (T. Chen, Hao, et al., 2015; Patil et al., 2016). Additionally, not much is known regarding the selectivity of readers, that is, whether different readers bind to distinct subsets of sites and how such specificity is achieved. Alternatively, the activity and selectivity of RNA demethylases could determine which marks can persist in a given RNA molecule. For m⁶A, at least two mRNA m⁶A methyltransferases and two demethylases have been uncovered. The level of target and functional redundancy between these factors is yet unknown. It is also unknown what enzyme(s) deposit(s)

methyl groups on purine rings in the 5' terminal m⁶A_m. Based on a recent report from Meyer et al., FTO prefers m⁶A_m rather than m⁶A (Mauer et al., 2017). This finding needs to be further integrated with other published results that link FTO nuclear function to m⁶A.

Taken together, a number of studies have increased significantly our awareness about the importance of methyl marks on coding RNA and have given rise to the new field of mRNA epitranscriptome. The field is still in its infancy, and there are exciting opportunities for new fundamental discoveries.

ACKNOWLEDGMENTS

We thank Mary O'Connell for reading the manuscript and Tomas Skalicky for phylogenetic analyses discussions. This work was supported by the Wellcome Trust (084316/B/07/Z to S.V.), the Czech Science Foundation (14-25884S to S.V., and H.C.-M. was supported from 305/11/1095), Ministry of Education, Youth and Sports of the Czech Republic under the project CEITEC 2020 (LQ1601), and the Brno City Municipality Scholarship for talented PhD students (to H.C.-M. and M.B.).

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

RELATED WIREs ARTICLES

[Mapping and significance of the mRNA methylome
RNA nucleotide methylation](#)

[mRNA methylation by NSUN2 in cell proliferation](#)

[Regulation and function of CMTR1-dependent mRNA cap methylation](#)

REFERENCES

- Aas, P. A., Otterlei, M., Falnes, P. O., Vagbo, C. B., Skorpen, F., Akbari, M., ... Krokan, H. E. (2003). Human and bacterial oxidative demethylases repair alkylation damage in both RNA and DNA. *Nature*, *421*(6925), 859–863. <https://doi.org/10.1038/nature01363>
- Adams, J. M., & Cory, S. (1975). Modified nucleosides and bizarre 5'-termini in mouse myeloma mRNA. *Nature*, *255*(5503), 28–33.
- Agafonov, D. E., Kastner, B., Dybkov, O., Hofele, R. V., Liu, W. T., Urlaub, H., ... Stark, H. (2016). Molecular architecture of the human U4/U6.U5 tri-snRNP. *Science*, *351*(6280), 1416–1420. <https://doi.org/10.1126/science.aad2085>
- Alarcon, C. R., Goodarzi, H., Lee, H., Liu, X., Tavazoie, S., & Tavazoie, S. F. (2015). HNRNPA2B1 is a mediator of m(6)A-dependent nuclear RNA processing events. *Cell*, *162*(6), 1299–1308. <https://doi.org/10.1016/j.cell.2015.08.011>
- Alarcon, C. R., Lee, H., Goodarzi, H., Halberg, N., & Tavazoie, S. F. (2015). N6-methyladenosine marks primary microRNAs for processing. *Nature*, *519*(7544), 482–485. <https://doi.org/10.1038/nature14281>
- Amort, T., Rieder, D., Wille, A., Khokhlova-Cubberley, D., Riml, C., Trixl, L., ... Lusser, A. (2017). Distinct 5-methylcytosine profiles in poly(A) RNA from mouse embryonic stem cells and brain. *Genome Biology*, *18*(1), 1. <https://doi.org/10.1186/s13059-016-1139-1>
- Baltz, A. G., Munschauer, M., Schwanhausser, B., Vasile, A., Murakawa, Y., Schueler, M., ... Landthaler, M. (2012). The mRNA-bound proteome and its global occupancy profile on protein-coding transcripts. *Molecular Cell*, *46*(5), 674–690. <https://doi.org/10.1016/j.molcel.2012.05.021>
- Bartosovic, M., Molares, H. C., Gregorova, P., Hrossova, D., Kudla, G., & Vanacova, S. (2017). N6-methyladenosine demethylase FTO targets pre-mRNAs and regulates alternative splicing and 3'-end processing. *Nucleic Acids Research*. <https://doi.org/10.1093/nar/gkx778>
- Batista, P. J., Molinie, B., Wang, J., Qu, K., Zhang, J., Li, L., ... Chang, H. Y. (2014). m(6)A RNA modification controls cell fate transition in mammalian embryonic stem cells. *Cell Stem Cell*, *15*(6), 707–719. <https://doi.org/10.1016/j.stem.2014.09.019>
- Belanger, F., Stepinski, J., Darzynkiewicz, E., & Pelletier, J. (2010). Characterization of hMTr1, a human Cap1 2'-O-ribose methyltransferase. *Journal of Biological Chemistry*, *285*(43), 33037–33044. <https://doi.org/10.1074/jbc.M110.155283>
- Boccaletto, P., Machnicka, M. A., Purta, E., Piatkowski, P., Baginski, B., Wirecki, T. K., ... Bujnicki, J. M. (2018). MODOMICS: A database of RNA modification pathways. 2017 update. *Nucleic Acids Research*, *46*(D1), D303–D307. <https://doi.org/10.1093/nar/gkx1030>
- Bringmann, P., & Luhrmann, R. (1987). Antibodies specific for N6-methyladenosine react with intact snRNPs U2 and U4/U6. *FEBS Letters*, *213*(2), 309–315.
- Camper, S. A., Albers, R. J., Coward, J. K., & Rottman, F. M. (1984). Effect of undermethylation on mRNA cytoplasmic appearance and half-life. *Molecular and Cellular Biology*, *4*(3), 538–543.
- Chen, K., Lu, Z., Wang, X., Fu, Y., Luo, G. Z., Liu, N., ... He, C. (2015). High-resolution N(6)-methyladenosine (m(6)A) map using photo-crosslinking-assisted m(6)A sequencing. *Angewandte Chemie*, *54*(5), 1587–1590. <https://doi.org/10.1002/anie.201410647>
- Chen, T., Hao, Y. J., Zhang, Y., Li, M. M., Wang, M., Han, W., ... Zhou, Q. (2015). m(6)A RNA methylation is regulated by microRNAs and promotes reprogramming to pluripotency. *Cell Stem Cell*, *16*(3), 289–301. <https://doi.org/10.1016/j.stem.2015.01.016>
- Clark, W. C., Evans, M. E., Dominissini, D., Zheng, G., & Pan, T. (2016). tRNA base methylation identification and quantification via high-throughput sequencing. *RNA*, *22*(11), 1771–1784. <https://doi.org/10.1261/rna.056531.116>
- Cozen, A. E., Quartley, E., Holmes, A. D., Hrabeta-Robinson, E., Phizicky, E. M., & Lowe, T. M. (2015). ARM-seq: AlkB-facilitated RNA methylation sequencing reveals a complex landscape of modified tRNA fragments. *Nature Methods*, *12*(9), 879–884. <https://doi.org/10.1038/nmeth.3508>
- Cui, Q., Shi, H., Ye, P., Li, L., Qu, Q., Sun, G., ... Shi, Y. (2017). m6A RNA methylation regulates the self-renewal and tumorigenesis of glioblastoma stem cells. *Cell Reports*, *18*(11), 2622–2634. <https://doi.org/10.1016/j.celrep.2017.02.059>
- Daffis, S., Szretter, K. J., Schriever, J., Li, J., Youn, S., Errett, J., ... Diamond, M. S. (2010). 2'-O methylation of the viral mRNA cap evades host restriction by IFIT family members. *Nature*, *468*(7322), 452–456. <https://doi.org/10.1038/nature09489>

