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Regulation of gene expression by *N*⁶-methyladenosine in cancer

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Abstract

As the most abundant mRNA modification in eukaryotic cells, *N*⁶-methyladenosine (m⁶A) has recently emerged as an important regulator of gene expression. m⁶A modification can be deposited by m⁶A methyltransferases, removed by m⁶A demethylases and recognized by different reader proteins. Numerous lines of evidence have shown that m⁶A methylation plays critical roles regulating gene expression in development and disease. In this review, we summarize the molecular and cellular function of m⁶A and highlight some key results which demonstrate the role of m⁶A in various cancers. Finally, we discuss future directions for research into m⁶A and its effects in cancer and the potential for targeting RNA modification in cancer treatment.

Keywords

RNA modification; *N*⁶-methyladenosine; gene regulation; cancer

*N*⁶-methyladenosine in messenger RNA

In the central dogma of molecular biology, genetic information is stored in DNA, transcribed into RNA, and then expressed through the function of protein. Parallel to the known roles of DNA and protein modifications in gene regulation, recent work has illuminated the regulation of RNA metabolism through processes such as RNA modification and editing as important posttranscriptional regulatory mechanisms [1]. Among hundreds of known RNA modifications, *N*⁶-methyladenosine (m⁶A) is the most prevalent internal modification in mRNA, present at an average of 1–2 m⁶A methylated sites per 1,000 nucleotides in mammalian cells [2]. The functions of m⁶A are regulated by writer, eraser and reader

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proteins (Box 1) (Figure 1). m⁶A is mainly installed by a large multiprotein writer complex that consists of the core METTL3 and METTL14 methyltransferase subunits and many other associated regulatory subunits (Box 1) [1]. m⁶A can also be deposited at internal sites by METTL16 [3, 4], and 2'-O-methyladenosines in the 5' cap of mRNAs are m⁶A methylated by PCIF1/CAPAM to generate N⁶,2'-O-dimethyladenosine (m⁶A_m) [5–8]. FTO [9] and ALKBH5 [10] are two demethylases that act as erasers. m⁶A can influence the metabolism of methylated transcripts though altering interactions with RNA binding proteins (RBPs) [1] (Box 1). m⁶A methylation directly recruits m⁶A reader proteins, which specifically bind m⁶A residues. m⁶A can also influence RBP binding indirectly through m⁶A's effects on RNA secondary structure [11] or by disrupting RBP binding motifs.

Although m⁶A has been known to exist in mRNA for decades [2, 12–14], its biological functions remained unknown for a long time. The discovery that FTO acts as a m⁶A demethylase in 2011 introduced the concept that RNA modifications could be reversible and provoked numerous studies on the dynamics of m⁶A modification and its regulatory functions in biology [9, 15]. The development of m⁶A antibody assisted immunoprecipitation based high-throughput sequencing methods in 2012 [16, 17] enabled mapping of m⁶A sites across the whole transcriptome and, in combination with other techniques to detect m⁶A (Box 2), accelerated the pace of research into the functions of m⁶A. Consistent with the important roles of m⁶A as a post-transcriptional gene regulatory mechanism, aberrant m⁶A methylation has been found to affect numerous cellular processes [18, 19], including many related to tumorigenesis and tumor progression. In this review, we will discuss the molecular and cellular functions of m⁶A in regulating gene expression, with a focus on its roles in cancer.

Molecular functions of m⁶A in post-transcriptional gene regulation

m⁶A has been shown to affect almost every stage of mRNA processing including splicing, export, translation and decay. Mechanistically, different sets of m⁶A-specific reader proteins recognize m⁶A methylated transcripts to effect these changes in mRNA metabolism (Figure 1).

