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REVIEW ARTICLE

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The role of N6-methyladenosine modification in the life cycle and disease pathogenesis of hepatitis B and C viruses

Geon-Woo Kim ¹ and Aleem Siddiqui¹

Abstract

N6-methyladenosine (m⁶A) is the most prevalent modification of mammalian cellular RNAs. m⁶A methylation is linked to epigenetic regulation of several aspects of gene expression, including RNA stability, splicing, nuclear export, RNA folding, and translational activity. m⁶A modification is reversibly catalyzed by methyltransferases (m⁶A writers) and demethylases (m⁶A erasers), and the dynamics of m⁶A-modified RNA are regulated by m⁶A-binding proteins (m⁶A readers). Recently, several studies have shown that m⁶A methylation sites have been identified in hepatitis B virus (HBV) transcripts and the hepatitis C virus (HCV) RNA genome. Here, we review the role of m⁶A modification in HBV/HCV replication and its contribution to liver disease pathogenesis. A better understanding of the functions of m⁶A methylation in the life cycles of HBV and HCV is required to establish the role of these modifications in liver diseases associated with these viral infections.

Introduction

Eukaryotic cellular RNAs contain diverse chemical modifications, including N6-methyladenosine (m⁶A), 5-methylcytidine (m⁵C), uridine to pseudouridine (U to Ψ), adenosine to inosine (A to I), and addition to N7-methylguanosine (m⁷G)¹. Among the diverse RNA modifications, m⁶A methylation, methylation of the adenosine base at the nitrogen 6 position, is the most well-characterized and the most common modification of cellular RNAs^{2,3}. This modification has been linked to various biological processes, including innate immune responses, sex determination, stem cell differentiation, circadian clock regulation, meiosis, stress responses, and cancer development³. m⁶A methylation was first identified in the 1970s but the technology to map individual-specific m⁶A sites in a given RNA became available only recently⁴. The development of highly sensitive detection methods with high-throughput sequencing revealed the topology of m⁶A in the cellular transcriptome^{2,5}. Over

25% of mammalian transcripts contain m⁶A modifications and m⁶A modification occurs within the consensus DRACH/RRACH motif (D = A, G, or U; R = G or A; H = A, C, or U)². Furthermore, this modification is typically enriched near the stop codon and the 3'-untranslated region (UTR)². Similar to DNA methylation, m⁶A methylation is reversibly catalyzed by various methyltransferases and demethylases (Fig. 1). The cellular m⁶A methyltransferase machinery is composed of methyltransferase-like 3 (METTL3), METTL14, and WT1-associated protein (WTAP)^{6,7}. Other additional subunits, such as Vir like m⁶A methyltransferase associated (VIRMA), zinc finger CCCH-type containing 13 (ZC3H13), and RNA-binding motif protein 15/15B (RBM15/15B), are also components of the m⁶A machinery^{8–11}. WTAP regulates the recruitment of the optimal substrate and nuclear localization of METTL3/14⁸. RBM15/15B interacts with the U-rich RNA regions, ZC3H13 is required for nuclear import of the METTL3/14 complex, and VIRMA is necessary for writing m⁶A in the 3'-UTR^{9–11}. Fat mass and obesity-associated protein (FTO) and AlkB homolog 5 (ALKBH5) are m⁶A