- David, R., Burgess, A., Parker, B., Li, J., Pulsford, K., Sibbritt, T., ... Searle, I. R. (2017). Transcriptome-wide mapping of RNA 5-methylcytosine in Arabidopsis mRNAs and noncoding RNAs. *Plant Cell*, 29(3), 445–460. <https://doi.org/10.1105/tpc.16.00751>
- Delatte, B., Wang, F., Ngoc, L. V., Collignon, E., Bonvin, E., Deplus, R., ... Fuks, F. (2016). RNA biochemistry. Transcriptome-wide distribution and function of RNA hydroxymethylcytosine. *Science*, 351(6270), 282–285. <https://doi.org/10.1126/science.aac5253>
- Desrosiers, R., Friderici, K., & Rottman, F. (1974). Identification of methylated nucleosides in messenger RNA from Novikoff hepatoma cells. *Proceedings of the National Academy of Sciences of the United States of America*, 71(10), 3971–3975.
- Dominissini, D., Moshitch-Moshkovitz, S., Schwartz, S., Salmon-Divon, M., Ungar, L., Osenberg, S., ... Rechavi, G. (2012). Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq. *Nature*, 485(7397), 201–206. <https://doi.org/10.1038/nature11112>
- Dominissini, D., Nachtergale, S., Moshitch-Moshkovitz, S., Peer, E., Kol, N., Ben-Haim, M. S., ... He, C. (2016). The dynamic N(1)-methyladenosine methylome in eukaryotic messenger RNA. *Nature*, 530(7591), 441–446. <https://doi.org/10.1038/nature16998>
- Du, H., Zhao, Y., He, J., Zhang, Y., Xi, H., Liu, M., ... Wu, L. (2016). YTHDF2 destabilizes m(6)A-containing RNA through direct recruitment of the CCR4-NOT deadenylase complex. *Nature Communications*, 7, 12626. <https://doi.org/10.1038/ncomms12626>
- Dubin, D. T., & Taylor, R. H. (1975). The methylation state of poly A-containing messenger RNA from cultured hamster cells. *Nucleic Acids Research*, 2(10), 1653–1668.
- Edmonds, M., Vaughan, M. H., Jr., & Nakazato, H. (1971). Polyadenylic acid sequences in the heterogeneous nuclear RNA and rapidly-labeled polyribosomal RNA of HeLa cells: Possible evidence for a precursor relationship. *Proceedings of the National Academy of Sciences of the United States of America*, 68(6), 1336–1340.
- Fedeles, B. I., Singh, V., Delaney, J. C., Li, D., & Essigmann, J. M. (2015). The AlkB family of Fe(II)/alpha-ketoglutarate-dependent dioxygenases: Repairing nucleic acid alkylation damage and beyond. *Journal of Biological Chemistry*, 290(34), 20734–20742. <https://doi.org/10.1074/jbc.R115.656462>
- Finkel, D., & Groner, Y. (1983). Methylations of adenosine residues (m6A) in pre-mRNA are important for formation of late simian virus 40 mRNAs. *Virology*, 131(2), 409–425.
- Fu, L., Guerrero, C. R., Zhong, N., Amato, N. J., Liu, Y., Liu, S., ... Wang, Y. (2014). Tet-mediated formation of 5-hydroxymethylcytosine in RNA. *Journal of the American Chemical Society*, 136(33), 11582–11585. <https://doi.org/10.1021/ja505305z>
- Fu, Y., Dominissini, D., Rechavi, G., & He, C. (2014). Gene expression regulation mediated through reversible m(6)A RNA methylation. *Nature Reviews. Genetics*, 15(5), 293–306. <https://doi.org/10.1038/nrg3724>
- Fu, Y., Jia, G., Pang, X., Wang, R. N., Wang, X., Li, C. J., ... He, C. (2013). FTO-mediated formation of N6-hydroxymethyladenosine and N6-formyladenosine in mammalian RNA. *Nature Communications*, 4, 1798. <https://doi.org/10.1038/ncomms2822>
- Fustin, J. M., Doi, M., Yamaguchi, Y., Hida, H., Nishimura, S., Yoshida, M., ... Okamura, H. (2013). RNA-methylation-dependent RNA processing controls the speed of the circadian clock. *Cell*, 155(4), 793–806. <https://doi.org/10.1016/j.cell.2013.10.026>
- Gerken, T., Girard, C. A., Tung, Y. C., Webby, C. J., Saudek, V., Hewitson, K. S., ... Schofield, C. J. (2007). The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. *Science*, 318(5855), 1469–1472. <https://doi.org/10.1126/science.1151710>
- Geula, S., Moshitch-Moshkovitz, S., Dominissini, D., Mansour, A. A., Kol, N., Salmon-Divon, M., ... Hanna, J. H. (2015). Stem cells. m6A mRNA methylation facilitates resolution of naive pluripotency toward differentiation. *Science*, 347(6225), 1002–1006. <https://doi.org/10.1126/science.1261417>
- Gruber, A. J., Schmidt, R., Gruber, A. R., Martin, G., Ghosh, S., Belmadani, M., ... Zavolan, M. (2016). A comprehensive analysis of 3' end sequencing data sets reveals novel polyadenylation signals and the repressive role of heterogeneous ribonucleoprotein C on cleavage and polyadenylation. *Genome Research*, 26(8), 1145–1159. <https://doi.org/10.1101/gr.202432.115>
