RNA m6A Methylation Across the Transcriptome

Erdem Sendinc[1,2] and Yang Shi[3,*]

[1]Stem Cell Program, Division of Hematology/Oncology, Boston Children's Hospital, Boston, MA 02115, USA

[2]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston MA 02115, USA

[3]Ludwig Institute for Cancer Research, University of Oxford, Roosevelt Dr, Headington, Oxford OX3 7DQ, UK,

Correspondence: Yang Shi (yang.shi@ludwig.ox.ac.uk).

Summary

Since the early days of foundational studies of nucleic acids, many chemical moieties have been discovered to decorate RNA and DNA in diverse organisms. In mammalian cells, one of these chemical modifications, N6-methyl adenosine (m6A), is unique in a way that it is highly abundant not only on RNA polymerase II (RNAPII) transcribed, protein-coding transcripts, but also on non-coding RNAs, such as ribosomal RNAs and snRNAs, mediated by distinct, evolutionarily conserved enzymes. Here we review RNA m6A modification in the light of the recent appreciation of nuclear roles for m6A in regulating chromatin states and gene expression, as well as the recent discoveries of the evolutionarily conserved methyltransferases, which catalyze methylation of adenosine on diverse sets of RNAs. Considering that the substrates of these enzymes are involved in many important biological processes, this modification warrants further research to understand the molecular mechanisms and functions of m6A in health and disease.

Introduction

Two years after the discovery of the double helix DNA structure [1,2], N6-methylated adenine (m6A) base was first described in bacterial DNA in 1955 [3]. Subsequently, m6A was also reported to be present in bacterial and yeast RNA [4,5]. Although not discussed in these papers, we now know that these initial m6A reports reflect abundant ribosomal RNA methylation in total RNA [6,7]. With the development of efficient methods for mRNA isolation in the early 1970s, m6A in mRNA from mammalian cells was identified and reported [8,9]. This discovery was quickly followed by the discovery of cap adjacent m6A methylation of ribose methylated adenosine, N6,2´-O-dimethyladenosine (m6Am), in mammalian and viral mRNA [10] (Figure 1).

It took a few more decades for the biological significance of RNA modifications to be widely investigated and appreciated. Although the sequence motif for mRNA m6A methylation was determined in 1977 [11,12], the corresponding methyltransferase METTL3 was identified two decades later [13]. The identification of the enzymes and “reader” proteins of RNA m6A facilitated studies of this modification in the context of regulation of gene expression. The phenotypes associated with loss of METTL3 in various organisms further highlighted the biological significance of mRNA methylation [14–16]. Importantly, the identification of FTO and ALKBH5 as demethylases for RNA m6A revealed a dynamic nature of m6A methylation regulation [17,18]. Another important milestone in the field was the development of m6A mapping technologies that provided global maps of m6A in many organisms [19–21], demonstrating that this modification marks specific mRNAs in a regulated fashion to potentially influence mRNA metabolism and function. This hypothesis was further strengthened by the identification of protein modalities (“reader” domains) that specifically recognize m6A methylated mRNA and influence its stability in the cytosol [19,22],

which is accepted as one of the main functions of METTL3-mediated mRNA m6A methylation. Additionally, in the nucleus, chromatin-associated RNAs have recently been shown to be m6A methylated, and the METTL3-mediated m6A methylation has been suggested to impact chromatin states and transcription dynamics, which will be discussed in this review. For a thorough review of the other roles of mRNA m6A methylation and proteins ("readers") that recognize this modification on mRNAs, readers are referred to published reviews [23–25].

Global maps of m6A methylation also made it clear that in addition to mRNAs, non-coding RNAs carry abundant m6A methylation. Importantly, significant progress has been made recently in identifying the corresponding enzymes that mediate N6-adenosine methylation on non-coding RNAs (ZCCHC4, METTL5, METTL16, METTL4) as well as on mRNAs (PCIF1). These new findings, which will also be discussed in this review, highlight the diverse nature of regulation of RNA metabolism and function through m6A methylation across the transcriptome (Figure 1).

mRNA m6A and m6Am modifications

1. m6A on the first adenosine—generation of m6Am on mRNA

Chemical modifications of mRNA contribute to regulation of gene expression throughout the life cycle of mRNA by influencing mRNA transcription and processing, stability, translation and localization. Many of these modifications start co-transcriptionally in the

nucleus, with short nascent mRNA transcript emerging from the exit tunnel of transcribing RNA Polymerase II (RNAPII) first acquiring m7G cap mediated by three sequential enzymatic reactions (Figure 2). In mammals, the RNGTT (RNA guanylyltransferase and 5' phosphatase enzyme) dephoshorylates the 5' end of nascent mRNA transcript and transfers a guanosine monophosphate moiety to the 5' end of mRNA [26], which is further methylated at the N7 position to generate m7G mRNA cap by the RNMT/RAMAC enzyme complex co-transcriptionally [27,28]. Along with protecting mRNA from exonucleases, facilitating pre-mRNA processing, nuclear export and translation, the mRNA capping enzymes interact with both the nascent transcript and RNAPII to facilitate RNAPII-mediated transcription [29]. Additional mRNA modifying enzymes that interact with RNAPII include CMTR1, which mediates ribose 2´-O-methylation of the first transcribed nucleoside of mRNAs [30,31]. CMTR1 is an interferon stimulated gene and the ribose 2´-O-methylation has been shown to influence expression of other interferon stimulated genes [32]. Moreover, viruses utilize this modification to evade host immune recognition of viral transcripts [33], highlighting a potential role of this modification in immunomodulatory pathways.

