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Regulation of Viral Infection by the RNA Modification *N6*-methyladenosine

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Abstract

In recent years, the RNA modification *N6*-methyladenosine (m^6A) has been found to play a role in the life cycles of numerous viruses and also in the cellular response to viral infection. m^6A has emerged as a regulator of many fundamental aspects of RNA biology. Here, we highlight recent advances in techniques for the study of m^6A, as well as advances in our understanding of the cellular machinery that controls the addition, removal, recognition, and functions of m^6A. We then summarize the many newly discovered roles of m^6A during viral infection, including how it regulates innate and adaptive immune responses to infection. Overall, the goals of this review are to summarize these roles of m^6A on both cellular and viral RNAs and to describe future directions for uncovering new functions of m^6A during infection.

Keywords

RNA modifications; *N6*-methyladenosine; m^6A; RNA viruses; DNA viruses; post-transcriptional regulation; innate immunity

1. INTRODUCTION

Post-transcriptional regulation heavily influences RNA fate and function (1, 2). Similarly, viral RNAs are regulated post-transcriptionally to control their function (2, 3). Cellular RNAs are also post-transcriptionally regulated during viral infection to generate either proviral or antiviral states (4, 5). A new post-transcriptional control of viral infection has now emerged: internal *N6*-methyladenosine (m^6A) RNA modification of both viral and cellular RNAs (6–10). In this review, we discuss how m^6A modification of both viral and cellular RNAs regulates their function to control infection and immunity.

m^6A is one of over 60 known covalent modifications in eukaryotic RNA (11). It is well known that both transfer RNAs and ribosomal RNAs contain many modifications that

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contribute to their function (12–14). Messenger RNAs (mRNAs) contain terminal modifications, such as the 5′ 7-methylguanosine cap and 2′O methylation of the first and second transcribed bases; this cap structure is critical for mRNA stability and translation (15, 16). mRNA also contains internal modifications, such as pseudouridine, 5-methylcytosine, *N4*-acetylcytidine, *N1*-methyladenosine, and m^6A, the focus of this review (11, 17–20). Viral RNAs were first described to have specific RNA modifications more than four decades ago (reviewed in 9). These studies identified m^6A in host and viral RNA from cells infected with viruses, including influenza A virus (IAV), Rous sarcoma virus, herpes simplex virus 1 (HSV-1), adenovirus, and simian virus 40 (SV40) (21–29). In fact, studies on Rous sarcoma virus RNA even suggested that m^6A was added to a consensus motif, [G/A]A*C (A* = m^6A) (24–26). However, the functional consequences of m^6A on cellular and viral RNA remained unknown for many years. Recently, the discovery of cellular proteins that add, remove, and recognize the modification, as well as the development of sequencing-based methods for transcriptome-wide m^6A mapping, has reignited m^6A research and advanced our interest in studying m^6A during viral infection. While this review focuses on the role of m^6A in viral infection, other RNA modifications such as 2′O methylation, terminal uridilyation, and deamination observed during adenosine-to-inosine editing have also been found in viral RNAs, revealing that many RNA modifications could play roles in viral infection (30–35).