- Gulati, P., Avezov, E., Ma, M., Antrobus, R., Lehner, P., O'Rahilly, S., & Yeo, G. S. (2014). Fat mass and obesity-related (FTO) shuttles between the nucleus and cytoplasm. *Bioscience Reports*, 34(5), 621–628. <https://doi.org/10.1042/BSR20140111>
- Harper, J. E., Miceli, S. M., Roberts, R. J., & Manley, J. L. (1990). Sequence specificity of the human mRNA N6-adenosine methylase in vitro. *Nucleic Acids Research*, 18(19), 5735–5741.
- Hausmann, I. U., Bodi, Z., Sanchez-Moran, E., Mongan, N. P., Archer, N., Fray, R. G., & Soller, M. (2016). m6A potentiates Sxl alternative pre-mRNA splicing for robust *Drosophila* sex determination. *Nature*, 540(7632), 301–304. <https://doi.org/10.1038/nature20577>
- Helm, M., Giege, R., & Florentz, C. (1999). A Watson-Crick base-pair-disrupting methyl group (m1A9) is sufficient for cloverleaf folding of human mitochondrial tRNA^{Lys}. *Biochemistry*, 38(40), 13338–13346.
- Helm, M., & Motorin, Y. (2017). Detecting RNA modifications in the epitranscriptome: Predict and validate. *Nature Reviews. Genetics*, 18(5), 275–291. <https://doi.org/10.1038/nrg.2016.169>
- Hocine, S., Singer, R. H., & Grunwald, D. (2010). RNA processing and export. *Cold Spring Harbor Perspectives in Biology*, 2(12), a000752. <https://doi.org/10.1101/cshperspect.a000752>
- Hoernes, T. P., & Erlacher, M. D. (2017). Translating the epitranscriptome. *WIREs RNA*, 8(1), e1375. <https://doi.org/10.1002/wrna.1375>
- Hsu, P. J., Zhu, Y., Ma, H., Guo, Y., Shi, X., Liu, Y., ... He, C. (2017). Ythdc2 is an N6-methyladenosine binding protein that regulates mammalian spermatogenesis. *Cell Research*, 27(9), 1115–1127. <https://doi.org/10.1038/cr.2017.99>
- Hussain, S., Aleksic, J., Blanco, S., Dietmann, S., & Frye, M. (2013). Characterizing 5-methylcytosine in the mammalian epitranscriptome. *Genome Biology*, 14(11), 215. <https://doi.org/10.1186/gb4143>
- Hussain, S., Sajini, A. A., Blanco, S., Dietmann, S., Lombard, P., Sugimoto, Y., ... Frye, M. (2013). NSun2-mediated cytosine-5 methylation of vault noncoding RNA determines its processing into regulatory small RNAs. *Cell Reports*, 4(2), 255–261. <https://doi.org/10.1016/j.celrep.2013.06.029>
- Ivanova, I., Much, C., Di Giacomo, M., Azzi, C., Morgan, M., Moreira, P. N., ... O'Carroll, D. (2017). The RNA m6A reader YTHDF2 is essential for the post-transcriptional regulation of the maternal transcriptome and oocyte competence. *Molecular Cell*, 67(6), 1059, e1054–1067. <https://doi.org/10.1016/j.molcel.2017.08.003>
- Iwanami, Y., & Brown, G. M. (1968a). Methylated bases of ribosomal ribonucleic acid from HeLa cells. *Archives of Biochemistry and Biophysics*, 126(1), 8–15.
- Iwanami, Y., & Brown, G. M. (1968b). Methylated bases of transfer ribonucleic acid from HeLa and L cells. *Archives of Biochemistry and Biophysics*, 124(1), 472–482.
- Jaffrey, S. R., & Kharas, M. G. (2017). Emerging links between m6A and misregulated mRNA methylation in cancer. *Genome Medicine*, 9(1), 2. <https://doi.org/10.1186/s13073-016-0395-8>
- Jia, G., Fu, Y., Zhao, X., Dai, Q., Zheng, G., Yang, Y., ... He, C. (2011). N6-methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. *Nature Chemical Biology*, 7(12), 885–887. <https://doi.org/10.1038/nchembio.687>
- Jia, G., Yang, C. G., Yang, S., Jian, X., Yi, C., Zhou, Z., & He, C. (2008). Oxidative demethylation of 3-methylthymine and 3-methyluracil in single-stranded DNA and RNA by mouse and human FTO. *FEBS Letters*, 582(23–24), 3313–3319. <https://doi.org/10.1016/j.febslet.2008.08.019>
- Ke, S., Alemu, E. A., Mertens, C., Gantman, E. C., Fak, J. J., Mele, A., ... Darnell, R. B. (2015). A majority of m6A residues are in the last exons, allowing the potential for 3' UTR regulation. *Genes & Development*, 29(19), 2037–2053. <https://doi.org/10.1101/gad.269415.115>
- Ke, S., Pandya-Jones, A., Saito, Y., Fak, J. J., Vagbo, C. B., Geula, S., ... Darnell, R. B. (2017). m6A mRNA modifications are deposited in nascent pre-mRNA and are not required for splicing but do specify cytoplasmic turnover. *Genes & Development*, 31(10), 990–1006. <https://doi.org/10.1101/gad.301036.117>

