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# Biological roles of adenine methylation in RNA

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## Abstract

$N^6$-Methyladenosine ($m^6A$) is one of the most abundant modifications of the epitranscriptome and is found in cellular RNAs across all kingdoms of life. Advances in detection and mapping methods have improved our understanding of the effects of $m^6A$ on mRNA fate and ribosomal RNA function, and have uncovered novel functional roles in virtually every species of RNA. In this Review, we explore the latest studies revealing roles for $m^6A$-modified RNAs in chromatin architecture, transcriptional regulation and genome stability. We also summarize $m^6A$ functions in biological processes such as stem-cell renewal and differentiation, brain function, immunity and cancer progression.

The RNA building block adenosine can be methylated at several distinct nitrogen atoms of the nucleobase (N1, N2 or N6) or on the 2′ oxygen of the ribose (BOX 1). RNA $N^6$-methyladenosine ($m^6A$) was identified in mammalian mRNA in the 1970s[1,2], but more sensitive detection and mapping techniques (TABLE 1) have facilitated transcriptome-wide mapping of $m^6A$ methylation in mRNA and have enabled its detection in nearly all types of RNAs, including ribosomal RNAs (rRNAs), mRNAs, small nuclear RNAs (snRNAs) and several species of regulatory RNAs. The expansion of new techniques coupled with the characterization of enzymes that methylate, demethylate and bind to $m^6A$ (TABLE 2) has accelerated research in the past decade, establishing $m^6A$ as a major determinant of RNA fate. This epitranscriptomic modification affects most aspects of RNA metabolism, increasing the versatility of and information encoded in RNA, and has fundamental roles in development, organism homeostasis and disease.

Approximately 25% of mammalian mRNAs contain $m^6A$, with an average of one to three modifications per transcript[3,4]. However, $m^6A$-modified transcripts seem to be highly heterogeneous from cell to cell[5]. For mRNA and other RNA Polymerase II-synthesized transcripts (including most small RNAs and microRNAs (miRNAs)), the METTL3–METTL14–WTAP methyltransferase complex is mainly responsible for the deposition of

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m$^6$A in newly synthesized transcripts in the cell nucleus[6] (FIG. 1a and TABLE 2). m$^6$A was found to be enriched in conserved locations in the 3′ untranslated regions (UTRs) and near stop codons of transcripts that regulate differentiation and development, whereas it is relatively depleted in housekeeping transcripts[3,4]. Mapping has refined the conserved sequence of m$^6$A to the motif DRACH (where D = A, G or U; R = A or G; H = A, C or U)[3,4,7,8]. Importantly, m$^6$A in mRNA is dynamic and reversible, as the methyl group can be removed by the action of the demethylases ALKBH5 (REF.[9]) and FTO[10], restoring the methylated base back to the canonical adenine base (FIG. 1a). By contrast, m$^6$A modification of rRNAs is thought to be constitutive. Unique highly conserved sites in the 28S and 18S rRNA of eukaryotes[11,12] are $N^6$-adenosine methylated by ZCCHC4 (REFS.[13–16]) and METTL5 (REFS.[16–23]), respectively.

m$^6$A elicits a wide range of effects on RNA through changes in RNA stability, conformation and folding or by directly modulating the interactions of modified RNAs with binding proteins that affect RNA fate and function. Although m$^6$A does not prevent Watson–Crick A:U base pairing, it does have a subtle destabilization effect on RNA[24], which has been proposed to be important in regulating certain types of interactions, such as between miRNAs and mRNA[25,26]. m$^6$A also destabilizes Hoogsteen base pairing[27] and can inhibit folding of RNA in a sequence-dependent fashion[28]. The functional consequences of m$^6$A are not restricted to the modified RNAs; once methylated RNAs are degraded, methylated adenosines can also function as signalling molecules by acting as ligands for the adenosine A3 receptor[29]. However, the majority of the functional consequences of m$^6$A methylation in RNA fate and function are thought to be mediated by m$^6$A-binding proteins (TABLE 2) (reviewed elsewhere[30,31]). In humans, the best characterized m$^6$A-binding proteins belong to the YTH family[3,4,30,32], but other binding proteins include insulin-like growth factor 2 binding proteins (IGF2BPs)[33] and fragile X mental retardation protein (FMRP)[34]. By contrast, the presence of m$^6$A has been shown to prevent binding of certain proteins, including the G3BP stress granule proteins[34].

In this Review, we focus on the functions of RNA m$^6$A methylation at the molecular, genomic and organismal level. We describe the functional consequences of m$^6$A deposition on RNA substrates, including mRNA, rRNAs and regulatory RNAs. We review recent advances that clarify the mechanisms by which RNA methylation has an impact on chromatin architecture, epigenetic regulation of gene expression and genome stability. Finally, we summarize the key roles of m$^6$A in fundamental biological processes during early development and in adult tissue homeostasis, including stem-cell differentiation, neurogenesis and brain function, and innate immune response, as well as m$^6$A dysregulation in diseases, such as cancer.

## Molecular consequences of m$^6$A on RNA

At the molecular level, m$^6$A participates in nearly every aspect of RNA metabolism, including mRNA expression[3,4], splicing[4,26,35], nuclear export[9,36], translation efficiency[37–40], RNA stability[41,42] and miRNA processing[43,44]. Its effect on these processes seems to be largely regulated by binding proteins. However, the specific determinants that

dictate whether an m$^6$A-modified RNA is regulated at the level of splicing, export, stability or translation, or at multiple levels, are still unclear.

## m$^6$A mediates pre-mRNA splicing and nuclear export.

m$^6$A affects the alternative splicing of mRNAs either through its direct interaction with nuclear reader YTHDC1 or through an 'm$^6$A switch' mechanism involving indirect binding of heterogeneous nuclear ribonucleoproteins (HNRNPs), which are abundant nuclear proteins known to regulate pre-mRNA processing (FIG. 1b). Work in *Drosophila melanogaster*[45,46] and human cell lines[47] demonstrated that YTHDC1 binds directly to m$^6$A-modified mRNAs and modulates alternative splicing by recruiting the splicing factor SRSF3, which promotes exon inclusion, and by inhibiting binding of splicing factor SRSF10 to drive exon skipping (FIG. 1b). In an example of the m$^6$A-switch mechanism, m$^6$A affects the conformation of local RNA structure by exposing the HNRNPC binding motif, which in turn promotes exon retention[26] (FIG. 1b). Future work will need to decipher how specificity of splicing is determined for interactions with YTHDC1 or HNRNPs.

m$^6$A-Binding proteins also facilitate interactions with nuclear export components to regulate the subcellular localization of modified mRNAs. YTHDC1 binds to the export proteins SRSF3 and NXF1, and FMRP physically associates with the export protein CRM1 to accelerate the export of $N^6$-adenosine methylated transcripts from the nucleus[48–50] (FIG. 1c). Thus, m$^6$A seems to facilitate shuttling of mRNAs to the cytoplasm for translation by multiple mechanisms. It remains to be determined how nuclear export specificity is determined and whether these two pathways function independently or coordinate to facilitate nuclear export and subsequent processing in the cytoplasm.

## m$^6$A regulates mRNA degradation and stability.

Initial work in mouse embryonic stem (mES) cells demonstrated that m$^6$A-modified mRNAs are less stable than unmodified transcripts, resulting in their reduced abundance and translation potential[51]. Subsequent studies showed that m$^6$A-marked transcripts are actively targeted for degradation through direct binding to YTHDF2 (REFS.[52,53]), which recruits the CCR4–NOT deadenylase complex, a complex responsible for mRNA decay[52] (FIG. 1d). Alternatively, YTHDF2 can physically interact with HRSP12 to recruit RNaseP/MRP endoribonucleases to cleave m$^6$A transcripts[53]. However, the effects of m$^6$A on stability seem to be complex, as binding of IGF2BPs to methylated mRNAs stabilizes them by protecting them from degradation[33] (FIG. 1d). Furthermore, work in the parasite *Trypanosoma brucei* found that when methylation of the polyA tail of transcripts occurs in conjunction with variant surface glycoproteins, there is an increase in mRNA stability[42]. Taken together, these data suggest that additional factors determine whether m$^6$A-containing transcripts are degraded or stabilized; additional work is needed to determine what these factors might be.

## m$^6$A promotes or inhibits mRNA translation in a context-dependent manner.

m$^6$A promotes translation rates by multiple mechanisms depending on the location of m$^6$A within the mRNA transcript. If m$^6$A is located at the 5′ UTR, it can promote cap-independent translation in response to stress through binding of eukaryotic translation

initiation factor 3 (eIF3), which directs the recruitment of the 43S translation complex to initiate translation[37] (FIG. 1e). When m$^6$A is located in the 3′ UTR, it can promote cap-dependent translation through direct interactions with YTHDF1 or YTHDF3, resulting in enhanced translation[38,54] (FIG. 1e). YTHDF1 is postulated to affect translation initiation at the 5′ end of the transcript through a physical interaction with eIF3 that results in the looping of YTHDF1 to the transcriptional start site[38] (FIG. 1e). However, m$^6$A on mRNAs has also been shown to inhibit translation by affecting the interaction of transcripts with tRNAs, thereby slowing translation elongation[55] (FIG. 1e).

### m$^6$A on rRNA refines ribosome binding.

rRNAs are modified at more than 200 nucleotides[56] (Box 1). However, they are $N^6$-adenosine methylated at only two conserved positions, corresponding to adenosine 1832 of human 18S rRNA and adenosine 4220 of human 28S rRNA. Recent discovery of the 28S rRNA methyltransferase ZCCHC4 in mammals[13,14] and the 18S rRNA methyltransferase METTL5 in various Metazoans[16–21] has begun to shed light on the functional role of these methylations in regulating translation (FIG. 2a). Genetic knockout of *ZCCHC4* in mammalian cell lines resulted in a reduction of global translation and inhibition of cell proliferation of hepatocellular carcinoma cell lines[13]. Loss of 18S m$^6$A methylation by genetic knockout or knockdown of *Mettl5* resulted in a wide range of phenotypes, including loss of pluripotency and decreased differentiation potential in mES cells[18,21], increased stress resistance in *Caenorhabditis elegans*[17] and behavioural defects in *D. melanogaster*[20]. Together, these findings raise the possibility that m$^6$A in rRNAs can expand ribosome heterogeneity, which could induce the translation of specific sets of transcripts under different contexts or in response to diverse stimuli to regulate specific cellular processes.