In addition to the 2´-O-methylation, the first nucleoside of the nascent mRNA, if it is adenosine, can undergo further methylation on the N6-position, generating what is referred to as "m6Am" modification [10]. This modification is catalyzed by the recently discovered, evolutionarily conserved RNA methyltransferase, PCIF1 [34–37], which was initially identified as a RNA polymerase II (Pol II)-associated protein [38]. Both CMTR1 and PCIF1 interact with RNAPII via their WW domains, which specifically recognize the serine 5 phosphorylated form of the RNAPII C terminal domain, suggesting that these enzymes are engaged with the promoter proximal RNAPII early in transcription [39,40]. The fact that PCIF1 binds RNAPII and mediates mRNA m6Am modification raises an interesting question of

whether PCIF1 may regulate RNAPII-mediated transcription in a way that involves its RNA methylation activity.

CMTR1 and PCIF1 methylate mRNA in a sequential manner in that PCIF1-mediated mRNA m6Am methylation occurs on the m7G cap adjacent initial nucleoside only after it has already been 2´-O-methylated (Am) by CMTR1 (Figure 2). Thus the adenosine immediately adjacent to the first guanine on mRNA transcripts can be in an unmodifed (A), Am or m6Am, but not m6A modified states [41,42]. The structure of PCIF1 offers mechanistic insight into the specificity of this enzyme resulting in this particular modification pattern on mammalian mRNAs. PCIF1 has a methyltransferase domain with a canonical Rossmann fold containing a conserved (D/N/S/H)-P-P-(Y/F/W) motif, which is shared by m6A methyltransferases [34]. Moreover, this methyltransferase domain contains alpha helical bundles adjacent to the conserved Rossmann fold domain [34]. These alpha helices, which are unique to PCIF1, create the m7G cap recognition site giving this enzyme its capped mRNA target specificity [34]. In addition, PCIF1 has higher binding affinity and activity towards Am methylated RNA compared to unmethylated RNA, further explaining its substrate preference [43].

The abundance of m6Am and Am transcripts varies in different tissues [41,42] and different transcripts in different cell types are m6Am methylated [35,44], although the functional significance remains to be fully understood. For instance, approximately 10% of all expressed genes in melanoma cells are m6Am methylated [35]. It is important to note that although RNAPII transcripts can start with any of the 4 nucleosides, transcription start sites of many genes are heterogenous in different cell types and developmental stages [45,46], suggesting that diverse RNAPII transcripts can potentially be methylated by PCIF1. But how selective m6Am methylation on mRNA is imparted and regulated remains unclear.

The m6Am modification is likely to be dynamically regulated because it can be demethylated by the FTO demethylase [47]. In contrast, whether 2´-O-methylation is dynamically regulated is unclear as no demethylases have been reported to date. m6Am modification has been implicated in regulating mRNA stability [36,47] and cap-dependent translation in different cell types [35]. Interestingly, m6Am modification also occurs on cap independently translated histone mRNAs and on non-coding RNAs including snRNAs (mediated by distinct enzymes) [35,48], suggesting that there are context dependent molecular functions of this modification, which are yet to be discovered.

2. m6A methylation in the body of mRNA--Co-transcriptional m6A methylation of mRNAs

As RNAPII travels along, methyl moieties are continually to be co-transcriptionally deposited [49–53] onto the N6 position of adenosines, predominantly at the last exon near the translation stop codon and 3´ untranslated region (3´ UTR) [19,20] (Figure 3). The development of recent m6A mapping methods uncovered low abundance m6A also on introns and 5' untranslated regions [54,55]. This modification is carried out by the METTL3 and its partner METTL14, which does not require 2´-O-methylation on the ribose moiety. Moreover, two oxidative demethylase enzymes, FTO and ALKBH5, have been reported to demethylate m6A modified mRNAs in the nucleus, suggesting that mRNA m6A methylation is a reversible modification [17,18]. Earlier studies reported that m6A and cap-proximal mRNA methylations exist on chromatin associated nascent pre-mRNAs with introns [53,56]. More recent studies mapped m6A onto both exons and introns of nascent transcripts, suggesting that methylation can happen before co-transcriptional splicing events are completed [49,57]. Moreover, the METTL3 complex was demonstrated to associate with chromatin at promoters [50,80], gene bodies [51,58] and DNA damage sites [97].