- Khoddami, V., & Cairns, B. R. (2013). Identification of direct targets and modified bases of RNA cytosine methyltransferases. *Nature Biotechnology*, *31*(5), 458–464. <https://doi.org/10.1038/nbt.2566>
- Knuckles, P., Carl, S. H., Musheev, M., Niehrs, C., Wenger, A., & Buhler, M. (2017). RNA fate determination through cotranscriptional adenosine methylation and microprocessor binding. *Nature Structural & Molecular Biology*, *24*(7), 561–569. <https://doi.org/10.1038/nsmb.3419>
- Knuckles, P., Lence, T., Haussmann, I. U., Jacob, D., Kreim, N., Carl, S. H., ... Roignant, J. Y. (2018). Zc3h13/Flacc is required for adenosine methylation by bridging the mRNA-binding factor Rbm15/Spenito to the m(6)A machinery component Wtap/Fl(2)d. *Genes & Development*, *32*(5–6), 415–429. <https://doi.org/10.1101/gad.309146.117>
- Kohli, R. M., & Zhang, Y. (2013). TET enzymes, TDG and the dynamics of DNA demethylation. *Nature*, *502*(7472), 472–479. <https://doi.org/10.1038/nature12750>
- Legrand, C., Tuorto, F., Hartmann, M., Liebers, R., Jacob, D., Helm, M., & Lyko, F. (2017). Statistically robust methylation calling for whole-transcriptome bisulfite sequencing reveals distinct methylation patterns for mouse RNAs. *Genome Research*, *27*(9), 1589–1596. <https://doi.org/10.1101/gr.210666.116>
- Lenasi, T., & Barboric, M. (2013). Mutual relationships between transcription and pre-mRNA processing in the synthesis of mRNA. *WIREs RNA*, *4*(2), 139–154. <https://doi.org/10.1002/wrna.1148>
- Lence, T., Akhtar, J., Bayer, M., Schmid, K., Spindler, L., Ho, C. H., ... Roignant, J. Y. (2016). m6A modulates neuronal functions and sex determination in *Drosophila*. *Nature*, *540*(7632), 242–247. <https://doi.org/10.1038/nature20568>
- Li, A., Chen, Y. S., Ping, X. L., Yang, X., Xiao, W., Yang, Y., ... Yang, Y. G. (2017). Cytoplasmic m(6)A reader YTHDF3 promotes mRNA translation. *Cell Research*, *27*(3), 444–447. <https://doi.org/10.1038/cr.2017.10>
- Li, X., Xiong, X., Wang, K., Wang, L., Shu, X., Ma, S., & Yi, C. (2016). Transcriptome-wide mapping reveals reversible and dynamic N(1)-methyladenosine methylome. *Nature Chemical Biology*, *12*(5), 311–316. <https://doi.org/10.1038/nchembio.2040>
- Li, X., Xiong, X., & Yi, C. (2016). Epitranscriptome sequencing technologies: Decoding RNA modifications. *Nature Methods*, *14*(1), 23–31. <https://doi.org/10.1038/nmeth.4110>
- Li, X., Xiong, X., Zhang, M., Wang, K., Chen, Y., Zhou, J., ... Yi, C. (2017). Base-resolution mapping reveals distinct m(1)A methylome in nuclear- and mitochondrial-encoded transcripts. *Molecular Cell*, *68*(5), 993–1005 e1009. <https://doi.org/10.1016/j.molcel.2017.10.019>
- Li, Z., Weng, H., Su, R., Weng, X., Zuo, Z., Li, C., ... Chen, J. (2017). FTO plays an oncogenic role in acute myeloid leukemia as a N6-methyladenosine RNA demethylase. *Cancer Cell*, *31*(1), 127–141. <https://doi.org/10.1016/j.ccell.2016.11.017>
- Lin, S., Choe, J., Du, P., Triboulet, R., & Gregory, R. I. (2016). The m(6)A methyltransferase METTL3 promotes translation in human cancer cells. *Molecular Cell*, *62*(3), 335–345. <https://doi.org/10.1016/j.molcel.2016.03.021>
- Linder, B., Grozhik, A. V., Olarerin-George, A. O., Meydan, C., Mason, C. E., & Jaffrey, S. R. (2015). Single-nucleotide-resolution mapping of m6A and m6Am throughout the transcriptome. *Nature Methods*, *12*(8), 767–772. <https://doi.org/10.1038/nmeth.3453>
- Liu, J., Yue, Y., Han, D., Wang, X., Fu, Y., Zhang, L., ... He, C. (2014). A METTL3-METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. *Nature Chemical Biology*, *10*(2), 93–95. <https://doi.org/10.1038/nchembio.1432>
- Liu, N., Dai, Q., Zheng, G., He, C., Parisien, M., & Pan, T. (2015). N(6)-methyladenosine-dependent RNA structural switches regulate RNA-protein interactions. *Nature*, *518*(7540), 560–564. <https://doi.org/10.1038/nature14234>
- Liu, N., Parisien, M., Dai, Q., Zheng, G., He, C., & Pan, T. (2013). Probing N6-methyladenosine RNA modification status at single nucleotide resolution in mRNA and long noncoding RNA. *RNA*, *19*(12), 1848–1856. <https://doi.org/10.1261/rna.041178.113>
- Liu, N., Zhou, K. L., Parisien, M., Dai, Q., Diatchenko, L., & Pan, T. (2017). N6-methyladenosine alters RNA structure to regulate binding of a low-complexity protein. *Nucleic Acids Research*, *45*(10), 6051–6063. <https://doi.org/10.1093/nar/gkx141>