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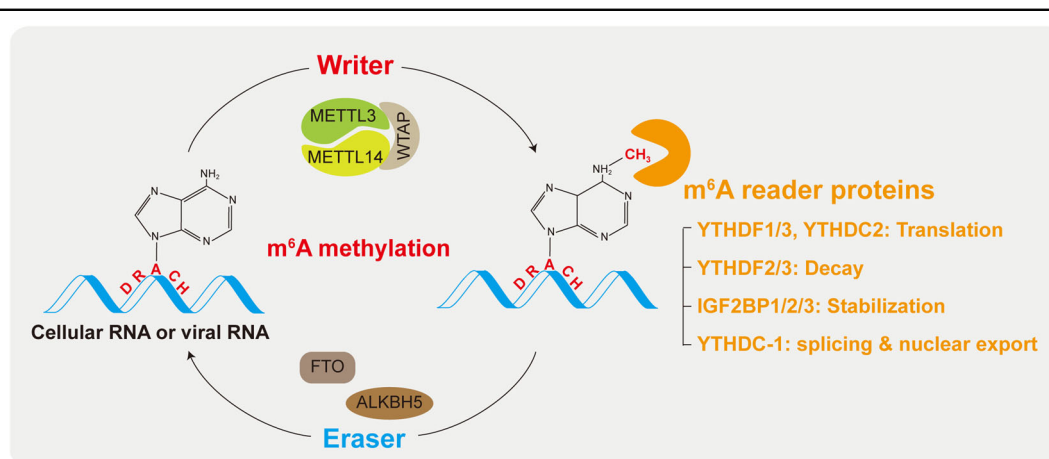


Fig. 1 The roles of the cellular m^6A machinery and m^6A reader proteins in regulating cellular RNA and viral RNA. m^6A modification occurs in consensus DRACH motifs of cellular and viral RNAs. This modification is reversibly catalyzed by an m^6A 'writer' or 'eraser'. The m^6A 'writer' (methyltransferase) complex is composed of METTL3, METTL14, and WTAP, and FTO or ALKBH5 is m^6A 'eraser' (demethylase). The dynamics of m^6A -modified RNAs are regulated by the m^6A 'reader' proteins, including YTHDF1/2/3, YTHDC1/2, and IGF2BP1/2/3.

demethylases that remove m^6A from cellular RNA (Fig. 1)^{12,13}. The dynamics of m^6A modified RNAs are regulated by m^6A readers, YT521-B homology (YTH) domain family proteins (YTHDF1/2/3 and YTHDC1/2)¹⁴. YTHDF3 first recognizes m^6A -modified RNA and recruits the YTHDF1 or 2 protein¹⁵. The YTHDF1-YTHDF3 complex induces the translation of m^6A -methylated mRNA, while the YTHDF2-YTHDF3 complex causes the degradation of its target mRNA degradation^{16,17}. Thus, YTHDF3 regulates mRNA degradation and translation by cooperating with YTHDF1 or 2. Because YTHDF2 has no RNase activity, it interacts with the CCR4-NOT (C-C motif chemokine receptor 4 negative on TATA-less) deadenylase complex to promote the degradation of its target RNA¹⁷. YTHDC1 regulates mRNA nuclear export in cooperation with the major export receptor NXF1, as well as RNA splicing^{18,19}. YTHDC2 is the only m^6A reader protein containing an RNA helicase domain and induces the translation of m^6A -modified mRNA by interacting with a small ribosomal subunit^{20,21}. The helicase activity of YTHDC2 is essential for YTHDC2-mediated mRNA translation, implying that YTHDC2 helps to resolve mRNA secondary structure²². Thus, m^6A -methylated RNAs are epigenetically regulated by diverse m^6A -binding proteins. However, the mechanism by which the m^6A site recruits specific m^6A binding proteins remains to be elucidated.

Several recent reports highlighted the role of m^6A in the genomes of RNA viruses as well as in the transcripts of DNA viruses^{23–32}. m^6A modification can affect viral life cycles in a complex way. Viral RNAs can be m^6A methylated; therefore, m^6A can play an antiviral or pro-viral role in the viral life cycle through the recruitment of different m^6A -binding proteins. In addition, m^6A can

indirectly affect viral replication by regulating the expression of specific genes involved in the viral life cycle. A better understanding of the biological functions of m^6A modification in viruses is important to establish their role in viral pathogenesis and to design innovative prevention measures to affect viral infection. In this review, we will summarize the emerging roles of m^6A modifications in HBV and HCV infections and discuss their functions and associated mechanisms related to the biological processes of viral infection.