mRNA splicing

Initial studies of m⁶A suggested a role in splicing because mRNAs that undergo alternative splicing contain more m⁶A sites than transcripts that do not undergo alternative splicing [17, 20]. Furthermore, deletion of METTL3 in mouse embryonic stem cells generally facilitates intron retention [21]. However, a study suggested that METTL3-dependent m⁶A methylation does not appear to be broadly required for splicing and affects only a subset of splicing events [22]. Recently, time-resolved, high-resolution sequencing of m⁶A on nascent RNA transcripts revealed that introns are frequently m⁶A modified and that pre-mRNA m⁶A methylation regulates the kinetics of RNA splicing [23]. Altered RNA splicing was also observed in ALKBH5 or FTO deficient cells [10, 24], including the observation that knockdown of FTO could promote inclusion of alternative exons by increasing m⁶A levels near splice sites [24]. Indeed, FTO may play roles in splicing as FTO preferentially binds to pre-mRNA in intronic regions, and FTO knockdown leads to substantial changes in pre-

mRNA splicing with exon skipping events [25]. Another m⁶A methyltransferase, METTL16, has been reported to regulate SAM homeostasis through regulating intron retention in the SAM synthetase MAT2A[4].

Studies of the m⁶A reader proteins present potential mechanisms for how m⁶A may regulate RNA splicing. YTHDC1 recruits SRSF3 while blocking binding by SRSF10, promoting exon inclusion [26]. m⁶A has also been shown to affect the binding of pre-mRNA splicing factor hnRNPC to the introns of its targeted mRNAs [11], and HNRNPA2B1 has also been implicated as an m⁶A reader that can regulate alternative splicing as well as pri-miRNA processing [27, 28].

mRNA export

Various studies suggest a role for m⁶A in regulating the export of methylated transcripts. For example, depletion of ALKBH5 enhances mRNA export to the cytoplasm [10], whereas depletion of METTL3 represses mRNA export [29]. The export of m⁶A methylated transcripts may be facilitated by the reader protein YTHDC1, which binds with nuclear transport receptors [30]. The m⁶A methyltransferase complex may also directly recruit the TREX mRNA export complex to m⁶A modified mRNAs, which can also aid in the recruitment of YTHDC1 and downstream nuclear transport receptors [31].

mRNA translation

m⁶A regulates translation through several different mechanisms. YTHDF1 contributes to the translation of m⁶A-modified mRNA by recruiting translation initiation factors, thereby enhancing the efficiency of cap-dependent translation [32]. YTHDF3 contributes to the translation of its target mRNA through interaction with YTHDF1 [33, 34]. m⁶A in the 5'UTR of transcripts may also directly recruit eukaryotic initiation factor 3 (eIF3), which may recruit the 43S ribosomal pre-initiation complex to facilitate cap-independent translation [35]. Upon heat shock stress, nuclear YTHDF2 can bind to the 5'UTR of some transcripts to prevent the demethylation by FTO, thus indirectly promoting cap-independent translation initiation [36]. m⁶A has also been found to cooperate with ABCF1 to facilitate cap-independent translation [37], and m⁶A may guide the selection of alternate translation start sites during stress responses [38]. Finally, cytoplasmic METTL3 was reported to promote cap-dependent translation in a specific subset of mRNA via directly recruiting eIF3H, and this activity is reported to be independent of its methyltransferase activity and YTHDF1 [39, 40].

mRNA decay

Depletion of METTL3 and METTL14 in human and mouse cells has been shown to increase the abundance of their mRNA targets, which indicates potential roles for m⁶A in regulating mRNA decay [41, 42]. Studies of the m⁶A reader protein YTHDF2 provided the first direct evidence of an m⁶A-dependent mRNA decay pathway [43]. YTHDF2 selectively binds m⁶A sites and mediates decay of these transcripts via recruiting the CCR4-NOT deadenylase complex, initiating deadenylation and degradation of targeted transcripts [43, 44]. YTHDF3 may accelerate mRNA decay through interacting cooperatively with YTHDF2 [33]. Tethering experiments suggest that all YTHDF1–3 can promote mRNA degradation [44].