- Liu, S. R., Hu, C. G., & Zhang, J. Z. (2016). Regulatory effects of cotranscriptional RNA structure formation and transitions. *WIREs RNA*, *7*(5), 562–574. <https://doi.org/10.1002/wrna.1350>
- Martinez-Perez, M., Aparicio, F., Lopez-Gresa, M. P., Belles, J. M., Sanchez-Navarro, J. A., & Pallas, V. (2017). Arabidopsis m6A demethylase activity modulates viral infection of a plant virus and the m6A abundance in its genomic RNAs. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(40), 10755–10760. <https://doi.org/10.1073/pnas.1703139114>
- Mauer, J., Luo, X., Blanjoie, A., Jiao, X., Grozhik, A. V., Patil, D. P., ... Jaffrey, S. R. (2017). Reversible methylation of m6Am in the 5' cap controls mRNA stability. *Nature*, *541*(7637), 371–375. <https://doi.org/10.1038/nature21022>
- Merkstein, M., Laber, S., McMurray, F., Andrew, D., Sachse, G., Sanderson, J., ... Cox, R. D. (2015). FTO influences adipogenesis by regulating mitotic clonal expansion. *Nature Communications*, *6*, 6792. <https://doi.org/10.1038/ncomms7792>
- Meyer, K. D., Patil, D. P., Zhou, J., Zinoviev, A., Skabkin, M. A., Elemento, O., ... Jaffrey, S. R. (2015). 5' UTR m(6)A promotes cap-independent translation. *Cell*, *163*(4), 999–1010. <https://doi.org/10.1016/j.cell.2015.10.012>
- Meyer, K. D., Saletore, Y., Zumbo, P., Elemento, O., Mason, C. E., & Jaffrey, S. R. (2012). Comprehensive analysis of mRNA methylation reveals enrichment in 3' UTRs and near stop codons. *Cell*, *149*(7), 1635–1646. <https://doi.org/10.1016/j.cell.2012.05.003>
- Molinie, B., Wang, J., Lim, K. S., Hillebrand, R., Lu, Z. X., Van Wittenberghe, N., ... Giallourakis, C. C. (2016). m(6)A-LAIC-seq reveals the census and complexity of the m(6)A epitranscriptome. *Nature Methods*, *13*(8), 692–698. <https://doi.org/10.1038/nmeth.3898>
- Muthukrishnan, S., Both, G. W., Furuichi, Y., & Shatkin, A. J. (1975). 5'-Terminal 7-methylguanosine in eukaryotic mRNA is required for translation. *Nature*, *255*(5503), 33–37.
- Nilsen, T. W., & Graveley, B. R. (2010). Expansion of the eukaryotic proteome by alternative splicing. *Nature*, *463*(7280), 457–463. <https://doi.org/10.1038/nature08909>
- Ougland, R., Zhang, C. M., Liiv, A., Johansen, R. F., Seeberg, E., Hou, Y. M., ... Falnes, P. O. (2004). AlkB restores the biological function of mRNA and tRNA inactivated by chemical methylation. *Molecular Cell*, *16*(1), 107–116. <https://doi.org/10.1016/j.molcel.2004.09.002>
- Patil, D. P., Chen, C. K., Pickering, B. F., Chow, A., Jackson, C., Guttman, M., & Jaffrey, S. R. (2016). m(6)A RNA methylation promotes XIST-mediated transcriptional repression. *Nature*, *537*(7620), 369–373. <https://doi.org/10.1038/nature19342>
- Pendleton, K. E., Chen, B., Liu, K., Hunter, O. V., Xie, Y., Tu, B. P., & Conrad, N. K. (2017). The U6 snRNA m6A methyltransferase METTL16 regulates SAM synthetase intron retention. *Cell*, *169*(5), 824–835 e814. <https://doi.org/10.1016/j.cell.2017.05.003>
- Perry, R. P., & Kelley, D. E. (1974). Existence of methylated messenger-RNA in mouse L cells. *Cell*, *1*(1), 37–42. [https://doi.org/10.1016/0092-8674\(74\)90153-6](https://doi.org/10.1016/0092-8674(74)90153-6)
- Ping, X. L., Sun, B. F., Wang, L., Xiao, W., Yang, X., Wang, W. J., ... Yang, Y. G. (2014). Mammalian WTAP is a regulatory subunit of the RNA N6-methyladenosine methyltransferase. *Cell Research*, *24*(2), 177–189. <https://doi.org/10.1038/cr.2014.3>
- Ramanathan, A., Robb, G. B., & Chan, S. H. (2016). mRNA capping: Biological functions and applications. *Nucleic Acids Research*, *44*(16), 7511–7526. <https://doi.org/10.1093/nar/gkw551>
- Roundtree, I. A., Luo, G. Z., Zhang, Z., Wang, X., Zhou, T., Cui, Y., ... He, C. (2017). YTHDC1 mediates nuclear export of N6-methyladenosine methylated mRNAs. *eLife*, *6*. <https://doi.org/10.7554/eLife.31311>
- Ruzicka, K., Zhang, M., Campilho, A., Bodi, Z., Kashif, M., Saleh, M., ... Fray, R. G. (2017). Identification of factors required for m6A mRNA methylation in Arabidopsis reveals a role for the conserved E3 ubiquitin ligase HAKAI. *The New Phytologist*, *215*(1), 157–172. <https://doi.org/10.1111/nph.14586>