Additionally, the m6A methylation patterns of mature, polyadenylated cytoplasmic mRNAs match the m6A distribution of nuclear pre-mRNA, suggesting that mRNAs do not gain or lose further methylation in the cytoplasm after they are transcribed and processed in the nucleus [49]. Collectively, these findings suggest that mRNA m6A modifications take place during transcription early in the lifecycle of mRNAs. In contrast, non-coding RNA species such as ribosomal RNAs, tRNAs and snRNAs can go through cytoplasmic modifications in addition to co-transcriptional modifications [48,59,60].

The mechanisms that underly the apparent 3´ UTR enrichment of m6A, mediated by METTL3/METTL14, are beginning to be understood, though perhaps still not completely. As discussed earlier, global mRNA m6A methylation mapping shows that methylation is enriched on the last exon of mRNAs around translation STOP codons with a DRACH consensus sequence motif [19,20]. Interestingly, this motif elsewhere in the mRNA is not as equally methylated [61]. Recent studies highlighted the importance of other co-transcriptional regulatory steps in controlling the target selectivity of METTL3 favoring adenosine residues near the 3´ end of mRNA. For instance, the 3´ UTR enriched pattern of mRNA m6A methylation is similar to the enrichment pattern of co-transcriptionally installed histone H3 lysine 36 trimethylation, mediated by the SETD2 methyltransferase complex, which directly interacts with RNAPII during transcriptional elongation [51]. Importantly, disruption of SETD2 activity and deposition of H3K36me3 or chemical inhibition of RNAPII results in a global decrease of mRNA m6A methylation [51], suggesting that co-transcriptional histone modifications play a role in dictating m6A modification of mRNAs. Moreover, METTL3-METTL14 is a part of a larger, evolutionarily conserved complex, which include additional components such as VIRMA/Virilizer, WTAP, Hakai and ZC3H13 [62,63]. VIRMA/Virilizer interacts with mRNA cleavage and polyadenylation specificity factors [64]. The depletion of VIRMA results in a reduced global 3´ UTR m6A mRNA methylation, suggesting a potential

crosstalk between transcription termination, polyadenylation and 3´ UTR m6A methylation via VIRMA [64,65]. Interestingly, the budding yeast genome codes for Ime4/METTL3 and Mum2/WTAP, but not homologs of the extended m6A machinery, such as METTL14 or VIRMA[21], suggesting that m6A 3´ UTR enrichment can take place, at least in budding yeast, without the proposed interactions between METTL14 and H3K36me3 or VIRMA and cleavage and polyadenylation factors. It should be noted that RNA secondary structure might also contribute to m6A distribution by preventing methylation by METTL3 in budding yeast [21]. Additionally, recent studies uncovered how splicing proteins impact co-transcriptional installation and distribution of m6A methylation by potentially blocking access of the METTL3 complex to nascent RNAPII transcripts during transcription [66,67] (Figure 3). This exclusion based m6A installation model explains the lack of m6A within proximity to exon-intron junctions on nascent transcripts [66,67]. Future work will provide further insights into the lack of m6A methylation away from the splice junctions other than the 3´ UTR and 3´ UTR m6A enrichment on intronless, single exon mammalian transcripts. Interestingly, the vast majority of budding yeast genes do not have introns [68] but their mRNAs also display 3´ UTR m6A enrichment [21], suggesting that there may be additional mechanisms that could also contribute to 3´ UTR enrichment of m6A methylation. Overall, while these discussed mechanisms (Figure 3), including interactions with chromatin architecture and 3´ cleavage-polyadenylation factors, RNA secondary structure and exclusion of m6A machinery by splicing factors, may contribute to 3´ UTR m6A enrichment and patterning, more work is necessary to fully understand the mechanisms behind the unique m6A distribution conserved across evolution.

m6A modification in the regulation of chromatin mediated processes

1.METTL3-mediated m6A of chromatin-associated RNAs

Recent studies highlighted potential nuclear functions of co-transcriptional RNA m6A methylation by the METTL3 complex. In addition to mRNAs, METTL3 also methylates non-coding RNAs, some of which are chromatin associated [69,72,80] and are collectively referred to as caRNAs [69] This plays a role in the regulation chromatin state and transcription dynamics (Figure 4). An example is XIST (X-inactive specific transcript) lncRNA, which is highly m6A methylated by METTL3 [61,70]. The METTL3 methyltransferase complex subunit RBM15 binds XIST lncRNA and recruits the enzyme complex to XIST. Upon RNA methylation, the nuclear m6A reader protein YTHDC1 is recruited to the methylated XIST RNA and this promotes XIST mediated transcriptional silencing of X chromosome. Knockdown of the nuclear m6A reader YTHDC1 or METTL3 impairs XIST-mediated silencing and leads to increased expression of X-linked genes [70]. Ectopic tethering of YTHDC1 to XIST in the absence of METTL3 mediated m6A methylation restores the suppression of X-linked genes, demonstrating the importance of recruitment of YTHDC1 in this process [70].