### m$^6$A mediates microRNA processing.

In mRNAs, m$^6$A and miRNA binding sites are both enriched in the 3′ UTR and near stop codons, leading to the proposal that crosstalk might occur between them in miRNA-dependent control of gene expression[3]. Indeed, m$^6$A is enriched at miRNA binding sites in mouse embryonic fibroblasts and human cancer cell lines[57–59], and depletion of Dicer, a regulator of biogenesis of mature miRNA, results in reduced m$^6$A deposition in these cells. Whether m$^6$A on mRNAs can have an impact on the targeting of miRNAs is still unclear. However, m$^6$A directly affects miRNA processing and biogenesis of mature miRNAs. The mirNa microprocessor complex subunit DGCR8 is recruited to m$^6$A-modified primary (pri)-miRNAs, usually via the HNRNPA2B1 m$^6$A-binding protein, and pri-miRNAs are then processed to mature miRNAs; depletion of m$^6$A from pri-miRNAs disrupts this process and leads to lower levels of mature miRNAs[43,44] (FIG. 2b). These findings begin to illustrate how m$^6$A could affect the biogenesis of specific miRNAs, and thereby add another level of regulation to miRNA-mediated gene silencing.

### m$^6$A promotes circular RNA biogenesis and function.

Circular RNAs (circRNAs) are frequently produced by back-splicing of exons, whereby a 5′ splice donor site is fused to an upstream 3′ splice acceptor site[60]. The functions of circRNAs are just beginning to be characterized, but some circRNAs suppress specific miRNAs by functioning as miRNA ‘sponges’[61,62]. Growing in vitro and in vivo evidence suggests

that $N^6$-adenosine methylation of circRNAs regulates their biogenesis and translation potential[63,64]. A single m$^6$A is sufficient to initiate cap-independent translation of an artificial circRNA reporter construct[63] and can increase circRNA biogenesis and stimulate cap-independent translation from an open reading frame in circ-ZNF609 via binding of YTHDF3 and eIF4G2 (REF.[64]) (FIG. 2c). Although the functions of polypeptides derived from circRNA-encoded open reading frames are still unknown, the fact that circRNA biogenesis and translation are modulated by m$^6$A implies that these by-products of alternative splicing of exons have important regulatory functions.

### m$^6$A modulates spliceosome assembly and function.

The deposition of m$^6$A in snRNAs, such as the spliceosome component U6 snRNA, is mediated by the METTL16 methyltransferase[65,66]. METTL16 specifically modifies adenosine 43 (A43) in U6 snRNA[65,66], but it is unclear whether this modification is constitutive or reversible (FIG. 2d). Interestingly, A43 of U6 snRNAs interacts with the 5′ splice site of pre-mRNAs and it has been proposed that A43 methylation might fine-tune snRNA–pre-mRNA interactions, thereby regulating spliceosome assembly at, or recognition of, the 5′ splice site[66]. These findings suggest that m$^6$A can have a dual role in pre-mRNA splicing: in addition to regulating alternative splicing in subsets of m$^6$A-modified transcripts as discussed above, it can also directly modulate spliceosome function.

## m$^6$A, chromatin and genome integrity

In the past decade, it has become increasingly clear that $N^6$-adenosine methylation is not only instrumental in regulating RNA fate and metabolism but also has broader roles in epigenetic regulation of gene expression, in shaping genomic architecture and maintaining genomic stability. These effects of m$^6$A on genome stability and function were found to be mediated by various types of RNAs, including nascent pre-mRNA transcripts, long non-coding RNAs (lncRNAs), chromosome-associated regulatory RNAs (carRNAs), endogenous retrovirus RNAs (ERVs) and R-loops.

### m$^6$A in pre-mRNA mediates crosstalk with chromatin modifications.

Several lines of evidence indicate extensive crosstalk between m$^6$A in pre-mRNAs and chromatin modifications, providing an additional layer of regulation in the control of gene expression and genome stability. Mapping experiments show that m$^6$A on mRNAs overlaps significantly with high levels of histone H3 trimethylated at lysine 36 (H3K36me3) at the corresponding genomic location, and deletion of the H3K36 trimethyltransferase SETD2 results in global reduction of m$^6$A levels and reduced levels of METTL3 complexes binding to target mRNAs[67]. Interestingly, METTL14 binds to H3K36me3-modified histones and recruits METTL3 to sites that are actively transcribed by RNA polymerase II so that m$^6$A is deposited co-transcriptionally[67]. Reciprocally, co-transcriptional $N^6$-adenosine methylation of nascent mRNA transcripts facilitates the removal of H3 dimethylated at lysine 9 (H3K9me2), a traditionally repressive histone modification[68], via recruitment of the H3K9me2 demethylase KDM3B, which physically interacts directly with the m$^6$A-binding protein YTHDC1 (REF.[68]) (FIG. 3a). The $N^6$-adenosine methyltransferase complex also directly regulates transcription by inducing the release of RNA polymerase II from a paused

state[69], and the presence of m$^6$A inhibits binding of the transcriptional termination integrator complex[70]. This coordinated communication between m$^6$A and the chromatin modifying machinery further highlights how epitranscriptomic and epigenetic modifications form a complex regulatory network with multiple modules and switches that increase the flexibility of gene expression control.

m$^6$A can also indirectly affect chromatin modifications, and presumably other methylated substrates, by regulating levels of the predominant methyl donor, *S*-adenosyl methionine (SAM). METTL16, whose homologue is essential for mouse embryonic development[71], mediates m$^6$A methylation of a hairpin in the 3′ UTR of *MAT2A* pre-mRNA (encoding the SAM synthetase protein). When unmodified, this hairpin inhibits splicing of *MAT2A* transcripts, causing intron retention and RNA decay, thereby limiting SAM levels[65]. However, when the levels of SAM are too low, METTL16 is recruited to and methylates the 3′ UTR hairpin, which facilitates efficient splicing of *MAT2A* pre-mRNA and stimulates SAM synthesis[65]. By contrast, in *C. elegans*, METT-10 (the worm ort-hologue of METTL16) inhibits endogenous SAM bio-synthesis via a negative feedback loop in response to changes in diet that increase intracellular SAM levels[72]; METT-10-mediated *N*$^6$-adenosine methylation of *sams3* transcripts (which encode SAM synthetase) at their 3′ splice sites inhibits their splicing, leading to lower levels of SAM[72]. Maintaining appropriate SAM levels via METT-10/METTL16 seems to be a conserved process, albeit through inhibition or promotion of SAM synthesis depending on the organism. This work exemplifies some of the crosstalk that mRNA methylation can have with other epigenetic cues including histone methylation.

### m$^6$A in lncRNAs facilitates oncogenesis and X chromosome gene silencing.

Initial mapping in mouse tissue and human cell line studies revealed that m$^6$A is prevalent in lncRNAs[3,4], and subsequent studies have revealed important biological consequences of m$^6$A for the functions of the lncRNAs *Xist* (which has a pivotal role in X chromosome inactivation)[73] and *Malat1* (an abundant nuclear lncRNA that is often upregulated in metastatic cancers).

Directed by the lncRNA *Rmb15*, METTL3 methylates 78 sites in lncRNA *Xist*[74], which causes YTHDC1 to bind to *Xist*, an interaction that is required for X chromosome inactivation (FIG. 3b). In an elegant experiment, the artificial tethering of YTHDC1 to *Xist* bypasses the requirement for m$^6$A for transcriptional repression of the X chromosome, indicating that the primary function of m$^6$A is to recruit YTHDC1 to *Xist*[74] (FIG. 3b). However, the extent of the involvement of m$^6$A in X chromosome inactivation and how YTHDC1 influences *Xist*-mediated gene silencing remain unclear, although it has been proposed that YTHDC1 facilitates the interaction of *Xist* with gene silencing proteins[74,75].

*Malat1* is known to localize to nuclear speckles, associate with splicing factors and affect the expression of oncogenes through interactions with transcription factors and chromatin modifying enzymes[76]. It has recently been shown that m$^6$A modifications in *Malat1* create a scaffold for the recruitment of YTHDC1 in nuclear speckles. Both the m$^6$A modifications in *Malat1* and the YTHDC1 localization to nuclear speckles seem to be necessary for the oncogenic effects of *Malat1* (REF.[77]). *N*$^6$-Adenosine methylation of *Malat1* has also

been proposed to destabilize an RNA hairpin, thereby exposing an RNA-binding motif and promoting interaction with HNRNPC[26] (FIG. 3c); although the functional consequences of this interaction remain unclear, binding presumably facilitates the localization of HNRNPC to nuclear speckles.

As lncRNAs have been shown to play roles in various processes, ranging from regulating the immune system, to development, to regulating genome stability and ageing[78], the prevalence of m$^6$A on lncRNAs could, in turn, affect each of these processes by altering binding of specific lncRNAs to proteins, RNAs or DNA or through modulation of local RNA structure.

## m$^6$A in carRNAs and ERVs suppresses expression of repetitive elements.

m$^6$A on regulatory RNA species could facilitate communication between RNAs and chromatin to help reinforce the functional consequences of m$^6$A. Deletion of the m$^6$A methyltransferase *Mettl3* or binding protein *Ythdc1* in mES cells resulted in reduced m$^6$A levels on several classes of carRNAs, such as promoter-associated RNAs, enhancer RNAs and repeat RNAs, and in broad increases in chromatin accessibility and transcriptional activation[79,80]. m$^6$A was shown to facilitate the degradation of certain carRNAs, such as long interspersed element 1 (LINE-1; also known as L1) repeat RNAs, by recruitment of the nuclear exosome targeting complex (NEXT)[79] (FIG. 3d). As L1 repeat RNAs promote chromatin accessibility during transcriptional activation, the m$^6$A-mediated decay of specific carRNAs might regulate the chromatin state during mES cell homeostasis.

Suppression of expression from large swaths of repetitive DNA is essential for cell viability and the regulated expression of appropriate transcripts. Repetitive elements and retrotransposons can cause genomic instability by inducing recombination-mediated chromosomal rearrangements, such as deletions, insertions and trans-locations, that affect the expression of nearby genes or by causing mutations through transposition events in the genome[81]. In a genome-wide CRISPR–Cas9 screen in mES cells, several components of the *Mettl3* complex were shown to be required for the repression of the intracisternal A-particle (IAP)-type family of ERVs[82]. The suppression of these IAPs is mediated by $N^6$-adenosine methylation of the 5′ UTRs of *IAP* mRNAs, which promotes their decay through an YTHDF2-dependent mechanism[82] (FIG. 3e). METTL3 also facilitates heterochromatin formation and subsequent transcriptional repression by binding and recruiting the H3K9me3 methyltransferase SETDB1 to IAP-encoding genomic regions[83] (FIG. 3e).

Taken together, these studies reveal that $N^6$-adenosine methylation can facilitate chromatin reorganization to regulate the appropriate suppression of retrotransposons to safeguard genome stability.

