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The Biogenesis and Precise Control of RNA m⁶A Methylation

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Abstract

N⁶-methyladenosine (m⁶A) is the most prevalent internal RNA modification in messenger RNA (mRNA) and has been found to be highly conserved and hard-coded in mammals and other eukaryotic species. The importance of m⁶A on gene expression regulation and cell fate decision has been well acknowledged in the past few years. However, it was only until recently that the mechanisms underlying the biogenesis and decision of m⁶A modification in cells were illustrated. Here, we review up-to-date knowledge on the biogenesis of RNA m⁶A modification, including the *cis*-regulatory element and *trans*-acting factors that determine general *de novo* m⁶A deposition and modulate cell type-specific m⁶A patterns, and discuss the biological significance of such regulations.

Keywords

RNA modification; m⁶A deposition; H3K36me3; biogenesis; epitranscriptomics or epitranscriptome; precise and dynamic control

The landmarks of the m⁶A Epitranscriptome

Similar to DNA and protein, the chemical structure of RNA could be modified in cells, serving as an epigenetic mechanism of gene expression control [1]. To date more than 170 types of RNA modifications have been identified [2–4], including those in messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), and long noncoding RNA (lncRNA). A global landscape of any type of RNA modification was described until recently with the advent of next-generation sequencing (NGS) methods that delineate epitranscriptome information in high resolution.

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As the most widespread and abundant internal modification on mRNAs, m⁶A modification has been a main focus in the field of epitranscriptomics during the past few years. In 2012, three decades after the first identification of the m⁶A modification, the m⁶A landscape in humans and mice was revealed by m⁶A-seq (also known as MeRIP-seq) [5, 6]. By utilizing an m⁶A specific antibody to immunoprecipitate fragmented RNA for subsequent deep sequencing, over 12,000 m⁶A-methylated peaks were identified associated with both coding and non-coding RNAs, and these peaks are highly conserved between human and mouse transcriptomes. Sequence analysis revealed that m⁶A modification congruously occurs in the consensus motif RRACH (R= G or A; H= A, C or U; A could be converted to m⁶A) [5, 6]. Despite the prevalence of RRACH sequences in the transcriptome, only 1-5% of them are methylated *in vivo*. Interestingly, m⁶A methylation on the RRACH motif does not randomly distribute across the transcript, but particularly occurs in the coding sequence (CDS), 3' untranslated region (UTR), and especially the region around the stop codon [5], indicating that the RRACH motif itself is not sufficient to determine m⁶A deposition. Accumulating data from various eukaryotic species including yeast [7], plant [8], fly [9, 10], zebrafish [11, 12] and mammals [5, 6, 13] have proved that the RRACH *cis*-regulatory motif and the CDS/3'UTR enrichment are two landmarks of the m⁶A epitranscriptome, which in turn strongly suggests the functional importance of m⁶A and the tight control of m⁶A deposition in eukaryotes.

Although widely used, m⁶A-seq detects m⁶A modification at a 100-200 nt resolution and is lacking of nucleotide-resolution information. While the SCARLET (site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography) method is able to determine m⁶A at a specific site in RNA samples [14], it is not a high-throughput or transcriptome-wide approach. To improve the resolution in m⁶A-profiling sequencing, a new method called Photo-crosslinking-assisted m⁶A-sequencing (PA-m⁶A-seq) was developed, which utilizes 4-thiouridine to crosslink the m⁶A antibody onto mRNA through 365 nm UV irradiation, and thus increases resolution up to ~23nt [7]. An alternative method, miCLIP (m⁶A individual-nucleotide resolution crosslinking and immunoprecipitation), was then developed to achieve single-nucleotide resolution by detecting the specific mutational signatures at m⁶A residues that were induced by crosslinking of the m⁶A antibody to m⁶A marks in RNA through 254 nm UV irradiation [15]. miCLIP-seq revealed that m⁶A residues tend to occur in clusters and frequently distribute in DRACH (D = A/G/U) motifs within CDS and 3'UTR, while N⁶2'-O-dimethyladenosine (m⁶A_m), a related base modification, was found within the BCA (B=C, U, or G) motif at the first encoded position of certain mRNAs [15]. However, miCLIP needs 20 µg of Poly(A)⁺ mRNA as starting material [15], and thus more effective methods with less starting material are warranted to be developed. More recently, a RNA endoribonuclease from *Escherichia coli*, MazF, was found to specifically cleave the ACA motif from the 5' side of first A only when it is not methylated [16]. Based on this finding, an antibody-independent method, called MAZTER-seq or m⁶A-sensitive RNA-Endoribonuclease-Facilitated sequencing (m⁶A-REF-seq), was developed by two independent groups to profile m⁶A [17, 18]. Although the m⁶A methylations detected by this new method are limited to the ones that occur at ACA sites, a distribution pattern enriched near stop codons is found, consistent with the pattern obtained by antibody-based profiling methods. Taken together,