2. METHODS FOR THE DETECTION OF m^6A-MODIFIED RNA

Determining the cellular and viral RNAs that contain m^6A has become feasible due to new techniques to map the modification (reviewed in 36). Prior to the development of these new techniques, modified nucleotides were detected by hydrolysis or nuclease digestion of radiolabeled RNA followed by chromatographic analysis. Now, sequencing-based methods can map m^6A across the cellular transcriptome (37–39). These methods can identify specific RNAs that contain m^6A and define the approximate position of m^6A within those RNAs. The most commonly used technique for transcriptome-wide m^6A mapping is MeRIP-seq (methylated RNA immunoprecipitation and sequencing, also known as m^6A-seq) (37, 38). In this method, total RNA or mRNA is fragmented (100–200 nucleotides), immunoprecipitated with an m^6A-specific antibody, and subjected to next-generation sequencing. m^6A-containing fragments are then identified by calculating the enrichment of sequencing reads in the immunoprecipitated sample relative to the input using specific peak calling algorithms such as MACS2 (40). The initial m^6A mapping studies identified an m^6A sequence motif, DRA*CH (where D = G/A/U, R = G > A, and H = U/C/A, and A* = m^6A), with GGACU being the most common motif (37, 38). This DRACH motif agrees with the originally proposed m^6A motif ([G/A]A*C) identified in Rous sarcoma virus RNA (24–26). While MeRIP-seq identifies m^6A-enriched fragments, it does not define which specific DRACH motifs in these fragments contain m^6A. A related technique, PA-m^6A-seq (photo-cross-linking-assisted m^6A sequencing), defines m^6A sites more precisely (41). In this method, RNA labeled with 4-thiouridine is immunoprecipitated with an anti-m^6A antibody. This immunoprecipitated, 4-thiouridine-containing RNA is treated with ultraviolet light to cross-link the antibody, and the RNA is then digested to 30-nucleotide fragments. When these fragments are reverse transcribed, the cross-linked antibody adducts introduce

mutations into the complementary DNA (cDNA) that ultimately reveal proximal m^6A sites. While this technique provides a more accurate m^6A map than MeRIP-seq, it does not always identify m^6A at single-nucleotide resolution.

Two recently developed methods for single-nucleotide resolution mapping of m^6A are miCLIP (m^6A individual-nucleotide-resolution-cross-linking and immunoprecipitation) and m^6A-CLIP (42–45). In these CLIP-based methods, fragmented RNA is cross-linked to anti-m^6A antibodies using ultraviolet light. If m^6A is present in the RNA, upon reverse transcription the cross-linked antibody adduct results in characteristic truncations or mutations directly adjacent to the exact m^6A site. These methods to detect m^6A do have some limitations: They are more labor intensive than MeRIP-seq and require a higher number of unique sequencing reads than traditional RNA-seq due to their reliance on detection of mutations or truncations within the cDNA pool (43).

All current m^6A mapping techniques have several shared limitations. They all rely on an antibody, which can lead to detection biases. Indeed, anti-m^6A antibodies from different suppliers have different specificities that can be altered by RNA structure (46, 47). Because anti-m^6A antibodies can also immunoprecipitate RNA with the similar modification 2′O-dimethyladenosine (m^6A_m), differentiating between these modifications can be difficult (43, 48–50). Also, while these methods can detect m^6A in mRNA, they have difficulty distinguishing condition-induced m^6A changes in a given RNA. This is because the current computational methods to call m^6A often do not adequately consider changes in transcript abundance or exon usage when mapping m^6A, and therefore they can provide divergent results regarding m^6A occupancy. In fact, most m^6A mapping studies have only been performed with one or two replicates, limiting the robustness of many genome-wide m^6A mapping studies. Therefore, more work is needed to develop new reagents, robust computational methods, and rigorous statistical analyses for accurate detection of both static and dynamic m^6A sites in the transcriptome. Despite these limitations, current transcriptome-wide m^6A mapping methods have provided remarkable information regarding the positioning of m^6A in viral and cellular RNA.

3. THE CELLULAR m^6A MACHINERY REGULATES RNA FUNCTION

Another major breakthrough that allowed for the study of m^6A in viral infection was the discovery of the m^6A machinery, which includes the cellular proteins that add, remove, or read m^6A (51–63) (Figure 1). Studying the m^6A machinery has revealed that m^6A regulates many aspects of RNA biology, including structure, splicing, alternative polyadenylation, localization, stability, and translation (64–66). Biologically, this translates into m^6A influencing physiological processes including organismal development, stem cell differentiation, hematopoiesis, immune cell function, oncogenesis, circadian rhythms, and neural function (64–66). In this section, we introduce the m^6A machinery and how it regulates RNA function.