## m$^6$A promotes genome stability at R-loops.

R-loops are frequently formed during transcription and have roles in transcription initiation and elongation, replication and DNA repair[84]. Interestingly, the RNA strand of R-loops is often $N^6$-adenosine methylated by METTL3 in human pluripotent stem cells[85]. Depletion of the m$^6$A-binding protein YTHDF2 results in accumulation of m$^6$A-methylated R-loops, leading to cell growth retardation and increased accumulation of histone γH2AX, a marker of DNA double-strand breaks[85]. These results suggest that m$^6$A in R-loops mediates R-loop

removal, thereby safeguarding genome stability, but the underlying mechanism of YTHDF2-mediated R-loop degradation remains unclear (FIG. 3f). There may be a dual or context-dependent role for m$^6$A in R-loop formation and maintenance, as an earlier study performed in HeLa cells suggested that m$^6$A promotes R-loop formation to facilitate transcription termination[86]. Interestingly, DNA damage signals induce the ataxia-telangiectasia mutated (ATM) protein to phosphorylate METTL3, which is then recruited to sites of double-strand breaks in R-loops where it methylates the RNA strand at $N^6$-adenosines[87]. Subsequent recruitment of YTHDC1 causes an increased accumulation of RNA:DNA hybrids, which results in the recruitment of RAD51 and BRCA1 to drive homologous recombination-mediated repair[87]. Therefore, the $N^6$-adenosine methylation of RNA in R-loops seems to induce a multipronged, context-specific response that can regulate numerous biological consequences of R-loop formation.

## m$^6$A in development and differentiation

Given the emerging roles of m$^6$A as a versatile modification that regulates RNA metabolism, protein synthesis, gene expression and genome stability, it is unsurprising that m$^6$A regulates diverse biological processes during development, differentiation and adult tissue homeostasis. The importance of m$^6$A in early developmental decisions is underscored by the fact that METTL3 and METTL14 are essential for mouse and plant embryonic development[88–90]. In this section, we explore the roles of m$^6$A in stem-cell renewal and differentiation, and in developmental transitions.

### m$^6$A facilitates ES cell self-renewal and differentiation.

The role of m$^6$A in regulating the stem-cell state is highlighted by the observation that genetic knockdown of components of the METTL3–METTL14 complex in mES cells causes a loss of self-renewal capacity owing to the increased stability of $N^6$-adenosine methylated pluripotency factors such as Nanog, SOX2 and KLF4 (REFS.[51,88,91]). Furthermore, genetic knockout of *METTL3* or *METTL14* in mES cells and human ES cells disrupts their exit from the self-renewal pathway and progression towards differentiation[91], resulting in a failure to undergo lineage priming and transition to the post-implantation primed stage leading to embryonic lethality[88,89]. METTL3 complex localization is regulated by two opposing zinc-finger proteins. ZFP217, a transcriptional activator of core pluripotency genes, binds to and sequesters METTL3, thereby limiting m$^6$A deposition on transcripts required for pluripotency, resulting in impaired stem-cell renewal[92]. Conversely, ZC3H13 anchors the m$^6$A methyltransferase complex in the nucleus of mES cells, facilitating m$^6$A deposition and promoting stem-cell renewal[93] (FIG. 4a). Importantly, the deposition of m$^6$A on transcripts encoding pluripotency factors is responsive to extracellular signalling cues. Activation of TGFβ–activin signalling in human pluripotent stem cells leads to the physical association of transcription factors SMAD2/3 with the METTL3–METTL14–WTAP methyltransferase complex, promoting m$^6$A deposition on a subset of mRNAs encoding pluripotency factors, including Nanog[94]. Conversely, inhibition of TGFβ–activin signalling destabilizes these mRNAs and causes their rapid degradation, allowing a timely exit from pluripotency and the induction of neuroectoderm differentiation[94]. Precisely how the changes in $N^6$-adenosine methylation are interpreted is still unclear. The

YTHDF2 binding protein is a good candidate for interpreting changes in m$^6$A in stem cells as it has recently been implicated in destabilizing neural-specific RNA transcripts during induced pluripotent stem cell differentiation[95]. However, owing to the functional redundancy of YTHDF proteins, it is plausible that other members of the family might also contribute to the degradation of core pluripotency factor transcripts[96]. Taken together, these findings underscore the importance of m$^6$A in regulating ES cell renewal, differentiation and pluripotency through marking key transcripts that maintain cell state or facilitate differentiation.

### m$^6$A facilitates developmental transitions.

During oocyte maturation and early embryogenesis, development is driven by the inherited pool of maternal mRNA transcripts. The activation of zygotic transcription occurs during the tightly regulated maternal-to-zygotic transition (MZT)[97]. In zebrafish, *N*$^6$-adenosine methylation of mRNAs plays a critical role in the clearance of maternal mRNAs, as approximately one third of maternal transcripts are m$^6$A-modified and elimination of YTHDF2 by genetic knockout limits the decay of the maternal mRNAs and delays development[98]. Interestingly, YTHDF2 may not function on its own to recognize m$^6$A-modified maternal transcripts, as all three YTHDF binding proteins act redundantly to control MZT and oogenesis[99] (FIG. 4b). Similarly, m$^6$A is important for mouse oocyte maturation, which is also characterized by a wave of degradation of maternal mRNAs when oocytes proceed through meiosis (M-decay) and another wave in the early zygote during MZT (Z-decay). The effects of METTL3-dependent m$^6$A deposition during mouse oocyte differentiation are mediated by the coordinated activity of YTHDF2, YTHDC1 and YTHDC2 (REFS.[100–104]). Oocyte-specific inactivation of YTHDF2 causes infertility owing to impaired degradation of a subset of maternal transcripts at or prior to the two-cell stage[103]. Moreover, knockdown of *Mettl3* in developing oocytes impairs maturation and MZT due to decreased mRNA translation and inefficient maternal mRNA clearance[105]. m$^6$A coordinates with other regulatory pathways to rapidly clear maternal mRNAs during MZT. In zebrafish, mir-430 is induced during MZT and facilitates the deadenylation and clearance of hundreds of maternal transcripts through binding to their 3′ UTRs[106]. The co-occurrence of m$^6$A modifications and the miR-430 seed in the 3′ UTR seems to accelerate the destabilization of maternal mRNAs[99] (FIG. 4b). Interestingly, other RNA modifications have also been implicated in maternal mRNA clearance during MZT in zebrafish. In contrast to m$^6$A, binding of YBX1 to a pool of C5-cytosine methylation (m$^5$C)-modified maternal mRNA was shown to increase their stability[86] (FIG. 4b). Moreover, addition of a uracil tail to maternal mRNAs by the terminal uridylyltransferases TUT4 and TUT7 facilitates their clearance during MZT[107]. It will be important in future studies to decipher how m$^6$A on maternal mRNAs communicates with miRNA-mediated regulation and to examine whether an epitranscriptomic code exists — a mechanistic crosstalk between m$^6$A and the other RNA modifications — which facilitates the read-out of multiple mRNA modifications to elicit a stronger biological consequence.

*N*$^6$-Adenosine methylation also seems to be critical for the endothelial-to-haematopoietic transition during zebrafish embryogenesis, a process governed by numerous interconnected signalling pathways, including NOTCH1 signalling, which drives haematopoietic stem and

progenitor cell specification[108]. Genetic knockout of *mettl3* in zebrafish embryos blocks the production of haematopoietic stem/progenitor cells during endothelial to haematopoietic transition[109,110], and these effects were shown to be mediated by YTHDF2-directed degradation of *Notch1a* mRNA, resulting in reduced NOTCH1 signalling. This m$^6$A-mediated regulation of endothelial-to-haematopoietic transition through NOTCH signalling is conserved in mice[109].

### m$^6$A regulates adult stem-cell differentiation.

$N^6$-Adenosine methylation of mRNA is also important for the homeostasis and differentiation of adult stem-cell lineages, including haematopoietic stem cells (HSCs) and neuronal stem cells (NSCs), which is underscored by the emerging roles of m$^6$A in cancer stem-cell biology especially in acute myeloid leukaemia (AML) and glioblastoma (see below). Deletion of *Mettl3* in adult mouse HSCs resulted in their impaired differentiation in vivo[111]. The role of m$^6$A in regulating HSCs seems to be mediated by the $N^6$-adenosine methylation of *Myc* mRNAs, which enhances their translation, with the resulting increase in MYC protein driving HSC differentiation[111] (FIG. 4c). Interestingly, the results observed for a *Mettl3*-knockout mouse contradict earlier studies in human HSC cells, which reported that knockdown of *METTL3* and *METTL14* impairs human progenitor haematopoietic stem and progenitor cell self-renewal and leads to myeloid differentiation by limiting expression of the MYB and MYC transcription factor[112,113]. These discrepancies might arise from differential effects of m$^6$A methylation in progenitor cells versus adult HSC cells or in differences between knocking out and knocking down the mRNA m$^6$A regulatory machinery. It will be important for future studies to resolve the role of m$^6$A in adult HSC differentiation and to identify which binding proteins mediate the effects of m$^6$A on MYC and other developmental regulators.

m$^6$A also affects NSC renewal and differentiation in the cortex of the mouse brain. m$^6$A depletion by *Mettl14* knockout or *Mettl3* knockdown prolongs the cell cycle and causes delayed differentiation of NSCs owing to increased stability of transcripts encoding neural developmental regulators that are normally kept at low levels[114]. Mouse NSCs lacking *Mettl14* show decreased proliferation and premature differentiation, suggesting a defect in their self-renewal potential[115]. Interestingly, this study demonstrated that m$^6$A destabilizes the mRNAs of histone modifying enzymes, suggesting a model by which m$^6$A influences NSC self-renewal and differentiation through regulation of chromatin structure[115]. The importance of m$^6$A in neural lineage differentiation is further highlighted by the revelation that *Ythdf2*-knockout mice exhibit delayed cortical neurogenesis owing to impaired degradation of m$^6$A-modified mRNAs associated with neuronal development and differentiation[116]. Moreover, knockout of the gene encoding the FTO demethylase causes defects in neurogenesis, which lead to impaired learning and memory owing to dysregulated gene expression in the hippocampus[117]. Taken together, these data suggest that m$^6$A can regulate NSC renewal and differentiation by multiple mechanisms that involve the destabilization of neural development regulators and alterations in chromatin structure.

## Organism-level effects of m$^6$A

m$^6$A is dynamically regulated in response to numerous physiological stimuli, and has been implicated in the control of numerous homeostatic mechanisms in the cell, including the UV-induced and acute restraint stress responses, circadian rhythm regulation, neurobehaviour and the innate immune system responses. Given the wide-ranging role of m$^6$A in maintaining homeostasis, when m$^6$A regulation goes awry, disease such as cancer develops[118,119]. In the following section, we focus on three emerging roles of m$^6$A: neuronal synaptic function and neurobehaviour; T cell homeostasis and immune responses; and cancer stem-cell establishment.