Besides the YTH binding proteins, the fragile X mental retardation protein FMRP has been reported to selectively bind m⁶A sites in its mRNA targets and interact with YTHDF2 in an RNA-independent manner [45]. Despite interacting with YTHDF2, however, FMRP binding seems to promote the stability of its targets [45]. G3BP1 has been found to bind unmethylated transcripts more strongly than methylated transcripts, and it positively regulates mRNA stability in an m⁶A-dependent manner [46], potentially through its roles in stress granules. Finally, a newly identified family of reader proteins IGF2BP1/2/3 has been reported to promote the stability and storage of their targeted mRNAs in an m⁶A-dependent manner [47].

These studies show that m⁶A-methylation can regulate RNA metabolism in a number of ways through a wide variety of reader proteins (Figure 1). Some reader proteins can promote very different functions (and in some cases opposite functions as in the case of YTHDF2, which promotes the degradation of methylated transcripts, and the IGF2BP family, which promotes the stabilization of methylated transcripts). Therefore, the functional outcome of m⁶A methylation depends on the reader proteins that target particular groups of transcripts and can change depending on the cell-type specific expression and activity of m⁶A reader proteins.

Multiple physiologic functions of m⁶A

Given the dynamic reversibility of m⁶A methylation and its many roles in post-transcriptional regulation, it has been reported to play an important role in numerous physiological processes.

m⁶A in stress responses

The modulation of RNA metabolism is particularly crucial under stress conditions such as hypoxia, extreme temperatures, nutrient deprivation, and exposure to ultraviolet irradiation. Considering the reversible nature of m⁶A modification, it could provide a good option for rapidly altering gene expression during stress. During the heat shock response, m⁶A methylation in the 5'UTR of certain transcripts enables selective cap-independent translation [36]. m⁶A methylation has also been shown to regulate the expression of heat shock proteins in normal and stress situations [48]. In addition to the heat shock response, m⁶A is transiently and rapidly induced at DNA damage sites in response to ultraviolet irradiation [49]. Oxidative stress also promotes the m⁶A methylation of transcripts, which facilitates their targeting to stress granules [50].

m⁶A influences stem cell differentiation

Many studies have reported key roles of m⁶A in embryonic and adult stem cell differentiation [1, 19]. Knockdown of METTL3 and METTL14 promoted the maintenance of self-renewal and impaired differentiation in both mouse and human embryonic stem cells (ESCs) [21, 51]. However, other groups found that decreased m⁶A led to loss of self-renewal capacity in mouse ESCs and murine embryonic fibroblasts [41, 52]. The reasons for the contradictory findings are because the regulatory role of m⁶A in stem cell differentiation is dependent on the cell state; different transcripts are expressed and methylated in naïve and

primed ESCs, so while *Mettl3* inactivation regulates the expression of genes that affect cell fate and identity, this activity leads to maintenance of pluripotency in naïve stem cells but promotes differentiation in primed stem cells [21]. These findings highlight the fact that the biological effects of m⁶A can be highly dependent on cell type and cell state. Interestingly, knockout of the nuclear m⁶A reader YTHDC1 also results in embryonic lethality similar to knockout of METTL3 [53], whereas knockout of the cytoplasmic readers YTHDF1 and YTHDF2 do not show the same effect, suggesting that the nuclear roles of m⁶A methylation are important in the regulation of early embryonic development. m⁶A has also been reported to play a critical role in transcriptome switching during the zebrafish maternal-to-zygotic transition through *Ythdf2*-dependent regulation of RNA decay [54], and determines cell fate during the endothelial-to-haematopoietic transition (EHT) to specify the earliest hematopoietic stem/progenitor cells (HSPCs) during zebrafish embryogenesis [55]. Recent work also showed that YTHDF2 knockout or knockdown can dramatically expand mouse and human hematopoietic stem cells, highlighting its potential in transplant related applications [56]. Additional studies reported the role of METTL16 in regulating embryonic development through the methylation of structured RNAs [57, 58]. Altogether, these findings suggest a cell state and cell type dependent function for m⁶A RNA signaling in stem cell maintenance and differentiation.