various strategies available for transcriptome-wide mapping of m⁶A confirm that m⁶A methylation is hard coded in mRNA transcripts, especially toward the 3' end.

RNA m⁶A machinery: from writing to interpreting

m⁶A methylation is introduced into mRNAs by a multicomponent m⁶A methyltransferase complex (MTC, also known as m⁶A 'writer'; see Figure 1). The core component of MTC is a ~200 kDa heterodimer comprised of methyltransferase-like 3 (METTL3) and methyltransferase-like 14 (METTL14) [19, 20]. Both METTL3 and METTL14 contain methyltransferase domains; however, structural studies revealed that METTL3 is the only catalytically active subunit with the bound donor substrate S-adenosyl-methionine (SAM) in the catalytic site, whereas METTL14 is degenerated and essential for stabilizing METTL3 conformation and binding substrate RNAs [21–23]. *In vitro* methylation assays revealed that the METTL3/METTL14 complex catalyzed m⁶A deposition on GGACU motif-containing RNAs most efficiently, and photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) also demonstrated the enriched binding of METTL3 and METTL14 to the GGAC motif, consistent with the previously identified RRACH consensus motif of m⁶A [19, 22]. Two CCCH motifs in METTL3 and the arginine 298 of METTL14 have been suggested to be critical for conferring RNA substrate specificity [22]. Silencing or genetic deletion of METTL3 and/or METTL14 could globally reduce the peak numbers and abundance of m⁶A in different cell contexts [24–31]. Given the importance of METTL3 and METTL14 in maintaining proper m⁶A deposition *in vivo*, abnormal expression or mutations of METTL3 and/or METTL14 usually leads to human diseases, including cancer [24, 25, 32–34]. In addition to METTL3 and METTL14, other regulatory subunits of MTC have also been identified, including WTAP and its cofactors KIAA1429 (VIRMA), ZC3H13 and RBM15/RBM15B, which play roles in anchoring MTC in nuclear speckles and U-rich regions adjacent to m⁶A sites in mRNAs [20, 31, 35–38]. Recently, a new methyltransferase, PCIF1, was also found to be responsible for methylation at N⁶-position of adenosine; however, PCIF1 catalyzes only cap-specific terminal m⁶A_m methylation of mRNAs but not internal m⁶A methylation [39, 40].

Another m⁶A writer protein, METTL16, was identified more recently and found to catalyze m⁶A in U6 spliceosomal RNA (snRNA) and some structured RNAs [41–44]. Unlike the METTL3/METTL14 complex, METTL16 functions alone and does not catalyze m⁶A deposition within a RRACH motif. Instead, it relies on a combination of the RNA sequence and structure to recognize its RNA substrates [42]. METTL16-dependent m⁶A marks are found in introns and at intron-exon boundaries [41, 43], implying a role related to pre-mRNA splicing. Most recently, a new m⁶A methyltransferase, ZCCHC4, was reported to mediate methylation of ribosomal RNA within the AAC motif [45].

