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Crosstalk between epitranscriptomic and epigenetic mechanisms in gene regulation

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Abstract

Epigenetic modifications occur on genomic DNA and histones to influence gene expression. More recently, the discovery that mRNA undergoes similar chemical modifications that powerfully impact transcript turnover and translation adds another layer of dynamic gene regulation. Central to precise and synchronized regulation of gene expression is intricate crosstalk between multiple checkpoints involved in transcript biosynthesis and processing. There are more than 100 internal modifications of RNA in mammalian cells. The most common is *N*⁶-methyladenosine (m⁶A) methylation. Although m⁶A is established to influence RNA stability dynamics and translation efficiency, rapidly accumulating evidence shows significant crosstalk between RNA methylation and histone/DNA epigenetic mechanisms. These interactions specify transcriptional outputs, translation, recruitment of chromatin modifiers, as well as the deployment of the m⁶A methyltransferase complex (MTC) at target sites. In this review, we dissect m⁶A-orchestrated feedback circuits that regulate histone modifications and the activity of regulatory RNAs, such as long noncoding (lnc)RNA and chromosome-associated regulatory RNA. Collectively, this body of evidence suggests that m⁶A acts as a versatile checkpoint that can couple different layers of gene regulation with one another.

Historical perspective and introduction of epitranscriptomics

Methylation of specific nucleotides on RNA was thought to be a phenomenon exclusive to ribosomal and transfer RNA species [1]. Multiple lines of evidence from over half a century showed that it is unlikely that mRNA undergoes substantial methylation. Bacterial mRNA contained less than one methyl group per 3500 nucleotides [2], and a series

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Declaration of interests

J.C. is a scientific advisor for Race Oncology and a scientific founder of Genovel Biotech Corp., in which he also holds equities.

of studies showed that incubation of eukaryotic cells with methyl-labeled methionine resulted in minimal incorporation of the label in mRNA precursors [3–5]. Ironically, it was the identification of another post-transcriptional modification that had a critical role in the discovery of mRNA methylation. The pioneering identification of widespread polyadenylation of transcriptional outputs led to the development of novel methods for large-scale isolation of purified mRNA [6–8]. Soon afterwards, Perry and Kelley demonstrated the existence of base-methylated mRNA in mouse L cells [3], followed by a study by Desrosiers *et al.* showing that, in contrast to the more promiscuous distribution of methylation on rRNA, methylated mRNA predominantly comprised m⁶A [9].

Although it was known for half a century that mRNA is chemically modified, it was only during the past decade that the field of ‘epitranscriptomics’ formally developed, mostly owing to technical and conceptual advances that allowed transcriptome-wide interrogation of m⁶A dynamics and, ultimately, galvanized interest in m⁶A function [10,11]. In particular, the identification of FTO as the first m⁶A demethylase in 2011 [12] revived this field, because it suggested that RNA modification can also be reversible and dynamic, analogous to DNA and histone modifications [13]. In 2012, the introduction of m⁶A-seq [14] and methylated RNA immunoprecipitation sequencing (MeRIP-Seq) [15] demonstrated the pervasive nature of chemical modification on mRNA and enabled functional interrogation, thus advancing our understanding of the significance of m⁶A modification in mammalian cell biology. Similar to epigenetics, the study of chemical modifications on DNA and histones, epitranscriptomics affects gene expression without altering the mRNA sequence (Figure 1). Although m⁶A is predominantly known to influence gene regulation through effects on mRNA stability and other post-transcriptional mechanisms, rapidly accumulating studies implicate m⁶A in epigenetic control. Here, we briefly introduce post-transcriptional effects of m⁶A on gene regulation and then discuss rapidly evolving evidence that highlights the significance of crosstalk between epitranscriptomic and histone/DNA epigenetics mechanisms.

Cellular regulation of m⁶A methylation

It is now established that methylation of the adenosine base at the nitrogen-6 position is the most abundant internal RNA modification [16]. Thousands of mRNAs undergo internal m⁶A modification of the consensus motif RRACH (R being either A or G, and H can be A, C or U) [17]. The frequency of this consensus motif significantly exceeds the m⁶A content on RNA in mammalian cells, hinting that additional ‘codes’ beyond sequence conservation impart m⁶A deposition [18]. In addition, m⁶A modifications are not randomly enriched on mRNA. Several studies have shown that most m⁶A sites cluster near the 3′-terminal exon, including the 3′-untranslated region (UTR), as well as long internal exons [19].

The installation of m⁶A occurs via a complex of enzymes known as ‘writers’ (see Glossary), comprising METTL14, METTL3, and WTAP [20]. Loss of function in any one of these components reduces m⁶A, which is critical for cell function [21,22]. The catalytic activity is contained in METTL3, with METTL14 acting as an allosteric activator. WTAP is an ubiquitously expressed nuclear protein that contains no catalytic activity but acts as a guide to recruit the methyltransferase complex to target transcripts [23]. Chemical

modifications on RNA are thought to be dynamic, with removal of m⁶A accomplished by ‘eraser’ proteins, which include FTO and ALKBH5. Recent evidence suggests that FTO also demethylates the related modification m⁶Am [24]. Although physiological contributions of m⁶A suggest a predominant role in cell differentiation and cancer regulation, m⁶A has been implicated in diverse biological systems, including neuroregulation, immune activation, and metabolic control [11,25–28].

m⁶A in RNA turnover dynamics

How does imparting m⁶A modifications alter the fate of mRNA? Shortly after its discovery, labeling studies in HeLa cells showed an association between m⁶A and mRNA stability [29]. The installation of m⁶A can powerfully alter the decay, nuclear export, and/or translation of a mRNA, a process that is executed by ‘reader’ proteins. Readers include HuR and YTH-containing domain proteins. For example, YTHDF2 selectively binds m⁶A, resulting in localization of bound mRNA from the translatable pool to mRNA decay sites [30]. Binding of YTHDF1 to m⁶A sites enhances the translation of mRNA [31]. Despite slightly different modes of function being ascribed to individual YTH domain-containing proteins, a more recent study suggests that the YTH group of proteins acts exclusively at the level of mRNA stability and have redundant rather than distinct roles in gene regulation in HeLa cells [32]. More systematical studies are warranted to test whether this is also true in different cellular contexts and experimental conditions.

