Sequencing, Physiological Regulation, and Representative Disease Research Progress of RNA m^{6}A Modification

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Abstract

To date, more than 150 chemical modifications have been disclosed in different RNA species, which are employed to diversify the structure and function of RNA in living organisms. The N^6-methyladenosine (m^{6}A) modification, which is found in the adenosine N^6 site of RNA, has been demonstrated to be the most heavy modification in the mRNA in cells. Moreover, the m^{6}A modification in mRNAs of mammalian and other eukaryotic cells is highly conserved and mandatorily encoded. Increasing evidence indicates that the m^{6}A modification plays a pivotal role in gene-expression regulation and cell-fate decisions. Here, we summarize the most recent m^{6}A-sequencing technology, as well as the molecular mechanism underlying its occurrence, development, and potential use as a target for the treatment of human diseases. Furthermore, our review highlights other newly discovered chemical modifications of RNA that are associated with human disease, as well as their underlying molecular mechanisms. Thus, significant advancements have been made in qualitative/quantitative m^{6}A detection and high-throughput sequencing, and research linking this RNA modification to disease. Efforts toward simplified and more accessible chemical/biological technologies that contribute to precision medicine are ongoing, to benefit society and patients alike.

Keywords
- RNA modification
- N^6-methyladenosine
- methyltransferase
- binding protein
- cancer

Introduction

The decades-long development of RNA innovations, from the discovery of the new functions of RNA (serving as a catalyst and regulator of many biochemical reactions) to its conventional role as a genetic-information carrier, linker in protein molecule synthesis, and structural scaffold of subcellular organelles, has contributed to the progress of nucleic acid research.[1–5] RNA plays an important role in protein synthesis, and RNA-based drug discovery has attracted great interest, as it contains only four types of nucleotides compared with the 20 different amino acid residues present in proteins.[6] To achieve diversity in RNA structure and function, nature uses a variety of chemical groups for its modification.[7] Since the discovery of the foremost RNA modification in yeast about 60 years ago, over 150 distinct modifications have been identified in all RNA species, including transfer RNA (tRNA), messenger RNA (mRNA), and ribosomal RNA (rRNA).[8–11] These modifications play crucial roles in genetics, growth, and disease

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development, including embryonic stem cell differentiation, oocyte meiosis, and cancer occurrence.[12–17]

RNA modifications enrich the diversity of RNA functions and of genetic information processing. An extensive range of RNA modifications has been identified (►**Fig. 1**), and the content and related functions of each type vary greatly. Among them, the methylation of RNA nucleotides is the main form of RNA modification, accounting for approximately two-thirds of the total number of RNA modifications. Currently, the methylation modifications found on mRNA include mainly 5-methylcytosine (m^{5}C),[18] 1-methyladenosine (m^{1}A),[19] N^6-methyladenosine (m^{6}A),[20] and pseudouridine (Ψ).[21] Compared with other types of RNA modifications, the m^{6}A internal modification occurs on the N^6 of the adenosine base and is most commonly encountered in eukaryotic mRNA, representing more than 80% of RNA base methylation and having been identified throughout numerous species.[10,12,22–27] It has been reported that m^{6}A can occur in the 5′-untranslated region (UTR), coding sequence (CDS), 3′-UTR, and introns of precursor mRNA, as well as in several noncoding RNAs and miRNAs.[28,29] In addition to the m^{6}A modification, other hydrophobic modifications of A also occur, such as 2-methylthio-N^6-isopentenyladenosine (ms^{2}i^{6}A),[30] N^6-isopentenyladenosine (i^{6}A),[30] N^6-methyl-threonylcarbamoyl adenosine,[31] and N^6-threonylcarbamoyladenosine (t^{6}A).[32] However, the molecular mechanism underlying these prenyl modifications remains poorly understood (►**Fig. 1**; i^{6}A, *S*-geranylated 2-thiouridines (ges^{2}U)). The biosynthesis mechanism, abundance, and distribution regularity of these prenyl modifications, and the molecular mechanism underlying their regulation network in cells, also remain elusive.

Sequencing Technology Used for m^{6}A Modification

Determining the location of chemical modification groups in transcripts is key information for understanding their basic functions, which is also true for the m^{6}A modification. An analysis of m^{6}A modification over the entire transcriptome indicated that m^{6}A modifications occur across an enormous spectrum of RNA transcripts in unique patterns.[28,29] In 2012, 30 years after the discovery of the m^{6}A modification, m^{6}A-seq (also known as MeRIP-seq) afforded the m^{6}A landscape in humans and mice. Two independent transcriptome studies have shown that the accurate proportion of m^{6}A-modified RNAs among the total mRNAs is 1 per 2,000 nucleotides,

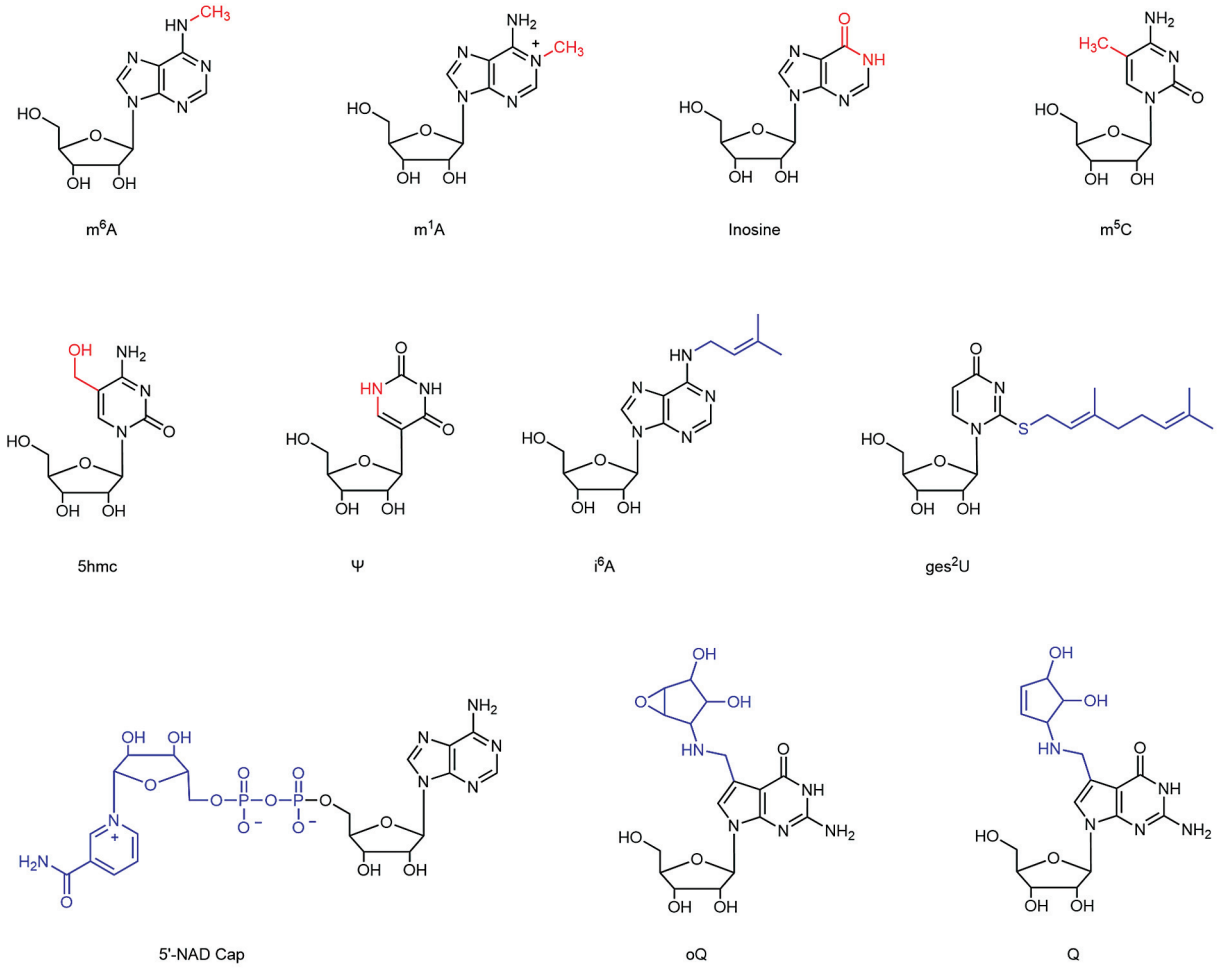

Fig. 1 Chemical structures of the typical modifications of RNA.

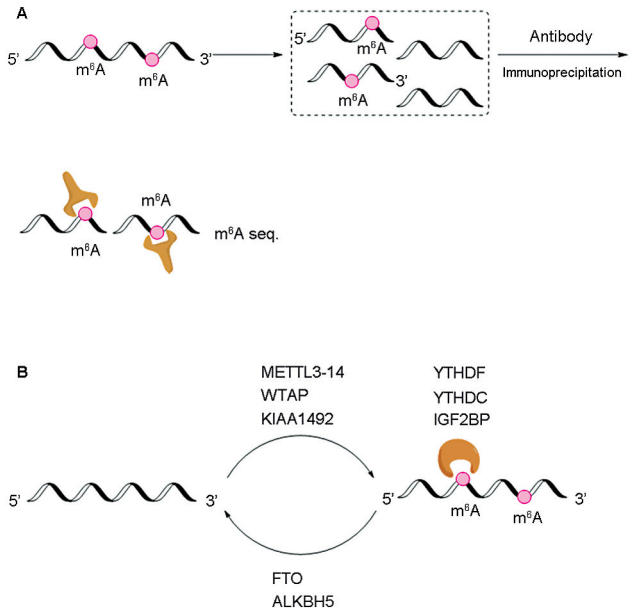

Fig. 2 Schematic illustrations of the molecular mechanism underlying the m^{6}A modification of RNA. (**A**) The molecular mechanism underlying the RNA m^{6}A modification involves regulatory enzymes, such as writers, readers, and erasers. (**B**) Pipeline of the m^{6}A sequencing technique.

on average.[28,29] Specifically, total RNA was divided into approximately 100 NTs and subjected to immunoprecipitation using anti-m^{6}A affinity-purified antibodies (►**Fig. 2A**). The library prepared from the immunoprecipitated NTs was sequenced, and an algorithm designed to detect peaks was employed to identify the m^{6}A sites.[28] A search of motif databases based on the identified m^{6}A peak sites revealed trends in m^{6}A occurrence in the RRACH consensus sequence (R = G or A; and H = A, C, or U).[28] Although RRACH sequences are ubiquitous in specific transcriptome species, instances of their methylation constitute only 1 to 5% of transcripts *in vivo*. Intriguingly, the RRACH motif that undergoes m^{6}A methylation is not randomly distributed on transcripts; rather, it occurs in specific CDSs and 3′-untranslated regions (3′-UTRs), and is most commonly observed close to the termination codon. These findings imply that the RRACH motif might not be solely responsible for modulating m^{6}A accumulation.[28] The results obtained in eukaryotic organisms, such as yeast,[33] plants,[34] fruit flies,[35] zebrafish,[36] and mammals,[37] have demonstrated that the *cis*-regulatory RRACH motif together with CDS/3′-UTR enrichment forms signature traits of the m^{6}A epigenetic transcriptome, thus illustrating the added value of m^{6}A in terms of its function. Given above, the accumulation of m^{6}A in eukaryotic organisms is strictly controlled.