m⁶A modulates gametogenesis

Another cell fate transition that seems particularly sensitive to m⁶A is spermatogenesis, in which diploid spermatogonial stem cells (SSCs) produce haploid spermatozoa, as knockout of ALKBH5 or germ-cell specific knockout of METTL3 or METTL14 all result in impaired male fertility [10, 59–61]. Dysregulation of m⁶A was observed to block the initiation of meiosis and alter the expression and splicing of key factors of spermatogonial stem cells and progenitor cells. The m⁶A reader YTHDC2 plays a critical role in these processes as knockout of YTHDC2 results in male and female fertility [62–65]. Knockout of the m⁶A reader YTHDF2 also results in female-specific infertility through similar mechanisms [66].

m⁶A influences T cell homeostasis

Loss of m⁶A has been reported to disrupt various aspects of T-cell homeostasis and differentiation. For example, in naïve T-cells, decreased levels of m⁶A modification on the mRNAs of suppressor of cytokine signaling (SOCS) family genes have been reported to slow mRNA decay and increase expression of the SOCS1, SOCS3 and CISH proteins [67]. These changes to SOCS gene expression lead to the inhibition of downstream IL-7/STAT5 signaling, which prevents naïve T cell proliferation and differentiation. In Treg cells, m⁶A also regulates SOCS family genes, this time modulating IL-2/STAT5 signaling, and knockout of METTL3 disrupts the immunoregulatory functions of Tregs [68]. Given the importance of immunity to cancer, these findings may have relevance to understanding the roles of m⁶A in cancer. Indeed, knockout of the m⁶A reader Ythdf1 enhances anti-tumor immunity in dendritic cells by increasing the cross-presentation of tumor antigens and the cross-priming of CD8⁺ T-cells [69].

Varying roles of m⁶A in cancer

Consistent with the important role of m⁶A mRNA modification in regulating gene expression in various biological processes, aberrant m⁶A modification is associated with a variety of human cancers. However, knowledge of the mechanistic link between m⁶A and human carcinogenesis is limited. While investigations addressing this issue are still at an early stage, efforts are underway to explore the biological impacts of m⁶A modifications in cancer. In particular, both elevated and decreased levels of m⁶A methylation have been associated with cancer, and it is unclear how these changes can both contribute to carcinogenesis. We will summarize recent reports describing our understanding of the biological functions and underlying molecular mechanisms of m⁶A regulatory proteins in various types of cancer (Figure 2, Key Figure).

Acute myeloid leukemia (AML)

AML is one of the most common haematopoietic malignancies and is associated with high mortality [70]. Various studies suggest an association between elevated expression of m⁶A writers and AML. Increased METTL3 levels have been observed in AML, which leads to higher m⁶A methylation levels of BCL2 and PTEN transcripts and thus promotes their translation, altering PI3K/AKT signaling to control cell differentiation and self-renewal [71] (Figure 2M). METTL3 also enhances the m⁶A modification and expression of SP1, an oncogene in AML that regulates c-MYC expression [72] (Figure 2M). Similarly, elevated METTL14 expression has also been observed in the disease, which may enhance MYB and MYC expression to block myeloid differentiation [73] (Figure 2L). Elevated WTAP promotes cell proliferation and inhibits cell differentiation of AML through links with Hsp90 [74] (Figure 2H). In contrast, other studies have explored AML subtypes with elevated FTO expression, which leads to the downregulation of m⁶A levels on the UTRs of ASB2 and RARA, thus reducing the mRNA and protein levels of these two genes and contributing to cell transformation and leukemogenesis [75] (Figure 2D). Additionally, FTO promotes the stability of MYC mRNA by demethylating it to inhibit its YTHDF2-mediated RNA decay [76] (Figure 2D). Overexpression of FTO in leukemia cells also helps to develop resistance during tyrosine kinase inhibitor therapy [77]. Genetic alterations of m⁶A regulatory genes are associated with worse survival in AML, suggesting METTL3, METTL14, WTAP and FTO are potential therapeutic targets [78].