Another example is RNA m6A methylation, often at the 5' ends, of transcripts emanating from endogenous retroviral elements in mouse embryonic stem cells (mESCs) [69,71–73]. This RNA methylation helps to recruit the nuclear m6A reader YTHDC1 and histone methyltransferase complexes to form heterochromatin and to suppress expression of these repetitive elements [72,73]. In the absence of METTL3 or METTL14, endogenous retroviral element IAP expression increases at both transcript [71,72] and protein level [71], demonstrating the importance of this methylation in suppression of endogenous retroviral elements. In one study, METTL3 was shown to bind genomic locations of repetitive

245 elements, specifically the IAP elements, to mediate methylation of the nascent repetitive
246 transcripts [72]. The methylated transcripts are recognized by the nuclear m6A reader
247 YTHDC1, and both METTL3 and YTHDC1 are necessary for the formation of the
248 heterochromatin structure at the IAP loci [72]. Mechanistically, YTHDC1 recruits the H3K9
249 methyltransferase SETDB1, known to be critical for heterochromatin formation at the IAP
250 loci [74], through protein–protein interactions to install H3K9 trimethylation at these repetitive
251 elements and suppress their expression in mouse embryonic stem cells (ESCs) [72].
252 Consistently, another study also demonstrated that recognition of m6A methylated
253 repetitive RNA by YTHDC1 is required for silencing of these repetitive heterochromatic loci
254 [73]. This and other studies also identified m6A methylation of additional repetitive RNA,
255 including the major satellite repeats (MSR) and long interspersed nuclear elements
256 (LINEs), suggesting that m6A may play a widespread role in the regulation of mouse
257 heterochromatin [73,75–78]. Interestingly, yet another study suggests that regulation of the
258 expression of these repetitive RNAs by m6A methylation works via a different mechanism
259 by mainly functioning to recruit the cytosolic YTHDF family of m6A readers to impact IAP
260 RNA stability [71]. Separately, the stability of the carRNA (chromatin-associated regulatory
261 RNA) has also been shown to be regulated by METTL3, FTO and the nuclear m6A reader
262 YTHDC1 [69]. It is possible that these different mechanisms, i.e., a direct role of m6A in
263 suppressing repetitive element expression at the chromatin level versus regulation of the
264 stability of the repetitive RNA transcripts, both contribute to the overall regulation of the
265 repetitive elements in mESCs. It is also possible that these different conclusions could be
266 due to the different experimental approaches used and the time scales when the analyses
267 were carried out. Specifically, genetic knockout of METTL3 was used to reveal a role of
268 METTL3 at the IAP repetitive elements [72], while the rapid degradation of both METTL3 and
269 its partner METTL14 protein by degron led to the finding of regulation of repetitive element

expression at the level of stability [71]. It is unclear whether genetic removal of just METTL3 versus removal of both METTL3 and METTL14 by the degron approach, potentially destabilizing the larger m6A complex, may have contributed to the differential findings. Additionally, the degron approach results in the reduction of m6A methylation on nascent RNA within hours [71,79], while traditional genetic knock-out/down or genome editing strategies take days to achieve substantial loss of m6A and METTL3 [72,73,80,81]. Therefore, the degron mediated rapid loss of METTL3 may result in a loss of de novo m6A on nascent RNA within hours, but changes in epigenetic landscape including chromatin marks and DNA methylation may take significantly longer to be detected.

In addition to histone methyltransferases, m6A also helps recruit histone demethylases to chromatin. In HEK293T cell lines, which carry a reporter system with either wild-type or mutant METTL3 methylation motifs, the locus that codes for m6A methylated reporter RNA has lower H3K9me2 compared to the one that codes for mutant methylation motifs [82]. This is because transcriptionally repressive H3K9me2 mark is maintained at lower levels through recruitment of the histone demethylase KDM3B to chromatin by YTHDC1 [82]. Lastly, regulation of chromatin states and gene expression by METTL3-mediated m6A could also be indirect through the regulation of transcript stability of chromatin modifying enzymes. For example, the loss of *Mettl14* in mouse embryonic neural stem cells results in changes in global histone modification levels accompanied by changes in gene expression. In the absence of *Mettl14*, the cells display a robust increase in acetylation of histone H3 at lysine 27, stemming from significantly increased stability of mRNAs encoding H3K27 acetyltransferases CBP and p300, indicating that m6A indirectly impacts histone modifications and gene expression by destabilizing transcripts that encode histone modifiers [83].Another example of this comes from epithelial tissues, where loss of METTL3

increases transcript stability and expression of chromatin modifying enzymes, resulting in widespread gene expression abnormalities [84].