- Safra, M., Sas-Chen, A., Nir, R., Winkler, R., Nachshon, A., Bar-Yaacov, D., ... Schwartz, S. (2017). The m1A landscape on cytosolic and mitochondrial mRNA at single-base resolution. *Nature*, 551(7679), 251–255. <https://doi.org/10.1038/nature24456>
- Salditt-Georgieff, M., Jelinek, W., Darnell, J. E., Furuichi, Y., Morgan, M., & Shatkin, A. (1976). Methyl labeling of HeLa cell hnRNA: A comparison with mRNA. *Cell*, 7(2), 227–237.
- Saneyoshi, M., Harada, F., & Nishimura, S. (1969). Isolation and characterization of N6-methyladenosine from *Escherichia coli* valine transfer RNA. *Biochimica et Biophysica Acta*, 190(2), 264–273.
- Schwartz, S., Agarwala, S. D., Mumbach, M. R., Jovanovic, M., Mertins, P., Shishkin, A., ... Regev, A. (2013). High-resolution mapping reveals a conserved, widespread, dynamic mRNA methylation program in yeast meiosis. *Cell*, 155(6), 1409–1421. <https://doi.org/10.1016/j.cell.2013.10.047>
- Schwartz, S., Mumbach, M. R., Jovanovic, M., Wang, T., Maciag, K., Bushkin, G. G., ... Regev, A. (2014). Perturbation of m6A writers reveals two distinct classes of mRNA methylation at internal and 5' sites. *Cell Reports*, 8(1), 284–296. <https://doi.org/10.1016/j.celrep.2014.05.048>
- Shimba, S., Bokar, J. A., Rottman, F., & Reddy, R. (1995). Accurate and efficient N-6-adenosine methylation in spliceosomal U6 small nuclear RNA by HeLa cell extract in vitro. *Nucleic Acids Research*, 23(13), 2421–2426.
- Sledz, P., & Jinek, M. (2016). Structural insights into the molecular mechanism of the m(6)A writer complex. *eLife*, 5, e18434. <https://doi.org/10.7554/eLife.18434>
- Slobodin, B., Han, R., Calderone, V., Vrieling, J. A., Loayza-Puch, F., Elkon, R., & Agami, R. (2017). Transcription impacts the efficiency of mRNA translation via co-transcriptional N6-adenosine methylation. *Cell*, 169(2), 326–337 e312. <https://doi.org/10.1016/j.cell.2017.03.031>
- Spitale, R. C., Flynn, R. A., Zhang, Q. C., Crisalli, P., Lee, B., Jung, J. W., ... Chang, H. Y. (2015). Structural imprints in vivo decode RNA regulatory mechanisms. *Nature*, 519(7544), 486–490. <https://doi.org/10.1038/nature14263>
- Squires, J. E., Patel, H. R., Nusch, M., Sibbritt, T., Humphreys, D. T., Parker, B. J., ... Preiss, T. (2012). Widespread occurrence of 5-methylcytosine in human coding and non-coding RNA. *Nucleic Acids Research*, 40(11), 5023–5033. <https://doi.org/10.1093/nar/gks144>
- Stoltzfus, C. M., & Dane, R. W. (1982). Accumulation of spliced avian retrovirus mRNA is inhibited in S-adenosylmethionine-depleted chicken embryo fibroblasts. *Journal of Virology*, 42(3), 918–931.
- Sundheim, O., Vagbo, C. B., Bjoras, M., Sousa, M. M., Talstad, V., Aas, P. A., ... Slupphaug, G. (2006). Human ABH3 structure and key residues for oxidative demethylation to reverse DNA/RNA damage. *EMBO Journal*, 25(14), 3389–3397. <https://doi.org/10.1038/sj.emboj.7601219>
- Tang, C., Klukovich, R., Peng, H., Wang, Z., Yu, T., Zhang, Y., ... Yan, W. (2018). ALKBH5-dependent m6A demethylation controls splicing and stability of long 3'-UTR mRNAs in male germ cells. *Proceedings of the National Academy of Sciences of the United States of America*, 115(2), E325–E333. <https://doi.org/10.1073/pnas.1717794115>
- Theler, D., Dominguez, C., Blatter, M., Boudet, J., & Allain, F. H. (2014). Solution structure of the YTH domain in complex with N6-methyladenosine RNA: A reader of methylated RNA. *Nucleic Acids Research*, 42(22), 13911–13919. <https://doi.org/10.1093/nar/gku1116>
- Tian, B., Hu, J., Zhang, H., & Lutz, C. S. (2005). A large-scale analysis of mRNA polyadenylation of human and mouse genes. *Nucleic Acids Research*, 33(1), 201–212. <https://doi.org/10.1093/nar/gki158>
- Tirumuru, N., Zhao, B. S., Lu, W., Lu, Z., He, C., & Wu, L. (2016). N(6)-methyladenosine of HIV-1 RNA regulates viral infection and HIV-1 Gag protein expression. *eLife*, 5. <https://doi.org/10.7554/eLife.15528>
- Topisirovic, I., Svitkin, Y. V., Sonenberg, N., & Shatkin, A. J. (2011). Cap and cap-binding proteins in the control of gene expression. *WIREs RNA*, 2(2), 277–298. <https://doi.org/10.1002/wrna.52>
- Tserovski, L., Marchand, V., Hauenschild, R., Blanloeil-Oillo, F., Helm, M., & Motorin, Y. (2016). High-throughput sequencing for 1-methyladenosine (m(1)A) mapping in RNA. *Methods*, 107, 110–121. <https://doi.org/10.1016/j.ymeth.2016.02.012>