After deposition, the m⁶A mark could be removed by RNA demethylases (also known as m⁶A 'erasers'), including fat mass and obesity-associated protein (FTO) and AlkB family member 5 (ALKBH5) [46, 47], therefore shaping the cellular m⁶A landscape dynamically (see Figure 1). As the first identified demethylase of m⁶A [46], FTO can also demethylate multiple other types of DNA and RNA methylations, such as m³T, m³U, m⁶A_m and m¹A [48, 49]. Nonetheless, m⁶A in mRNA is the major substrate of FTO *in cellulo* [48–52]. The

demethylase activity of ALKBH5 seems to be specific for m⁶A methylation in RNA [47, 53].

The m⁶A marks influence RNA fate in multiple aspects by recruiting different m⁶A-binding proteins (also known as m⁶A ‘readers’) (see Figure 1). Through recognizing m⁶A on pre-mRNA in a direct or indirect (structural switch) manner, YTHDC1, hnRNPC, hnRNPG and hnRNPA2B1 may regulate mRNA splicing [54–57]. YHDC1 could also mediate nuclear export of processed RNAs into cytoplasm [58]. In the cytoplasm, m⁶A containing transcripts are sorted for degradation in p-bodies when bound by YTHDF2, YTHDF3 or YTHDC2, whereas IGF2BP1/2/3, FMRP and PRRC2A play an opposite role in stabilizing the m⁶A-modified transcripts [26, 59–63]. Besides the regulation on RNA stability, binding of m⁶A by reader proteins, including YTHDF1, YTHDF3, IGF2BP1/2/3, YTHDC2 and METTL3, also promotes mRNA translation [26, 33, 60, 61, 64–66]. Thus, the m⁶A modification is involved in every aspect of post-transcriptional gene regulation and RNA metabolism, dependent on the functions of different readers.

Evidence for co-transcriptional m⁶A deposition

Transcription is the first step of gene expression, in which the genetic information is transferred from DNA to RNA by the enzyme RNA polymerase. Before separation from the DNA template and RNA polymerase (RNA pol), the nascent transcripts (known as nascent RNAs or chromatin-associated RNAs) are a class of unstable RNAs with heterogeneous size, and will undergo a series of processing events, such as 5' capping, 3' polyadenylation and splicing. Back in 1976, it was found that m⁶A existed in pre-mRNA-containing high molecular nuclear DNA-like RNA (dRNA, also known as hnRNA) [67]. The presence of m⁶A on immature RNAs was further confirmed by direct analyses of m⁶A on the chromatin-associated RNA (CA-RNA) fraction or bromouridine (BrU) labeled nascent RNA [27, 68, 69], indicating a co-transcriptional mechanism of m⁶A deposition.

Nonetheless, there is a discrepancy on the distribution pattern of m⁶A on nascent RNAs. In one study a urea solution was used to isolate CA-RNAs from Hela cells, and this study found that m⁶A modifications on CA-RNAs were mostly in exons but very rarely in introns or splice sites, extremely similar to m⁶A on nucleoplasmic and cytoplasmic RNAs. They thus claimed that m⁶A methylations are added to exons in pre-mRNA during transcription and are not required for most splicing [68]. In contrast, another study [69] captured BrU-labeled nascent pre-mRNA for a time-resolved high-resolution assessment of m⁶A and found that more than a half (57%) of the early m⁶A peaks reside within introns, whereas most of the m⁶A peaks of steady-state mRNA reside in CDS and 3' UTR. More importantly, the co-transcriptional m⁶A deposition regulates splicing kinetics and alternative splicing of immature RNAs [69], in agreement with a previous study showing that the m⁶A demethylase FTO bound preferentially to intronic m⁶A to remove m⁶A modifications and regulate alternative splicing [70]. The two later studies together suggested a transient function of co-transcriptional early m⁶A deposition on splicing and a transition of the distribution and function of m⁶A from nascent RNAs to mature RNAs. Indeed, a high level of m⁶A was observed on chromatin-associated RNAs, which was dramatically reduced on nucleoplasmic and cytoplasmic RNAs [27]. In addition, several other studies have pointed out that m⁶A

modification is involved in splicing regulation in nuclei [20, 54, 57, 71, 72], and thus support the notion of co-transcriptional deposition of m⁶A.