It should be noted that, although m^{6}A-seq can detect m^{6}A modifications at a resolution of 100–200 nt, it lacks information at the single-nucleotide resolution. Site-specific cleavage and radioactive labeling followed by ligation-assisted extraction and thin-layer chromatography (SCARLET) can locate m^{6}A-modification sites down to the individual nucleotide level in RNA samples. Although it is effective for limited applications, it cannot be easily scaled up for the analysis of entire transcriptomes.[38] To boost the granularity of the m^{6}A sequencing information, a technique called photo-crosslinking-assisted m^{6}A sequencing (PA-m^{6}A-seq) has been developed recently. This method uses UV irradiation at 365 nm to crosslink 4-thiouracil with m^{6}A antibodies against mRNA, thereby increasing its resolution to ~23 nt.[33] miCLIP-seq m^{6}A single-nucleotide resolution crosslinking and immunoprecipitation (CLIP) is another method that can be used in this context, which incorporates the implementation of cutting-edge sequencing strategies for precise genomic characterization. By tracing the distinct alterations at the m^{6}A site that are induced by the UV irradiation of anti-m^{6}A antibodies crosslinked to m^{6}A labeling in RNA, it has been shown that m^{6}A residues often occur in clusters and are frequently distributed in the DRACH (D = A/G/U) motif of CDSs and 3′-UTRs.[39] However, miCLIP requires an input of 20 μg of poly(A)$^+$ mRNA[39,40]; therefore, it is necessary to optimize its protocols toward a lower input of the initial sample.

The RNA endonuclease *MazF* from *Escherichia coli* only cleaves the ACA motif specifically from the 5′ side in the absence of methylation. Based on this discovery, researchers developed a new method termed MAZTER-seq (short for m^{6}A-REF-seq) as an antibody-independent method for analyzing m^{6}A.[41,42] Although this new method detects m^{6}A methylation only at the ACA site, it identified the enrichment of a distribution pattern near the termination codon, which is consistent with the conclusions drawn from the antibody-based experiments. Furthermore, a sequencing method termed deamination adjacent to RNA modification targets (DART-seq) was developed as an antibody-free m^{6}A-seq method[43] in which the cytosine deaminase apolipoprotein B mRNA editing enzyme catalytic subunit 1 (APOBEC1) is fused to the YT521-B homology (YTH) domain, which binds to m^{6}A to induce C-U deamination at the m^{6}AC sequence, followed by its detection using standard sequencing methods targeting RNA molecules. DART-seq can pinpoint numerous m^{6}A sites, even among small quantities of total RNA from cells (down to 10 ng), and can measure temporal fluctuations in m^{6}A concentrations. However, because effective transfection remains crucial for the performance of the DART-seq method, its usage in living organisms is currently curtailed.[43]

The antibody-free m^{6}A-seq techniques (MAZTER-seq, m^{6}A-REF-seq, and DART-seq) rely on m^{6}A sequencing or cell transfection.[41,43] A recent study proposed a revolutionary m^{6}A-detection method, i.e., m^{6}A-SEAL, which offers antibody-free fat-mass- and obesity-associated (FTO) protein–assisted chemical labeling for specific detection.[44] In m^{6}A-SEAL, the FTO enzyme promotes the conversion of m^{6}A into a transient hm^{6}A intermediary molecule through thiol addition reactions mediated by DL-dithiothreitol (DTT), thus generating the more stable sulfide-containing dm^{6}A using a simplified installation of biotinylation and other functional tags. The profiling of human and plant transcriptomes using m^{6}A-SEAL confirmed the expected m^{6}A distributions. Moreover, based on comparative analyses against the existing m^{6}A sequencing techniques and the confirmation of specific m^{6}A sites using single-base elongation- and ligation-based qPCR

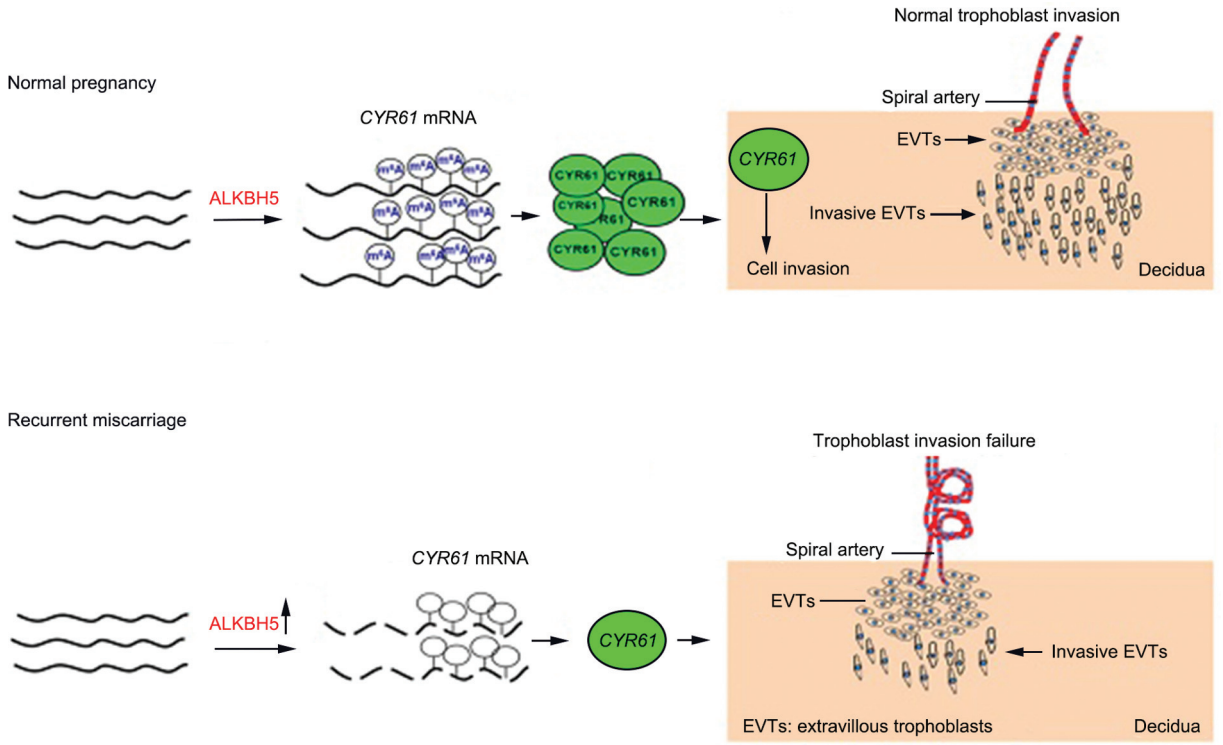

Fig. 3 Functional roles of ALKBH5 and mRNA m^{6}A methylation in trophoblasts. (Reproduced with permission from Li XC, Jin F, Wang BY, et al. The m^{6}A demethylase ALKBH5 controls trophoblast invasion at the maternal–fetal interface by regulating the stability of the *CYR61* mRNA. Theranostics 2019;9(13):3853–3865[50].)

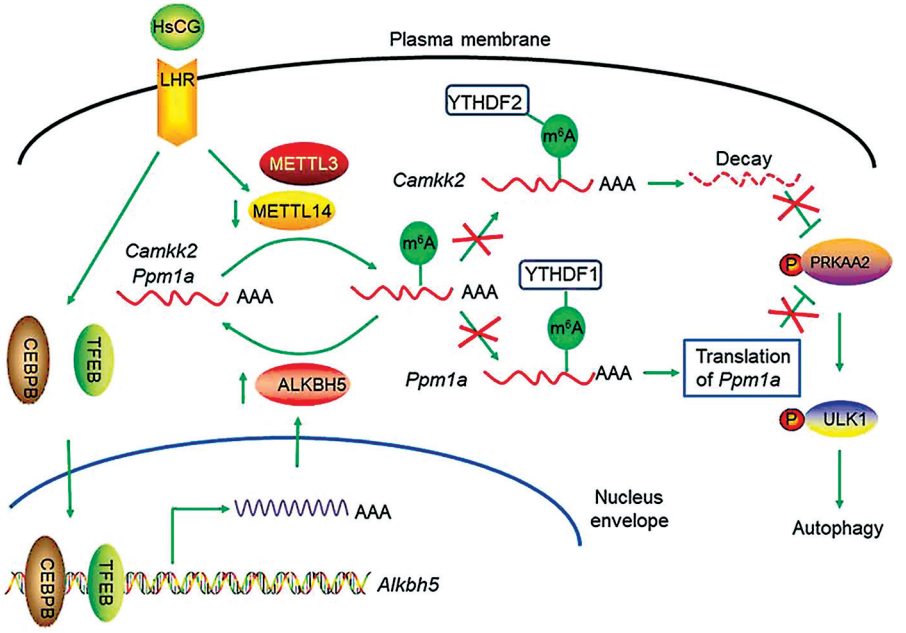

Fig. 4 The proposed model outlines the connection between METTL14/ALKBH5 and autophagy in human chorionic gonadotropin-treated Leydig cells. (Reproduced with permission from Chen Y, Wang J, Xu D, et al. m^{6}A mRNA methylation regulates testosterone synthesis by modulating autophagy in Leydig cells. Autophagy 2020:1–19[64].)

amplification (SELECT), it has been demonstrated that whole-transcriptome scanning via m^{6}A-SEAL yields excellent sensitivity, specificity, and reliability. The versatility of m^{6}A-SEAL, with its FTO-based oxidation and labeling abilities, renders it suitable for diverse applications, ranging from sequencing to enrichment and imaging, thereby advancing m^{6}A research.[44] The combination of this labeling and enrichment strategy with highly specific chemical reactions has attracted great research interest because of its high discrimination ability at the single-base level; furthermore, it has been widely used in studies of the 5-hydroxymethylcytosine, 5-formylcytosine, and pseudouridine (Ψ) RNA modifications.