2. m6A on enhancer and promoter nascent RNAs

In addition to mRNAs and heterochromatic repetitive RNAs, other RNA pol II transcripts are also co-transcriptionally m6A methylated. These include chromatin associated enhancer RNAs (eRNAs) and promoter upstream transcripts (PROMPTs)[69,80,] and are collectively referred to as caRNA [69]. eRNAs are short, rapidly degraded RNAs that are often not spliced or polyadenylated [85] and their presence [86] correlates well with enhancer activity [86–88]. Transcription at enhancers provide an open chromatin and enable deposition of active chromatin marks and maintain transcriptionally permissive chromatin landscape. Similar to promoters, enhancerss also recruit Mediator, general transcription factors and RNA pol II for eRNA transcription [85]. eRNA m6A methylation is also mediated by METTL3 and methylation of eRNAs might be another regulatory step in gene expression. Specifically, the nuclear m6A reader protein YTHDC1 has been shown to be recruited to eRNAs dependent on METTL3 mediated m6A methylation [89]. siRNA knock-down or rapid degron loss of YTHDC1 results in a decrease in eRNA transcription. Moreover, loss of YTHDC1 leads to a decrease in BRD4 at enhancers, suggesting that m6A methylated eRNAs and YTHDC1 plays a role in the regulation of chromatin landscape and transcription at enhancer loci [89].

METTL3 complex has also been implicated in the regulation of transcription dynamics at promoters in cancer cell lines. Knockdown of components of the METTL3 complex including METTL3, WTAP or METTL14 orthologs in Drosophila and mammalian cells results in promoter proximal RNAPII accumulation [81] and reduction of nascent RNA

synthesis [80]. While RNAPII accumulates near transcription start sites, the RNAPII occupancy in gene bodies decrease upon knock down of METTL3 complex. This is also accompanied by a decrease in elongation-associated serine 2 phosphorylated RNAPII signal along gene bodies, which indicates that knock down of METTL3 complex results in a defect in entry into productive transcription elongation [81]. Knock down of YTHDC1 also leads to RNAPII accumulation at promoters suggesting that this effect is mediated through m6A methylation [81].

The loss of METTL3 complex results in the accumulation of not just RNA pol II but also integrator complex at the promoters [80]. Cleavage of nascent RNA by the Integrator complex is required for snRNA and eRNA biogenesis [90]. On the other hand, Integrator negatively impacts transcription of protein coding mRNA genes by triggering premature transcription termination [91] and recruiting PP2A to oppose CDK9 activity, which is required for productive elongation [92,93]. Knockdown of METTL3 complex results in increased binding of Integrator complex to promoters contributing to premature transcription termination [80]. These findings led to the hypothesis that m6A methylation of nascent RNA may play a role in marking transcripts for productive transcription [80].

Collectively, the finding that METTL3-mediated m6A regulates heterochromatin in mouse ES cells but impacts transcription dynamics at promoters in the established cancer cell lines highlights potential context-dependent functions of this important RNA modification.

3. m6A at R-loops and DNA damage sites

Recent evidence suggests that METTL3 mediated m6A methylation occurs not only on mRNAs but also on RNAPII transcripts that are part of R-loops. R-loops are nucleic acid structures that are composed of an RNA:DNA hybrid and a complementary single stranded DNA. These structures participate in DNA damage repair and transcriptional

346 regulation, including initiation, elongation and termination, but also may act as a source of
347 replication stress leading to genomic instability [94]. R-loops have been observed around
348 transcription start sites and also transcription termination sites of many RNAPII transcribed
349 genes [94]. Knock down of METTL3 results in an increase in R-loop formation at
350 transcription termination sites and this phenotype is rescued by only catalytically active
351 METTL3, suggesting that m6A methylation plays a role in R-loop formation at RNAPII
352 transcription termination sites [95]. In addition to METTL3, knock down of the m6A reader
353 YTHDF2 also results in increased R-loop levels, suggesting that m6A methylation
354 regulates accumulation of R-loops [96]. In the absence of this m6A reader, gamma-H2AX, a
355 marker for DNA double strand breaks, accumulates, underlying the importance of m6A
356 methylation in genome integrity [96].
357
358 RNAPII transcription plays an important role in transcription-coupled DNA damage repair.
359 Upon UV irradiation, METTL3 is rapidly recruited to DNA damage sites [97].
360 Since METTL3 methylates nascent RNA co-transcriptionally, recruitment of this complex to
361 damaged loci might be mediated by RNAPII complex. The detection of METTL3 at DNA
362 damage sites suggests that RNA m6A methylation might have a role in transcription
363 coupled DNA damage repair pathway. More work is necessary to determine the biological
364 significance of RNA and its methylation in DNA damage response.
365
366 **m6A on ribosomal and other non-coding RNA**
367 Early biochemical studies in the 1960s determined that ribosomal RNA carries diverse
368 RNA modifications including m6A methylation [6,7]. Recent global m6A maps highlighted the
369 abundance of m6A methylation on ribosomal RNA [61]. In fact, most of the global m6A
370 content of the transcriptome is contributed by rRNA m6A methylation on the small and