Precisely how RNA methylation can alter transcript fate is poorly understood, but multiple mechanisms have been proposed. It is thought that RNA methylation can: (i) perturb the secondary structure of a mRNA to expose or mask RNA-binding motifs; (ii) influence the affinity of m⁶A-binding proteins for methylated mRNAs; (iii) reduce the solvation penalty of hydrophobic side chains of RNA-binding proteins that allows for increased affinity; and (iv) enhance phase separation of mRNAs [16,33]. Regardless of the mechanism, it is clear that alterations of RNA turnover dynamics are a principal mechanism by which m⁶A impacts transcript levels. The presence of m⁶A modifications increase RNA turnover and reduce protein levels, but there are exceptions in which the opposite may also be true depending on the context [30,34]. For instance, IGF2BP proteins (IGF2BP1/2/3) have been reported to enhance the stability and translation of m⁶A-modified mRNA targets by recruiting the mRNA stabilizers HuR, MATR3, and PABPC1, as well as localizing to stress granules during stress [35]. Hence, the consequence of the presence of m⁶A on any individual mRNA will potentially be driven by the location of this modification on the mRNA, interplay between writer and eraser complexes, and specific reader proteins involved [36].

Although m⁶A methylation has been strongly associated with RNA stability, it has also been linked with almost every step in RNA homeostasis, from pre-mRNA processing to translational efficiency. As an example, m⁶A is deposited co-transcriptionally and several studies have shown that m⁶A methylation regulates pre-mRNA splicing [37,38]. This evidence is consistent with colocalization of m⁶A writers and erasers at **nuclear speckles**, with their depletion impacting alternative splicing [39,40]. However, other investigations found that m⁶A deposited on nascent transcripts dictates transcript decay but is not required for splicing [41]. Similarly, other studies found conflicting evidence for the roles of m⁶A

methylation in regulating translational efficiency [32,42,43]. The distinct conclusions are likely due to the different cell contexts and experimental models used for the studies. Taken together, there is strong evidence suggesting that m⁶A impacts transcripts stability, but its precise mode of regulation in impacting other steps in RNA processing is still emerging.

m⁶A and epigenetic control

A hint that m⁶A methylation may influence physiological regulation beyond post-transcriptional mechanisms comes from knockout studies of m⁶A-modulating machinery. Knockout of cytoplasmic YTHDF proteins, critical regulators of m⁶A-imparted transcript decay, does not recapitulate the loss of nuclear m⁶A-installing enzymes or other nuclear readers, which are developmentally lethal at an early stage [20,44]. This effect may be due, in part, to compensation from other readers [32], but also hints that m⁶A modifiers in the nucleus may have other roles independent of the executive actions of cytoplasmic readers. Indeed, rapidly accumulating evidence suggests that m⁶A methylation is integral to the proper maintenance of RNA biogenesis through feedback on epigenetic circuits. We discuss herein the diverse mechanisms by which m⁶A may crosstalk with chromatin modification. A summary of the effect of m⁶A on specific modifications is provided in Table 1.

Direct impact of m⁶A on heterochromatin regulation

Heterochromatin is an essential component of the genome because it maintains genome integrity and stable gene expression [45]. A critical function of heterochromatin is to restrain the activity of embedded satellite repeats and transposable elements [46]. In humans and mice, endogenous retroviruses (ERVs) are a prominent class of retrotransposons that require constitutive silencing by regulation machineries. Recently, three groups reported a role for m⁶A in the regulation of ERVs, specifically a type of repetitive element known as intracisternal A particle (IAP), in mouse embryonic stem cells (mESCs) (Figure 2) [47–49]. Knockout of *Mettl3* and rescue by catalytically inactive *Mettl3*^{APPA} failed to restore H3K9me3 levels, a defining molecular feature of heterochromatin, at IAP elements. Conversely, knockout of *Alkbh5*, a m⁶A eraser, significantly increased H3K9me3 levels at these sites. The m⁶A deposited by *Mettl3* is read by *Ythdc1* and contributes to heterochromatin formation. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) of *Ythdc1* confirmed the enrichment at H3K9me3-rich transposable elements, and its role in mediating retrotransposon silencing and maintaining mESC identity [49]. Both IAP mRNA and protein levels are inversely correlated with that of m⁶A. The deposition of m⁶A onto the 5'UTR of the IAP mRNA recruits the YTHDF protein family for mRNA degradation. Mechanistically, *Ythdc1* appears to act as an important guide for *Mettl3*, facilitating its interaction with chromatin, *Setdb1*, and *Trim28*, which in turn regulate H3K9me3 deposition at IAPs. Liu *et al.* demonstrated that, in addition to the involvement of *Setdb1*, m⁶A deposition represses *Dux*, the master regulator of the two-cell stage in ESCs [49]. Chelminski *et al.* also observed an increase in IAP mRNA when both *Mettl3* and *Mettl14* were depleted; however, unlike other studies [49], H3K9me3 levels remained similar, with only a moderate increase [48]. A key difference is that Chelminski *et al.* used an auxin-inducible degron (AID) system that can rapidly deplete *Mettl3* and/or *Mettl14* at will. Thus, it is conceivable that tampering *de novo* m⁶A deposition has a different effect

on H3K9me3 levels at IAP elements than observed by Xu *et al.*, who knocked out *Mettl3* entirely. Despite some differences in the proposed mechanisms, these three studies [47–49] provide convincing evidence that links m⁶A and heterochromatin formation.