### m$^6$A in neural synaptic function and behaviour.

In addition to the roles of m$^6$A in regulation of neurogenesis, and consistent with the elevated levels of m$^6$A in mRNAs in adult brains[3], several lines of evidence suggest a role for m$^6$A in synapse activity and plasticity during learning and memory. Learning and the formation and maintenance of memory depend on activity-dependent changes in the strength of synapses in the hippocampus, which is achieved through changes in local protein synthesis[120]. Several regulatory mechanisms involving polyA tail length and the activity of miRNAs and RNA-binding proteins have been implicated, but recent evidence suggests that m$^6$A on mRNA can impact activity-induced local translation at the synapse. Low-input sequencing-based analysis of m$^6$A in synaptosomal RNA in the forebrain revealed that ~3,000 synaptic $N^6$-adenosine methylated transcripts are enriched for functions in synapse synthesis and modulation of synaptic transmission[121]. Interestingly, the $N^6$-adenosine methyltransferase and demethylase and m$^6$A-binding proteins are located in dendrites and adjacent to synapses, raising the possibility that subcellular modification or recruitment of modified mRNAs could have a role in synaptic activity[121] (FIG. 5a). Excitingly, knockdown of the genes encoding the m$^6$A-binding proteins YTHDF1 or YTHDF3 in cultured hippocampal pyramidal neurons reduces translation of the dendritically localized *Arc* mRNAs, which encode a synaptic protein that promotes internalization of specific glutamate receptors important for fast excitatory neurotransmission; the knockout cells also showed synaptic dysfunction and impaired synaptic transmission[121] (FIG. 5a). Conversely, axon-specific knockdown or chemical inhibition of FTO promotes local translation of mRNAs in axons of cultured neurons[122].

Numerous recent studies report that dynamic regulation of m$^6$A in response to experience or acute stress is required for regulation of complex behaviours such as fear conditioning[123–125]. Fear conditioning results in a rapid decrease in expression of FTO demethylase in the mouse medial prefrontal cortex and near synapses in the hippocampal CA1 neurons, with a concomitant increase in m$^6$A levels[123,124]. Targeted depletion of FTO or METTL3 in the medial prefrontal cortex or hippocampus results in enhanced fear memory consolidation following behavioural training[123–125]. Given the opposing activities of FTO and METTL3 with respect to m$^6$A deposition, and the fact that FTO can also demethylate the cap-adjacent dimethyladenosine (m$^6$Am) modification[126] (Box 1), it is possible that some of the FTO-dependent behavioural effects are mediated by m$^6$Am. These results raise the possibility that, following experience, changes in m$^6$A deposition near

synapses could result in enhanced local translation of synaptic transcripts that facilitate memory consolidation. In support of this hypothesis, conditional inactivation of *Mettl3* specifically in the hippocampus results in long-term memory defects owing to impaired translation of early response synaptic transcripts, whereas *Mettl3* overexpression enhances learning efficacy and long-term memory formation[127]. Similarly, *Ythdf1*-knockout mice display defects in translation of m$^6$A-methylated transcripts following neuronal stimulation which correlate with defects in hippocampal synaptic transmission and learning and memory[128]. Taken together, these studies support a model whereby m$^6$A facilitates local translation of synaptic transcripts to facilitate synaptic signalling and confer memory consolidation.

### m$^6$A in T cell homeostasis and innate immune response.

m$^6$A on mRNA has been shown to have critical roles in the immune response, including modifying T cell proliferation and differentiation, regulating the turnover of essential host transcripts and methylating viral transcripts to facilitate evasion of the host immune system. Deletion of *Mettl3* or *Mettl14* in naive T cells blocks proliferation and differentiation to effector T cells[129]. Loss of $N^6$-adenosine methylation on transcripts encoding inhibitors of STAT5 increased their stability, causing inhibition of STAT5 and subsequent disruption of T cell homeostasis[129]. Additionally, the mRNA encoding interferon-β (IFNβ) (the main cytokine in the type I interferon response) is $N^6$-adenosine methylated, enabling its fast turnover following activation of the immune response[130] (FIG. 5b). CRISPR-induced depletion of m$^6$A (through deletion of *METTL3*) or reducing the ability of cell to detect and respond to m$^6$A (through depletion of the m$^6$A-binding protein *YTHDF2*) in both human and mouse cells results in interferon stabilization and activation of interferon-stimulated genes, thereby facilitating protection against viral proliferation[130].

Host immune systems have used m$^6$A methylation to effectively fight viral infections. In response to infection with vesicular stomatitis virus (VSV), macrophages inhibit the enzymatic activity of ALKBH5 (an m$^6$A demethylase) resulting in a concomitant increase in m$^6$A levels on RNA[131]. Mechanistically, it was shown that increased m$^6$A methylation on mRNA encoding 2-oxyglutarate dehydrogenase (OGDH) reduces its stability and OGDH protein expression, which leads to reduced levels of a metabolite (itaconate) required for viral replication[131] (FIG. 5b). Therefore, m$^6$A-mediated degradation of OGDH transcripts in macrophages prevents viral proliferation.

Unsurprisingly, viruses have also evolved mechanisms that take advantage of m$^6$A mRNA methylation to evade the host immune system. The RIG-I and MDA5 RNA receptor sensing pathways, which are normally activated upon recognition of unmethylated viral RNA, trigger the expression of type I interferons[132]. However, human metapneumovirus (HMPV), a negative-sense single-strand RNA virus, possesses m$^6$A on its viral RNAs, which promotes viral replication and facilitates evasion of the innate immune response in human cells. Interestingly, depletion of m$^6$A from HMPV RNA enhances binding and activation of RIG-I and the interferon response pathway[133]. In a separate example, VSV infection causes the translocation of METTL3 to the cytoplasm and m$^6$A deposition on viral RNAs, which facilitates its evasion of the RIG-I and MDA5 sensing pathways. Depletion

of m$^6$A from VSV viral RNAs enhances the formation of double-stranded RNA domains that are recognized by RIG-I and MDA5 to trigger the activation of the interferon response pathway[134]. Double-strand DNA viruses and certain single-strand RNA viruses can also generate viral circRNAs, which similarly to viral RNAs trigger the RIG-I innate immune response pathway when unmethylated[135]. Taken together, these findings suggest that diverse viruses use m$^6$A to abrogate the host cell immune response.

### m$^6$A in cancer stem cells in acute myeloid lymphoma and glioblastoma.

m$^6$A is central to the regulation of RNA metabolism and fate in numerous developmental and homeostatic cellular processes, and thus disruption of the genetic networks that establish, modulate and bind to m$^6$A ultimately leads to pathologies such as cancer, psychiatric disorders and metabolic disorders[118,119]. m$^6$A dysregulation has been implicated in various human cancers including breast cancer, hepatic cancers, brain cancer, cervical cancer, lung cancer and leukaemias (for a review of m$^6$A role in cancer, see REF.[118]). Here, we briefly highlight some of the roles of m$^6$A in cancer stem cells, using AML and glioblastoma as examples.

Given the importance of $N^6$-adenosine methylation for the homeostasis of the HSC and NSC lineages, it is unsurprising that m$^6$A plays a critical role in AML, an aggressive clonal disorder of HSCs, and in the tumorigenesis of glioblastoma stem-like cells (GSCs). METTL3 and METTL14 promote the m$^6$A-dependent translation of MYC and MYB transcription factors, which are both activated and causal for the development of AML, indicating that m$^6$A can enable the maintenance and self-renewal of leukaemia stem/initiation cells and the survival of AML cell lines[112,113]. However, *(R)*-2-hydroxyglutarate (R-2HG), which inhibits FTO activity and increases m$^6$A levels, inhibits the proliferation of leukaemia cells by inducing the degradation of m$^6$A-modified transcripts, including those encoding MYC[136]. YTHDF2 has been implicated in mediating some of the effects of m$^6$A in the progression of AML; inactivation of YTHDF2 increases the half-life of m$^6$A-modified transcripts and, specifically, compromises cancer stem-cell initiation and propagation[137]. YTHDC1 has also been shown in ex vitro studies to be required for AML tumorigenesis by sequestering $N^6$-adenosine methylated transcripts to specific regions of the cell to prevent their degradation[138].

Similarly, knockdown or overexpression of the genes encoding METTL3/METTL14 or FTO, respectively, alters the self-renewal and tumorigenesis of GSCs[139]. In addition, the ALKBH5 demethylase is required for the proliferation and tumorigenesis of GSCs, as inactivation of ALKBH5 inhibits the proliferation of GSCs. ALKBH5 demethylates transcripts encoding the FOXM1 transcription factor, leading to increased FOXM1 expression and stimulation of cell proliferation[140]. Taken together, these findings underscore the critical roles of m$^6$A in tumorigenesis of cancer stem cells and provide a rationale for targeting components of the m$^6$A machinery for the development of cancer therapeutics.

## Conclusions and future perspectives

In this Review, we have delineated the chemical consequences of adenine methylations on RNA, described the functional effects of methylation of various different substrates and

provided illustrative examples of how these methylation events affect various biological processes, including stem-cell pluripotency, memory formation, immune responses and tumorigenesis. It has become clear that the addition of this small chemical moiety to RNA (which can physically alter the RNA or signal to binding proteins to treat the RNA molecule differently) plays an outsized role in regulating virtually every aspect of biology.

With so many diverse functions assigned to $m^6A$, the next step for the field will be to distinguish direct consequences of this modification from secondary or tertiary consequences that have been inappropriately ascribed to $m^6A$. Independent validation of $m^6A$ functions using orthogonal approaches will be necessary, and we believe that analysing the functional consequences of directed RNA methylation or demethylation of specific residues[79] will play an important part in confirming the biological importance of $m^6A$. Moreover, complementing directed editing with directed chemical labelling of specific $m^6A$ modifications[141], for accurate visualization and tracking, will further bolster confidence in specific biological consequences of $m^6A$.

Moving forwards, it will also be important to determine how different epitranscriptomic modifications communicate with each other and with chromatin modifications, and whether an epitranscriptomic code exists that can help integrate $m^6A$ modifications with other epitranscriptomic or epigenetic signalling pathways. In addition, questions remain about how this abundant modification, which has been implicated in regulating so many diverse processes, can achieve specificity: do methyl-binding proteins alone provide the required specificity or are other systems involved? Precision editing and an improved understanding of $m^6A$ stoichiometry should help provide answers. Finally, RNA methylation has already been demonstrated to have a role in subcellular localization[48–50], so it is exciting to speculate that $m^6A$ could also mark specific RNAs for inheritance through cell divisions or even across generations, similar to what our recent preprint reports $m^{6,2}A$ to do on 18S rRNA[141].