Biological Function of the M^{6}A Modification: Physiological Regulation and Disease Occurrence

RNA methylation plays an essential role in the regulation of many biological functions, with *N*^{6}-methyladenosine being a prominent example for controlling gene expression, translation, and physiology in many organisms, including humans.[45,46] Three factors govern the reversible m^{6}A modifications (►**Fig. 2B**): (1) methyltransferases (Writers), such as methyltransferase-3 (METTL3), methyltransferase-14 (METTL14), and their cofactors, Wilms tumor 1-associated protein (WTAP), KIAA1429 (VIRMA), HAKAI, ZC3H13, RBM15, and RBM15B[47]; (2) m^{6}A-binding proteins (Readers), such as YTH-domain-family proteins and YTH domain-containing protein 1 (YTHDC1)[48]; and (3) demethylases (Erasers), such as the FTO protein and alkylation repair homolog protein 5 (ALKBH5) (►**Fig. 3**).[49,50] Being abundant within eukaryotic cells, m^{6}A controls crucial processes, such as embryonic growth, stem cell specialization, brain-cell generation, and malignancy.

mRNA m^{6}A modulation imparts singular control over transcripts, and its correct deposition in mRNA is crucial for embryonic development.[51] Research has shown that methyltransferase affects the meiosis process in yeast. The fundamental structure of yeast RNA methyltransferase contains three building blocks that form the Mum2–Ime4–Slz1 (MIS) complex: Mum2 (homologous to mammalian WTAP), Ime4 (homologous to mammalian METTL3), and Slz1 (MIS). Mutating any slz1 in yeast does not cause death, but it can lead to impaired meiosis.[52,53] *Arabidopsis thaliana* mRNA contains m^{6}A, which is similar to that in animal cells. This modification is necessary for embryogenesis.[54] Inactivation of METLL3 in *A. thaliana* leads to the failure of continuous transformation throughout the early developmental stages of embryos.[34,54] During the development of mouse follicles, the ability of RNA metabolism mediated by the m^{6}A methyltransferase KIAA1429 to maintain oocytes is retained.[55] Moreover, m^{6}A determines the transition of endothelial cells to hematopoietic cells during zebrafish embryogenesis, and m^{6}A modification in endothelial cells can specifically regulate the inhibition of the endothelial Notch signals that

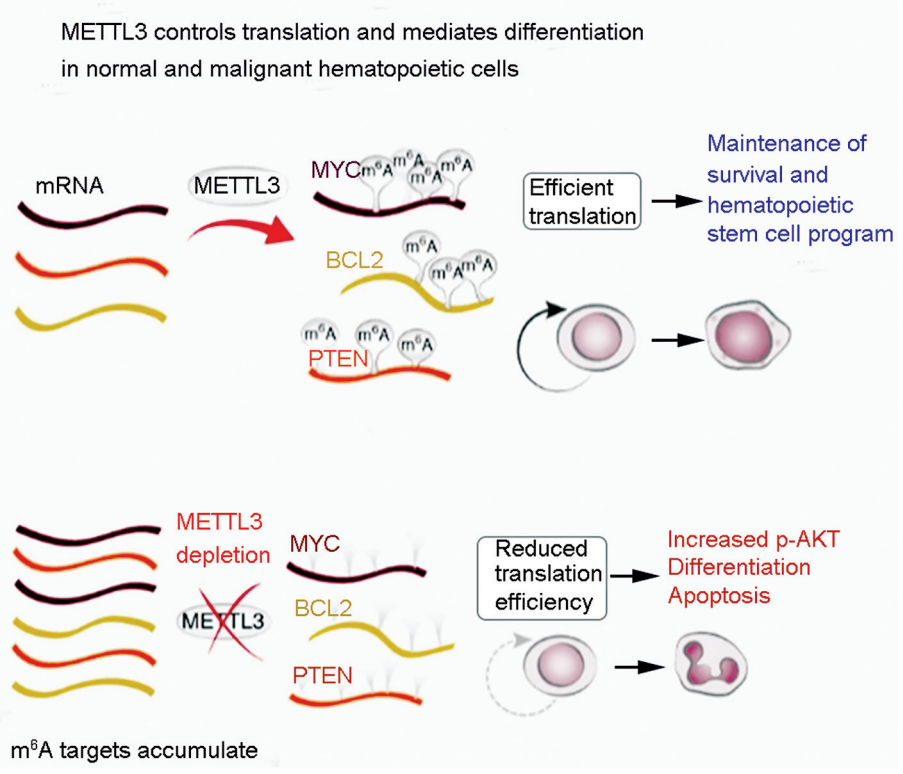

Fig. 5 Proposed model describing the role of METTL3 in the regulation of myeloid differentiation. (Reproduced with permission from Vu LP, Pickering B, Cheng Y, et al. The *N*^{6}-methyladenosine (m^{6}A)-forming enzyme METTL3 controls myeloid differentiation of normal hematopoietic and leukemia cells. Nat Med 2017;23(11):1369–1376[65].)

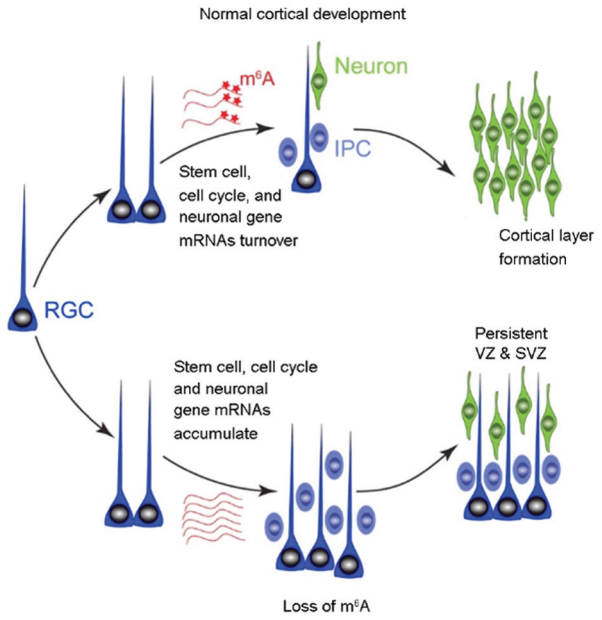

Fig. 6 Illustrations of the downregulation of m^{6}A caused by *METTL14* nullification during the development of mouse brains, which prompted an expanded cell cycle in radial nerve cells and postponed cortical neurogenesis into postnatal stages. (Reproduced with permission from Yoon KJ, Ringeling FR, Vissers C, et al. Temporal control of mammalian cortical neurogenesis by m^{6}A methylation. Cell 2017;171(04):877–889[66].)

trigger the emergence of blood-forming progenitors/stem cells. Furthermore, maternal mRNAs that are dependent on m^{6}A are recognized and cleared by YTHDF2, thus promoting the process from zebrafish-fertilized eggs through the maternal-to-zygotic transition.[56] In turn, YTHDC1 guides splicing and polyadenylation decisions during mouse oocyte maturation.[57] Mice lacking YTHDC2 are infertile because their germ cells do not progress past the zygote stage and are essential for sperm production.[58] Absence of ALKBH5 results in higher concentrations of m^{6}A-modified RNAs in male mice, abnormal apoptosis, and reduced fertility.[59] Recently, a transcriptomics investigation identified links between m^{6}A marks in 5′ UTRs and fetal development/pre-eclampsia in the human placenta.[60]

The presence of m^{6}A affects embryonic stem cell maintenance and fate determination.[13,61] The primordial pluripotent genes of embryonic stem cells, as well as many lineage-specific regulatory genes, carry m^{6}A modifications in their mRNAs.[12,61] Moreover, the maintenance of stem cell characteristics and destiny relies on proper mRNA m^{6}A modification.[62] The inactivation or depletion of METTL3 in mice and humans leads to prolonged expression of NANOG (which is a transcription factor that is involved in the self-renewal of embryonic stem cells) and delays the turnover of embryonic stem cells caused by self-renewal, thereby preventing their differentiation into downstream lineages in the absence of m^{6}A. This study underscored the significance of m^{6}A for stem cell signaling and homeostasis.[12] Knockout of *METTL3* in mice downregulates the m^{6}A levels, causing embryo demise during early gestation.[62] In turn, loss of METTL14 impedes the proliferation and hastens the differentiation of mouse embryonic neural stem cells (NSCs), suggesting that the m^{6}A modification can enhance NSC self-renewal.[63] Recent research indicates that m^{6}A RNA methylation may serve as a promising therapeutic target for various diseases with reduced serum testosterone levels, including azoospermia and oligospermia. A negative correlation exists between the m^{6}A modification and autophagy processes in Leydig cells during testosterone synthesis (►**Fig. 4**).[64] These studies demonstrate the significance of m^{6}A during embryonic growth and starting cell difference projects.

m^{6}A and Acute Myeloid Leukemia

The presence of the m^{6}A modification on mRNA is vital for the advancement and upkeep of acute myeloid leukemia (AML), similar to that observed for the self-restoration of leukemia stem or initiating cells (LSCs/LICs) (►**Fig. 5**).[65] Compared with sound hematopoietic undeveloped/progenitor cells (HSPCs) or different types of malignancy cells, the METTL3 quality in AML cells shows an abundance of mRNA and protein expression.[65] The absence of the shaping compound METTL3 in HSPCs advances cell separation and diminishes cell multiplication. METTL3 controls myeloid separation in AML cells through conditional exhaustion, thus prompting cell separation and apoptosis in living beings and postponing the improvement of leukemia in recipient mice.[65] An examination performed at the

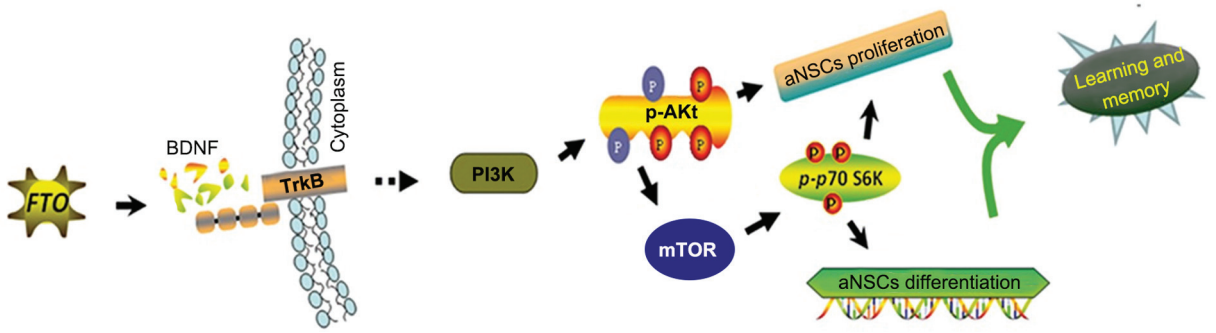

Fig. 7 Proposed mechanism of FTO protein regulation of adult neurogenesis. FTO, fat-mass- and obesity-associated. (Reproduced with permission from Li L, Zang L, Zhang F, et al. Fat mass and obesity-associated (FTO) protein regulates adult neurogenesis. Hum Mol Genet 2017;26(13):2398–2411[71].)