Histone modification guides m⁶A deposition in CDS and 3' UTR

The unique pattern of the m⁶A epitranscriptome indicates that there could be a general yet dedicated mechanism for the precise deposition of m⁶A *in cellulo*. Strikingly, it was found that histone H3 lysine 36 trimethylation (H3K36me3), classically associated with active transcription and deposited co-transcriptionally, shows similar CDS and 3' UTR distribution pattern with m⁶A, implicating some unappreciated link between H3K36me3 and m⁶A RNA modification [27]. Further analysis of the m⁶A epitranscriptome and H3K36me3 ChIP-seq data in the same cell context confirmed the co-occurrence of these two epigenetic modifications, with approximately 70% of m⁶A peaks being overlapped with H3K36me3 sites. More importantly, changes in cellular H3K36me3 level by manipulation of the major H3K36me3 methyltransferase SETD2 or the H3K36me3 demethylase KDM4A led to corresponding alterations of m⁶A modification in human and mouse transcriptomes, while epigenetic editing at specific genomic loci using dCas9-fusions (dCas9-SETD2 or dCas9-KDM4A) resulted in expected and remarkable changes in m⁶A abundance at the corresponding RNA sites, demonstrating a causal regulation of H3K36me3 on m⁶A deposition [27]. To gain a global view, transcriptome-wide m⁶A-seq and miCLIP-seq analyses were performed, revealing a global m⁶A hypomethylation upon SETD2 knockdown, predominantly enriched in the CDS and 3'UTR with a GGAC motif [27]. Notably, more than 80% of H3K36me3-dependent m⁶A sites are also the target sites of METTL3, METTL14 or/and WTAP, suggesting that H3K36me3-mediated regulation on m⁶A deposition has a broad effect in the transcriptome.

Mechanistically, the m⁶A MTC could interact with H3K36me3 and the actively transcribing RNA Pol II [27], consistent with the previously recognized model of co-transcriptional installation of m⁶A. More specifically, H3K36me3 is directly recognized and bound by METTL14, which recruits other components of the m⁶A MTC and mediates deposition of m⁶A on the newly synthesized RNAs when encountering RNA Pol II [27]. Although it is still unclear how METTL14 recognizes H3K36me3, it has been shown that the N-terminal of METTL14 is critical for binding with H3K36me3 [27]. Therefore, it appears that the recognition of H3K36me3 by METTL14 together with the specific recognition of RRACH motif by m⁶A MTC enable precise and dynamic writing (deposition) of the m⁶A marks on the transcriptome (Figure 2, Key Figure). This model is also in compliant with reported distinct functions of METTL3 and METTL14, where METTL3 is the catalytic subunit while METTL14 is responsible for substrate recognition, and reveals the functional importance of METTL14 in selective and precise decision of *de novo* m⁶A deposition.

H3K36me3-m⁶A cross-talk in mESC pluripotency

The aforementioned data link the epigenome with the epitranscriptome and add an additional complexity to the gene expression network. In fact, it was shown that decrease of H3K36me3 suppressed global gene expression, likely through m⁶A-mediated influence on mRNA stability [27]. H3K36me3 is most commonly associated with transcription of active

euchromatin, and has been implicated in diverse cellular processes, including alternative splicing, DNA repair and recombination [73, 74]. Given the importance of m⁶A in regulation of mRNA stability, translation and splicing, the crosstalk between H3K36me3 and m⁶A provides a new way to coordinate multiple aspects of gene expression regulation.