3.1. Cellular Writers of m^6A

The primary enzyme that adds, or writes, m^6A to mRNA is the methyltransferase METTL3 (Figure 1). METTL3 is predominantly localized in the nucleus and co-transcriptionally

methylates adenosine residues within specific DRACH motifs in nascent RNA (45, 52, 67). It can also directly promote of translation (68, 69). METTL3 stability, catalytic efficiency, localization, and RNA targeting are all regulated by numerous interacting RNA-binding proteins. These proteins include METTL14, WTAP, ZC3H13, VIRMA (KIAA1429), and RBM15/15B. METTL14 complexes with METTL3 to stabilize its expression and enhance its methyltransferase activity (52, 58, 70, 71). WTAP localizes METTL3-METTL14 to transcription sites where it promotes RNA binding, while ZC3H13 maintains the nuclear localization of this complex (54, 55, 72). Targeting of METTL3-METTL14 to mRNA occurs through accessory factors such as VIRMA and RBM15/15B (56, 72, 73). The complete set of features in RNA that lead to methylation of specific DRACH motifs, including the RNA structures or secondary m^6A recognition motifs that influence this selectivity, are unknown. Elucidating how this selectivity occurs and identifying additional proteins within the methyltransferase complex will be essential for understanding how both cellular and viral RNAs are selected for m^6A modification.

In addition to METTL3, three other enzymes have been shown to act as m^6A methyltransferases in eukaryotes: METTL16 adds m^6A to U6 small nuclear RNAs as well as some mRNAs; ZCCHC4 adds m^6A to 28S ribosomal RNA; and PCIF1, a cap-specific m^6A methyltransferase, catalyzes the formation of m^6A_m (48, 49, 74–76). These enzymes have only recently been characterized and have not at present been studied during viral infection; however, it is possible that they may also deposit m^6A on viral RNAs.

3.2. Cellular Erasers of m^6A

The enzymes that remove, or erase, m^6A from mRNA are FTO and ALKBH5 (50, 53, 57, 77, 78) (Figure 1). FTO demethylates both m^6A and terminal m^6A_m, while ALKBH5 specifically demethylates m^6A (50, 57, 78). The discovery of these m^6A demethylases suggested for the first time that m^6A could be added or removed from mRNAs under specific conditions, setting the stage for the study of RNA epigenetics or the epitranscriptome (79, 80).

3.3. Cellular Readers of m^6A

The RNA-binding proteins that bind to m^6A are referred to as m^6A readers. These m^6A readers, whose RNA-binding activity can be modulated by the presence of m^6A and/or RNA structure, elicit the regulatory functions of m^6A on modified RNAs (62, 63, 81, 82; reviewed in 83). These readers can regulate the stability, splicing, polyadenylation, nuclear export, and translation efficiency of their target RNAs (59–61, 84–89) (Figure 1). The most well-described m^6A readers—including YTHDF1, YTHDF2, YTHDF3, YTHDC1, and YTHDC2—all possess a YTH domain that contains an m^6A-binding pocket (90–93). The YTHDF proteins and YTHDC2 all act as readers of m^6A-containing mRNAs in the cytoplasm. In m^6A-containing mRNAs, YTHDF1 promotes translation, YTHDF2 increases RNA decay, and YTHDF3 and YTHDC2 can regulate both of these processes (59–61, 85, 94–102). The nuclear reader YTHDC1 regulates the splicing and alternative polyadenylation of specific transcripts (86, 88).

Besides the YTH domain-containing m^{6}A readers, we now know of dozens of additional proteins that preferentially bind to or are repelled by m^{6}A-containing RNA (62, 63, 82, 103). Proteins with enhanced specificity for m^{6}A-modified RNA include eIF3D, FMR1, IGF2BP1, IGF2BP2, IGF2BP3, HNRNPC, HNRNPG, and HNRNPA2B1. Proteins repelled by m^{6}A include G3BP1, G3BP2, and CAPRIN1 (62, 63, 81, 82, 84, 89, 103). The RNA regulatory functions of many of these newly identified m^{6}A readers remain incompletely defined, and an understanding of which m^{6}A-containing RNAs they regulate will undoubtedly reveal new functions for m^{6}A in RNA biology.