Another role of m⁶A in stabilizing heterochromatin lies in major satellite repeats (MSR). In mouse chromatin, the centromeres are occupied by heterochromatin, which contains MSR and is characterized by H3K9me3, HP1 proteins, and DNA methylation [45]. Some MSR can be transcribed by RNA polymerase II (Pol II) and the resulting RNA is categorized as heterochromatic RNA (hetRNA) [50]. These hetRNA form RNA:DNA duplexes that help retain HP1 and Suv39h, which are histone lysine methyltransferases. MSR RNA, especially the sense strand, which contains six RRACH motifs compared with one in the antisense strand, is a preferred substrate for the METTL3/METTL14 complex *in vitro*. In *Mettl3/Mettl14* knockout mESCs, MSR transcripts have reduced m⁶A levels, impaired chromatin association, as assayed by RT-qPCR of different cellular fractions, as well as decreased RNA:DNA hybrid formation [51]. Although the biological effect of this phenomenon is unclear, this study shows another mode by which m⁶A can regulate chromatin structure.

m⁶A and chromosome-associated regulatory RNA

Various RNA species can physically associate with chromatin, including promoter-associated RNAs (paRNA), enhancer RNAs (eRNA), and repeat RNAs transcribed from transposons. These chromosome-associated regulatory RNAs (carRNAs) have been shown to contribute to genome organization, transcript regulation, and nuclear subdomains [52]. A study by Liu *et al.* expanded our understanding of the destabilizing nature of m⁶A, showing that RNA modifications on carRNAs regulate local chromatin accessibility and downstream transcription (Figure 3, Key figure) [21]. Deletion of *Mettl3* in mESCs enhanced the stability of carRNAs, leading to more-open chromatin and active transcription [21]. Congruent with these observations, the authors found elevations in histone marks associated with active transcription, such as H3K4me3 and H3K27ac. A similar phenotype was observed with nucleus-localizing *Ythdc1* conditional knockout, but not cytoplasmic *Ythdf2*. YTHDC1 associated with RBM7 and ZCCHC8, key components of the Nuclear Exosome Targeting (NEXT) complex [53]. Hence, the study suggests that METTL3 tags carRNAs with m⁶A for degradation by YTHDC1 through the NEXT complex.

An intriguing observation is that an increase in chromatin accessibility following *Mettl3* knockout was more prevalent at genes that normally had upstream m⁶A-tagged carRNAs. The biggest transcription rate differences were observed at genes that were involved in stem cell maintenance (*Esrrb* and *Ranbp17*) and chromatin remodeling (*Prdm9* and *Kmt2d*), forming a positive-feedback loop in opening up chromatin [21]. Hypomethylation of carRNAs enhances the recruitment of EP300, a histone acetyltransferase, and YY1, a multifunctional transcription factor. In addition, RNA methylation loss decreased the binding of JARID2, a component of the Polycomb Repressive Complex 2 (PRC2), to carRNAs. Together, these findings suggest that methylation of carRNAs stabilizes closed chromatin by recruiting deactivating factors and parrying activating ones, thus adding another twist to the role of m⁶A in gene regulation.

Among the repeat RNA families that showed m⁶A-enrichment changes, are Long Interspersed Element –1 (LINE1) elements. These abundant retrotransposons showed more significant fold changes in methylation and altered decay upon *Mettl3* depletion. Consistent with other carRNAs, LINE1 was highly enriched upon *Mettl3* knockout and contributed to the global increase in chromatin accessibility. The authors provide direct evidence of functional effects of LINE1 methylation through use of CRISPR/dCas13b-fused FTO. Analogous to modified cas9 guides for DNA, the dCas13b-FTO fusion protein can achieve site-specific RNA demethylation when deployed with a guide RNA [21]. Using this approach, Liu *et al.* demonstrated a specific decrease in m⁶A levels at LINE1 RNAs as well as enhanced chromatin accessibility, carRNA abundance, and downstream transcription. Finally, the authors provide evidence of the functional relevance of RNA modifications on LINE RNAs by showing that this targeted strategy can decrease differentiation potential and increase self-renewal in mESC. Collectively, this work strongly supports the idea that m⁶A modifications gate chromatin accessibility and downstream transcription through modulation of carRNAs [21].

Key figure

Mechanisms of crosstalk between N⁶-methyladenosine (m⁶A) and epigenetic checkpoints