large ribosomal RNA transcripts [61]. Both the small and the large subunit rRNA carry m6A modification on a single adenosine residue [61]. While the mRNA m6A methyltransferase METTL3 was identified in the 1990s, the enzymes catalyzing rRNA methylation have not been discovered until recently. METTL5 and ZCCHC4 enzymes are the methyltransferases that catalyze 18S and 28S rRNA m6A, respectively [98–103]. The loss of these enzymes does not affect mRNA m6A methylation level or distribution, suggesting that METTL5 and ZCCHC4 enzymes are specific for rRNA methylation. Although majority of global m6A level is contributed by rRNA methylation by METTL5 and ZCCHC4, these enzymes are not as essential as METTL3/METTL14 or METTL16, which contribute to the remaining total global m6A level. Although the loss of ribosomal small subunit m6A methylation does not impact global translation, the loss of large ribosomal subunit m6A impairs global translation, mostly by impacting polysomes [102,103].

There are many other chemical modifications on rRNA [104], which contribute to processing and folding of rRNA during ribosome biogenesis. Both METTL5 and ZCCHC4 display nucleolar localization where rRNA transcription and most of the ribosome maturation and modifications take place [103,105,106]. This suggests that rRNA m6A methylation is an early event that takes place in the nucleolus before ribosomes are exported to the cytoplasm. Moreover, methylations of the target adenosine residues on both the large and the small subunit rRNA were shown to be close to 100% in different cell lines and organisms, suggesting that m6A on ribosomes is probably not dynamic [100,107]. Consistent with this idea, ribosomal m6A methylation by METTL5 and ZCCHC4 takes place in *C. elegans* where the m6A demethylases ALKBH5 and FTO are absent [101], although the possibility of yet-to-be-identified rRNA m6A demethylases in *C. elegans* cannot be excluded. Moreover, both the small and large ribosomal m6A modifications are located inside the ribosome structure and would not be easily accessible to potential m6A reader proteins [98,104].

Although the worm ribosomes carry m6A modifications on both rRNA subunits, the lack of YTH domain m6A readers in *C. elegans* genome further suggests that ribosomal m6A modifications are unlikely to be recognized by canonical m6A reader proteins [101].

In addition to rRNA, other non-coding RNAs are also m6A or m6Am methylated. Adenosines of the U2 and U6 spliceosomal snRNAs are m6Am or m6A methylated by METTL4 and METTL16, respectively [108–111]. As components of the spliceosome, snRNAs are highly modified non-coding RNAs that are required for pre-mRNA splicing by recognizing the consensus motifs in introns primarily through base pairing interactions with pre-mRNAs [112]. METTL16 mediated U6 snRNA methylation is evolutionarily conserved in many organisms including fission yeast, where loss of this modification impacts splicing of many pre-mRNAs, especially the ones with introns that pair with the m6A in U6 [113]. The proposed role for m6A methylation on U6 snRNA is to facilitate base pairing with the 5' splice site on pre-mRNA [26,111,113]. In addition to U6 snRNA, METTL16 also catalyzes m6A methylation of the SAM synthetase MAT2A mRNA and impacts intron retention and hence expression of this enzyme, which regulates cellular SAM levels [110]. METTL16 recognizes a specific RNA sequence in a structural hairpin context that is shared between the 3´ UTR of MAT2A mRNA and U6 snRNA, highlighting the importance of RNA structure in m6A methylation by METTL16 [114], which is in contrast to the preference of METTL3 for unstructured RNA [52,115]. The loss of METTL16 alters not only m6A level of its direct enzymatic targets, but also global m6A methylome, impacting even the METTL3 mRNA targets through altering cellular SAM levels [116,117]. Lastly, METTL16 might impact translation in an m6A-independent way through direct interaction with translation initiation factors [116].

In addition to U6, U2 snRNA is also m6A methylated. METTL4 methylates U2 snRNA at adenine position 30 of the U2 snRNA. Adenine 30 is already 2´-O-methylated (Am) by FBL in mammalian cells, and thus the addition of m6A leads to the generation of m6Am [108,109] on the U2 snRNA. While m6Am modification of the U2 snRNA is conserved from *Arabidopsis thaliana* [118] *to mammals* and 2´-O-methylation is necessary for m6A methylation by METTL4, in other organisms such as worms and fruit flies this adenine is not Am methylated, and their METTL4 orthologs nonetheless methylates the N6-position of adenine, leading to m6A instead of m6Am methylation of U2 snRNA [101,119]. This m6Am/m6A residue on U2 snRNA is located immediately upstream of the intronic branch point recognition sequence, suggesting that this modification might play a role in the recognition of this splicing motif. Indeed, loss of METTL4 activity results in defects in splicing, mainly increasing splicing of retained introns [108,109,119]. A recent crystal structure of *Arabidopsis* METTL4 provided mechanistic insights into this enzyme's requirement for the target sequence motif and the prior Am methylation of U2 snRNA [118]. In addition to U2 snRNA, recent studies also suggested the single stranded mitochondrial DNA as a substrate for methylation by METTL4 [120,121]. Further work on this recently discovered enzyme is expected to lead to a full understanding of the potentially diverse substrates of METTL4.