Hepatocellular carcinoma (HCC)

HCC is a major type of primary liver cancer, and it is associated with a low survival rate, thus the mechanism and pathogenesis of HCC are urgent to be addressed. One study found elevated expression of METTL3 in human HCC, which leads to increased m⁶A modification levels on the tumor suppressor SOCS2, accelerates the degradation of SOCS2 through a YTHDF2-dependent pathway and alters the proliferation of HCC cells [79] (Figure 2M). Another report, however, found decreased METTL14 expression in HCC, which reduces m⁶A modification levels on microRNA126 (miR126), impairs its processing, and inhibits miR126-mediated suppression of the metastatic potential of HCC [80] (Figure 2B). Additionally, YTHDF2 is reported to be associated with the malignance of HCC and it is

regulated by the microRNA miR-145, which is commonly downregulated in HCC patients [81] (Figure 2E).

Glioblastoma

Glioblastoma is an invasive malignant primary brain tumor associated with a short survival time and poor quality of life. Knockdown of METTL3 or METTL14 leads to decreased m⁶A and enhances the growth, self-renewal, and tumorigenesis of glioblastoma stem cells (GSCs) [82] (Figure 2C). Treatment with the FTO inhibitor MA2 suppresses GSC proliferation and self-renewal [82]. Accordingly, enhanced ALKBH5-mediated demethylation also contributes to GSC maintenance and survival through inhibiting the methylation and decay of FOXM1 [83] (Figure 2C). However, one study observed increased METTL3 expression in GSCs and found that METTL3 mediates GSCs maintenance and differentiation by regulating the stability of the SOX2 mRNA through installing m⁶A on its 3' UTR [84] (Figure 2M).

Breast cancer

Among all malignant tumors in women, breast cancer has the highest incidence and is responsible for the largest number of deaths. In breast cancer, hypoxia can stimulate the expression of ALKBH5 and ZNF217, which both inhibit the methylation of NANOG and KLF4 mRNAs to enhance their stability [85, 86] (Figure 2A). Elevated NANOG and KLF4 expression increases the stemness of breast cancer cells, resulting in a breast cancer stem cell phenotype [85, 86]. Another report indicated a potential positive feedback loop between the oncogene HBXIP and METTL3—in which HBXIP promotes the expression of METTL3 through suppression of the miRNA let-7g and METTL3 methylates HBXIP mRNA to promote its translation—that may contribute to breast cancer progression [87] (Figure 2I). Finally, a study suggests that low levels of m⁶A modification on MAGI3 mRNA could lead to premature polyadenylation, switching its functional role from a tumor suppressor gene to a dominant-negative oncogene in breast cancer cells [88] (Figure 2A).

Cervical cancer

In cervical cancer, reduced levels of m⁶A is associated with poorer survival and knockdown of m⁶A regulators increases the proliferation and tumorigenicity of cervical cancer cells [89]. In particular, the expression of FTO is significantly higher in cervical tumors than that in normal tissues, resulting in lower levels of m⁶A modification in CTNNB1 transcripts, which causes decreased expression of β -catenin and increased chemoradiotherapy resistance [90] (Figure 2D). The discovery of this mechanism suggests that MA2, a novel small molecule inhibitor of FTO, may increase the chemoradiotherapy sensitivity of cervical cancer [90].

Lung cancer

Lung cancer includes small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), and NSCLC accounts for approximately 85% of all cases. Although the incidence and death rates have declined, the 5-year survival rates remain poor. METTL3 is elevated in NSCLC and may contribute to the tumorigenicity of lung cancer cells by associating with

the translation machinery to enhance the translation of oncogenic mRNAs (e.g. RGFR and TAZ) independent of its methyltransferase activity [39, 40]. Consistent with this observation, another report indicated that miR-33a reduces NSCLC cell proliferation by inhibiting METTL3 expression [91] (Figure 2J). However, SUMOylation of METTL3, which decreases m⁶A levels on mRNAs, also appears to promote the tumorigenicity of NSCLC cells [92] (Figure 2F).