These are exciting times for the field. With the rapid improvement of existing methods and development of new technology, the study of $m^6A$ is poised to uncover new biological regulatory systems and $m^6A$ may even find use as a therapeutic target[142].

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## Glossary

**Hoogsteen base pairing**
An alternative base pairing in which the purine is flipped and form different hydrogen bonds with partner bases. For adenines, the second hydrogen bond with the pyrimidine base is formed with N6 rather than N1. These alternative base pairs allow for additional structures beyond double helix including triplexes and quadruplexes.

**miRNA microprocessor complex**

A protein complex involved in the early stages of processing microRNA (miRNA) and RNA interference in animal cells.

**Spliceosome**
A large RNA–protein complex that catalyses the removal of introns from nuclearpre-mRNA.

**Long non-coding RNAs**
(lncRNAs). Non-coding RNAs longer than 200 nucleotides.

**Chromosome-associated regulatory RNAs**
(carRNAs). regulatory RNAs associated with the chromatin.

**Endogenous retrovirus RNAs**
(ERVs). The prevalent endogenous viral elements that are derived from retroviruses that have become integrated into the genome.

**R-loops**
These RNA:DNA hybrids form three-stranded structures when nascent RNA transcripts hybridize with one strand of the DNA template.

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**Box 1 |**

## Functions of other adenosine methylation modifications

Adenosines can be monomethylated in either the N1, N2 or N6 position of the nucleobase, resulting in $N^1$-methyladenosine ($m^1A$), $N^2$-methyladenosine ($m^2A$) or $N^6$-methyladenosine ($m^6A$), respectively, or can be 2′-O-methylated in the ribose yielding 2′-*O*-methyladenosine (Am). Additionally, adenosines can be dimethylated as is the case for $N^6$,2′-*O*-dimethyladenosine ($m^6Am$) or $N^6$,$N^6$-dimethyladenosine ($m^{6,2}A$) (see the figure). $m^2A$ has thus far only been identified on adenosine 2503 of the 23S ribosomal RNA (rRNA) in prokaryotes where it helps confer a fitness advantage[198], whereas Am (and other 2′-O-methylated bases) is prevalent on rRNAs[56] and the first and second nucleosides of mRNAs directly after the $N^7$-methylguanosine ($m^7G$) cap[1,199]. The other modified adenosines are discussed in more detail below.

### $m^1A$ in tRNAs, rRNA and mRNA

$m^1A$ is deposited by the TRM10 and TRM6/61A methyltransferases on conserved nucleosides 9, 14, 22, 57 and 58 in cytosolic and mitochondrial tRNAs to regulate structural stability and proper folding (reviewed elsewhere[200]). These methylation events are dynamic, increasing in response to glucose, and A58 can be demethylated by ALKBH1 to slow translation initiation and elongation[191].

$m^1A$ has also been found to be methylated by RRP8 at a conserved adenosine in the 28S rRNA (A1322) from yeast to humans, which is required for appropriate translation initiation and translation of specific sets of transcripts[201–204].

The presence and prevalence of $m^1A$ in mRNAs has been highly debated. Initial $m^1A$-sequencing mapping studies reported $m^1A$ to be abundant in mammalian mRNAs and enriched near start codons, and it has been proposed to regulate translation[149,150]. However, subsequent single-nucleotide resolution mapping studies found limited $m^1A$ in mRNAs at low stoichiometry, and its presence leads to translation repression[151,152,205]. A study using multiple $m^1A$ mapping techniques confirmed the very low prevalence of $m^1A$ in mRNAs and suggested that conflicting reports were due to non-specific antibody binding[153]. It has been proposed[151,152,205] that cells may tend to avoid mRNA $m^1A$ deposition because $m^1A$ disrupts base pairing, which blocks translation.

### $m^6Am$ in mRNA and snRNA

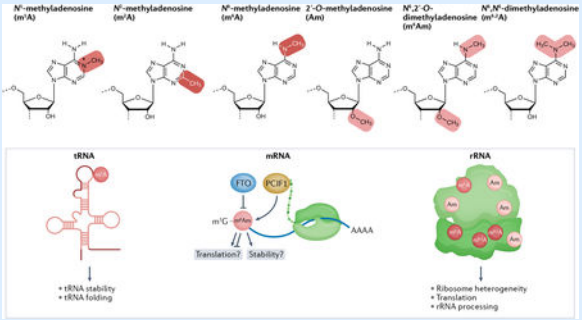
$m^6Am$ is an abundant RNA modification, found on at least 25% of vertebrate mRNAs and certain viral RNAs, but is restricted to the first transcribed nucleotide following the $m^7G$ cap[206,207]. 2′-*O*-Methyladenosines are $N^6$-methylated by PCIF1 (REFS.[172–175]). The phenotypic consequences and effects on RNA fate of PCIF1 deletion under basal conditions seem to be minimal, and the effects on RNA fate seem to be modest and often not in agreement in different studies[172,174,175,208]. $m^6Am$ has been reported to enhance translation[126,172], inhibit translation[175] or promote the stability of a subset of transcripts[126,174]. $m^6Am$ is a dynamic modification as FTO can remove $m^6Am$ both in vitro and in mammalian cells[126]. Recently, $m^6Am$ has been linked to obesity in mice because a high-fat diet stimulates FTO-mediated demethylation of $m^6Am$ in selected

metabolic genes in the liver resulting in decreases in their translation[209]. Interestingly, viruses can hijack host PCIF1 to methylate viral RNAs to evade the immune system[210].

Several small nuclear RNAs (snRNAs), including the U1 and U2 snRNAs, are also $m^6Am$ methylated on their caps and this methylation is removed by FTO[211] (FIG. 2d). Cap $m^6Am$ modifications in U1 and U2 snRNAs cause altered alternative splicing[211]. In addition, U2 snRNAs are internally $N^6$,2′-O-dimethylated at adenosine 30 by METTL4, which also increases splicing[189,190] (FIG. 2d). Future experiments are needed to determine whether these snRNA modifications communicate with each other to create an epitranscriptomic code and how adenosine methylations help determine splicing specificity.

**$m^{6,2}A$ in rRnA**

$m^{6,2}A$ occurs at two conserved adjacent adenosines of eukaryotic 18S rRNAs (1850, 1851)[12] and the consequences on rRNA function are just beginning to be deciphered. Although these methylation events have a role in the processing of 18S rRNA[186,212], recent reports demonstrated that 18S rRNA $N^6$,$N^6$-dimethylation is induced in response to sulfur starvation in yeast and mammalian cells[213] or starvation in *Caenorhabditis elegans*[141], suggesting that rRNA $m^{6,2}A$ is not constitutive and could therefore contribute to ribosome heterogeneity to facilitate the translation of unique sets of stress resistance genes. DIMT1 is the 18S rRNA $N^6$,$N^6$-dimethyltransferase both in vitro and in vivo[141,186–188]. Recent work from our laboratory suggests that worms transmit elevated $m^{6,2}A$ 18S rRNA to their naive progeny in response to starvation, which helps confer an intergenerational hormesis phenotype (whereby low exposure to a stress induces the stress response pathway and allows a more favourable response to stress in the future)[141].



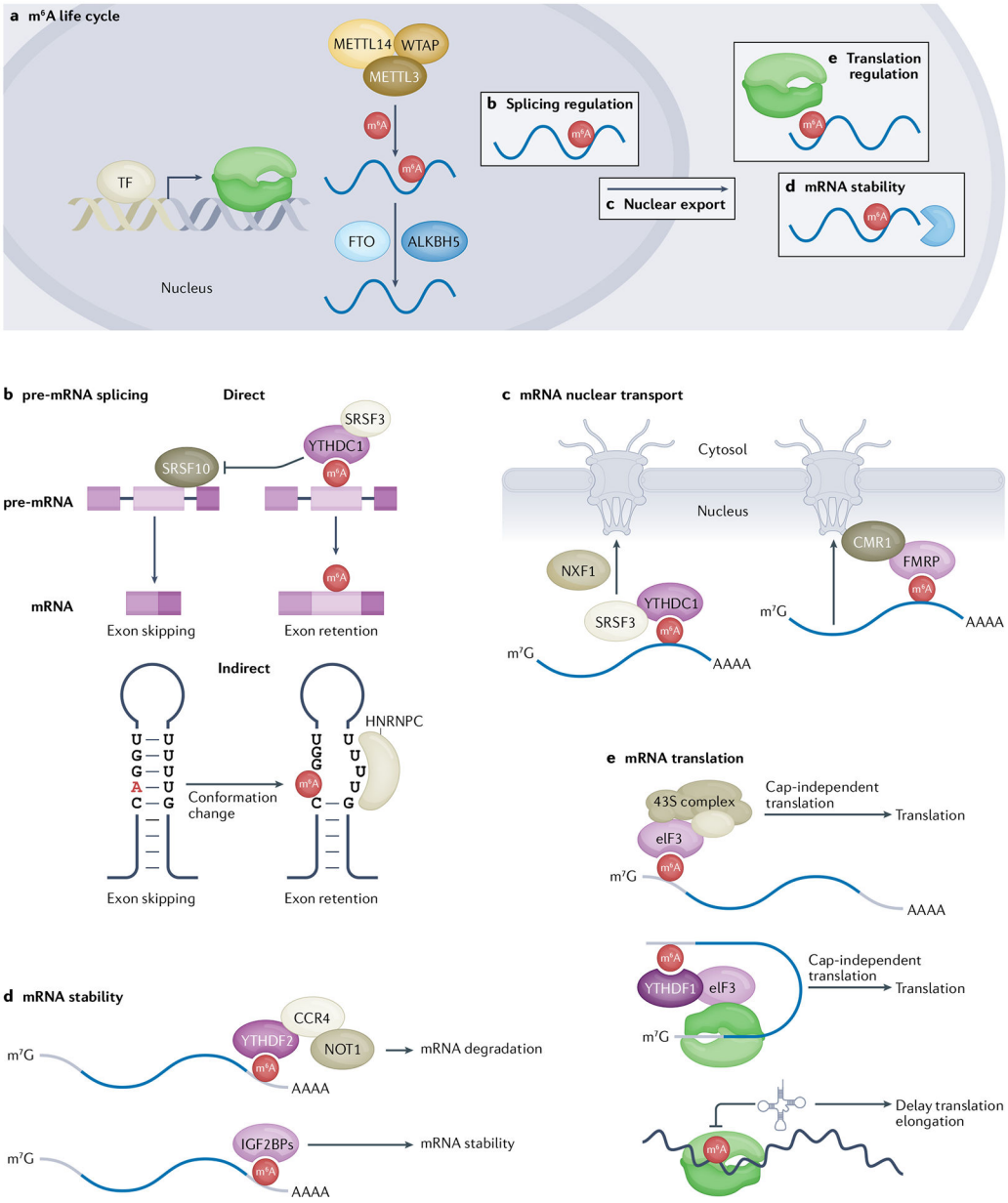



**Fig. 1 |. Molecular consequences of m$^6$A modification of mRNA.**

**a** | *N*$^6$-Adenosine methylation is a dynamic modification that occurs in the nucleus and is regulated by both a methyltransferase complex (METTL3–METTL14–WTAP) and by demethylases (FTO and ALKBH5). *N*$^6$-Methyladenosine (m$^6$A) has been implicated in regulating various nuclear and cytoplasmic processes in the mRNA life cycle, including subcellular localization, splicing (part **b**), export (part **c**), stability or degradation (part **d**) and translation (part **e**). **b** | m$^6$A regulates pre-mRNA splicing by both direct and indirect mechanisms. In a direct mechanism, YTHDC1 binding to m$^6$A leads to exon inclusion by blocking binding of SRSF10 and recruitment of SRSF3. In the absence of *N*$^6$-adenosine methylation, SRSF10 binds resulting in exon skipping. In an indirect mechanism, termed an m$^6$A switch, m$^6$A induces a conformational change in RNA folding,