It has been shown that Setd2, as an H3K36me3 writer, was critical for mouse embryonic stem cells (mESCs) differentiation via multifaceted functions [75]. Along these lines it was reported that depletion of Setd2 delayed endodermal differentiation in mESCs by regulating transcription initiation of *Egfr3* and activation of the downstream Erk pathway, as well as the transcriptional activity of a number of infrequently transcribed genes and alternative splicing of a subset of pre-mRNAs [75]. Additionally it was shown that silencing of Setd2 in mESCs resulted in a global hypomethylation of m⁶A, and more specifically, decreased m⁶A level and delayed reduction of mRNA transcripts of several critical pluripotency factors during differentiation, including *Oct4*, *Sox2*, *Nanog* and *Klf4* [27]. m⁶A-mediated prolonged expression of pluripotency transcripts is critical for the maintenance of the naïve phenotype in Setd2 knockdown mESCs, which was underlined by the similar phenotype in Mettl3 and Mettl14 knockdown mESCs and a series of epistasis experiments [27]. Collectively, regulation on m⁶A emerges as a novel mechanism underlying the critical role of H3K36me3 in mESCs differentiation (Figure 2, Key Figure). It is anticipated that the significance of H3K36me3-m⁶A cross-talk in controlling gene expression and cell fate could be fully revealed in various biological processes.

Transcription factors modulate cell context-specific m⁶A methylation

Similar to the epigenome, the epitranscriptome is highly plastic and reacts to changing external conditions [76, 77], thus allowing for dynamic gene expression regulation. Beyond the general regulation of the m⁶A epitranscriptome by H3K36me3, dynamic regulation of m⁶A could be achieved by recruitment of m⁶A MTC by transcription factors to specific chromatin loci in specific cell contexts (Figure 2, Key Figure).

ZFP217, a chromatin-associated zinc finger protein that plays a role in maintaining self-renewal of mESCs, was shown to activate transcription of key pluripotency genes and interact with several epigenetic regulators, including METTL3 [78]. Sequestration of METTL3 by ZFP217 blocked the formation of functional m⁶A MTC and thus prevented m⁶A deposition on ZFP217 target transcripts [78]. In contrast to ZFP217, activated transcription factors SMAD2 and SMAD3 could facilitate the recruitment of METTL3-METTL14-WTAP MTC onto nascent mRNAs by a yet unknown mechanism and promotes m⁶A deposition on their target transcripts [79]. Both ZFP217 and SMAD2/3 modulate m⁶A deposition in ESCs, but lead to different fate decisions of ESCs owing to the distinct functions of m⁶A on the target transcripts. ZFP217 maintains the pluripotency state by coupling active transcription with prolonged RNA stability of the mRNA transcripts of pluripotency factors, including *Nanog*, *Sox2*, *Klf4* and *Myc* [78]. In contrast, SMAD2/3 enables timely exit from the naïve state by introducing rapid clearing of the *Nanog* transcript upon differentiation [79]. In acute myeloid leukemia (AML) cells, METTL3 associated with the CAATT-box binding protein CEBPZ at the transcriptional start sites and induces m⁶A to enhance translation of associated mRNAs essential for the maintenance of the leukemic state

[32]. Unlike the transcriptome-wide regulation of H3K36me3 on m⁶A deposition, these transcription factors modulate m⁶A deposition on largely a subset of transcripts under specific cell context to achieve dynamic regulation of gene expression.

Concluding remarks and future perspectives

Inspired by the discovery of histone and DNA demethylases, RNA methylation was shown to be reversible as early as 2008 when FTO was reported as the demethylase of m³U [48]. Later on, m⁶A was found to be the dominant substrate of FTO in nucleus that undergoes reversible regulation *in cellulo* [46]. The biological significance of RNA modifications, especially of m⁶A, has been well acknowledged in various physiological and pathological processes [1, 80–84]. However, how *de novo* m⁶A RNA methylation is precisely deposited was not known until very recently, when accumulating evidence suggest that histone and transcription factor signatures on chromatin together shape the m⁶A epitranscriptome. Generally, m⁶A deposition on most m⁶A-containing transcripts occurs in a sequence and histone H3K36me3 mark dependent manner, which maintains the conservation and CDS /3' UTR enrichment features of m⁶A. During specialized processes, transcript factors stand out, and through different actions on m⁶A MTC, to mediate enrichment or exclusion of m⁶A in certain transcripts that are need to execute specific biological functions. Together, these two distinct mechanisms enable the inheritance and plasticity of m⁶A RNA methylation.