Other cancers

METTL3 plays an inhibitory role in renal cell carcinoma, where depletion of METTL3 promotes cell proliferation, growth, and colony formation through the PI3K-AKT-mTOR pathway activation and enhances cell migration and invasion through the epithelial-mesenchymal transition (EMT) pathway [93] (Figure 2G). In endometrial cancer, reduced m⁶A due to either METTL14 mutation or decreased METTL3 expression promotes cell proliferation also through activation of the PI3K-AKT pathway [94] (Figure 2G). In pancreatic cancer, YTHDF2 expression is elevated and seems to play a dual role in the disease; it reduces adhesion, invasion, migration and EMT through the activation of YAP signaling, but also promotes proliferation via activation of the AKT/GSK3b/CyclinD1 pathway [95] (Figure 2K).

Concluding remarks

RNA modification is a new layer of post-transcriptional gene regulation that is important in various aspects of biology, including cancer. In many cases, the roles of m⁶A in regulating gene expression in cancer mirror the physiological roles of m⁶A in development and normal tissue homeostasis. A variety of studies have identified essential roles for m⁶A methylation in regulating cell differentiation, for example, mediating transcriptome turnover during critical cell fate transitions in the differentiation of pluripotent stem cells [21, 41, 51] and the maternal-to-zygotic transition in embryos [54]. Similarly, aberrant m⁶A methylation appears to block the differentiation of hematopoietic stem cells and contribute to leukemogenesis in AML [71–73]. During these developmental transitions, m⁶A methylation also regulates the expression of important genes and pathways that regulate cell fate, and the misregulation of these pathways by aberrant m⁶A methylation may contribute to cancer. For example, m⁶A regulates the PI3K/AKT pathway during T-cell development [67] as well as in endometrial cancer, renal cell carcinoma, and AML [71, 93, 94]. Finally, m⁶A methylation has important roles in adapting cells to stress, and these stress-induced changes to m⁶A may also occur in cancer, such as in the hypoxia-induced, m⁶A-dependent changes to cell physiology observed in breast cancer cells [85, 86]. Just as studies of the roles in m⁶A in normal development can inform our understanding of m⁶A in cancer, we expect that studies of m⁶A in cancer will be relevant to understanding the broader biology of m⁶A-dependent gene regulation.

Different studies have found differing effects of m⁶A modification on tumor progression. Various studies suggest that m⁶A has tumor-promoting roles through enhancing either the translation of oncogenes or the degradation of suppressor genes (Figure 2H–M). However, other studies show that m⁶A can also exert tumor-suppressing functions through inhibiting the expression of oncogenes (Figure 2A–G). Because the biological effects of m⁶A

modification depend both on the identity of the methylated transcripts being expressed and the expression and activity of m⁶A reader proteins, the effects of m⁶A modification on cell physiology are likely to be cell type and cell state dependent. However, given the multiple roles of m⁶A in the regulation of RNA metabolism and gene expression, it may also be the case that both increased and decreased levels of m⁶A modification can alter gene expression from a “healthy” state and thus promote cancer. More research will be needed to clarify the specific circumstances in which increases and decreases of m⁶A modification will be beneficial or deleterious to carcinogenesis and tumor progression.

The role of aberrant m⁶A modification in a variety of cancers suggests that regulators or targets of m⁶A methylation could be tested as new candidates for clinical therapies. For example, the FTO inhibitor R-2-hydroxyglutarate (R-2HG) exhibits an antitumor activity in AML cells [76]. In some conditions, inducing the expression of m⁶A writers may also suppress cancer cell growth, for example, overexpressing METTL3 repressed GSC growth [82] and cell proliferation in endometrial cancer [94]. Future research is necessary to identify ways to pharmacologically target the regulators of m⁶A-dependent gene regulation. However, it should be noted that because m⁶A mRNA methylation (as well as expression of METTL3 and METTL14) is essential to cells, a simple knockdown or METTL3 or METTL14 could inhibit cell proliferation in both cancer cells and healthy cells. Thus, in different cancers with different genetic causes, it will be important to analyze the precise role of m⁶A on key transcripts that are specifically important to cancer progression but not healthy control cells. Finally, to broaden and better our understanding of how m⁶A affects tumorigenesis and cancer development, the basic mechanism of how m⁶A functions in gene regulation needs to be further explored, and improvements in the techniques for detecting m⁶A and validating its functions are also required (see Outstanding Questions).