single-nucleotide level revealed that m^{6}A drives the translation of the cellular myelocytomatosis (*c-MYC*), B-cell lymphoma 2 (*BCL2*), and lipid and protein phosphatase and tension homolog (*PTEN*) mRNAs in a human AML cell line (MOLM-13 cells).[65] Moreover, in AML cells, METTL3 is associated with the CAATT-box binding protein C/EBPZ (CCAAT/enhancer-binding protein zeta) at the transcription initiation site, thus triggering m^{6}A to enhance the translation of related mRNAs, which is crucial for the maintenance of the leukemic state.[11] These investigations provide a hypothetical basis for focusing on METTL3 in AML therapeutically.

m^{6}A and Neurological Diseases

Previous research has confirmed that the m^{6}A modification of mRNA can control neurological outcomes and is important for human brain development and neurological diseases.[66–68] Studies have found that *METTL14* knockout or *METTL3* depletion in the cerebrums of developing mouse hatchlings results in the depletion of m^{6}A, thus lengthening the cell cycle and keeping up with the presence of radial nerve cells (►**Fig. 6**).[66] The sequencing of m^{6}A in the cerebral mantle of mice revealed an overflow of mRNA particles connected with transcription factors, neurogenesis, the cell cycle, and neuronal separation, whereas m^{6}A marking advanced its disintegration. The presence of m^{6}A also manages human cortical neurogenesis in the organoids of the prefrontal cortex. The examination of the results of m^{6}A-mRNA sequencing during mouse and human cortical neurogenesis revealed a wealth of human-explicit m^{6}A-marked transcripts connected with qualities that favor illnesses of the mind.[66]

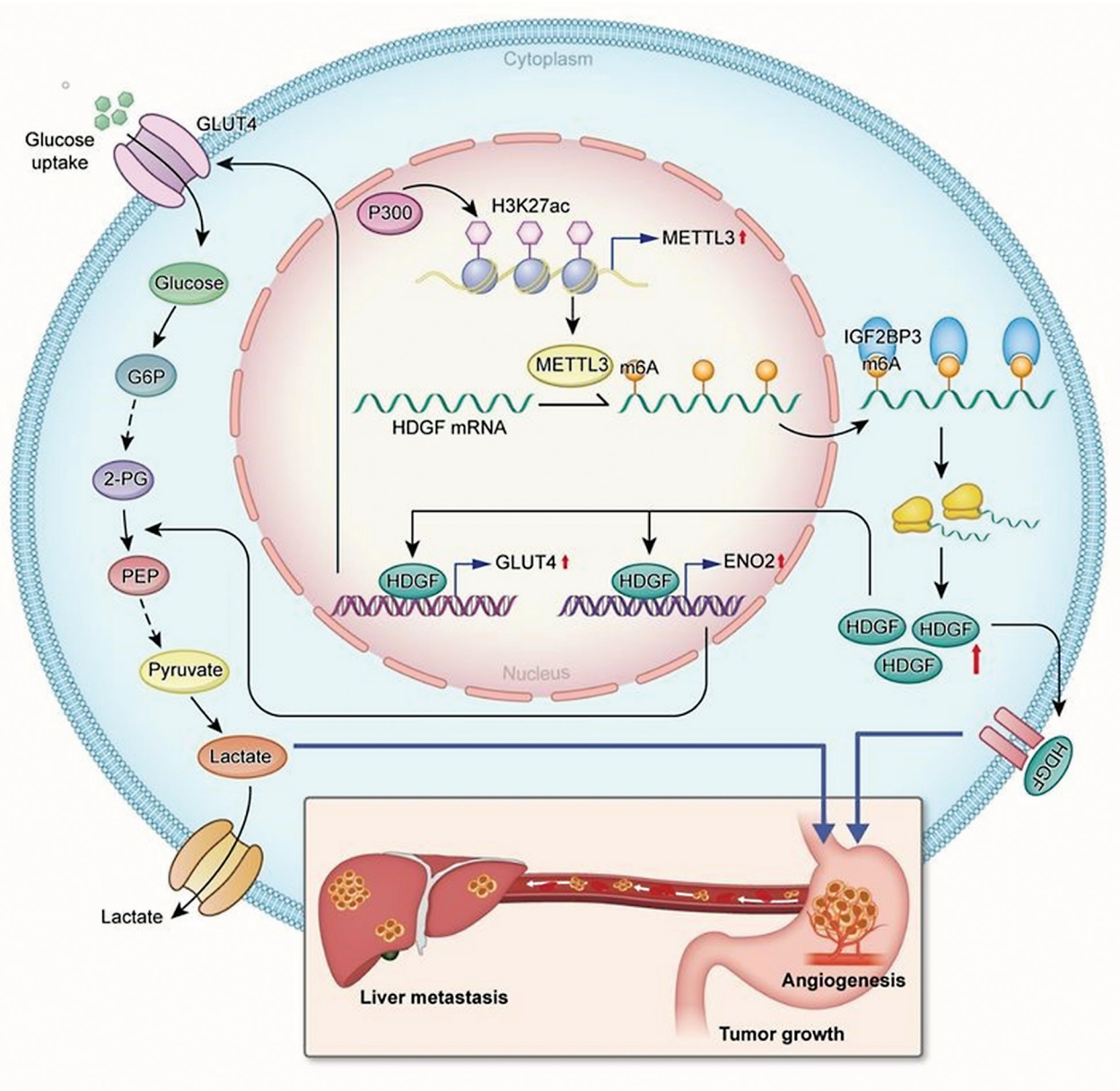

Fig. 8 Graphical representation of the manner in which METTL3 affects tumor glycolysis and angiogenesis, leading to enhanced growth and liver metastasis of gastric cancer tumors. (Reproduced with permission from Wang Q, Chen C, Ding Q, et al. METTL3-mediated m^{6}A modification of HDGF mRNA promotes gastric cancer progression and has prognostic significance. Gut 2020;69(07):1193–1205[82].)

Conditional knockout of *METTL14* in animals prompted a decline in the number of oligodendrocytes and diminished the myelin layers in the focal sensory system. *In vitro*, *METTL14* depletion can disrupt the maturation of oligodendrocytes after mitosis and has a significant effect on the transcriptomes of precursor cells and oligodendrocytes. In turn, abnormalities of oligodendrocytes can trigger not only demyelinating infections of the focal sensory system, but also neuronal harm or mental issues, and, cerebrum tumors. Moreover, the deletion of *METTL14* in oligodendrocyte cell lines induces a strange joining of various RNA molecules.[67,69] Via behavioral and functional magnetic resonance imaging studies, genetic variations in the m^{6}A demethylase FTO can affect the response of the dopamine-dependent midbrain to reward learning, as well as behavioral responses related to learning from negative outcomes.[70]

Based on whole-genome m^{6}A analysis and the observation of dynamic m^{6}A modifications during postnatal neurodevelopment, it was determined that FTO deficiency causes changes in the articulation of specific fundamental parts of the brain-prompted neurotrophic factor pathway, denoted by m^{6}A (►**Fig. 7**).[71] These examinations suggest that FTO plays a significant role in neurogenesis, learning, and memory. Concomitantly, previous research has found that the m^{6}A demethylase genes *FTO* and *ALKBH5* are associated with major depressive disorder.[72] Furthermore, it has been reported that the insufficiency of FTO can lessen uneasiness and melancholy-like behavior in rodents by changing the gut flora.[73,74] Moreover, FTO is firmly associated with insulin-inadequacy-related Alzheimer's disease, and conditional deletion of *FTO* in neurons can decrease intellectual disability in diseased mice.

m^{6}A and Cancers

The m^{6}A modification of mRNA is deeply implicated in the origins and progression of malignancies.[75,76] The downregulation of the m^{6}A methyltransferase METTL3 or METTL14 significantly promotes the growth, self-renewal, and tumorigenesis of human glioblastoma stem cells (GSCs).[77,78] METTL3 coordinates the successful execution of carcinogenic pathways in GSCs.[79] An increasing number of studies have confirmed that METTL3 promotes the progression of liver cancer,[80] bladder cancer,[81] gastric cancer (►**Fig. 8**),[82,83] breast cancer (►**Fig. 9**),[84] colorectal cancer (►**Figs. 10** and **11**),[85,86] prostate cancer,[87] and other tumors in an m^{6}A-dependent manner.

A recent study confirmed that the miR-186–METTL3 axis promotes the growth of colorectal cancer through the Wnt–*β*-catenin signaling pathway. The *β*-catenin signaling pathway promotes the development of hepatoblastoma (►**Fig. 12**).[88] In addition, METTL3 can promote chemotherapy and radiation resistance in various tumors, such as pancreatic cancer.[89]

Recently, another study found that the silencing of *METTL3* downregulates the mesenchymal–epithelial transition factor (c-Met), phosphorylated Akt (p-Akt), and cell-cycle-related proteins in uveal melanoma (UM) cells, leading to G1 arrest of the cell cycle and restricting UM cell multiplication, aggregation, and mobility.[90] The Epstein–Barr virus (EBV) is a ubiquitous cancer-causing virus that can induce various types of tumors. The reprogramming of EBV antigenic determinants through METTL14 is crucial for the development of virus-associated tumors (►**Fig. 13**).[91] The aforementioned studies revealed that the m^{6}A methyltransferase METTL3 or METTL1 may be a potential target for cancer treatment.