The role of m^6A during hepatitis B and C virus infections

The role of m^6A in the HBV life cycle

HBV infection leads to chronic hepatitis and carries a risk for the development of hepatocellular carcinoma (HCC)^{33,34}. HBV belongs to the *Hepadnaviridae* family and contains a partially double-stranded DNA genome. Although HBV is a DNA virus, it replicates by reverse transcription of an RNA intermediate termed pregenome RNA (pgRNA) to ultimately produce viral genomic DNA in a covalently closed circular conformation termed cccDNA³⁵. Initially, pgRNA is reverse transcribed to relaxed circular DNA (rcDNA) in the cytoplasmic core particles, and rcDNA is subsequently converted to cccDNA in the nucleus, where it functions as a template for transcription³⁴. Transcription from cccDNA is achieved through the cellular polymerase II machinery to synthesize viral RNAs. Synthesis of HBV transcripts is initiated from different transcription start sites in the HBV genome, but it terminates at a common transcription termination signal³⁴. Hence, HBV transcripts have different 5' termini but share a common 3' terminal sequence. These HBV transcripts encode the following

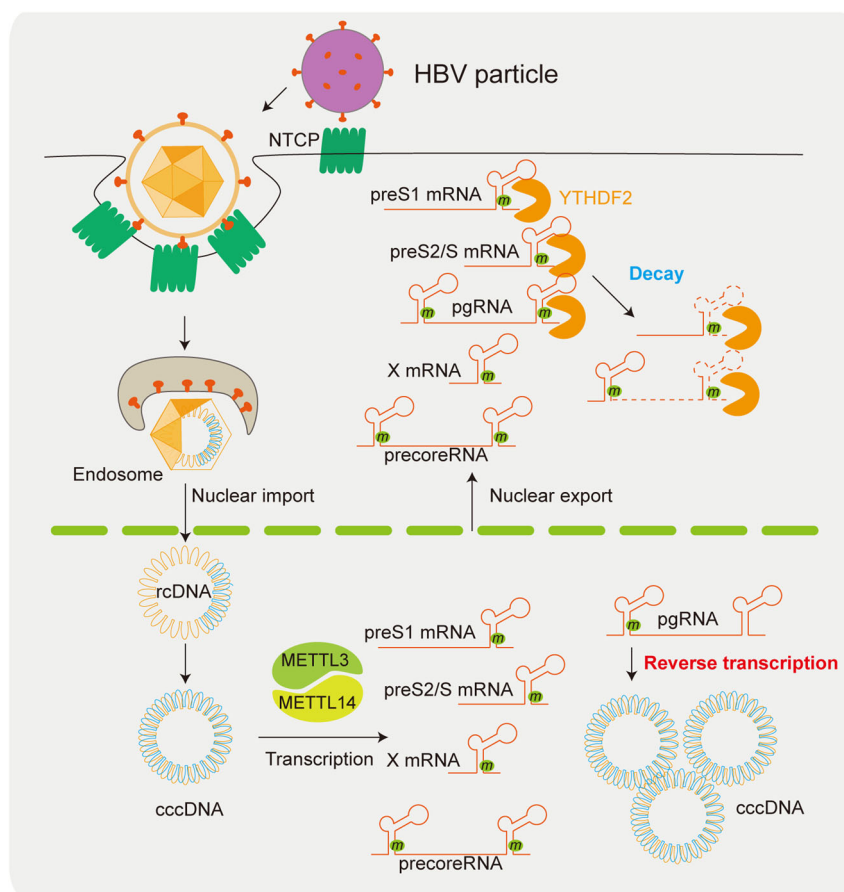


Fig. 2 The role of m⁶A modification in differentially regulating the HBV life cycle. HBV transcripts are cotranscriptionally m⁶A-methylated at a consensus DRACH motif in the epsilon stem-loop region. HBV pgRNA contains two such motifs at the 5' and 3' termini owing to terminal redundancy, but other viral transcripts contain only one such motif, in the 3' terminal sequence. m⁶A methylation of the 5' terminus occurs in the area surrounding the priming site for reverse transcription initiation and induces reverse transcription of HBV DNA from pgRNA, whereas m⁶A at the 3' terminus in all viral transcripts reduces RNA stability by interacting with YTHDF2.

proteins: surface (HBs), precore or 'e' (HBe), and core (HBc) antigen, polymerase, and X (HBx) proteins.