Physiological and molecular impact of RNA N6-adenine methylation

1. Biological functions of the newly identified RNA m6A methyltransferases

Among the six evolutionarily conserved RNA enzymes that methylate adenines at the N6 position, two of those (METTL16 and METTL3) appear to be common essential genes. Loss of METTL16 impacts cell viability and growth the most among these enzymes according to the recent high throughput cancer cell line CRISPR screens (Figure 5) [122]. In addition to a direct impact on SAM synthase mRNA splicing regulation, loss of METTL16

448 also results in a global splicing defect possibly due to loss of m6A methylation of U6
449 snRNA [110,113], which may be the basis for METTL16 as a common-essential gene.
450 Additionally, METTL3 is also required for cell viability except in stem cells [123–125]. Impacting
451 cancer cell viability upon knock-out makes METTL3 a potential therapeutic target for
452 cancer treatment. Recently, METTL3 small molecule inhibitors have been developed [126–
453 128], and they display anti-leukemic properties *in vitro* and in mice *in vivo,* indicating
454 METTL3 as a promising target against myeloid malignancies [126]. Further research will
455 uncover whether a therapeutic window exists for these inhibitors, which target this
456 ubiquitously expressed essential enzyme in different cancers [129].
457
458 In a transgenic mouse model, the global removal of *Mettl14* by ubiquitously expressed Cre
459 leads to embryonic lethality, which is also observed upon *Mettl3* loss [83,123]. On the other
460 hand, conditional *Mettl14* deletion in the nervous system results in lethality within the first
461 neonatal week [83]. These animals display defects in brain development and have smaller
462 cerebral cortex, highlighting the importance of this complex in physiology.
463
464 The loss of the other four N6-adenine methylase enzymes, PCIF1, METTL4, ZCCHC4 and
465 METTL5, by and large does not impact viability of various cell types as significantly as
466 METTL16 or METTL3 loss (Figure 5). However, new data reported in the DepMap
467 suggests that METTL5 may in fact be a selectively essential gene. Additionally, *Mettl5*
468 deficient mice are born at non-Mendelian ratios and display developmental defects [99]. The
469 mice that lack METTL5 and hence small subunit ribosomal m6A methylation have defects
470 in craniofacial development, hearing, fertility and behavior [99]. In worms, the loss of
471 METTL5 also results in fertility defects[101]. In addition, METTL5 null worms display
472 resistance to a variety of stresses [100]. A recent genetic study in a large cohort of individuals

suggests that frameshift variants of METTL5 are associated with intellectual disability, facial dysmorphism and microcephaly in an autosomal-recessive fashion, underlying the importance of small subunit rRNA m6A methylation in human health [130]. The loss of the large subunit rRNA m6A methyltransferase ZCCHC4 also leads to different phenotypes in various organisms. In worms, deficiency of both rRNA m6A enzymes resulted in a decrease in fertility with the loss of ZCCHC4 leading to a more substantial decrease [101]. This rRNA methyltransferase was also shown to be overexpressed in hepatocellular carcinoma and its knock out significantly reduced tumor size in a xenograft liver cancer mouse model [103]. More studies are needed to understand the physiological impact of loss of the evolutionarily conserved rRNA m6A methylation and its impact on protein translation in different cell types and tissues.

At the organismal level, the pair of the m6Am methyltransferases, METTL4 and PCIF1, also shows varying phenotypes, Specifically, the *Mettl4* deficient mice display embryonic sub-lethality, craniofacial dysmorphism and splenomegaly resulting in aberrant hematopoiesis underlining the functional significance of METTL4-mediated U2 snRNA methylation [121]. Given that METTL16 and METTL4 both methylate snRNAs and impact splicing, it will be important to investigate potential roles of these snRNA modifications in different tissues. Genetic knockout of the mRNA m6Am enzyme *Pcif1* results in viable mice with a reduced body weight [123,131]. However, whether PCIF1 plays a role in specific development processes remains unclear. While PCIF1 methylates only the cap-adjacent adenine residue of RNAPII transcripts, METTL3 methylates multiple adenines across the RNAPII transcript [35,61]. Based on the associated phenotypes, the N6-adenosine methyl groups in the body of RNAPII transcripts seem to have a distinct physiological significance compared to the N6-methyl groups on the first adenosine of RNAPII transcripts, although both methyltransferases seem to be expressed in many cell types.