The m^{6}A-modified mRNA-binding protein YTHDF1 is overexpressed in various cancers, including non-small-cell lung cancer (►**Fig. 14**),[92] colorectal cancer,[93] liver cancer,[94] and ovarian cancer (►**Fig. 15**)[95]; moreover, the depletion of *YTHDF1* can inhibit tumor development. Furthermore, *in vitro* studies have shown that *YTHDF1* knockdown can facilitate the responsiveness to chemotherapy agents, such as fluorouracil and oxaliplatin.[96] Snail is a key transcription factor in the epithelial–mesenchymal transition. Studies of loss and gain of function have confirmed that the YTHDF1-mediated m^{6}A modification of the *snail* mRNA enhances its translation.[97] Research has demonstrated that by promoting the translation of the m^{6}A-modified cathepsin mRNA, YTHDF1 enhances antigen degradation in phagosomes and limits the cross-presentation of new antigens in dendritic cells, which may become a target for tumor immunotherapy.[98] Moreover, YTHDC2 upregulates HIF-1α and other factors, thus driving tumor spread through the modulation of gene/protein expression.[99] In turn, the inhibition of YTHDC2 can greatly promote the proliferation of tumors, including esophageal squamous cell carcinoma, by affecting several cancer-related signaling pathways.[100,101]

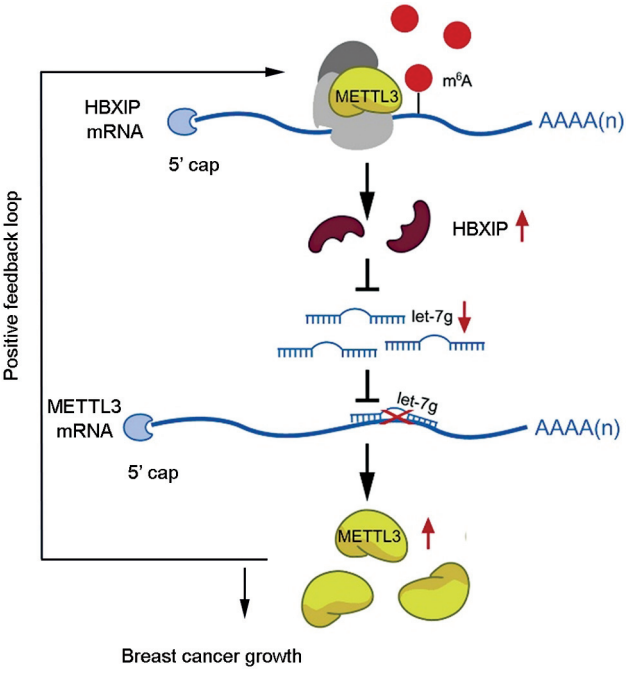

Fig. 9 Schematic representation of the manner in which the oncoprotein HBXIP upregulates METTL3 by suppressing the levels of the tumor suppressor let-7g, which contributes to breast cancer progression. (Reproduced with permission from Cai X, Wang X, Cao C, et al. HBXIP-elevated methyltransferase METTL3 promotes the progression of breast cancer via inhibiting tumor suppressor let-7g. Cancer Lett 2018;415:11–19[84].)

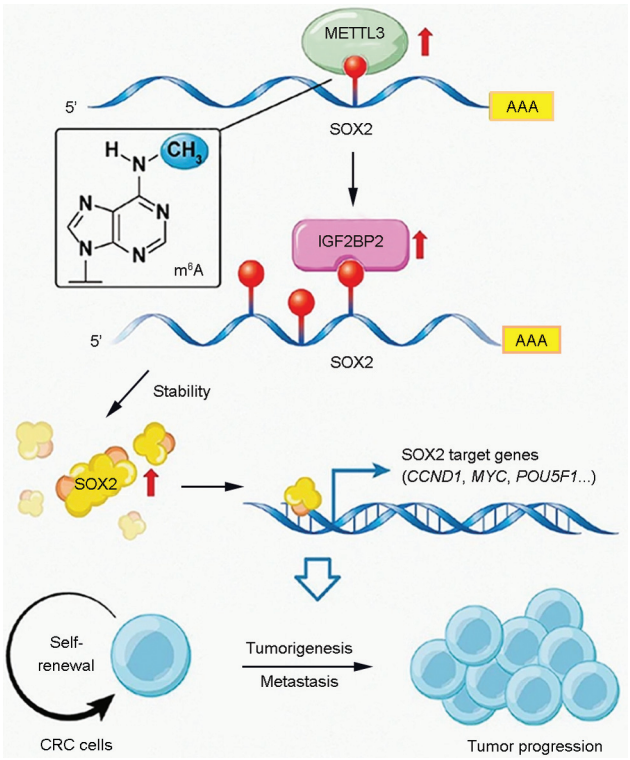

Fig. 10 Proposed model outlining the role of *METTL3* as an oncogene in the maintenance of SOX2 expression via an m^{6}A-IGF2BP2-dependent process in colorectal cancer cells. The model also suggests a potential biomarker panel for predicting outcomes in patients with CRC. (Reproduced with permission from Li T, Hu PS, Zuo Z, et al. METTL3 facilitates tumor progression via an m^{6}A-IGF2BP2-dependent mechanism in colorectal carcinoma. Mol Cancer 2019;18(01):112[85].)

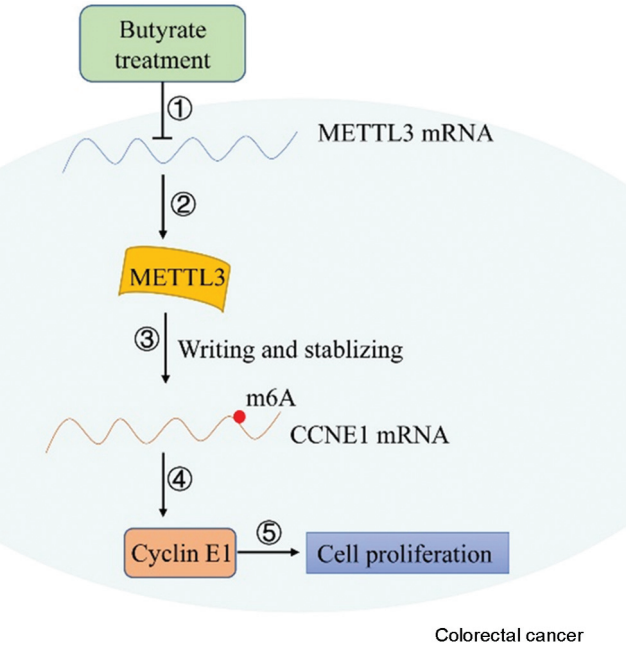

Fig. 11 Proposed model of the manner in which METTL3 enhances CRC cell proliferation via the stabilization of *CCNE1* mRNAs in an m^{6}A-dependent manner. CRC, colorectal cancer; METTL3, methyltransferase-like 3. (Reproduced with permission from Zhu et al. Methyltransferase 3 promotes colorectal cancer proliferation by stabilizing CCNE1 mRNA in an m^{6}A-dependent manner. J Cell Mol Med 2020;24(06):3521–3533[86].)

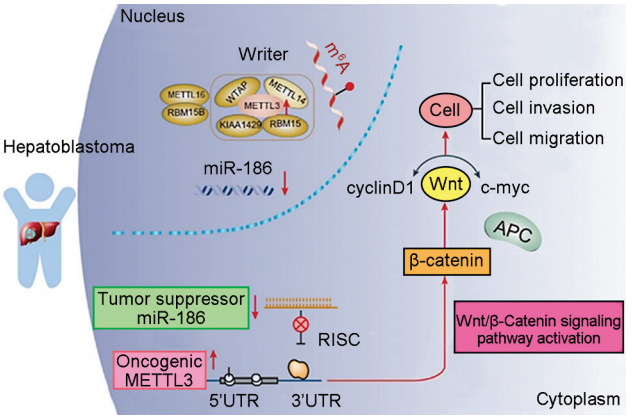

Fig. 12 Suggested model outlining the functional role of the miR-186–METTL3 axis in hepatoblastoma. (Reproduced with permission from Cui X, Wang Z, Li J, et al. Cross-talk between RNA *N*^{6}-methyladenosine methyltransferase-like 3 and miR-186 regulates hepatoblastoma progression through Wnt/beta-catenin signaling pathway. Cell Prolif 2020;53(03):e12768[88].)

The insulin-like growth factor-2 mRNA-binding proteins 1, 2, and 3 (IGF2BP1/2/3) are unique families of m^{6}A "readers" that target thousands of mRNA transcripts by recognizing the common GG (m^{6}A) C sequence. These factors may play a carcinogenic role in cancer cells by stabilizing the methylation of mRNAs that target carcinogenic targets, such as c-MYC (►**Fig. 16**).[102–104] In addition, IGF2BP1 can disrupt the stability of the highly upregulated lncRNA in liver cancer through the CCR4–NOT (a highly conserved specific gene silencer) adenylate kinase complex, which is highly linked to liver cancer progression and severity.[105] The *IGF2BP3* gene is a downstream target of the carcinogenic effector Lin28b. In a mouse model, it was found that the overexpression of IGF2BP3 drives liver tumorigenesis.[106] Finally, IGF2BP2 and IGF2BP2-2 can promote the occurrence of liver cancer.[107,108]

It has been reported that the m^{6}A demethylases FTO and ALKBH1 are associated with various malignant tumors,[109–113] such as the carcinogenic role of FTO in AML (►**Fig. 17**).[114] Through autophagy and NF-κ, nutrient-deprivation-triggered

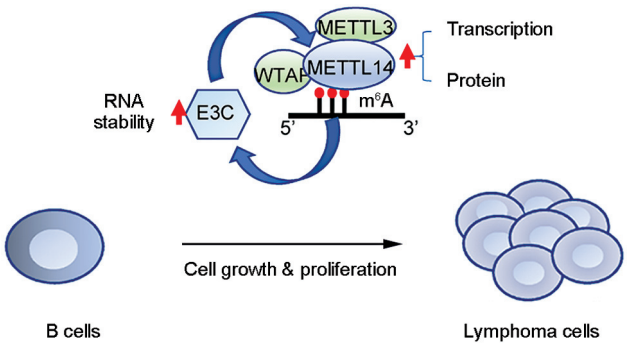

Fig. 13 Illustration of the interaction between the EBV antigens EBNA3C and METTL14 and EBV transcripts that are modified by METTL14 and its associated proteins. EBV, Epstein–Barr virus; METTL14, methyltransferase-14. (Reproduced with permission from Lang F, Singh RK, Pei Y, Zhang S, Sun K, Robertson ES. EBV epitranscriptome reprogramming by METTL14 is critical for viral-associated tumorigenesis. PLoS Pathog 2019;15(06):e1007796[91].)