Crosstalk between m⁶A and epigenetics

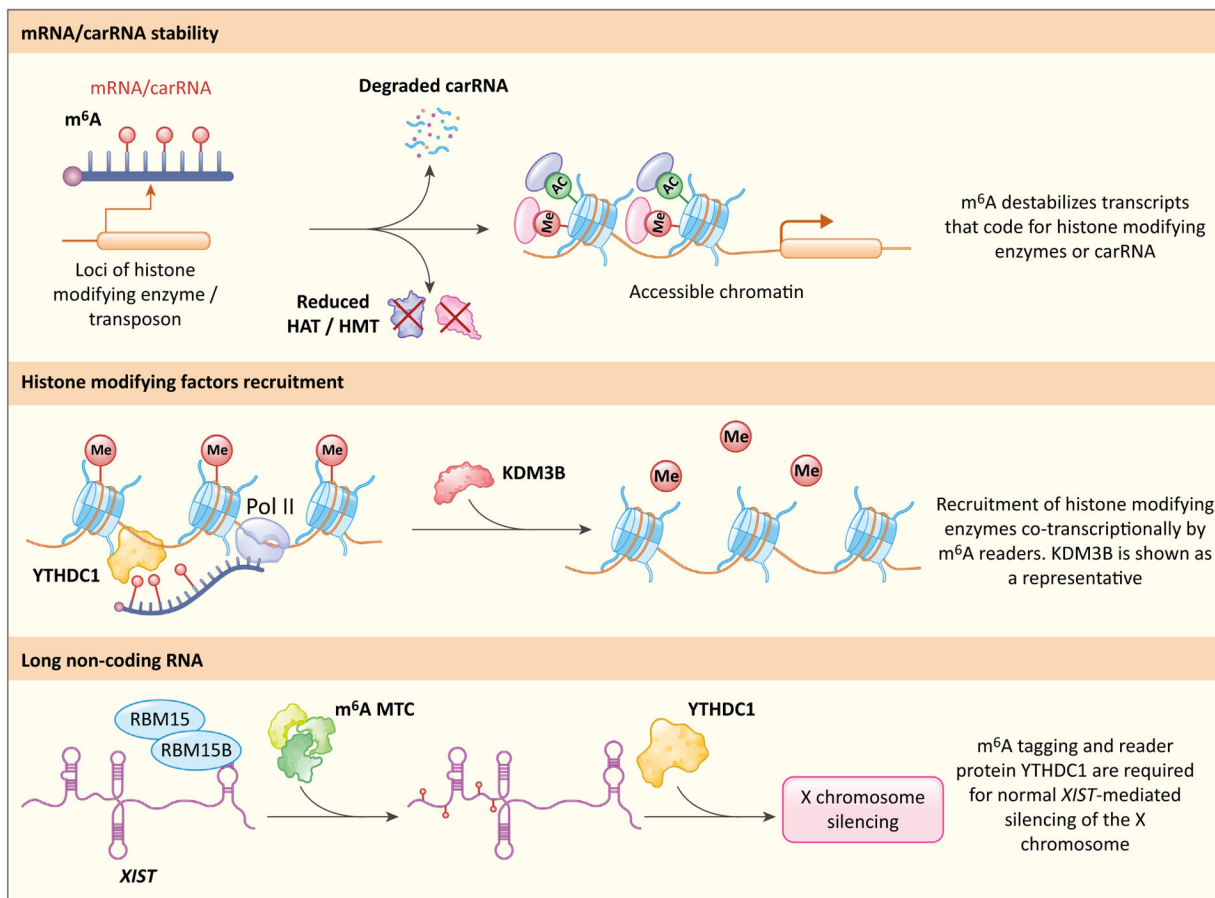


Figure 3.

Chromosome-associated regulatory RNAs (carRNAs) or mRNA that encode histone-modifying enzymes are methylated and tagged for degradation, causing a decrease in carRNA and histone acetyltransferases (HATs) or histone methyltransferases (HMTs), ultimately leading to accessible chromatin. KDM3B is shown as a representative enzyme that is recruited by m⁶A. Other histone-modifying factors, such as Setdb1 and Trim28, are recruited in the same fashion. RBM15/RBM15B-mediated m⁶A deposition and subsequent YTHDC1 recruitment are essential for X-chromosome silencing.

m⁶A and H3K27ac

One mechanism by which RNA modifications can feed back on critical epigenetic checkpoints is through deposition of m⁶A on transcripts that encode histone-modifying proteins (Figure 3). Wang *et al.* discovered that m⁶A regulates gene expression in neural stem cells (NSCs) by destabilizing transcripts that encode the critical histone acetyltransferases P300/CBP [54]. The loss of *Mettl14* was associated with developmental abnormalities, including reduced proliferation and premature differentiation. In homozygous *mettl14^{fl/fl}nes-cre* mice, levels of H3K27ac and H3K27me3 were significantly upregulated. Compared with controls, *mettl14^{fl/fl}nes-cre* mice showed increased expression of CBP and P300, with m⁶A-RNA immunoprecipitation showing minimal m⁶A enrichment of CBP and P300 transcripts. These results hint that the change in the activity of P300/CBP may be due to the destabilizing effects of m⁶A installation and that changes in these histone-modifying regulators underlie the alteration of chromatin architecture. Two key histone marks, H3K27ac and H3K4me3, were associated with gene activation, whereas H3K27me3 was associated with gene repression. In line with these results, unbiased transcriptome analysis did not favor global gene activation or repression in *Mettl14*-knockout NSCs, but Gene Ontology (GO) analysis indicated that the upregulated genes have a role in NSC differentiation, while the downregulated genes relate to proliferation. Treatment with the small-molecule Ezh2 inhibitor GSK343 partially rescued the proliferation defect in the loss-of-function cells. Although P300/CBP mediate acetylation of H3K27 at gene promoters and enhancers, it is unclear how a change in these factors specifically influences the methylation state of histone. Notably, the authors did not observe changes in the methylation state of PRC2 components, hinting that there must be distinct mechanisms by which m⁶A regulates histone modifications. Nonetheless, the aforementioned evidence suggests that a key mechanism by which m⁶A regulates the pluripotency of NSC is by altering the transcript stability of proteins that deposit histone chemical modifications [54].