- Wang, P., Doxtader, K. A., & Nam, Y. (2016). Structural basis for cooperative function of Mett13 and Mett14 methyltransferases. *Molecular Cell*, 63(2), 306–317. <https://doi.org/10.1016/j.molcel.2016.05.041>
- Wang, X., Feng, J., Xue, Y., Guan, Z., Zhang, D., Liu, Z., ... Yin, P. (2016). Structural basis of N(6)-adenosine methylation by the METTL3–METTL14 complex. *Nature*, 534(7608), 575–578. <https://doi.org/10.1038/nature18298>
- Wang, X., Lu, Z., Gomez, A., Hon, G. C., Yue, Y., Han, D., ... He, C. (2014). N6-methyladenosine-dependent regulation of messenger RNA stability. *Nature*, 505(7481), 117–120. <https://doi.org/10.1038/nature12730>
- Wang, X., Zhao, B. S., Roundtree, I. A., Lu, Z., Han, D., Ma, H., ... He, C. (2015). N(6)-methyladenosine modulates messenger RNA translation efficiency. *Cell*, 161(6), 1388–1399. <https://doi.org/10.1016/j.cell.2015.05.014>
- Wang, Y., Li, Y., Toth, J. I., Petroski, M. D., Zhang, Z., & Zhao, J. C. (2014). N6-methyladenosine modification destabilizes developmental regulators in embryonic stem cells. *Nature Cell Biology*, 16(2), 191–198. <https://doi.org/10.1038/ncb2902>
- Warda, A. S., Kretschmer, J., Hackert, P., Lenz, C., Urlaub, H., Hobartner, C., ... Bohnsack, M. T. (2017). Human METTL16 is a N6-methyladenosine (m6A) methyltransferase that targets pre-mRNAs and various non-coding RNAs. *EMBO Reports*, 18, 2004–2014. <https://doi.org/10.15252/embr.201744940>
- Wei, C., Gershowitz, A., & Moss, B. (1975a). N6, O2'-dimethyladenosine a novel methylated ribonucleoside next to the 5' terminal of animal cell and virus mRNAs. *Nature*, 257(5523), 251–253.
- Wei, C. M., Gershowitz, A., & Moss, B. (1975b). Methylated nucleotides block 5' terminus of HeLa cell messenger RNA. *Cell*, 4(4), 379–386.
- Wei, C. M., Gershowitz, A., & Moss, B. (1976). 5'-Terminal and internal methylated nucleotide sequences in HeLa cell mRNA. *Biochemistry*, 15(2), 397–401.
- Wei, C. M., & Moss, B. (1975). Methylated nucleotides block 5'-terminus of vaccinia virus messenger RNA. *Proceedings of the National Academy of Sciences of the United States of America*, 72(1), 318–322.
- Wei, C. M., & Moss, B. (1977). Nucleotide sequences at the N6-methyladenosine sites of HeLa cell messenger ribonucleic acid. *Biochemistry*, 16(8), 1672–1676.
- Werner, M., Purta, E., Kaminska, K. H., Cymerman, I. A., Campbell, D. A., Mitra, B., ... Bujnicki, J. M. (2011). 2'-O-ribose methylation of cap2 in human: Function and evolution in a horizontally mobile family. *Nucleic Acids Research*, 39(11), 4756–4768. <https://doi.org/10.1093/nar/gkr038>
- Wilusz, J. E., Freier, S. M., & Spector, D. L. (2008). 3' end processing of a long nuclear-retained noncoding RNA yields a tRNA-like cytoplasmic RNA. *Cell*, 135(5), 919–932. <https://doi.org/10.1016/j.cell.2008.10.012>
- Wojtas, M. N., Pandey, R. R., Mendel, M., Homolka, D., Sachidanandam, R., & Pillai, R. S. (2017). Regulation of m(6)A transcripts by the 3'→5' RNA helicase YTHDC2 is essential for a successful meiotic program in the mammalian germline. *Molecular Cell*, 68(2), 374–387 e312. <https://doi.org/10.1016/j.molcel.2017.09.021>
- Xiang, Y., Laurent, B., Hsu, C. H., Nachtergaele, S., Lu, Z., Sheng, W., ... Shi, Y. (2017). Corrigendum: RNA m(6)A methylation regulates the ultraviolet-induced DNA damage response. *Nature*, 552(7685), 430. <https://doi.org/10.1038/nature24007>
- Xiao, W., Adhikari, S., Dahal, U., Chen, Y. S., Hao, Y. J., Sun, B. F., ... Yang, Y. G. (2016). Nuclear m(6)A reader YTHDC1 regulates mRNA splicing. *Molecular Cell*, 61(4), 507–519. <https://doi.org/10.1016/j.molcel.2016.01.012>
- Xu, L., Liu, X., Sheng, N., Oo, K. S., Liang, J., Chionh, Y. H., ... Fu, X. Y. (2017). Three distinct 3-methylcytidine (m(3)C) methyltransferases modify tRNA and mRNA in mice and humans. *Journal of Biological Chemistry*, 292(35), 14695–14703. <https://doi.org/10.1074/jbc.M117.798298>
- Yang, X., Yang, Y., Sun, B. F., Chen, Y. S., Xu, J. W., Lai, W. Y., ... Yang, Y. G. (2017). 5-Methylcytosine promotes mRNA export—NSUN2 as the methyltransferase and ALYREF as an m5C reader. *Cell Research*, 27(5), 606–625. <https://doi.org/10.1038/cr.2017.55>