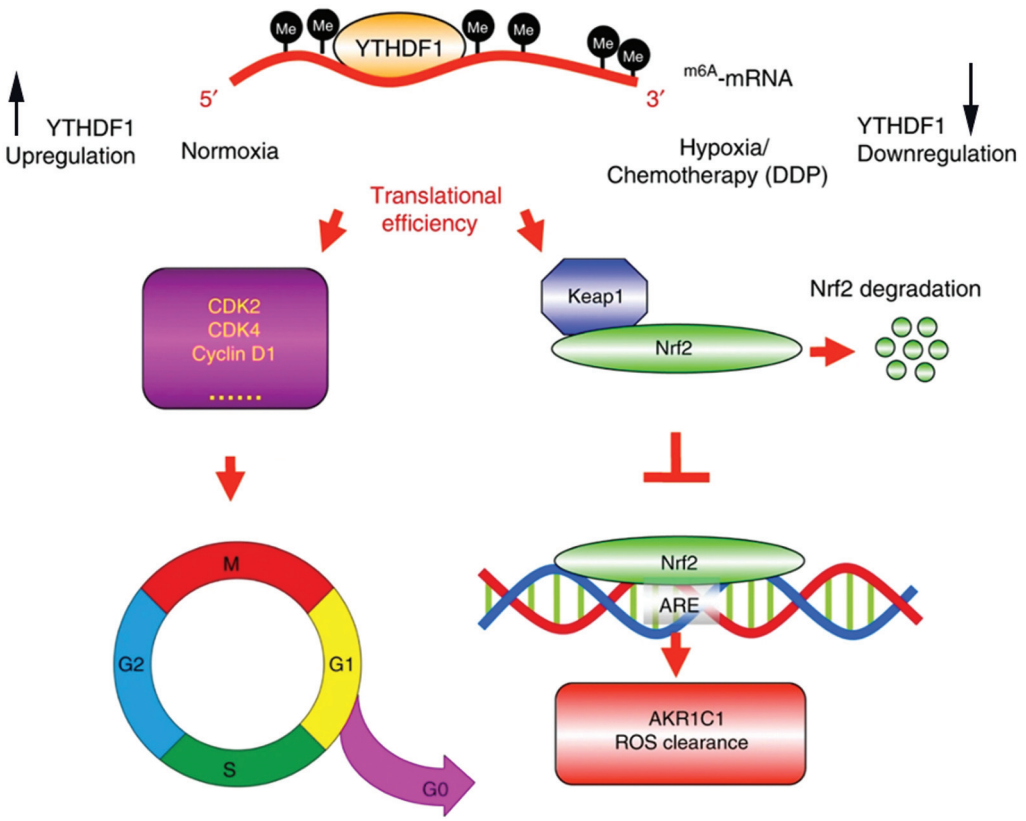

Fig. 14 Proposed model of non-small-cell lung cancer development under normal oxygen conditions: increased or high expression of YTHDF1 primarily boosts the translation efficiency of m^{6}A-modified target transcripts, such as the cell–cycle regulators CDK2, CDK4, and Cyclin D1, within cancerous cells, resulting in uncontrolled cancerous cell proliferation. (Reproduced with permission from Shi Y, Fan S, Wu M, et al. YTHDF1 links hypoxia adaptation and non-small cell lung cancer progression. Nat Commun 2019;10(01):4892[92].)

FTO in the B pathway leads to heightened melanoma onset in human and murine models. This study suggests that FTO inhibition combined with anti-programmed death-1 (anti-PD-1) blockade may reduce the resistance of melanoma to immunotherapy (►**Fig. 18**).[115] By modulating the m^{6}A status of the *E2F1* and *MYC* transcripts, FTO potentiates the proliferation and movement of cervical cancer cells.

Intrahepatic cholangiocarcinoma (ICC) is second only to primary liver cancer as the deadliest form of primary liver cancer with a high prevalence. A Kaplan–Meier survival

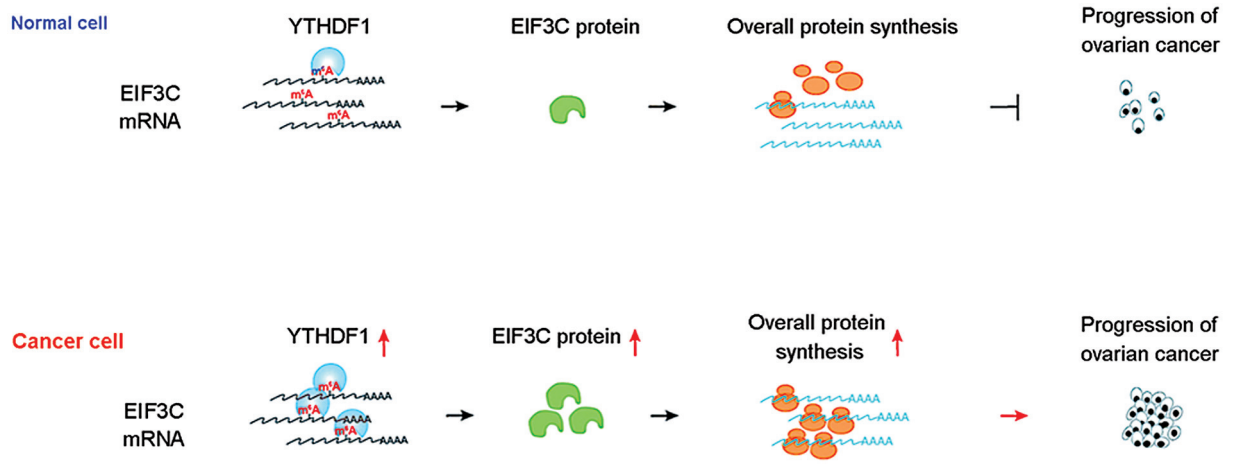

Fig. 15 The proposed model explorations of the role of YTHDF1-mediated EIF3C translation in ovarian cancer. (Reproduced with permission from Liu T, Wei Q, Jin J, et al. The m^{6}A reader YTHDF1 promotes ovarian cancer progression via augmenting EIF3C translation. Nucleic Acids Res 2020;48(07):3816–3831[95].)

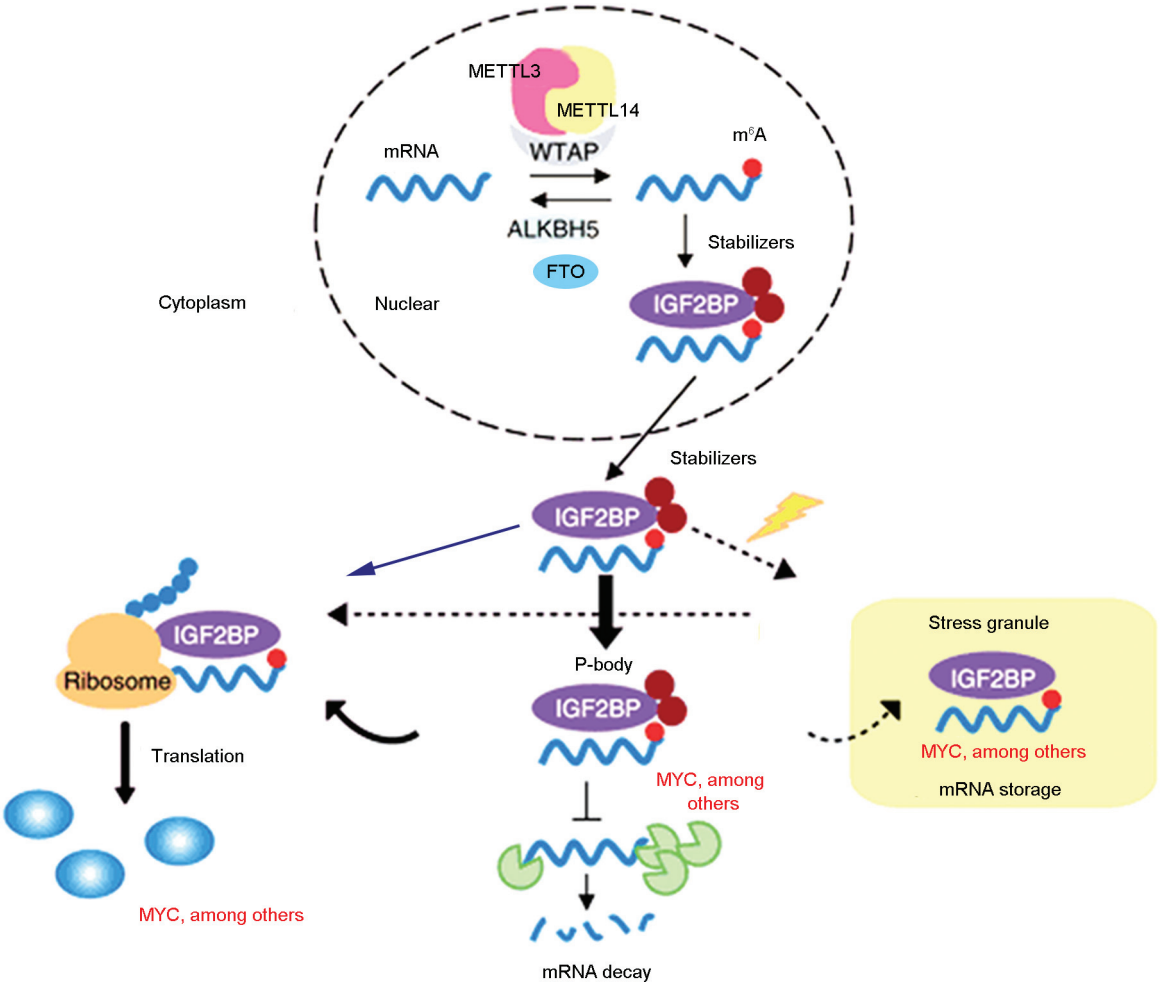

Fig. 16 IGF2BP-mediated control of m^{6}A mRNA expression. mRNAs undergo methylation in the nucleus and selectively associate with IGF2BPs, thus maintaining the stability of P-bodies and enhancing translation until their sequestration in SGs under heat shock. (Reproduced with permission from Huang H, Weng H, Sun W, et al. Recognition of RNA *N*^{6}-methyladenosine by IGF2BP proteins enhances mRNA stability and translation. Nat Cell Biol 2018;20(03):285–295[102].)

analysis showed that low expression of FTO promotes the development of ICC, indicating a poor prognosis of ICC.[116,117] Head and neck squamous cell carcinoma (HNSCC) is the sixth most-common cancer worldwide, with oral squamous cell carcinoma (OSCC) being an aggressive form of HNSCC. Recent research has found that DDX3 (a highly conserved subfamily of the DEAD-box proteins) regulates ALKBH5 to downregulate the m^{6}A methylation level in FOXM1 (Forkhead box, FOX) and NANOG (Nanog Homeobox) new transcripts, thus promoting cisplatin resistance in OSCC.[118] A recent study confirmed that decreased levels of the *ALKBH5* and *FTO* mRNAs are associated with shorter overall survival and cancer-specific survival after nephrectomy.[119] These investigations offer possibilities for novel therapeutic and prognostic avenues for cancer management. The implication of FTO in cancer advancement has prompted interest in drugs such as FB23 and FB23-2, which bind to FTO and inhibit its m^{6}A function.[114] In addition, fluorescein-based compounds have shown potential for targeting FTO demethylation.[120] Meclofenamic acid has been verified as a potent inhibitor that is specific to FTO, with a higher selectivity for FTO versus ALKBH5.[121] The physiological regulation and occurrence of human diseases discussed above focus only on m^{6}A, which is by far the most important chemical modification. In addition to chemical modifications, such as methylation (for example, m^{6}A, m^{5}C, etc.), there is a great need for basic research on the molecular mechanisms of other chemical modifications, together with their links to illness onset and progression in humans, especially the geranyl modification and nicotinamide adenine dinucleotide (NAD) cap modification reported recently.