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Box 1.**m⁶A regulators in humans****Writers—m⁶A methyltransferase enzymes**

- The primary m⁶A methyltransferase complex includes: METTL3, the catalytic component [42, 96]; METTL14 which helps METTL3 to recognize its RNA substrates [97–99]; WTAP [20, 41, 42, 100], VIRMA [101], HAKAI and ZC3H13 [102–104] are adaptor proteins which may aid in guiding the METTL3-METTL14 heterodimer to its target mRNAs; RBM15 and RBM15B may also help to determine which DRACH sites are methylated [105, 106].
- METTL16 methylates small nucleolar RNAs and a number of mostly intronic sites in pre-mRNAs [3, 4].
- PCIF1/CAPAM methylates 2'O-methyladenines in the 5' cap of mRNAs [5–8].

Erasers—m⁶A demethylase enzymes

FTO [9] and ALKBH5 [10] function as m⁶A demethylase in some specific mRNA targets. FTO shows a preference for demethylating m⁶A_m but also demethylates m⁶A [107, 108].

Readers—m⁶A binding proteins

YTH family proteins contain a YTH domain that specifically recognizes m⁶A versus A. YTHDC1 [26, 30] is predominantly found in the nucleus, and YTHDF1 [32], YTHDF2 [43], YTHDF3 [33, 34] and YTHDC2 [62–65] are mostly located in the cytoplasm. In addition to the YTH family of proteins, HNRNPA2B1 serves as a direct reader which regulates miRNA splicing [27]; eIF3 directly binds with m⁶A and promotes cap-independent translation [35]; the IGF2BP family of proteins bind m⁶A to increase mRNA stability [47]; FMR1/FMRP can also read m⁶A to regulate mRNA stability [45].

The binding of HNRNPC [11] and HNRNPG [109] to certain sites can be regulated by m⁶A indirectly through a structural switch mechanism; their binding site is occluded by RNA secondary structure, and m⁶A methylation destabilizes the secondary structure, exposing the RNP binding site.

Repelled proteins—RBPs repelled by m⁶A.

ELAVL1 [41] and G3BPs [46] function as repelled proteins that bind unmodified A over m⁶A and help to increase mRNA stability in response to m⁶A methylation.

Box 2:**Techniques to detect m⁶A**

The ability to quantify the amount of m⁶A in mRNA and identify the locations of m⁶A in RNA is important for understanding its biological functions. To date, several major methods have been developed:

m⁶A-seq/MeRIP-seq

In 2012, the transcriptome-wide profile of m⁶A in mammalian cells was first revealed by immunoprecipitation of mRNA with an m⁶A-specific antibody followed by high-throughput sequencing [16, 17]. Briefly, purified mRNA is fragmented to 100–150 nt and immunoprecipitated by m⁶A-specific antibodies. The input RNA and enriched m⁶A-containing RNA fragments are subjected to library construction and high-throughput sequencing. This method has a resolution of 100–200 nt and cannot determine the specific base location of m⁶A in RNA nor can it measure the stoichiometry of the modification at a particular site. However, this method is still among the most commonly used techniques for profiling m⁶A sites across the transcriptome.