- Yang, Y., Fan, X., Mao, M., Song, X., Wu, P., Zhang, Y., ... Wang, Z. (2017). Extensive translation of circular RNAs driven by N6-methyladenosine. *Cell Research*, 27(5), 626–641. <https://doi.org/10.1038/cr.2017.31>
- Yue, Y., Liu, J., Cui, X., Cao, J., Luo, G., Zhang, Z., ... Liu, J. (2018). VIRMA mediates preferential m(6)A mRNA methylation in 3'UTR and near stop codon and associates with alternative polyadenylation. *Cell Discovery*, 4, 10. <https://doi.org/10.1038/s41421-018-0019-0>
- Zhang, G., Huang, H., Liu, D., Cheng, Y., Liu, X., Zhang, W., ... Chen, D. (2015). N6-methyladenine DNA modification in *Drosophila*. *Cell*, 161(4), 893–906. <https://doi.org/10.1016/j.cell.2015.04.018>
- Zhang, S., Zhao, B. S., Zhou, A., Lin, K., Zheng, S., Lu, Z., ... Huang, S. (2017). m6A demethylase ALKBH5 maintains tumorigenicity of glioblastoma stem-like cells by sustaining FOXM1 expression and cell proliferation program. *Cancer Cell*, 31(4), 591–606 e596. <https://doi.org/10.1016/j.ccell.2017.02.013>
- Zhao, B. S., Wang, X., Beadell, A. V., Lu, Z., Shi, H., Kuuspalu, A., ... He, C. (2017). m6A-dependent maternal mRNA clearance facilitates zebrafish maternal-to-zygotic transition. *Nature*, 542(7642), 475–478. <https://doi.org/10.1038/nature21355>
- Zhao, X., Yang, Y., Sun, B. F., Shi, Y., Yang, X., Xiao, W., ... Yang, Y. G. (2014). FTO-dependent demethylation of N6-methyladenosine regulates mRNA splicing and is required for adipogenesis. *Cell Research*, 24(12), 1403–1419. <https://doi.org/10.1038/cr.2014.151>
- Zheng, G., Dahl, J. A., Niu, Y., Fedorcsak, P., Huang, C. M., Li, C. J., ... He, C. (2013). ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. *Molecular Cell*, 49(1), 18–29. <https://doi.org/10.1016/j.molcel.2012.10.015>
- Zhong, S., Li, H., Bodi, Z., Button, J., Vespa, L., Herzog, M., & Fray, R. G. (2008). MTA is an Arabidopsis messenger RNA adenosine methylase and interacts with a homolog of a sex-specific splicing factor. *Plant Cell*, 20(5), 1278–1288. <https://doi.org/10.1105/tpc.108.058883>
- Zhou, J., Wan, J., Gao, X., Zhang, X., Jaffrey, S. R., & Qian, S. B. (2015). Dynamic m(6)A mRNA methylation directs translational control of heat shock response. *Nature*, 526(7574), 591–594. <https://doi.org/10.1038/nature15377>
- Zhu, T., Roundtree, I. A., Wang, P., Wang, X., Wang, L., Sun, C., ... Xu, Y. (2014). Crystal structure of the YTH domain of YTHDF2 reveals mechanism for recognition of N6-methyladenosine. *Cell Research*, 24(12), 1493–1496. <https://doi.org/10.1038/cr.2014.152>
- Zust, R., Cervantes-Barragan, L., Habjan, M., Maier, R., Neuman, B. W., Ziebuhr, J., ... Thiel, V. (2011). Ribose 2'-O-methylation provides a molecular signature for the distinction of self and non-self mRNA dependent on the RNA sensor Mda5. *Nature Immunology*, 12(2), 137–143. <https://doi.org/10.1038/ni.1979>

How to cite this article: Covelo-Molares H, Bartosovic M, Vanacova S. RNA methylation in nuclear pre-mRNA processing. *WIREs RNA*. 2018;9:e1489. <https://doi.org/10.1002/wrna.1489>