exposing a heterogeneous nuclear ribonucleoprotein C (HNRNPC) binding motif, which leads to HNRNPC recruitment and exon retention. **c** | $N^6$-Adenosine methylation facilitates mRNA nuclear export. Binding of YTHDC1 to $N^6$-adenosine methylated transcripts promotes export by mediating interactions with SRSF3 and nuclear export mediator NXF1. Alternatively, binding of fragile X mental retardation protein (FMRP) to $m^6A$ facilitates export by mediating a physical association with the nuclear export protein CMR1. **d** | $m^6A$ can promote or inhibit mRNA degradation. YTHDF2 promotes the degradation of $m^6A$-modified transcripts by recruiting the CCR4–NOT1 deadenylase complex. By contrast, insulin-like growth factor 2 binding proteins (IGF2BPs) bind $m^6A$-containing mRNAs and protect them from degradation. **e** | $m^6A$ either promotes or slows translation depending on its location within the mRNA. If the methylated adenosine is located within the 5′ untranslated region (UTR), binding of eukaryotic translation initiation factor 3 (eIF3) recruits the 43S translation initiation complex to promote cap-independent translation. If $m^6A$ occurs in the 3′ UTR, YTHDF1 or YTHDF3 will bind and stimulate translation. However, if $m^6A$ is localized in the coding region it can slow translation elongation rates by inhibiting efficient tRNA selection. $m^7G$, $N^7$-methylguanosine; TF, transcription factor.

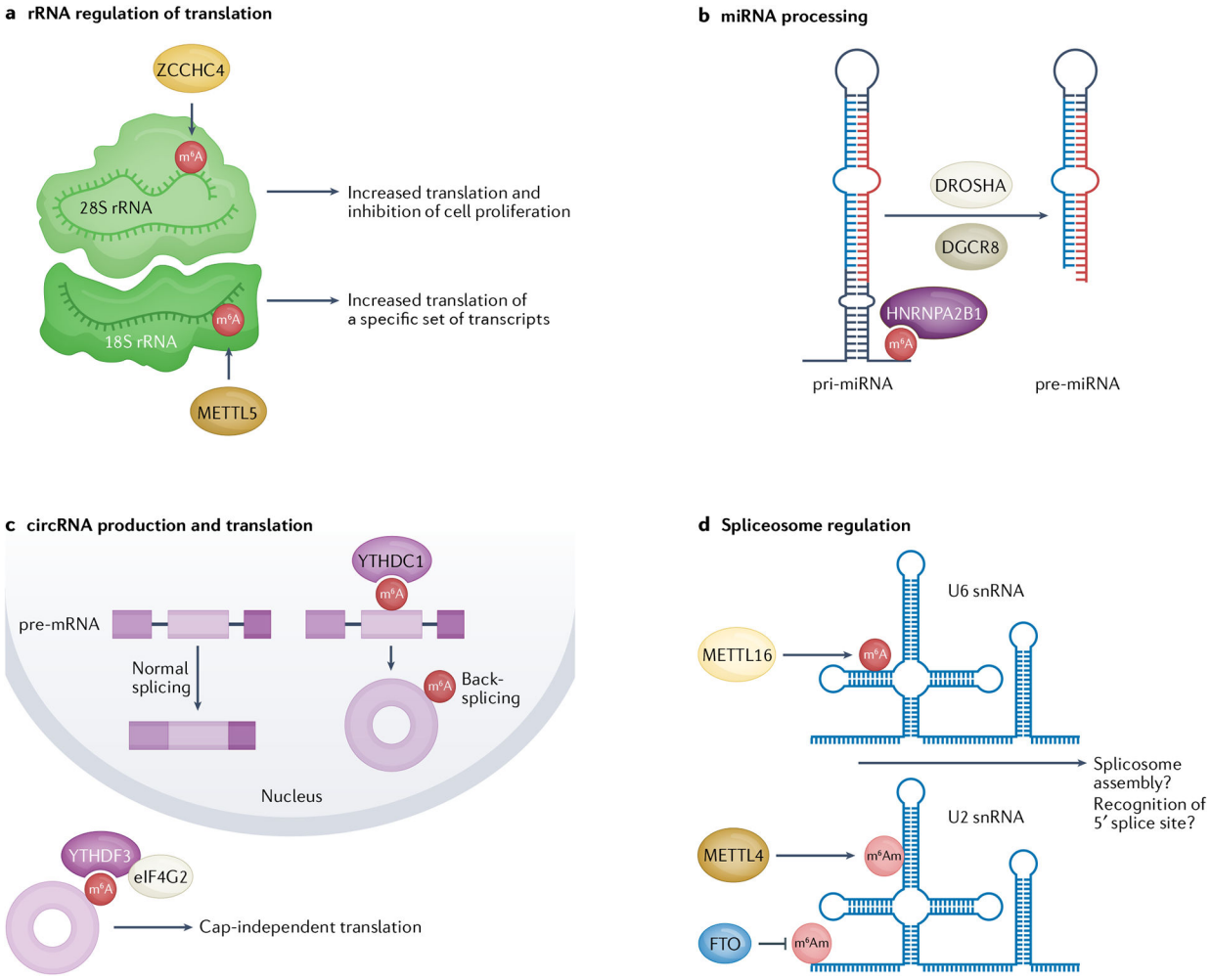



**Fig. 2 |. Molecular consequences of m$^6$A modification of non-coding RNAs.**

**a** | $N^6$-Adenosine methylation of ribosomal RNAs (rRNAs) can promote translation or specify mRNAs to be translated. ZCCHC4 $N^6$-methylates adenosine 4220 in human 28S rRNA resulting in increased translation and inhibition of cell proliferation. METTL5-mediated $N^6$-methylation of adenosine 1832 on human 18S rRNA seems to result in the selective increased translation of a unique sets of transcripts. **b** | $N^6$-Adenosine methylation facilitates microRNA (miRNA) processing. $N^6$-Methyladenosine (m$^6$A) deposition on primary (pri)-miRNAs is recognized by the heterogeneous nuclear ribonucleoprotein HNRNPA2B1, which in turn facilitates their processing to precursor (pre)-miRNAs via the Drosha–DGCR8 microprocessor complex. **c** | m$^6$A regulates circular RNA (circRNA) biogenesis and function. m$^6$A induces back-splicing of circRNA through a YTHDC1-dependent mechanism. In addition, YTHDF3 binds to m$^6$A-modified circRNAs to enhance cap-independent translation, resulting in protein synthesis from circRNA transcripts. **d** | m$^6$A regulates small nuclear RNAs (snRNAs) to facilitate spliceosome assembly. METTL16-dependent m$^6$A deposition on U6 snRNAs regulates spliceosome assembly or 5′ splice site recognition. $N^6$,2′-*O*-Dimethyladenosine (m$^6$Am) modification of U2 snRNAs by METTL4 increases splicing whereas FTO demethylation of U2 snRNAs inhibits splicing.