m⁶A and H3K27me3

A recent study interrogated bacteria-induced inflammatory responses, finding that tight m⁶A control guards against unrestrained inflammation [55]. The authors found that loss of the m⁶A reader protein YTHDF2 led to enhanced stability of the histone demethylase KDM6B transcript, thereby increasing KDM6B translation and decreasing H3K27me3 levels at loci that encode proinflammatory cytokines, such as *IL6*, *IL12B*, and *ICAM1*. Enhanced hypomethylation of H3K27me3 in turn increases the transcription of inflammatory cytokines. In addition, the authors surmised that bidirectional crosstalk between m⁶A and H3K27me3 acts as a critical regulatory checkpoint that controls

inflammatory gene expression. In heat-killed *Salmonella typhimurium* (HKST)-treated THP-1 cells, m⁶A peaks were mostly enriched in regions in which H3K27me3 was sparse. Chemically inhibiting KDM6B by GSK-J4 also reduced global m⁶A levels. Furthermore, HA-KDM6B co-immunoprecipitated with FLAG-METTL3 and FLAG-METTL14, hinting that KDM6B acts as guide for m⁶A installing machinery. Indeed, GSK-J4-treated cells showed impaired binding of METTL14 at the *KDM6B* locus, forming a negative-feedback loop to regulate H3K27me3 levels by modulating the stability of KDM6B [55]. Thus, it appears that H3K27me3 deposition, which is tightly regulated by the demethylase KDM6B, protects against m⁶A installation. In summary, this study suggests that the demethylation of H3K27me3 is crucial for the co-transcriptional recruitment of the methyltransferase complex METTL3/METTL14.

m⁶A and H3K9me2

Similar to the mode of regulation imparted by H3K27me3 and KDM6B crosstalk, a recent study by Li *et al.* suggests that METTL3/METTL14 regulates the repressive histone mark H3K9me2 [56]. The authors utilized an elegant screening system using a tetracycline-inducible reporter in human Flp-In HEK293 cells to directly investigate the effects of m⁶A on histone modifications. The reporter system comprised two plasmids, one with the m⁶A consensus motif GGAC repeats and the other with GGTC repeats as a control. An array of histone modifications was measured and analyzed after tetracycline treatment, and H3K9me2 showed the most drastic decrease (approximately fourfold) compared with the control reporter. The authors also observed minor upregulation of H3K27ac and H3K27me3, which would conflict with results reported by Wang *et al.* [54], who observed upregulation of H3K27me3 in Mettl14-knockout NSCs. However, the experimental conditions and contexts used by the two studies were drastically different. Li *et al.* validated their main findings showing that a loss of METTL3 or METTL14 increased global levels of H3K9me2, and such upregulation was reversed by induced expression of wild-type METTL3, but not the catalytically inactive METTL3^{D395A}. m⁶A was shown to recruit KDM3B to demethylate H3K9me2, typically a repressive mark, through the reader protein YTHDC1. YTHDC1 coimmunoprecipitated with KDM3B, and a large fraction of endogenous YTHDC1 colocalized with KDM3B within the nucleus. *Ythdc1* knockdown also showed a similar decrease in KDM3B binding to the chromatin as METTL3^{D395A}. In addition, specific targeting of dPspCas13b-YTHDC1 increased local KDM3B abundance and a collateral decrease in H3K9me2 levels. KDM3B-occupied genes and m⁶A-modified genes showed large overlap, and distance analysis of KDM3B-ChIP showed enrichment at m⁶A RIP and METTL3 peak centers. Furthermore, such recruitment occurred cotranscriptionally, as supported by the enrichment of KDM3B at Pol II peaks, and addition of the Pol II inhibitor 5,6-dichlorobenzimidazole-1-β-D-ribofuranoside (DRB) significantly decreased the abundance of chromatin-bound KDM3B. Collectively, the body of work in this study provides direct mechanistic insights that link m⁶A deposition with functionally relevant histone modifications.

m⁶A and long noncoding RNA

Long noncoding RNAs (lncRNAs) have emerged as critical regulators of epigenetic mechanisms [57]. Expression of *Xist*, arguably the best-characterized lncRNA, ‘coats’

the X chromosome in females to silence gene expression [58]. Thus, *Xist* is critical for maintaining dose compensation between genders. A recent study by Patil *et al.* suggested that *Xist* is a target of m⁶A regulation and that chemical modifications on *Xist* are required for its function [59]. Mapping m⁶A sites at high resolution using m⁶A individual nucleotide-resolution crosslinking and immunoprecipitation (miCLIP) showed 78 putative m⁶A sites on *XIST* near the A repeat region. The authors further surmised that *Xist*-binding partners, RBM15 and RBM15B, are required for gene silencing by guiding the m⁶A methylation complex to the RNA. Co-immunoprecipitation assays showed a robust interaction between RBM15/RBM15B and METTL3 in a WTAP-dependent manner. Using a doxycycline-inducible *Xist* expression system, knockdown of either RBM15 or RBM15B did not achieve silencing of the *Xist* targets *Gpc4* and *Atrx*, whereas *Rbm15/Rbm15b* double knockdown resulted in a substantial increase in *Gpc4* and *Atrx* transcription. These results suggest that RBM15 and RBM15B have redundant functions. In addition, knockdown of *Rbm15/Rbm15b* significantly reduced the amount of m⁶A on *XIST*, a result recapitulated with knockdown of METTL3. Finally, the authors showed that the reader protein YTHDC1 is critical for m⁶A recognition on *Xist* and downstream transcription silencing. Altogether, these data support the model that RBM15/RBM15B recruit the METTL3-WTAP complex to *XIST* for targeted methylation. In addition, this work positions m⁶A as a dynamic regulator of physiological gene silencing and that the methylation state of ncRNA may be critical to their function.

m⁶A and H3K36me3

Accumulating evidence suggests that m⁶A feeds back on chromatin modifiers and histones, but the reverse is also true. H3K36me3, a transcription elongation marker that is enriched mainly in coding sequences and near the 3' end, has been shown to guide m⁶A deposition [60]. Consistent with previous results, H3K36me3, but not H3K9me3 or H3K27me3, is over-represented at m⁶A peaks under distance analysis. This distribution is also consistent with the observation that H3K9me3 and H3K27me3 are mainly found in heterochromatin, in which transcription activity is inherently low. In various normal and cancer cell lines, the mRNA expression of SETD2, the histone methyltransferase of H3K36, positively correlated with the expression of the m⁶A writers METTL3, METTL14, and WTAP. Knockdown of SETD2 or overexpression of the histone demethylase KDM4A drastically decreased global m⁶A levels, as well as representative genes, such as *MYC* and *ACTB*.