4. m^{6}A AND m^{6}A-REGULATORY PROTEINS REGULATE VIRUS INFECTION

While m^{6}A was first identified in viral RNA in the 1970s, the specific roles of m^{6}A in virus replication remained unclear. These roles for m^{6}A in viral infection are now beginning to be uncovered, and the studies that define these roles reveal that m^{6}A is a new regulatory control of viral infection (6–10). In this section we describe the recent work that has defined these regulatory roles for m^{6}A during virus infection, including positive-sense RNA viruses, negative-sense RNA viruses, retroviruses, and DNA viruses (Figure 2).

4.1. Flaviviridae

The *Flaviviridae* family of viruses is composed of positive-sense, single-stranded RNA viruses that replicate in the cytoplasm. We and others mapped m^{6}A on the viral RNA genomes of *Flaviviridae* members, including hepatitis C virus (HCV), Zika virus (ZIKV), dengue virus, yellow fever virus, and West Nile virus (WNV) (104, 105). These viral genomes contain multiple m^{6}A sites along their genomes, as determined by MeRIP-seq, and their presence was validated by mass spectrometry (34, 104, 105). Interestingly, each virus had a high concentration of sites present in the last viral gene (NS5B for HCV or NS5 for the other viruses) (104, 105).

As the canonical m^{6}A methyltransferase complex is predominantly nuclear, it is unclear how these cytoplasmic RNA genomes gain m^{6}A (52). At least some portion of METTL3-METTL14 is in the cytoplasm, where it could interact with and methylate viral RNA (104, 105). In the case of HCV, nuclear pore complex proteins are recruited to the membranous sites of replication, and it is possible that METTL3, which contains a nuclear localization signal, is also recruited to HCV replication sites by these nuclear pore complex proteins (106).

During HCV infection, m^{6}A negatively regulates the production of infectious particles (104). Specifically, m^{6}A in the E1 gene of HCV RNA inhibits the packaging of viral RNA into infectious particles, likely by facilitating competition between YTHDF proteins, which bind to m^{6}A, and the HCV core protein, which is repelled by m^{6}A. Interestingly, during HCV infection these YTHDF proteins relocalize to virion assembly sites at lipid droplets. Together, these findings support the idea that m^{6}A recognition by the YTHDF proteins has a negative effect on viral RNA packaging. Indeed, intracellular HCV RNA contains quantitatively more m^{6}A than extracellular, virion-associated HCV RNA (34, 104). This suggests that the m^{6}A RNA profile on HCV, and likely the other *Flaviviridae* members, may be different at varying life cycle stages. However, the mechanisms that would underlie this

differential modification at different life cycle stages are still unknown. Importantly, while altering expression of the m^{6}A machinery changed the levels of HCV, it did not affect the expression of several known interferon- (IFN) stimulated genes (ISGs), suggesting that manipulation of m^{6}A levels does not alter the overall antiviral response in HCV infection.

Similar to HCV, ZIKV infection is also inhibited by m^{6}A, implying that features of m^{6}A regulation may be shared among *Flaviviridae* members (105). Some *Flaviviridae* members are transmitted by mosquitoes, so it is possible that m^{6}A regulates vector-borne transmission. Going forward, more work is required to uncover the role of m^{6}A in other *Flaviviridae* and establish the function of each m^{6}A site in mammalian and vector models of infection. Further, a kinetic analysis of m^{6}A on viral RNA may give important clues to how m^{6}A regulates *Flaviviridae* RNA at different stages of the viral life cycle.