We first reported that HBV transcripts were m⁶A methylated at an m⁶A consensus motif (A1907) located within the epsilon stem-loop region present in all HBV RNAs²³. pgRNA of HBV acquires this m⁶A motif at two locations-at the 5' and 3' termini due to terminal redundancy, but this motif is presented only once in the 3' termini of the other subgenomic viral transcripts³⁴. Importantly, m⁶A modification of HBV RNAs differentially regulates the viral life cycle depending on its position in the viral RNAs (Fig. 2)²³. m⁶A modification at the 3' epsilon stem-loop of HBV RNA transcripts reduces their RNA stability, leading to decreased viral protein expression²³. The reduction in viral RNA stability resulting from m⁶A is mediated by the recognition of the m⁶A site at the 3' epsilon stem-loop by YTHDF2 and 3 (m⁶A binding proteins). On the other hand, the m⁶A site located in the 5' epsilon stem-loop of pgRNA positively

regulates reverse transcriptase activity, but the exact mechanism remains to be characterized. Therefore, these results reveal that m⁶A modification in the epsilon stem-loop structure of HBV regulates effects on HBV RNA stability and reverse transcription.

We have recently discovered that HBV utilizes a specific mechanism to guide m⁶A modification on viral RNAs³⁶. During HBV infection, HBx interacts with m⁶A methyltransferases, which in turn stimulates their nuclear import and thereby delivers the m⁶A methyltransferases to HBV cccDNA to achieve cotranscriptional m⁶A modification of HBV RNAs. On the other hand, infection with HBx-defective HBV fails to produce m⁶A-modified viral transcripts³⁶. In this role, HBx regulates the HBV life cycle by modulating m⁶A modification of viral RNAs. These findings highlight the unique role of HBx in the cotranscriptional RNA modification at the sites of transcription initiation, in addition to its transactivating function affecting the Smc5/6 complex and HBx-DDB-mediated degradation activity^{37–39}.

In addition, m⁶A modification plays an important role in interferon (IFN)-mediated inhibition of HBV replication⁴⁰. IFN treatment of HBV-infected cells promotes the reduction of viral replication through the degradation of viral RNAs by the exonuclease activity of the IFN-stimulated gene 20 (ISG20)⁴¹. ISG20 induced by IFN treatment is recognized by YTHDF2, and YTHDF2 then delivers ISG20 to the m⁶A-methylated HBV RNAs for their degradation⁴⁰. Mutation of the m⁶A site of HBV RNA abrogates ISG20-mediated viral RNA degradation. This study shows a new function of m⁶A reader proteins in IFN-mediated HBV RNA degradation.

The role of m⁶A in the HCV life cycle

Hepatitis C virus (HCV) belongs to the *Flaviviridae* family⁴². HCV is a positive-sense single-stranded RNA virus and encodes a polyprotein of ~3010 amino acids that is cleaved by cellular and viral proteases into structural and nonstructural proteins. The viral polymerase has RNA-dependent RNA polymerase activity to replicate viral RNA from a template RNA.