The authors used a catalytically inactive CRISPR/dCas9 system fusion protein targeted to ectopic loci on the genome to test the causal relationship between H3K36me3 and m⁶A. Targeting of dCas9-KDM4A to the coding region instability determinant (CRD) region of *MYC*, in which H3K36me3 is enriched, resulted in a substantial decrease in H3K36me3 and m⁶A levels; conversely, targeting of dCas9-SETD2 to the gene body of *GNG4*, where there is no H3K36me3, resulted in an increase in H3K36me3 and m⁶A. In addition, the authors found that METTL14 acts as a key player to recognize and bind H3K36me3 in the MTC, linking H3K36me3 and m⁶A deposition. HA-METTL14 showed significant chromatin binding in HepG2 cells, as shown by ChIP-seq and, not surprisingly, co-enrichment with H3K36me3 mostly at gene bodies. Photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) followed by sequencing also

confirmed that METTL14-binding sites overlapped with H3K36me3 and m⁶A. METTL3/METTL14/WTAP co-immunoprecipitated with H3K36me3 and the elongating form of Pol II (phosphorylated at Ser2 of the CTD). In mESCs, doxycycline-induced knockdown of *Setd2* recapitulated the loss of m⁶A, as seen in other cell types, as well as an increase in the mRNA and protein levels of pluripotency factors Oct4, Sox2, and Nanog, consistent with the role of m⁶A in destabilizing transcripts. Collectively, this supports the model that H3K36me3 guides the binding of the m⁶A MTC to elongating transcripts and methylates nascent RNA co-transcriptionally [60].

Concluding remarks

In recent years, we have witnessed an explosion in the number of studies dissecting the mechanisms of m⁶A and its physiological contributions. Accumulating evidence has substantiated the significance of m⁶A in controlling gene expression and disease progression. While not the initial focus of m⁶A studies, the interaction between RNA methylation and other epigenetic regulatory players has received well-deserved attention and has inspired several intriguing questions moving forward. It is of great interest to understand whether and how the defects in such interactions or crosstalk contribute to the development of various types of disease. For example, altered ERV regulation is associated with abnormal development, cancer, and neurodegeneration, pathological states highly linked to m⁶A. Thus, the recent link between MTC activity and repetitive element requires further probing as to the extent to which m⁶A-endowed epigenetic mechanisms contribute to normal development and pathological states. While most studies have focused on roles of m⁶A in protein-coding transcripts, it is clear that we are just scratching the surface as far as appreciating the role of m⁶A in noncoding gene regulation is concerned (see Outstanding questions). The poor signal-to-noise ratio associated with commonly used m⁶A-mapping techniques is a major barrier to progress on that front, particularly since noncoding transcripts tend to be less abundant, short lived, and expressed in a context-specific fashion. Newer techniques are rapidly being disseminated, which should improve signal at a base pair resolution and provide detailed stoichiometric analysis. Given that noncoding gene modulation is known to have a regulatory role in epigenetic control, further probing of this area will likely be fruitful. Currently available m⁶A-profiling methods often need a large amount of RNA material, which hampers the application of such methods to profiling m⁶A distributions in the transcriptome of primary patient samples, especially cancer stem cells and the primitive/progenitor cells of normal tissues. Development of novel technologies that only need a limited amount of RNA material and provide base-resolution m⁶A profiles with better quantitative information will substantially advance research in this field. In addition, the use of CRISPR genome editing and CRISPR-mediated RNA modification can help clarify the epistatic relationship between RNA methylation and chromatin dynamics. More recently, extensive evidence has shown that the introduction or removal of m⁶A at precise RNA sites is readily achievable using a guide RNA and dCas13-tethered modified methylase complex (e.g., METTL3:METTL14) with minimal off-target effects [21,61,62]. This opens exciting possibilities for RNA modification-based therapy and testing directly for crosstalk with other layers of gene regulation at precise transcripts. In addition, it is evident that more-refined definitions of epigenetic and epitranscriptomic landscapes are necessary

[63]. For example, single-cell technologies are slowly being leveraged in epitranscriptomic applications, which can unmask heterogeneity and enable deeper investigation of spatial differences in RNA methylation patterns. Finally, it is unclear how m⁶A can have seemingly opposing effects on epigenetic control under different contexts. It could make the chromatin more or less accessible, as well as increase or decrease transcription and translation. Despite an increase in the number of studies and depth, we clearly need continued investment to investigate the molecular underpinnings of m⁶A effects.

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Glossary

Erasers	proteins that remove m ⁶ A; include ALKBH5 and FTO, although FTO may preferentially target m ⁶ A _m
Nuclear speckles	subnuclear domains enriched in pre-mRNA splicing factors that are located in the interchromatin regions of the nucleoplasm of mammalian cells
Readers	proteins that bind m ⁶ A and impact the stability or localization of mRNAs
Writers	protein complexes that install m ⁶ A and that include the core protein METTL3 and the adaptor protein METTL14

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Highlights

Chemical modifications on histones, DNA, and RNA robustly impact gene regulation. Installation of the RNA modification m⁶A leads to altered mRNA stability and translation. Emerging data suggest that perturbations in m⁶A feedback on epigenetic checkpoints and vice versa.

RNA methylation of intracisternal A particle, a type of repetitive element, is required for proper heterochromatin formation and maintain genome integrity.