Compared to the well-studied DNA cytosine methylation (5-methylcytosine, 5-mC), m⁶A RNA methylation has its unique characteristics. The *de novo* DNA methylation and maintenance were achieved by different DNA methyltransferases, DNMT3A/B and DNMT1, respectively [85, 86]. DNMT3A/B generates new methylation sites to fulfill the requirement of dynamic regulation [86], whereas DNMT1 faithfully copies the methylation pattern from the mother strand during DNA replication [85]. Unlike DNMT3A/B and DNMT1, which can catalyze DNA methylation alone but function in different processes, METTL3 and METTL14 must work together to catalyze m⁶A RNA methylation, and may exhibit new functions other than methyltransferases. For example, METTL3 alone could function as an m⁶A reader to promote translation by recruiting eIF3 to the translation initiation machinery (Figure 1) [33, 66], allowing for more complicated and flexible regulation of gene regulation. Compared to 5-mC, a modified nucleotide more similar to m⁶A, which is however catalyzed by a different methyltransferase N6AMT1 and known as N⁶-methyl-2'-deoxyadenosine (m6dA), was also found in DNA [87]. It is interesting to investigate how METTL3/METTL14 and N6AMT1 could specifically deposit methyl groups on adenosines in RNA and DNA, respectively.

Collectively, the crosstalk between histone H3K36me3 and RNA m⁶A methylation uncovers a new layer of gene expression regulation, adding m⁶A methylation into the complicated epigenetic regulation network. By carefully studying the relationship between m⁶A RNA modification and other epigenetic modifications, more and more cross-talk is expected to be uncovered in the near future, enabling a comprehensive understanding of the entire epigenetic regulation network in various essential biological processes.

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Glossary

N⁶-methyladenosine

N⁶-methyladenosine (m⁶A) refers to methylation of the adenosine base at the nitrogen-6 position. It is the most abundant internal modification in messenger RNA (mRNA) of most eukaryotes, and is also found in other types of RNAs, including transfer RNA (tRNA), ribosome RNA (rRNA), small nuclear RNA (snRNA), and long non-coding RNA (lncRNA)

Epigenome

Epigenome refers to all the chemical modifications of DNA and histones. Such modifications regulate the expression of genes within the genome

Epitranscriptome and epitranscriptomics

Epitranscriptome includes all the chemical modifications of the transcriptome within a cell. Epitranscriptomics refers to the study of all functionally relevant changes to the transcriptome without alteration of the RNA sequence

CLIP

Cross-linking and immunoprecipitation, an antibody-based technique developed for studying RNA-protein interactions. Instead of using formaldehyde that is commonly used for DNA-protein crosslinking, CLIP uses ultraviolet (UV) light and the crosslinking is irreversible

MeRIP-seq

MeRIP-seq is also known as m⁶A-seq, which utilizes the specific m⁶A antibody to immunoprecipitate fragmented RNA for subsequent deep sequencing. Although the resolution is not very high, MeRIP-seq provides a good way to reveal the m⁶A profile in the transcriptome

SCARLET

SCARLET stands for site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography, a method developed to accurately determine m⁶A status at any site in mRNA or long non-coding RNAs

miCLIP-seq

miCLIP stands for m⁶A individual-nucleotide resolution crosslinking and immunoprecipitation, a method developed to increase resolution of m⁶A-seq. Different to m⁶A-seq, a UV-crosslinking step is added after m⁶A antibodies are introduced. miCLIP-seq maps m⁶A locations in the transcriptome with single-nucleotide resolution

MAZTER-seq

A newly developed m⁶A profiling method that is independent of m⁶A antibody but instead taking advantage of the specific cleavage of the unmethylated ACA motif by the MazF

endoribonuclease. The coverage of MAZTER-seq is therefore lower than the m⁶A antibody based sequencing methods as it only detects m⁶A within the ACA motif

Epigenetic editing

A type of genetic engineering in which specific sites of the epigenome is modified without changing the actual DNA sequence. In addition to Zinc finger proteins and Transcription activator-like effectors (TALEs) proteins, nuclease deficient Cas9 fusions (dCas9-fusions) were recently developed, in which the enzyme for specific epigenetic modification is fused to dCas9 and guided by guide RNA (gRNA) to the targeted DNA loci for editing