Horner and colleagues reported that the HCV RNA genome is m⁶A methylated at approximately 19 regions and that all YTHDF proteins broadly interact with the HCV genome²⁴. Interestingly, m⁶A modification in the HCV genome decreased extracellular viral RNA levels and viral particle production without affecting viral replication or protein translation. YTHDF1-3 proteins recognized the m⁶A-methylated HCV genome and relocalized HCV RNAs to the lipid droplet fraction to inhibit HCV RNA packaging into virions (Fig. 3)²⁴. To elucidate the functional relevance of a specific m⁶A site in the HCV genome, Gokhale et al. mutated m⁶A sites within the HCV E1 coding region. Mutations of these m⁶A sites in the HCV E1 gene increased HCV virion production by abolishing HCV E1 recognition by YTHDF1-3 proteins. These results suggest that m⁶A modifications of the HCV E1 gene regulate viral RNA packaging into virions via interactions with YTHDF1-3 proteins²⁴.

Gokhale et al. further analyzed m⁶A motifs in the RNA genomes of other members of the *Flaviviridae* family, including dengue, yellow fever, West Nile, and Zika viruses²⁴. Among these viruses, some m⁶A sites were enriched within the NS3 and NS5 regions. Furthermore, HCV, Zika virus, and dengue virus contained similar m⁶A sites in the E1 region. Therefore, these data suggest that potentially conserved m⁶A sites in flaviviruses could regulate the virion maturation process.

The role of m⁶A in the modulation of host response by HBV and HCV infections

Activation of host pattern recognition receptors (PRRs) by viral infection allows the detection of pathogen-associated molecular patterns and initiates innate immune responses

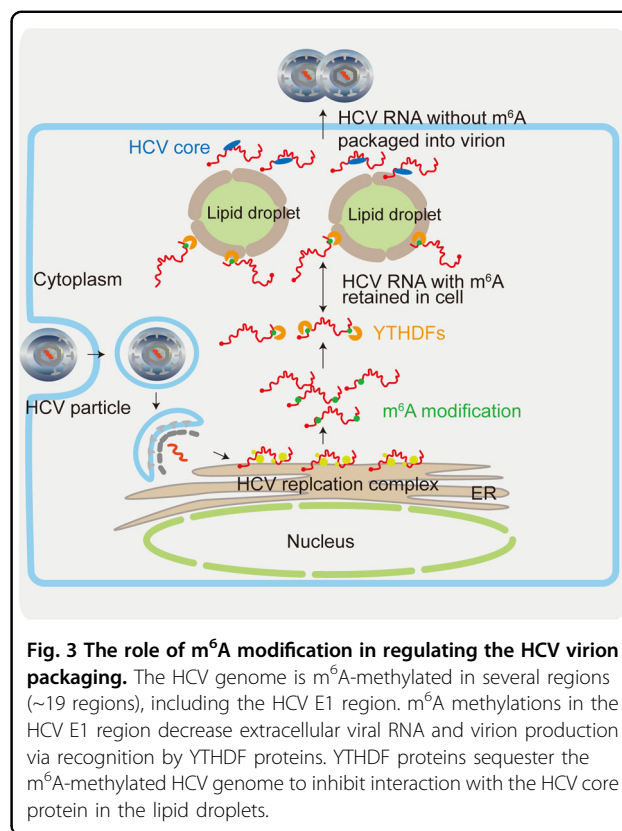


Fig. 3 The role of m⁶A modification in regulating the HCV virion packaging. The HCV genome is m⁶A-methylated in several regions (~19 regions), including the HCV E1 region. m⁶A methylations in the HCV E1 region decrease extracellular viral RNA and virion production via recognition by YTHDF proteins. YTHDF proteins sequester the m⁶A-methylated HCV genome to inhibit interaction with the HCV core protein in the lipid droplets.