Loss of m⁶A in mouse embryonic stem cells enhances the stability of chromatin-associated RNAs, leading to more-open chromatin and active transcription.

m⁶A destabilizes transcripts that encode histone-modifying enzymes and complexes, including KDM6B, CBP, and P300.

The histone elongation mark H3K36me3 guides m⁶A deposition.

Xist-mediated silencing of the X chromosome requires m⁶A deposition and recognition by reader proteins.

m⁶A influence extends beyond mRNA stability, and investigation of functional effects of m⁶A on RNA biogenesis should be considered in physiological and mechanistic studies.

Outstanding questions

What is the role of RNA methylation of noncoding transcripts in gene regulation? Newer techniques will provide a better resolution of the m⁶A interactome of non-coding transcripts and enhance applications in multiple fields.

How does the deposition of m⁶A on RNA integrate with different layers of gene regulation at precise transcripts?

The recent evidence that m⁶A is required to restrain aberrant ERV activity provides evidence that effects of METTL3 on heterochromatin architecture may underlie its role in organismal development. However, what are the relative contributions of heterochromatin-dependent versus independent effects to mammalian physiology? What underlies the differences in heterochromatin formation in different cells and conditions? How does the MTC interface with transcriptional enablers at heterochromatin?

Are there other RNA modifications beyond m⁶A that feed back on epigenetic checkpoints?

Are the effects of m⁶A on epigenetic landscapes durable over a range of conditions and cell types? Can they override discordant effects on transcript stability or gene translation or are their biological effects more subtle?

Methylation residues on *Xist* tether other proteins that promote transcriptional repression. How does the binding of collaborative factors, such RBM15 and YTHDC1, lead to gene silencing? Are there other regulatory RNAs that exploit RNA modification sites as decoys or guides for binding of epigenetic factors?

Given the development of novel approaches that enable targeted *in vivo* control of RNA modification, can we exploit these technologies to alter the epigenetic landscape relevant in health and disease states?

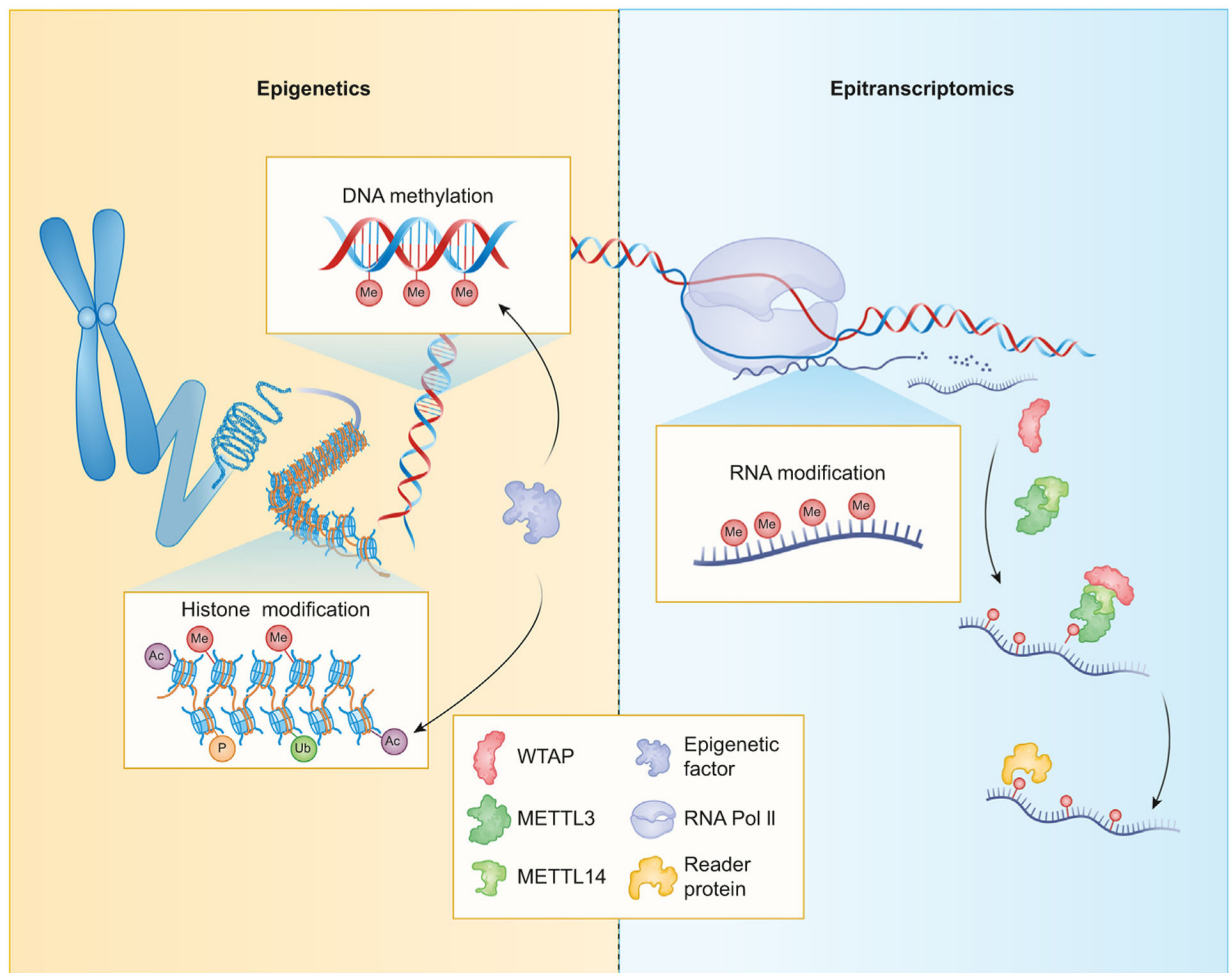


Figure 1. Schematic of epigenetic and epitranscriptomic mechanisms.