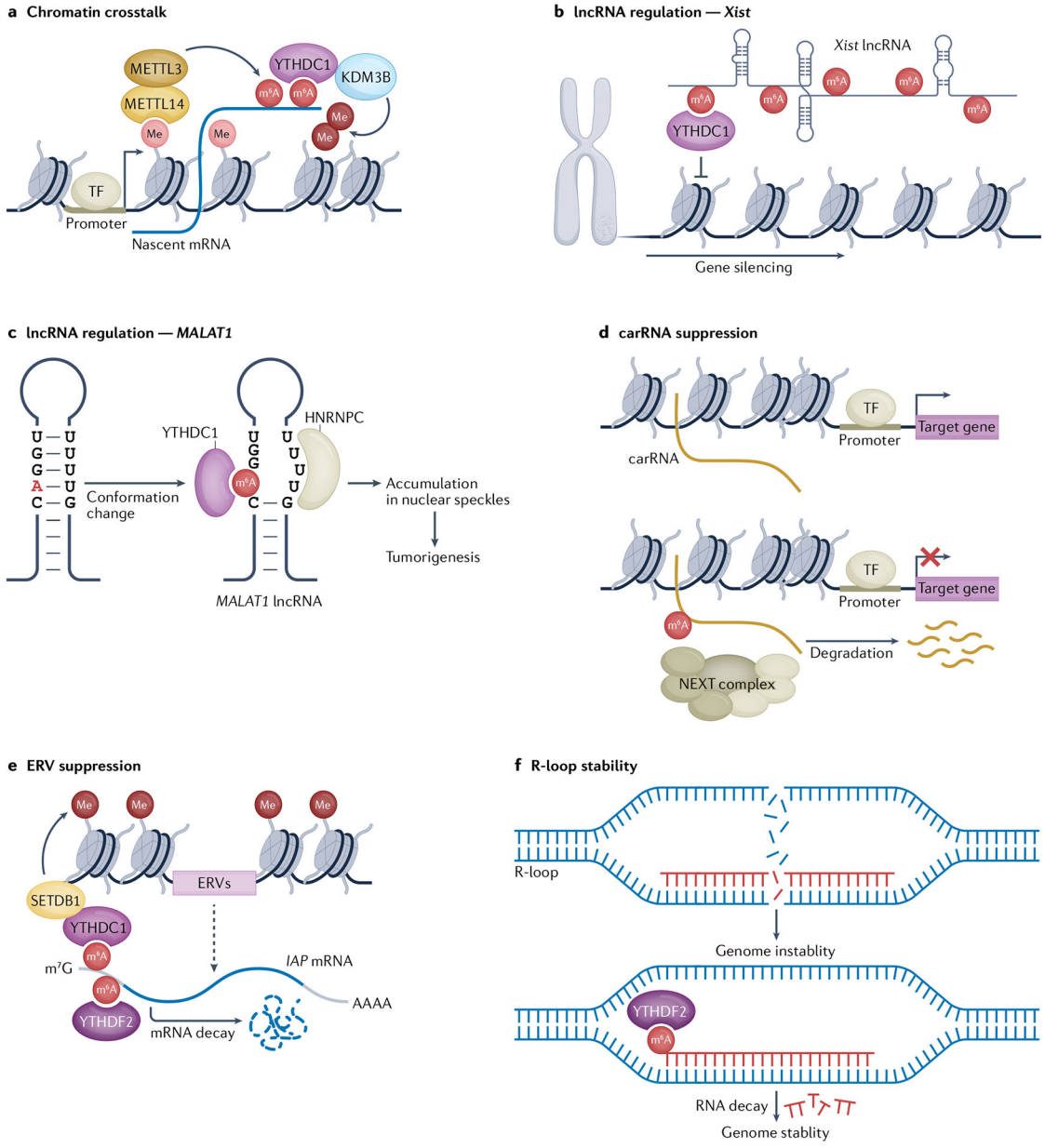



**Fig. 3 |. Genomic consequences of m$^6$A.**

**a** | Crosstalk between $N^6$-methyladenosine (m$^6$A) and chromatin modifications reinforces epigenetic signatures. YTHDC1 can bind to both m$^6$A-modified transcripts and to the histone H3 lysine 9 (H3K9) demethylase KDM3B to induce histone demethylation to reinforce chromatin accessibility in regions being actively transcribed. Moreover, methylation of H3 lysine 36 (H3K36) can recruit the METTL3–METTL14 $N^6$-adenosine methylation complex to m$^6$A-methylate nascent transcripts in regions of active chromatin. **b** | m$^6$A regulates activity and folding of long non-coding RNAs (lncRNAs). *Xist* lncRNA is $N^6$-adenosine methylated at many locations and m$^6$A promotes *Xist*-mediated gene silencing and X chromosome inactivation in a YTHDC1-dependent manner. **c** | $N^6$-Adenosine methylation of *MALAT1* lncRNA induces a conformational change, which leads

to heterogeneous nuclear ribonucleoprotein C (HNRNPC) binding and alterations in nuclear organization and tumorigenesis. **d** | $N^6$-Adenosine methylation of chromosome-associated regulatory RNAs (carRNAs) causes their degradation via the nuclear exosome targeting complex (NEXT) complex, resulting in gene repression. **e** | $m^6A$ facilitates endogenous retrovirus RNA (ERV) suppression by multiple mechanisms to maintain genome stability. $m^6A$-modified intracisternal A-particle (IAP) mRNA is bound by YTHDC1, which recruits the histone H3K9 methyltransferase SETDB1, resulting in the deposition of repressive chromatin marks. In addition, YTHDF2 binds to $m^6A$-modified IAP transcripts to induce degradation of IAP mRNAs. **f** | $m^6A$ prevents genome instability caused by RNA:DNA hybrids (R-loops). $N^6$-Adenosine methylation of RNA in R-loops results in YTHDF2-dependent RNA degradation, which suppresses the formation of R-loops and prevents genomic instability. $m^7G$, $N^7$-methylguanosine; TF, transcription factor.

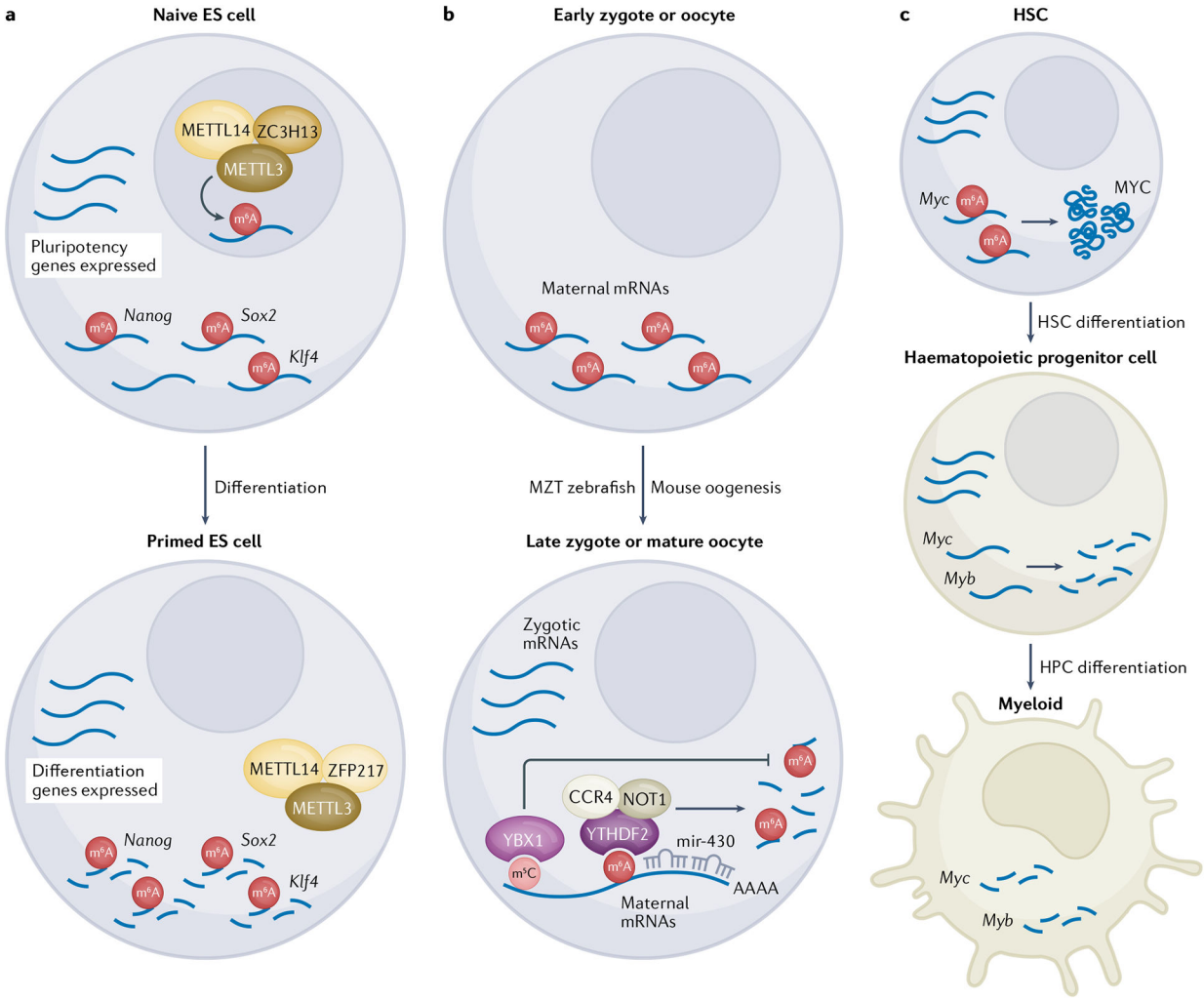



**Fig. 4 |. m$^6$A is a critical modification during early development and embryogenesis.**
**a** | *N*$^6$-Methyladenosine (m$^6$A) modulates embryonic stem (ES) cell renewal and differentiation. Transcripts encoding ES cell pluripotency factors, such as SOX2 and NANOG, are selectively m$^6$A-modified in the nucleus of naive ES cells by the METTL3–METTL14 methyltransferase complex in conjunction with the zinc-finger protein ZC3H13. m$^6$A-dependent degradation of pluripotency factor mRNAs drives ES cell differentiation to the primed state, whereas new m$^6$A deposition is inhibited by sequestration of the METTL3–METTL14 complex to the cytoplasm by the zinc-finger protein ZFP217. **b** | m$^6$A facilitates clearance of maternal mRNAs during the earliest developmental stages of zebrafish and mice. During zebrafish maternal-to-zygotic transition (MZT), YTHDF2 binds to m$^6$A-modified transcripts to direct degradation via the CCR4–NOT deadenylase complex. Binding of the microRNA mir-430 to maternal transcripts further promotes their clearance. By contrast, C5-cytosine methylation (m$^5$C) of maternal transcripts promotes stabilization. **c** | m$^6$A promotes haematopoietic stem cell (HSC) differentiation at several points in the differentiation pathway. m$^6$A deposition on *Myc* mRNA stimulates MYC protein synthesis, which promotes HSC differentiation to haematopoietic progenitor cells. Subsequently, m$^6$A-

dependent degradation of *Myc* and *Myb* transcripts drives haematopoietic progenitor cell (HPC) differentiation to the myeloid cell lineage.