to ultimately eliminate viral infection⁴³. PRRs, which detect foreign RNAs, rely on specific molecular signatures and structures to distinguish these RNAs from cellular RNAs and this recognition of foreign RNA is an important cellular surveillance strategy⁴⁴. Interestingly, the ability to use m⁶A to distinguish self from non-self RNAs has been recently highlighted during HBV and HCV infection based on the finding that m⁶A suppresses recognition by retinoic acid-inducible gene I (RIG-I)-like PRRs⁴⁵. The 5' epsilon structure of HBV pgRNA and the 3'-end poly(U/UC) region of HCV are high-affinity RIG-I ligands^{46,47}. m⁶A modifications at the 5' epsilon stem-loop of HBV pgRNA and the adenosine nucleotide at position 8766 of HCV reduced the sensing activity of RIG-I, while abrogation of these m⁶A sites in HBV and HCV enhanced RIG-I signaling⁴⁵. YTHDF2 interacted with m⁶A sites within RIG-I ligand regions of the HBV and HCV RNAs and inhibited RIG-I signaling. Thus, YTHDF2 may inhibit the sensing of m⁶A-modified viral RNAs by RIG-I by sequestering these RNAs from RIG-I. A similar role of m⁶A in preventing the sensing of viral RNAs by PRRs was also studied in human metapneumovirus (HMPV)⁴⁸. The genome and antigenome of HMPV were m⁶A-methylated and m⁶A modification of the HMPV genomes suppressed RIG-I sensing and subsequent IFN production. In contrast, deficient m⁶A modification in the HMPV genomes increased the recognition by RIG-I,

leading to enhanced IFN synthesis. Together, these studies indicate that m⁶A modification of viral RNAs contributes to inhibiting RIG-I sensing through its sequestration by m⁶A binding proteins.

In addition to regulating the host immune response, viral infection can regulate host gene expression and cellular processes to optimize long-term survival^{49–52}. As m⁶A methylation can regulate many cellular pathways, including stress responses and cancer development, its role in viral infection-related cellular gene expression is an important aspect of virus–host interactions³. Indeed, several studies have shown that diverse viral infections modulate the m⁶A profile within the host transcriptome^{53–56}. We recently analyzed changes in the m⁶A profile of cellular RNAs during HBV infection⁵³. Among the host genes whose m⁶A status was dramatically altered by HBV infection was the phosphatase and tensin homolog (PTEN) transcript, which exhibited enhanced levels of m⁶A methylation during HBV infection. Importantly, increased m⁶A modification of PTEN mRNA by HBV decreased its stability, affecting its protein expression. PTEN is a phosphatase of both proteins and lipids that functions as a metabolic regulator as well as a tumor suppressor^{57,58}. Chronic HBV infection causes HCC via diverse pathways, including inflammation and oxidative stress pathways⁵⁹. Thus, the decreased PTEN expression by HBV partially explains its role in HBV-associated hepatocarcinogenesis. In addition to its role as a tumor suppressor, PTEN plays an important role in the innate immune response during viral infections⁶⁰. PTEN promotes IRF-3 nuclear translocation to activate the IFN signaling pathway by inducing dephosphorylation at the Ser96 residue of IRF-3. Based on these findings, HBV inhibits the host immune response through upregulation of m⁶A modification of PTEN⁵³. Interestingly, the HBx protein cotranscriptionally regulates m⁶A modification of cellular RNA, including that of PTEN³⁶. HBx promoted the recruitment of m⁶A methyltransferases (METTL3/14) to the PTEN chromosomal locus to add m⁶A to PTEN transcripts. In addition to its role as a viral protein, the HBx protein is widely acknowledged to be indirectly involved in the development of HCC and viral immune evasion^{61,62}. These studies highlight the unique role of the HBx protein in regulating virus/host gene expression, immune responses, and HBV-associated hepatocarcinogenesis by modulating m⁶A modification of cellular RNAs.