4.2. Picornaviridae

Enterovirus 71 (EV71) and poliovirus, both in the family *Picornaviridae*, are positive-sense, single-stranded RNA viruses that replicate in the cytoplasm and contain m^{6}A (34, 107). While neither the specific location of the m^{6}A sites in the poliovirus RNA nor their importance in replication have been assessed, this has been done for EV71. The m^{6}A sites in the EV71 genome are in genes encoding the VP capsid proteins, the 3D RNA-dependent RNA polymerase, and the nonstructural 2C protein (107). Two m^{6}A-abrogating mutations in VP1 and 2C reduced EV71 replication, suggesting that m^{6}A positively regulates EV71 infection. In support of this, METTL3 depletion reduced viral replication, while depletion of FTO, all three of the YTHDF proteins, or YTHDC1 had the opposite effect (107). Interestingly, in EV71-infected cells, several m^{6}A machinery proteins changed localization: METTL3 and METTL14 were upregulated and moved to the cytoplasm, the cytoplasmic readers YTHDF1 and YTHDF2 were partly relocalized to the nucleus, and the nuclear reader YTHDC1 was also moved to the cytoplasm. Consistent with this change in localization of the m^{6}A writers, METTL3 directly interacted with 3D, indicating that 3D could recruit METTL3 to sites of viral RNA replication.

4.3. Orthomyxoviridae

IAV, in the *Orthomyxoviridae* family, is a negative-sense, segmented, single-stranded RNA virus that replicates in the nucleus. m^{6}A was first discovered on IAV RNA in the 1970s, but now its role in regulating IAV infection has been more clearly defined (21, 108, 109). Mapping m^{6}A and YTHDF protein binding sites in IAV-infected A549 lung epithelial cells revealed a high density of m^{6}A sites in negative- and positive-sense IAV RNA. The number of m^{6}A sites found in each RNA segment, including HA, is similar to the number of m^{6}A residues per IAV mRNA predicted 30 years ago (108, 109). Unlike in the *Flaviviridae* viruses already discussed, m^{6}A promotes IAV infection. Overexpression of METTL3 and YTHDF2 during IAV infection increased viral protein expression and viral titer, while mutational inactivation of these m^{6}A sites in either strand of IAV lowered HA mRNA and protein levels (109). These m^{6}A mutant viruses were also attenuated in mouse models of infection. Together, this suggests that m^{6}A enhances HA expression during infection, ultimately promoting viral replication and pathogenesis. Future studies are needed to reveal the molecular mechanisms underlying m^{6}A enhancement of IAV gene expression, such as

RNA stability, nuclear export, translation, and how it may regulate the assembly and packaging of the negative-sense genomic RNA segments.

4.4. Retroviridae

m^{6}A has also been identified in retroviruses, including Rous sarcoma virus, feline leukemia virus, and more recently human immunodeficiency virus-1 (HIV-1) (reviewed in 9, 110). Early studies found that m^{6}A sites on RSV RNA were within [G/A]A*C (A* = m^{6}A) motifs within distinct regions of the viral RNA, indicating that there was a mechanism by which m^{6}A could be specifically added to these sites (24–26, 111). These early studies also suggested that m^{6}A may alter the splicing of the RSV *Env* transcript (112).