S-adenosyl-methionine (SAM)

An important methyl donor derived from ATP and methionine via the one-carbon metabolism. SAM is the main methyl donor in cellular methylation reactions, including DNA methylation, RNA methylation, and histone methylation

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Highlights

- m⁶A modification is a type of abundant and conserved RNA modification in eukaryotic RNAs that affects RNA fate, and is characterized by the preferential deposition within RRACH motif and enrichment in CDS and 3' UTR of mRNA.
- The RNA methyltransferase complex, comprising of the METTL3-METTL14 core subunit and other cofactors, is responsible for the deposition of m⁶A on mRNA, with METTL3 being the catalytic subunit while METTL14 being critical for target recognition.
- Histone H3K36me3 emerges as a general determinant for m⁶A deposition by recruiting METTL14 and the associated RNA methyltransferase complex to guide m⁶A deposition co-transcriptionally.
- By recruiting or sequestering m⁶A methyltransferase components, transcription factors exhibit new roles in controlling m⁶A level of specific transcripts under specific cell context.

Outstanding Questions

While tremendous advances have been made in our knowledge of m⁶A modification biogenesis, function and mechanisms during the past few years, there are still many essential questions to be addressed in this field. For instance:

How common is the cross-talk in RNA epigenetic regulation?

Is there cross-talk between RNA m⁶A modification and other histone modifications (such as histone methylation, acetylation and phosphorylation) or DNA modifications (such as DNA methylation and hydroxymethylation)?

Is there epigenetic cross-talk between other RNA modifications, such as m¹A, m⁵C and hm⁵C?

What is the structural basis of the interaction between METTL14 and H3K36me3?

Do cells coordinate histone, DNA, and RNA modifications for the fine control of gene expression in physiological and pathological conditions?

How does the interruption of H3K36me3-m⁶A cross-talk contribute to cancer development and drug response?

Figure 1. The m⁶A methylation machinery and the biological functions of m⁶A.

The m⁶A modification is installed onto mRNA by the methyltransferase complex (Writers) comprising of the METTL3-METTL14 heterodimer core subunit and other cofactors, including WTAP, KIAA1429, ZC3H13, and RBM15/RBM15b. METTL16 alone can catalyze m⁶A formation in U6 snRNA and some structured RNAs, while ZCCHC4 is responsible for deposition of m⁶A on rRNA. The RNA m⁶A modification can be reversibly removed by RNA demethylases (Erasers) FTO and ALKBH5. The biological functions of m⁶A modification are achieved by specific recognition and binding by RNA binding proteins (Readers), which affects RNA fate by regulating RNA splicing, export, decay, stabilization, and translation.

Figure 2. Key Figure: Regulation on m⁶A deposition.

H3K36me3 is installed onto histone tails by SETD2 co-transcriptionally. METTL14, acts as an H3K36me3 reader, binds directly to the H3K36me3 mark and recruits the m⁶A methyltransferase complex, which deposits m⁶A onto newly transcribed nascent RNAs. Given that H3K36me3 is enriched in 3' end of gene bodies, such a mechanism allows for the shaping of the m⁶A landscape characterized by the enrichment of m⁶A in CDS and 3' UTR, especially around stop codons. In addition to the H3K36me3 mediated global regulation, transcription factors act to fine tune m⁶A levels of specific transcripts under specific cell

contexts. ZFP217 recruits METTL3 to the promoters of pluripotency transcripts, including *Nanog*, *Sox2*, *Klf4* and *Myc*. Binding of METTL3 by ZFP217 prevents its binding to METTL14, leading to the hypomethylation and increasing stability of these target transcripts that are needed to maintain the pluripotent state of ESCs. During ESC differentiation, activated SMAD2/3 recruits the RNA methyltransferase complex to the promoter of *Nanog*, facilitating deposition of m⁶A and the rapid degradation of the *Nanog* transcripts. In leukemia cells, CEBPZ recruits METTL3 to deposit m⁶A on transcripts critical for leukemia maintenance and promote their translation.