HCV infection also regulates host gene expression by modulating m⁶A modification of cellular mRNAs⁵⁴. HCV infection increased the m⁶A level of cellular RIOK3 mRNA, promoting its translation⁵⁴. RIOK3 is a serine/threonine kinase that may be involved in antiviral signaling⁶³. Importantly, viral activation of the innate immune response contributed to the increased m⁶A levels

of RIOK3, and the increase in RIOK3 expression by m⁶A promoted the production of IFN, leading to inhibition of HCV replication. In addition, the m⁶A level of CIRBP, a stress-induced RNA-binding protein, was changed during HCV infection, although this transcript lost m⁶A modification^{54,64}. In the case of CIRBP, m⁶A deficiency promoted alternative splicing to its shorter isoform. Interestingly, endoplasmic stress induced by viral infection promoted the loss of m⁶A in CIRBP, and the expression of the short isoform of CIRBP positively regulated HCV replication^{54,65}. The precise mechanisms by which HCV infection changes the m⁶A status of individual transcripts are not clear, but these data suggest that activation of host cell pathways during infection may affect the m⁶A status of individual cellular RNAs.

Conclusion and future perspectives

New roles of m⁶A in epigenetically regulating cellular RNAs and viral RNAs are constantly emerging. Recently, reports have demonstrated that the genomes of several RNA viruses, as well as the RNA transcripts of DNA viruses, are modified by m⁶A methylation and that this modification of viral transcripts regulates various aspects of the viral life cycle and the development of pathogenesis^{23–31}. In this review, we discussed the recently identified functions of m⁶A modification during HBV and HCV infections. m⁶A modification regulates the HBV and HCV life cycles in a complex way because it can differentially affect both viral and host RNAs depending on their location in the genome. Eventually, the regulation of HBV and HCV infections by m⁶A affects the development of liver disease, suggesting that m⁶A modification plays previously undefined roles in regulating the hepatitis B and C virus life cycles.

Generally, histone H3 trimethylation at lysine 36 (H3K36me3) is bound directly by the cellular m⁶A machinery, which in turn promotes the binding of the m⁶A machinery to adjacent RNA polymerase II molecules, thereby transporting the m⁶A machinery to the transcribed nascent RNA to add m⁶A cotranscriptionally⁶⁶. Importantly, m⁶A methyltransferases are present in the cytoplasm as well as the nucleus⁶⁷. Because HCV replication occurs in the cytoplasmic fraction⁴², it is conceivable that m⁶A methylation of the HCV genome may be accomplished by the cytoplasmic methyltransferases. However, the functional roles of cytoplasmic methyltransferases in mammalian cells are not yet clear. To gain a broad understanding of the mechanism by which the m⁶A machinery and its bound cellular proteins regulate viral infection, future research must address the roles of both the cytoplasmic and nuclear m⁶A machinery in the regulation of viral infection and cellular pathways. Furthermore, an understanding of how and whether viral infections regulate the function of the cellular m⁶A

machinery and the m⁶A profiles of host RNAs are needed to enhance our understanding of the role of m⁶A in virus–host interactions. This understanding may offer novel avenues for possible m⁶A-based therapeutic interventions to promote viral genome clearance from infected cells. In addition, m⁶A reader proteins are known to interact with many RNA-binding proteins, suggesting that these interactions can affect viral replication and translation^{17,21}. Hence, the interactome of the m⁶A binding proteins during viral infection needs to be identified, which may reveal the unique roles of the RNA-binding protein network that affects the viral life cycle.

Recent studies have highlighted the distinct role of m⁶A methylation in differentiating self RNAs from non-self RNAs based on the findings that m⁶A modification reduces recognition by Toll-like receptor 3 (TLR3), TLR7, and RIG-I^{68–70}. In this respect, m⁶A methylation may allow self RNAs to be distinguished from non-self RNAs to evade recognition by cellular RNA sensor proteins, which trigger the immune response. In addition to m⁶A modification, several other chemical modifications, including 5-methylcytosine (m⁵C), uridine to pseudouridine (U to Ψ) editing, and adenosine to inosine (A to I) editing, occur in viral transcripts, and the functions of these modifications are being characterized^{70–73}. These modifications can also be used by viruses to mimic self RNA and disrupt the host immune response. This interesting issue is currently under further investigation.

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Conflict of interest

The authors declare no competing interests.

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