Three independent groups have now reported multiple roles for m^{6}A during HIV-1 infection. While they all found that m^{6}A is present in both HIV-1 genomic RNA and mRNA, they uncovered somewhat different mechanisms by which the m^{6}A machinery regulates HIV-1 infection (113–116; reviewed in 110). The first study to examine m^{6}A in HIV-1 found that the m^{6}A machinery regulates infection by controlling the methylation of the Rev response element (RRE). m^{6}A in the RRE increased Rev protein binding to this RNA structure, which is present on and critical for nuclear export of some HIV-1 mRNAs (113). HIV-1 gene expression is also regulated by the YTHDF proteins, which bind to m^{6}A-containing 3′UTRs of viral mRNAs to increase their mRNA levels and protein expression. Consistent with this, YTHDF proteins also positively regulated HIV-1 replication (114). Two more studies found both proviral and antiviral roles for m^{6}A in HIV-1 infection (115, 116). Specifically, YTHDF proteins bound to incoming viral genomic and also reduced HIV-1 reverse transcription products, implying a negative role for YTHDF proteins in these stages of the viral life cycle. Despite this, m^{6}A-abrogating mutations in the 5′UTR of HIV-1 genomic RNA, which prevent binding by YTHDF proteins, reduce infectivity. Additionally, YTHDF proteins promoted Gag protein expression in infected cells. Taken together, these data indicate that m^{6}A and its machinery can have many roles in regulating HIV-1 infection depending on the stage of the viral life cycle and on the position of m^{6}A in both HIV-1 genomic RNA and mRNA. Interestingly, HIV-1 increases m^{6}A in cellular RNA through gp120 protein interacting with the HIV-1 receptor CD4, although its functional consequence for HIV-1 infection remains unclear (113, 117). Further work is needed to fully define the molecular mechanisms that drive m^{6}A regulation of host and viral RNA during HIV-1 infection and define how m^{6}A regulates various aspects of HIV-1 infection.

4.5. Hepadnaviridae

The replication of hepatitis B virus (HBV), a nuclear-replicating, double-stranded DNA virus in the *Hepadnaviridae* family, is also regulated by m^{6}A. HBV copies its viral DNA using a replication intermediate RNA called the pregenomic RNA (pgRNA), which is reverse transcribed to make viral DNA. Both METTL3-METTL14 and YTHDF2 knockdown increased the stability of viral transcripts, resulting in greater expression of viral proteins (118). All viral transcripts had a single m^{6}A peak within the epsilon stem loop. This stem loop is present at the 3′ end of all viral transcripts, including pgRNA, and also in the 5′ end of the pgRNA. Interestingly, an m^{6}A-abrogating mutation in the 3′ epsilon stem loop increased the stability of viral transcripts, but abrogating the m^{6}A site in the 5′ epsilon

stem loop in pgRNA decreased reverse transcription. Taken together, this suggests that m^{6}A can have different effects on the HBV life cycle; therefore, it would be interesting to decipher how the balance of m^{6}A between 3′ and 5′ epsilon stem loop sites facilitates the HBV life cycle.

4.6. Polyomaviridae

The polyomavirus SV40, a small, double-stranded DNA virus that replicates in the nucleus, also contains m^{6}A in its RNA. While the presence of m^{6}A in SV40 mRNA has been known for decades (29, 119), the precise location of m^{6}A on the viral mRNAs and how it may regulate viral replication remained incompletely understood until recently. We now know that m^{6}A is located in both SV40 transcripts; there are 2 m^{6}A sites in the early transcript, while there are 11 m^{6}A sites in the late transcript (120). Loss of m^{6}A from the SV40 early transcript had no effect on infection. Mutation of m^{6}A sites on the late transcript reduced infection. This loss of m^{6}A in the late transcript prevents its nuclear export, resulting in reduced expression of the encoded structural protein VP1 (120). This is in concordance with earlier studies that showed that the SAM inhibitor cycloleucine blocked nuclear export of SV40 late RNAs (119). In support of these results, depletion of METTL3 reduced SV40 replication, while YTHDF2 overexpression increased viral replication, further suggesting that m^{6}A promotes SV40 infection. Future studies are needed to understand the molecular mechanisms by which m^{6}A affects nuclear export of the late transcripts and whether m^{6}A functions similarly in human polyomaviruses.