Modifications of DNA or histone proteins alter RNA polymerase II (RNA Pol II) transcriptional output. Chemical modifications on RNA, including N^6 -methyladenosine (m^6A), are deposited cotranscriptionally by writer proteins. WTAP guides METTL3/METTL14 to m^6A -binding motifs. The m^6A sites are recognized by nuclear or cytoplasmic reader proteins, which alter mRNA localization, degradation, or translation.

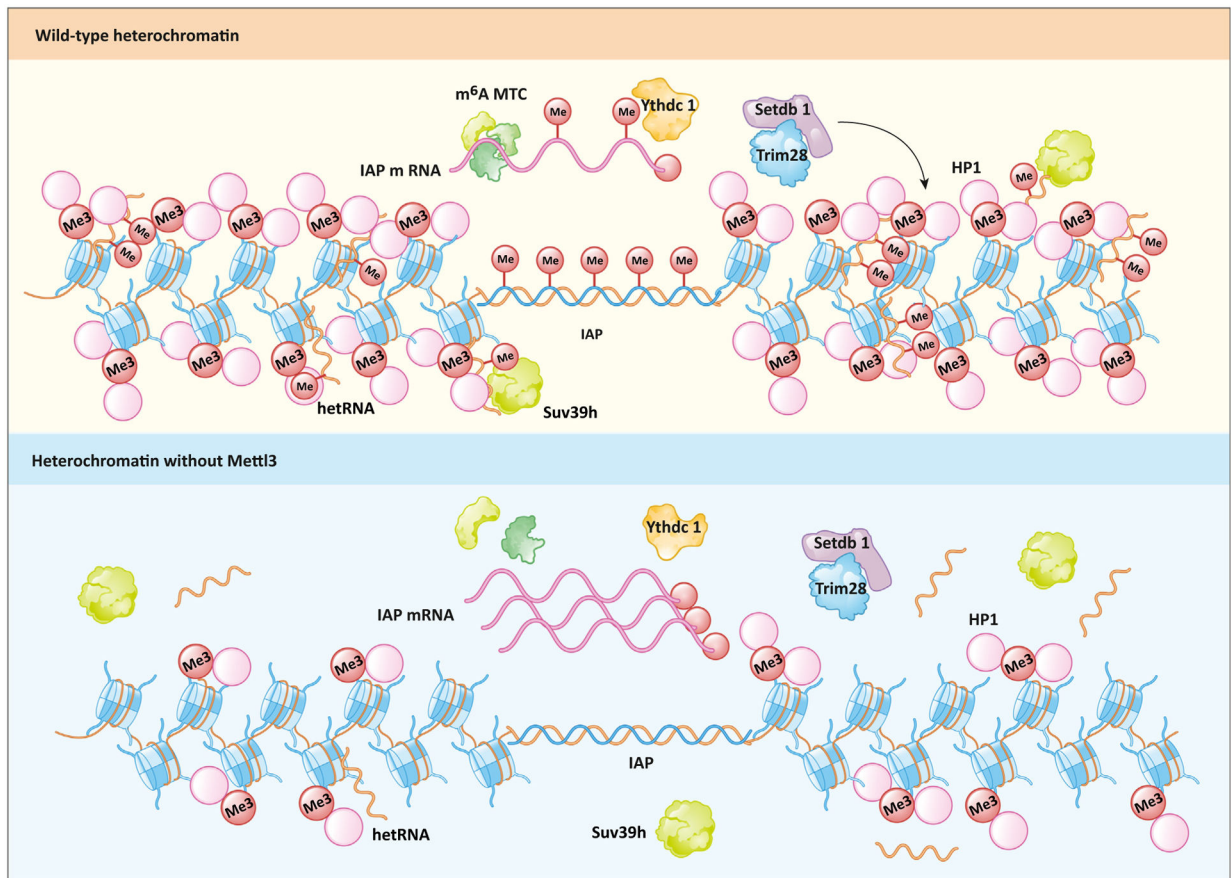


Figure 2. Role of METTL3 at heterochromatin.

The *N*⁶-methyladenosine (*m*⁶A) methyltransferase complex (MTC) that includes METTL3 methylates intracisternal A particle (IAP) mRNA and heterochromatic RNA (hetRNA). Binding of *m*⁶A by Ythdc1 is essential for the downstream deposition of H3K9me3 by Setdb1/Trim28. Methylated hetRNA associates with chromatin and facilitates the retention of HP1 and Suv39h proteins. H3K9me3, HP1, hetRNA, and Suv39h are lost upon Mettl3 depletion.

Table 1.

Histone modifications impacted by m⁶A

Modification	Model system(s)	Genetic manipulation	Effect on modification	Refs
H3K4me3	Mouse ES cell	Mettl3 KO	Increased	[21,48]
		Ythdc1 KO		[21,49]
	Mouse neural stem cell	Mettl14 KO		[54]
H3K27ac	Mouse ES cell	Mettl3 KO	Increased	[21]
		Ythdc1 KO		[21]
	HEK293	Increasing m ⁶ A		[56]
	Mouse neural stem cell	Mettl14 KO		[54]
H3K27me3	Mouse neural stem cell	Mettl14 KO	Increased	[54]
	HEK293	Increasing m ⁶ A		[56]
	THP-1	YTHDF2 KO	Decreased	[55]
H3K9me2	mESC and HEK293	METTL3 KO	Increased	[56]
		Mettl14 KO		[56]
		YTHDC1 KD		[56]
		FTO KO	Decreased	[56]
		ALKHB5 KO		[56]
H3K9me3	mESC	Mettl3KO	Decreased	[47,48]
		Alkbh5KO	Increased	[47]
		Mettl3 KO	Decreased	[47]