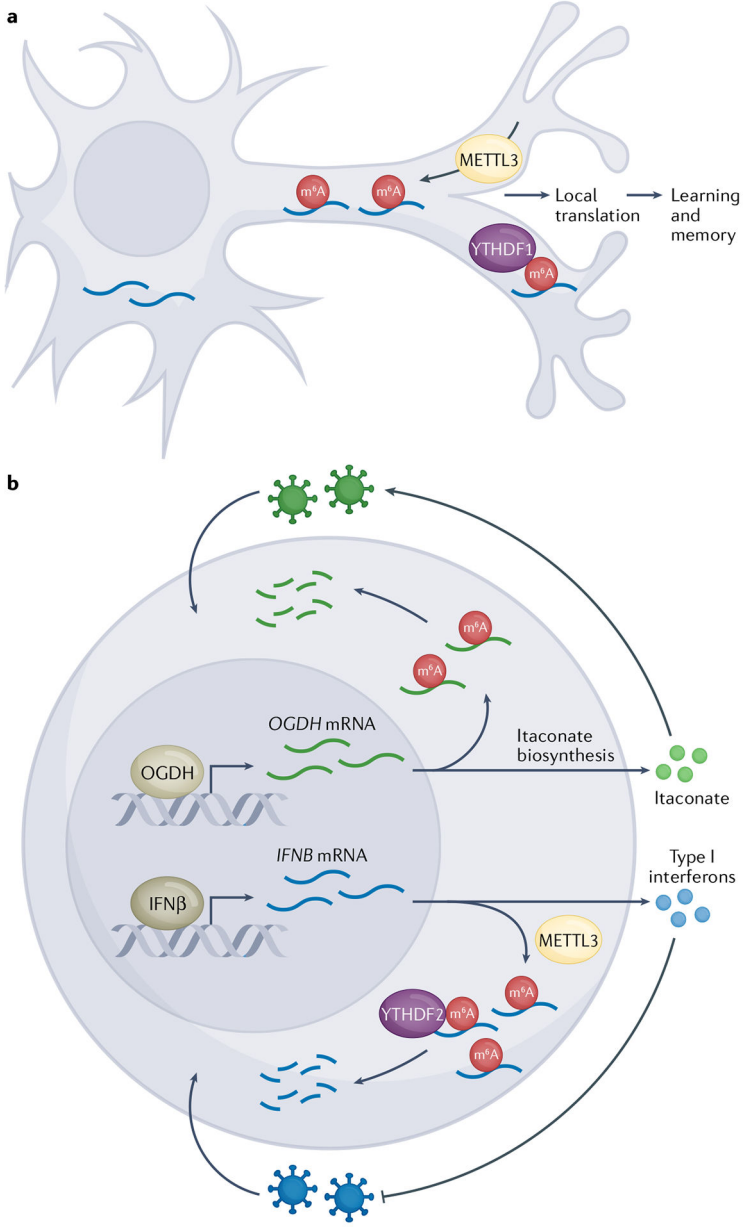



**Fig. 5 |. m$^6$A function in adult cell homeostasis in the nervous system and immune reactions.**

**a** | In neurons, $N^6$-methyladenosine (m$^6$A) is proposed to regulate local translation of selected dendritic transcripts encoding proteins that facilitating memory consolidation during learning and fear conditioning, such as ARC, a synaptic protein important for fast excitatory neurotransmission. The $N^6$-adenosine methyltransferase METTL3, demethylase FTO and binding proteins YTHDF1–YTHDF3, as well as m$^6$A-modified transcripts, are enriched near synapses, raising the possibility of localized regulation of these transcripts. **b** | m$^6$A facilitates degradation of specific transcripts in immune cells to allow a rapid response to viral infection and a return to basal states once the virus has been inhibited. Viral infection induces type I interferon activation to inhibit additional viral infection and, subsequently, $N^6$-adenosine methylation of mRNA encoding interferon-β (IFNβ) elicits

its decay, facilitating a return to basal IFNβ levels. In macrophages, following vesicular stomatitis virus (VSV) infection, mRNA encoding 2-oxyglutarate dehydrogenase (OGDH) are $N^6$-adenosine methylated, which causes their degradation and blocks synthesis of the naturally produced itaconate, which is an activator of VSV replication.

**Table 1 |**

Techniques for detecting adenine methylation

| Technique | Description | Sensitivity | Limitations | Refs. |
|---|---|---|---|---|
| 2D TLC | RNase T1 digestion and radioactive labelling followed by 2D TLC to separate different modified bases | High relative quantification | No qualitative info | 90,143 |
| UHPLC-MS/MS | Chemical separation by UHPLC followed by detection with tandem MS/MS | Highly quantitative | Need to ensure that methylated bases are not being contributed by contaminating mycoplasma or digestion enzymes | 144,145 |
| SCARLET | RNase H site-specific cleavage, splinted ligation, p32 labelling, digestion and TLC to separate and quantify specific methylation sites. | Highly quantitative and qualitative | Only assesses one specific site per assay; the assay is laborious | 146 |
| MazF and MAZTERseq | Site-specific fluorescent quantification of $m^6A$ found in specific context upon MazF cleavage or can be expanded to transcriptome-wide mapping by sequencing in MAZTERseq | High qualitative info, can provide stoichiometry information | Can only detect methylation in specific context of MazF cleavage sites (ACA triplet) | 147,148 |
| meRIP-seq and meRIP-qPCR | Immunoprecipitation followed by sequencing or qPCR | Medium | Depends on immunoprecipitation efficiency<br>Not single-nucleotide resolution<br>Can be prone to misreading other modifications (for example, $m^1A$) as $m^6A$ | 3,4,51, 149–153 |
| RT-based detection methods | Several techniques detect signatures created by RT enzymes (such as 4SedTTP-RT, Tth polymerase and RT-KTQ polymerase) that terminate reverse transcription, or incorporate an incorrect base, when they encounter $m^6A$ | Medium to low | Higher false-positive rate than other methods | 154–156 |
| miCLIP and $m^6A$-CLIP | Antibody crosslinking followed by sequencing to detect mutations | Qualitative but dependent on mutations being accurately detected | Large amount of starting material required | 8,157 |
| $m^6A$-LAIC-seq | Immunoprecipitation of polyA selected transcripts with $m^6A$ antibody followed by sequencing of eluate and supernatant<br>Calling a methylated site requires both enrichment in eluate and depletion of the same region in the supernatant | Medium | Dependent on antibody specificity | 158 |
| $m^6A$ ELISA | ELISA-based method to detect the amount of $m^6A$ antibody-enriched RNA | Medium | Dependent on antibody specificity and lack of contaminations in normalization controls | 159 |
| Nm-seq | Harnesses chemical properties of 2′-O-methylation to expose base and then adaptors added and sequenced | Nucleoside resolution, more sensitive than RiboMeth-seq which takes advantage of 2′-O-methylation resistance to alkaline hydrolysis | – | 160 |
| $m^6A$ melting-qPCR | Harnesses capacity of different polymerases to retrotranscribe $N^6$-adenosine methylated or unmethylated regions to do comparative RT-qPCR | Medium sensitivity | – | 161 |
| SMRTseq and nanopore | Transcriptome-wide mapping of modifications based on detecting change of kinetic incorporation of new bases or travelling through pores | High qualitative information if optimized for appropriate base detection | High error rates if not optimized | 162–165 |

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| Technique | Description | Sensitivity | Limitations | Refs. |
|---|---|---|---|---|
| meClickseq | Click chemistry to attach small molecules to methylated substrates for targeted degradation<br>Uses enzymes to introduce alkyne rather than methyl group | Highly accurate | Measures depletion rather than enrichment | 166 |
| scDARTseq | Single-cell m$^6$A profiling approach that can map methylomes of thousands of cells<br>Reveals that most transcripts are methylated in a small fraction of cells and there is a high degree of methylation heterogeneity | Highly sensitive for single-cell analyses | – | 5 |
| T3 DNA ligase-qPCR and SELECT | Harness changes in ligation efficiency of methylated bases<br>T3 DNA ligase displays significantly higher selectivity for unmethylated adenosines than m$^6$A, which can be used with qPCR to compare methylation levels in different samples<br>In SELECT, m$^6$A hinders Bst DNA polymerase elongation and ligation efficiency of nicks and uses qPCR for quantification | Highly sensitive at specific sites | – | 167,168 |

Detection and high-throughput approaches for mapping of m$^6$A in RNA offer the advantage that they can inform on both the presence of a modification in RNA and its precise location at single-nucleotide resolution. Several of the antibody-based techniques, however, have the caveat that antibodies may bind to non-specific sequences resulting in a high degree of false-positives sites. m$^6$A, $N^6$-methyladenosine; MazF, RNA endonuclease cleaves only non-methylated RNA in ACA sequence but not m$^6$ACA; MAZTERseq, RNA digestion via m$^6$A sensitive RNase; miCLIP, m$^6$A individual nucleotide resolution crosslinking and immunoprecipitation; MS, mass spectrometry; qPCR, quantitative PCR; RT, reverse transcriptase; SCARLET, site-specific cleavage and radioactive labelling of the modified nucleotides followed by ligation-assisted extraction and thin-layer chromatography; scDARTseq, single-cell deamination adjacent to RNA modification targets; SELECT, single-base elongation-based and ligation-based qPCR amplification; SMRTseq, single-molecule real-time sequencing; TLC, thin-layer chromatography; UHPLC, ultra-high performance liquid chromatography.

**Table 2 |**

Enzymes that regulate adenine methylation

| Enzymes | Substrates | Prevalence | Refs. |
|---|---|---|---|
| *Methyltransferases* | | | |
| METTL3–METTL14 | $m^6A$ mRNAs | 25% of mRNAs[3,4] | 6,169 |
| IME4 | $m^6A$ mRNAs | NA | 170 |
| METTL16 | $m^6A$ snRNA, mRNAs, and other ncRNAs | NA | 65,66,171 |
| PCIF1 | $m^6Am$ mRNA cap | 30% of mRNAs | 172–175 |
| CMTR1 | Am mRNA | NA | 176 |
| FBL | Am rRNA | 40–100%[56] | 177–180 |
| TRMT61A | $m^1A58$ tRNA | Most tRNAs[181] | 182 |
| TRMT61B | $m^1A$ nuclear encoded tRNA | NA | 183 |
| TRMT10B | $m^1A$ nuclear encoded tRNA | NA | 184 |
| TRMT10C | $m^1A$ mitochondrial mRNA | NA | 185 |
| DIM1 | $m^{6,2}A$ 18S rRNA A1850, 1851 | NA | 186–188 |
| METTL4 | $m^6Am$ snRNA | NA | 189,190 |
| *Demethylases* | | | |
| FTO | $m^6A$, $m^6Am$ RNA | NA | 10,126 |
| ALKBH5 | $m^6A$ RNA | NA | 9 |
| ALKBH1 | $m^1A$, $m^5C$ and $hm^5C$ tRNA | NA | 191,192 |
| ALKBH3 | $m^1A$ RNA and DNA | NA | 193 |
| *Binding proteins* | | | |
| YTHDC1 | $m^6A$ mRNAs | NA | 194,195 |
| YTHDC2 | $m^6A$ mRNAs | NA | 100,101 |
| YTHDF1 | $m^6A$ mRNAs | NA | 38,196 |
| YTHDF2 | $m^6A$ mRNAs | NA | 4,41 |
| YTHDF3 | $m^6A$ mRNAs | NA | 4 |
| ELAVL1 | $m^6A$ mRNAs | NA | 4 |
| eIF3 | $m^6A$ mRNAs | NA | 37 |
| HNRNPC/G | $m^6A$ mRNAs | NA | 26,197 |
| HNRNPA2B1 | $m^6A$ mRNAs | NA | 44 |
| IGF2BP1–3 | $m^6A$ mRNAs | NA | 33 |
| FMRP | $m^6A$ mRNAs | NA | 34 |

Am, 2′-*O*-methyladenosine; eIF3, eukaryotic translation initiation factor 3; FMRP, fragile X mental retardation protein; $hm^5C$, 5-hydroxymethylcytosine; HNRNP, heterogeneous nuclear ribonucleoprotein; IGF2BP, insulin-like growth factor 2 binding protein; $m^1A$, $N^1$-methyladenosine; $m^5C$, C5-cytosine methylation; $m^6A$, $N^6$-methyladenosine; $m^{6,2}A$, $N^6$,$N^6$-dimethyladenosine; $m^6Am$, $N^6$,2′-*O*-dimethyladenosine; NA, not available; ncRNA, non-coding RNA; rRNA, ribosomal RNA; snRNA, small nuclear RNA.