4.7. Herpesviridae

Herpesviruses have large, double-stranded DNA genomes and replicate in the nucleus. m^{6}A has been studied in HSV-1, human cytomegalovirus (HCMV), and Kaposi's sarcoma-associated herpesvirus (KSHV) (28, 121–125). While decades ago, HSV-1 was found to contain m^{6}A (28), m^{6}A has now been mapped and studied during HCMV and KSHV infection (121–125). While the studies on HCMV found m^{6}A on multiple viral transcripts, they focused on how m^{6}A impacts antiviral innate immunity through regulating IFN production. Therefore, we discuss these HCMV studies later in Section 5.2, and here we focus on the studies that define how m^{6}A regulates KSHV infection.

Three studies (121–123) found that m^{6}A was present on many KSHV transcripts during lytic reactivation, including the KSHV *ORF50/RTA* mRNA, which encodes a major transactivator required for the reactivation of latent KSHV. While all three studies showed that m^{6}A regulated this transactivator, they present conflicting roles for how m^{6}A and its machinery control *ORF50/RTA* RNA levels and/or expression, often depending on the cell type used. In epithelial cells, Hesser et al. (123) found that METTL3 and YTHDF2 depletion suppressed KSHV *ORF50/RTA* expression, lytic reactivation, and virion release, suggesting that m^{6}A promotes KSHV lytic reactivation. Conversely, Tan et al. (122) found that in similar epithelial cells, YTHDF2 depletion increased the half-life of many viral mRNAs including *ORF50/RTA* during reactivation, which may or may not change its expression, pointing to a potential inhibitory role for m^{6}A in KSHV lytic reactivation. However, in B cells, Hesser et al. (123) found that m^{6}A negatively regulated *ORF50/RTA* and KSHV lytic reactivation. On the other hand, in the same cell type, Ye et al. (121) showed that m^{6}A on *ORF50/RTA* is

read by YTHDC1, which acts with the splicing factors SRSF3 and SRSF10 to promote its splicing and expression. Together, these results demonstrate that while m^{6}A clearly plays a role in KSHV lytic reactivation, the whole picture is not yet clear. Further, these seemingly opposing results in different cell lines underscore the importance of studying the effects of m^{6}A in diverse cell lines and limiting conclusions only to the cell line tested.

5. m^{6}A IN INNATE AND ADAPTIVE IMMUNITY

In addition to acting directly on viral RNA to regulate viral replication, m^{6}A can also regulate the immune response to infection. It has been shown to do this by regulating sensing of foreign RNAs, by altering the expression and stability of the transcripts of innate immune signaling molecules, and by affecting adaptive immune responses.

5.1. m^{6}A Regulates Sensing of Foreign Nucleic Acids

Viral RNA present in the cytoplasm can be sensed as foreign or nonself by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and RIG-I-like receptors (reviewed in 126). These receptors bind to viral RNAs, often containing specific features, such as a double-stranded region or a 5′ triphosphate, to drive signaling programs that ultimately induce cytokines such as IFNs, leading to an antiviral response. When added to in vitro transcribed RNAs, m^{6}A and other modified nucleotides suppress the activation of these PRRs (127, 128). Indeed, the potential of modified nucleotides for suppressing antiviral innate immune sensing pathways has been harnessed for generation of mRNA vaccines to viruses, including IAV, ZIKV, and HIV-1 (129–132). Therefore, this suggests that m^{6}A addition to viral RNAs could be a strategy that viruses use for innate immune evasion. While this has not been demonstrated yet for m^{6}A in viral infection, it has proven to be true for another RNA modification, 2′O methylation, which normally occurs in mRNA cap (133). Specific viruses (e.g., WNV and vesicular stomatitis virus) use virally encoded methyltransferases to 2′O methylate their RNA caps to prevent sensing by IFIT1, which inhibits the translation of viral RNAs lacking this modification (31, 134). So far, no virus is known to encode an m^{6}A methyltransferase, and at least for flaviviruses, the viral 2′O methyltransferase domain cannot add m^{6}A (135). Interestingly, the cellular methyltransferase FTSJ3 catalyzes internal 2′O methylation in HIV-1 RNA, which prevents IFN activation (35). Therefore, it is also possible that m^{6}A deposited on viral RNA by host methyltransferases could present a pattern that either evades detection by host PRRs or is bound by proviral cellular RNA-binding proteins.