Recent Research on Newly Discovered RNA Modifications and Their Implications for Cellular Biology

Although the mechanism underlying the RNA chemical modifications remains unclear, especially that of the m^{6}A modification, its role in biological processes has gained increasing

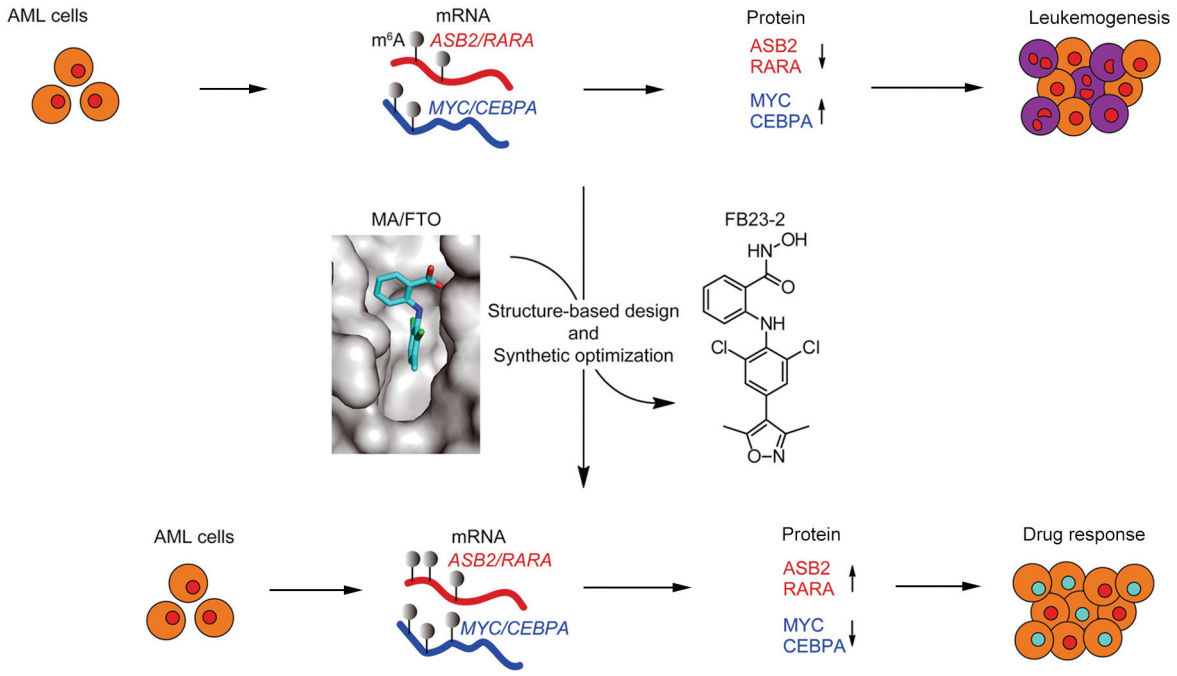

Fig. 17 Illustrations of FTO as a viable drug target. The use of small-molecule inhibitors to target FTO may offer potential therapeutic benefits for AML. AML, acute myeloid leukemia; FTO, fat-mass- and obesity-associated. (Reproduced with permission from Huang Y, Su R, Sheng Y, et al. Small-molecule targeting of oncogenic FTO demethylase in acute myeloid leukemia. Cancer Cell 2019;35(04):677–691[114].)

recognition, despite the uncertainty about its impact on humans. This review focuses on the progress of high-throughput sequencing technology for the m^{6}A modification; its physiological regulation; and its role in cancer occurrence, cancer development, and tumor suppression. Accumulating evidence indicates that RNA m^{6}A modifiers offer promising biomarker prospects for cancer prognosis and therapy planning, such as in the context of rectal cancer,[122] non-small-cell lung cancer,[123] and renal cell carcinoma.[124] Recent research has found that m^{6}A-mediated long noncoding RNA00958 (LINC00958) upregulation increases adipogenesis and represents a promising nanomedicine target for hepatocellular carcinoma.[125] Because of its pivotal role in multiple diseases, targeting m^{6}A may prove beneficial for the diagnosis and management of illnesses such as AML, glioblastoma, and breast cancer. In addition, scientists recently used the human metapneumovirus (HMPV) as a representative sample, and it was discovered that m^{6}A confers evasion of RNA sensors such as RIG-I on viral RNA (RIG-I), thus confirming that viral RNA m^{6}A can be used as a target for the development of attenuated HMPV vaccines (►**Fig. 19**).[126]

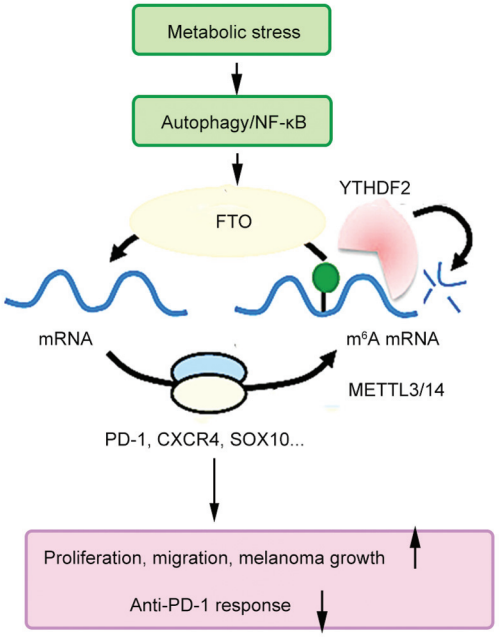

Fig. 18 Proposed model outlining the regulatory and functional impact of FTO on melanoma development and the response to anti-PD-1 blockade therapy. anti-PD-1, anti-programmed death-1; FTO, fat-mass- and obesity-associated. (Reproduced with permission from Yang S, Wei J, Cui YH, et al. m^{6}A mRNA demethylase FTO regulates melanoma tumorigenicity and response to anti-PD-1 blockade. Nat Commun 2019;10(01):2782[115].)

In summary, unraveling the significance of and the mechanisms underlying m^{6}A in nucleic acids holds promise for designing new antitumor treatments, which can provide insights for the development of novel therapeutic strategies.

As a popular research field in recent years, the recognition of new types of RNA modification, the investigation of gene activities involved in RNA modifications, and the elucidation of pathogenic mechanisms are valuable avenues for drug development. A typical example is the NAD variant of the RNA cap that commonly occurs outside of the canonical m^{7}G type reported by Liu et al in 2009 (►**Fig. 20**). With the advancement of detection technology, NAD cap structures were first discovered in *E. coli*,[127] followed by yeast, cell lines, plants, and mouse tissues.[128–132] According to the reported results, NAD cap formation occurs simultaneously with RNA transcription. Concomitantly, the existing RNA sequencing

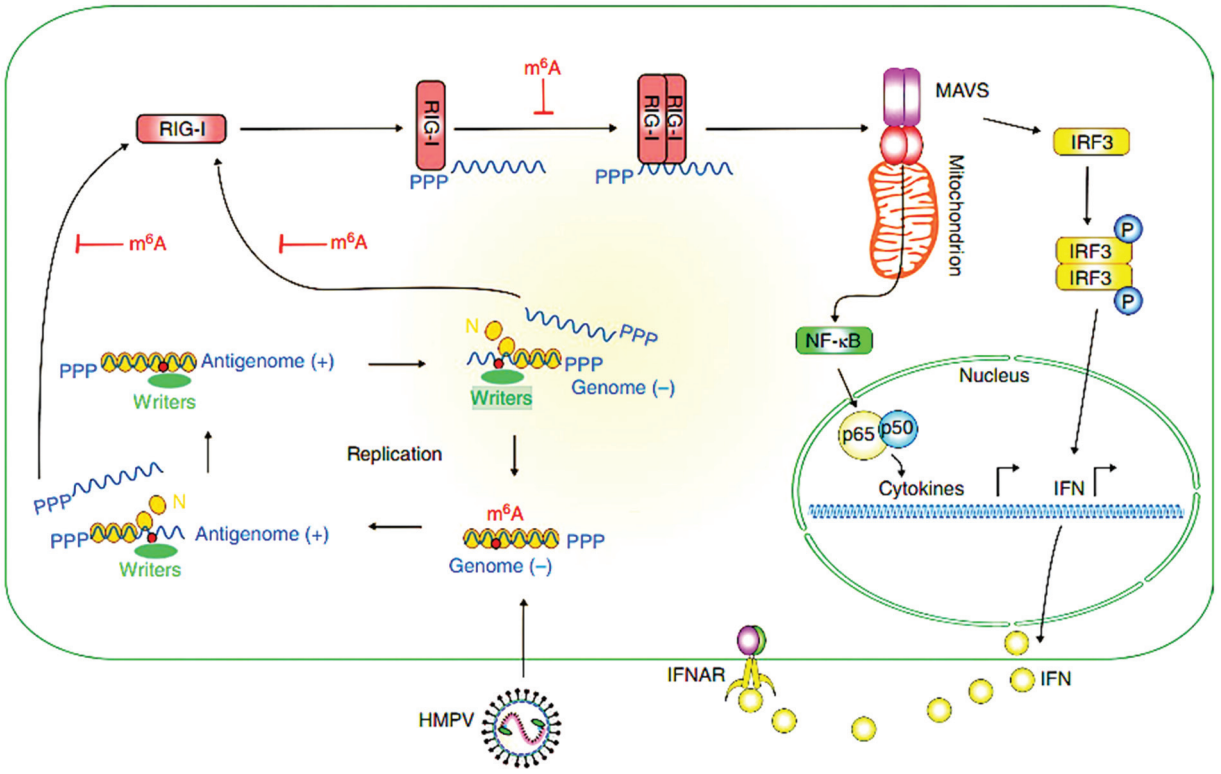

Fig. 19 Schematic representation outlining the RIG-I-triggered interferon response after HMPV entry. Viral particles travel to the cytoplasm, and RdRp transcribes starting at the terminal 3′ genomic section, forming full antigenomes that serve as templates for producing copycat viral RNA. (Reproduced with permission from Lu M, Zhang Z, Xue M, et al. N^6-methyladenosine modification enables viral RNA to escape recognition by the RNA sensor RIG-I. Nat Microbiol 2020;5(04):584–598[126].)

technology has shown that the enzyme NUDT12 (a deNADding enzyme), which removes the NAD cap structure from RNA, has a close regulatory effect on the circadian clock in the liver. Although significant achievements have been reached, e.g., for NAD tagSeq., research on the underlying molecular mechanism remains very limited.