PA-m⁶A-seq (photo-crosslinking-assisted m⁶A sequencing strategy) and miCLIP (m⁶A individual-nucleotide-resolution crosslinking and immunoprecipitation)

In these two methods, RNA fragments are immunoprecipitated and crosslinked to antibody using 365-nm (PA-m⁶A-seq) [110] or 254-nm (miCLIP) UV light [111]. These two methods can map m⁶A sites at single-base-resolution across the transcriptome.

m⁶A-LAIC-SEQ (m⁶A-level and isoform-characterization sequencing)

This method uses excess antibody to pull down all m⁶A containing full-length RNAs, and though the use of methylated and non-methylated spike-in RNAs, enables the measurement of the stoichiometry of the modification [112]. However, because RNAs are not fragmented, information about the location of m⁶A on the transcript is lost. This technique has been used to show that most genes exhibit less than 50% m⁶A-methylation levels and transcripts marked by m⁶A are coupled with proximal alternative polyadenylation sites, leading to shortened 3'UTRs.

SCARLET (site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography)

The method combines RNase H site-specific cleavage, splinted ligation, ribonuclease digestion, and thin-layer chromatography (TLC) [113, 114]. SCARLET is capable of directly determining the m⁶A modification stoichiometry of a particular m⁶A site; however, it is time consuming, requires the use of radioactive reagents, and is not subject to high-throughput transcriptome-wide applications.

SELECT (single-base elongation and ligation-based qPCR amplification method)

The method takes advantages of the fact that m⁶A hinders both single-base elongation activity of DNA polymerases and nick ligation efficiency of ligases, and uses qPCR-based detection [115]. SELECT enables quantification of m⁶A stoichiometry at a

particular m⁶A site. However, it is not yet capable of high-throughput transcriptome-wide applications.

Antibody-based quantification assays vs LC-MS/MS

The global changes of m⁶A levels in total RNA or a particular class of RNAs can be measured by dot blot- or ELISA-based methods using m⁶A specific antibodies or by LC-MS/MS (liquid chromatography coupled to tandem mass spectrometry). The antibody-based methods for detection of global m⁶A changes are relatively straightforward methods to quantitate m⁶A modification but suffer from low sensitivity when the fraction of m⁶A-modified RNA is low in the analyzed samples [9]. Furthermore, m⁶A antibodies non-specifically bind and cross-react with other modifications, such as 6mA residues in DNA. Because 6mA is common in bacterial genomes, care must be taken to avoid contamination of the sample with bacterial DNA. In comparison, LC-MS/MS is a more sensitive and accurate quantification method, though it requires access to a relatively expensive instrument. Triple quadrupole-based detection methods allow for limits of quantification in the low femtomole range and avoid issues with the cross-reactivity of antibodies with other similar modifications [9, 116]. For both antibody-based and LC-MS/MS-based quantification of m⁶A in mRNA, it is important to rigorously purify mRNA from other RNA species, as abundant RNA species like rRNAs and snRNAs also contain m⁶A.

Highlights

- m6A is the most abundant mRNA modification, and it regulates many aspects of RNA metabolism, including RNA splicing, export, translation and decay.
- Numerous recent studies indicate an important role for m6A in regulating gene expression in multiple physiologic processes, such as stress responses, stem cell differentiation, gametogenesis and T cell homeostasis.
- Aberrant m6A mRNA methylation, through the altered expression of m6A writer, eraser or reader proteins, has also been associated with several cancers.
- m6A has been reported to affect gene expression in cancer cells through many different pathways. In some studies, increased m6A methylation appears to contribute to cancer cell tumorigenicity by enhancing translation of oncogenes or degrading tumor suppressor genes. Other studies observe that m6A helps inhibit the expression of oncogenes, suggesting tumor suppressing roles for m6A methylation.

Figure 1. The regulation of m⁶A by writers, erasers and readers.
The diagram depicts various writer (green), eraser (red) and reader (blue) proteins involved in the regulation of gene expression by m⁶A methylation.

Figure 2, Key Figure. The regulation of gene expression by m⁶A in cancer.

The diagram depicts the tumor suppressing (A-G) and tumor promoting (H-M) roles of m⁶A methylation in a variety of cancers. It proposes how writers, erasers and readers are involved in the regulation of various genes and pathways.