2. Diverse functions of METTL3-mediated m6A methylation

The molecular functions attributed to METTL3 mediated m6A methylation are highly diverse [24]. Different studies have concluded that METTL3 mediated m6A is involved in regulation of transcription, splicing, polyadenylation, translation, nuclear export, RNA localization, stability and turnover, DNA damage response, apoptosis, and chromatin organization [25,132]. These seemingly unrelated roles of m6A stem from the fact that most, if not all, RNAPII transcripts are m6A methylated and these transcripts play important roles in various cellular pathways. There are also exceptions to each of the proposed functions. For example, although m6A has been shown to impact cellular translation, many untranslated long non-coding RNAs are also m6A methylated [126,133,134]. Additionally, while m6A has been shown to play roles in alternative polyadenylation, non-polyadenylated transcripts, including histone mRNAs and lncRNAs, are also methylated by METTL3 [64,135]. Furthermore, although there is evidence that m6A impacts splicing and/or nuclear export, unspliced long non-coding RNAs, which reside in the nucleus are also methylated by METTL3 [79,135,136], suggesting that this modification does not always participate in the regulation of these processes. Finally, while multiple studies suggest that m6A regulates expression of mRNAs that code for specific signaling or stress pathways under certain conditions, there are thousands of other transcripts including those of housekeeping genes that are also m6A methylated in these same cells under the same conditions [61,137]. Although molecular roles of m6A seem to be diverse with many exceptions, one unifying feature of METTL3 mediated m6A methylation is that it happens on RNAPII transcripts. Although RNAPI transcripts (pre-ribosomal RNAs) are also m6A methylated, these modifications are carried out by rRNA m6A methylase enzymes.

The other unifying feature of METTL3 methylation is that it adds a methyl group to RNAPII transcripts, which in turn functions as a chemical moiety for recognition by reader proteins. Independent of what the methylated RNAPII transcripts code for, the m6A reader proteins can specifically bind them and impact the expression and fate of these transcripts. Co-transcriptionally placing this chemical modification on RNAPII transcripts enables evolutionarily conserved m6A reader proteins to regulate fates of these RNAs independent of transcript identity, sequence, or function. For instance, while the nuclear YTHDC1 m6A reader could interact with m6A methylated non-coding nuclear XIST transcripts to impact X-chromosome inactivation, YTHDF2 could target m6A methylated mRNAs for degradation in the cytoplasm [70,138]. This way, m6A modification provides a new layer to regulate the fates of RNAPII transcripts via specific reader proteins. Future research will determine how functions and specificity of these various reader proteins are regulated to influence diverse RNAPII transcripts via METTL3 mediated m6A.

Given that m6A modification can impact many aspects of RNAPII transcripts, it is important to untangle the direct functions of m6A from the indirect effects. Since METTL3 is an essential gene, long term knock-down of this gene would indirectly impact many cellular processes due to the loss of cellular viability. In addition to its direct role in regulating chromatin state and transcription dynamics, METTL3-mediated m6A modification could also indirectly impact chromatin states and gene expression, in part by destabilizing transcripts that encode histone modifying enzymes [83,84]. New molecular approaches such as rapid protein degradation systems and fast acting inhibitors resulting in impact in short durations will help to differentiate direct vs indirect effects of the loss of m6A [71,79,126].

Summary and future directions

There are more than 150 chemical modifications that decorate diverse cellular RNAs. Methylation of adenosine at N6 position is unique in that it occurs not only on coding and non-coding RNAPII transcripts abundantly, but also on snRNAs and ribosomal RNAs by distinct enzymes. Recent studies discussed in this review identified and characterized the enzymes that carry out m6A on these RNAs. Although evolutionary conservation of these methyltransferases suggests biological significance of these methylation events, the regulation and biological roles of these enzymes are largely understudied. Furthermore, the mechanisms of recruitment of the enzymes to their specific targets remain unknown. Importantly, although multiple studies characterized the loss of these enzymes using different cell lines and methods, interpretation of the data would have to take into account potential indirect effects. In this regard, studies using rapid degrader systems and small molecule inhibitors will potentially help differentiate indirect and direct effects.

Although genome wide CRISPR screens employing cancer cell lines offer clues as to which enzymes could be potential therapeutic targets, small molecule inhibitors of these enzymes will also help to determine the significance of targeting these methylation events in cancer or other potential diseases. Moreover, some of these m6A methyltransferase enzymes are only associated with modest phenotypes in cell culture. More work is therefore necessary to understand the biological significance of these evolutionary conserved enzymes especially in model organisms *in vivo*.

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Declaration of Interests

Y.S. is a co-founder and member of the Scientific Advisory Broad of K36 Therapeutics and Alternative Bio, Inc. Y.S. is also a member of the Scientific Advisory Board of EPICRISPR BIOTECHNOLOGIES, INC, and a member of the MD Anderson External Advisory Board. Y.S. holds equity in Alternative Bio, Inc, Active Motif and K36 Therapeutics. E.S. declares no competing interests.

Figure Legends

Figure 1: Timeline of major discoveries

Fig 2: Cap proximal mRNA methylation. After RNMT catalyzed cap m7G methylation, RNAPII interacting CMTR1 and PCIF1 enzymes methylate first nucleotide of mRNAs

Fig 3. METTL3 methylates RNAPII transcripts co-transcriptionally

Fig 4. m6A modification in gene regulation and heterochromatin formation

Fig 5. N6-methyl adenine methyltransferases and their RNA targets. On the right, gene dependency scores of these enzymes across 1086 cancer cell lines from DepMap database

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