Another typical example was reported in 2012 by Liu et al in which the strongest hydrophobic geranyl (pentenyl dimer)

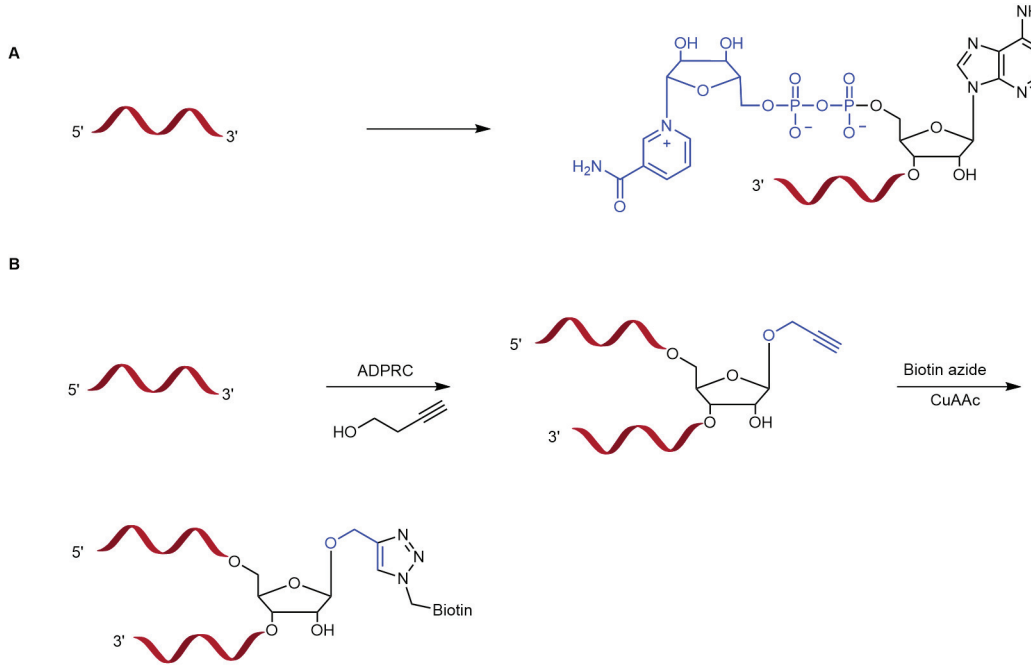

Fig. 20 (**A**) Schematic illustrations of the RNA 5′-NAD capping process and (**B**) its capturing sequencing approach (NAD tagSeq).

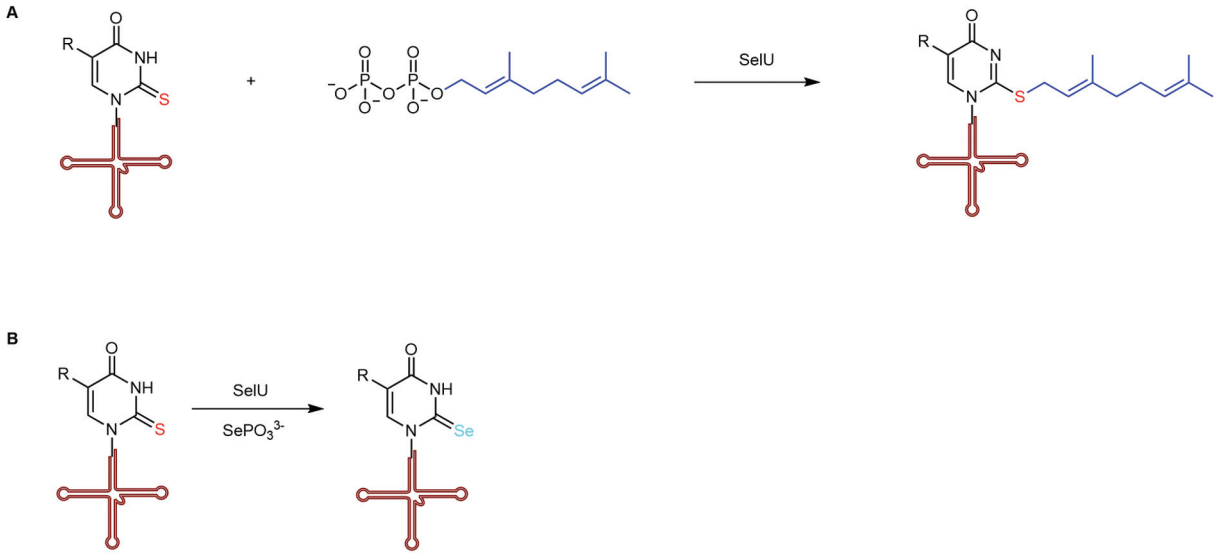

Fig. 21 Overall illustrations of the possible pathway for geranylated RNA generation. (**A**) Geranylated RNA (ges^{2}U-RNA) was synthesized from s^{2}U-RNA, which was mediated by the SelU enzyme. (**B**) Proposed pathway of selenide RNA (se^{2}U-RNA) generation through the geranylated RNA intermediate (ges^{2}U-RNA).

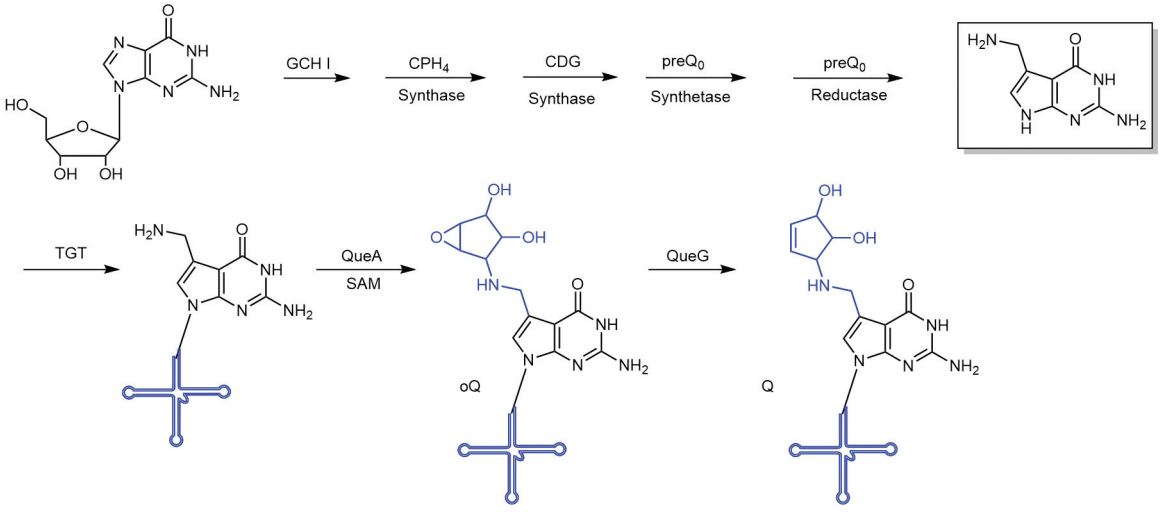

Fig. 22 Proposed biosynthetic pathway for the oQ modification of RNA.

modification to date exists at position 34 of the bacterial tRNA. Geranylation of RNA *in vivo* is achieved by the enzyme tRNA 2-selenouridine synthase (SelU) (►**Fig. 21**). Preliminary studies revealed that the geranyl-modified group can shape the structure and operational properties of RNAs after binding to a specific RNA, as well as its localization within the cell. However, the molecular mechanism underlying this lipid modification is completely unknown.[133] The development of specific markers and localization and enrichment technologies for aryl-modified RNAs will be of great research significance, by laying a solid foundation for explaining its molecular mechanism and even exploring its association with human diseases.

Another noteworthy example is the epoxy-queuosine (oQ) modification reported by Bandarian et al in 2011. The biosynthetic pathway for this modification is shown in ►**Fig. 22**; however, the molecular mechanism via which the modifying enzymes QueA and QueG regulate the levels of modified oQ and Q remains unknown, especially regarding its possible link to human diseases.[134]

Summary

With the in-depth development of multidisciplinary and interdisciplinary research, an increasing number of new chemically modified RNAs have been reported using comprehensive methods, such as those based on chemistry, life sciences, medicine, and pharmacy. Although these new chemically modified groups perform diverse structural and functional roles in RNA, little is known about their regulatory molecular mechanisms. The development of simple and highly selective chemical and biological methods for their

detection, labeling, and localization is of practical significance for elucidating their underlying molecular mechanisms. In particular, the development of highly specific chemical and biological labeling and detection tools for these new chemically modified groups, as well as high-throughput sequencing, will shed light on the molecular underpinnings of RNA modifications. Thus, the development of reliable tools targeting these RNA modifications has great application potential.

In addition, the manner in which these new chemical modification groups are implicated in the pathogenesis of human ailments is particularly noteworthy. The development and application of inhibitors, regulation strategies, and corresponding mRNA-targeted degradation technologies for this new chemically modified enzyme (including encoding and decoding) targets will have practical research significance.

In recent years, in particular, COVID-19 has been rampant worldwide, with very few specific therapeutic drugs or vaccines being available for this condition.[135] Research has shown that there are many unknown chemical modifications in the RNA of this virus that have not been reported. The impact of these modifications on viral characteristics remains elusive, as well as the types, molecular mechanisms, and targets of these modifications.[136] At present, the role played by these new chemical modifications (NAD, prenyl, and oQ modifications) remains unknown,[137–145] although it has been speculated that they may help the virus avoid host attacks (for example m^{6}A, m^{1}A, and Ψ). Therefore, elucidating the types and characteristics of these chemical modifications is expected to provide new clues for the fight against COVID-19 and many other RNA-relevant viruses. In addition to the application of mature technological tools, the development of new technological toolsets to identify them and the implementation of highly specific small-molecule inhibitors are of great importance to human society.

In summary, significant advancements have been made in qualitative/quantitative m^{6}A detection, high-throughput sequencing, and research linking it to disease. Efforts continue toward the development of simplified and more accessible chemical/biological technologies that will contribute to precision medicine, thus benefiting society and patients alike.

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Conflict of Interest
None declared.

Acknowledgments
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