5.2. m^{6}A Regulates the Abundance and Induction of Antiviral Signaling and Effector Molecules

Antiviral gene expression must be tightly controlled to promote effective immunity while preventing cellular toxicity. Indeed, m^{6}A and its machinery contribute to this control by post-transcriptionally regulating the mRNAs of several key molecules that determine the innate immune response to viral infection. Specifically, m^{6}A has been identified in the 3′UTRs of *TRAF3*, *TRAF6*, *MAVS*, and *IFNB1* mRNA (124, 125, 136). During innate immune stimulation *TRAF3*, *TRAF6*, and *MAVS* transcripts all lose m^{6}A. This loss of m^{6}A reduces their nuclear export and translation and is catalyzed by the eraser ALKBH5, which

is recruited to these mRNAs by DDX46 (136). Interestingly, other DDX family members have been found to interact with ALKBH5, suggesting that this family of proteins may regulate virus infection by altering the abundance and stability of antiviral signaling molecules (137, 138). The *IFNB1* transcript is also negatively regulated by m^{6}A in its 3′UTR (124, 125). Loss of m^{6}A in this region due to METTL3/METTL14 depletion increases expression of IFN-β protein during HCMV infection, suggesting again that m^{6}A may be used by the cell to turn off antiviral responses (124, 125). On the other hand, METTL3 promotes the splicing of the TLR signaling adaptor *MYD88* and the induction of several cytokines in response to lipopolysaccharide stimulation, which reveals that m^{6}A can also positively regulate innate immunity (139).

The IFN response leads to the transcriptional induction of hundreds of ISGs and is likely regulated by m^{6}A and its machinery. The expression of ISGs is negatively regulated by YTHDF3, which promotes the translation of a transcriptional repressor of ISGs called FOXO3 (140). On the other hand, the expression of some ISGs can be post-transcriptionally regulated by RNA-binding proteins such as G3BP1, G3BP2, and CAPRIN1, which are known to be repelled by m^{6}A-containing RNAs and therefore could regulate ISGs in an m^{6}A-dependent manner (62, 63, 141). Also, some ISG-encoded RNA-binding proteins, including IFIT1, ZAP, and SLFN11, can be directly antiviral (3, 31); others, such as FMR1 and IGF2BP3, selectively recognize m^{6}A (62, 63, 142). Therefore, it is conceivable that uncharacterized IFN-induced RNA-binding proteins could selectively recognize the presence of m^{6}A in viral or cellular transcripts and modulate their function during an antiviral response. An increased understanding of how m^{6}A regulates antiviral innate immunity will have implications for host-directed therapeutics to limit virus infection.

5.3. m^{6}A Regulates Adaptive Immunity

In addition to having several roles in antiviral innate immunity, m^{6}A also regulates adaptive immunity (143). In particular, T cell differentiation and proliferation in mice are regulated by m^{6}A modification of *Socs1* and *Socs3*, which positively regulates their abundance (143). This leads to increased expression of the encoded Socs proteins, thereby reducing T cell differentiation and proliferation (143). Mettl3 also influences T regulatory cell generation and suppressive function (144). Because many diseases are caused by defects in T cell processes, some of which we now know are regulated by m^{6}A-containing mRNAs, m^{6}A in these specific mRNAs may be a therapeutic target.

6. FUTURE FOCUSES IN m^{6}A BIOLOGY DURING VIRAL INFECTION

We anticipate that future work in this rapidly advancing field will paint a more complete picture of the mechanisms by which m^{6}A influences viral infection. Here, we discuss future directions for studying m^{6}A during viral infection, including the impact of m^{6}A on RNA structure, methods for improving m^{6}A mapping techniques, and how the field will move toward understanding the ways m^{6}A and its associated machinery mechanistically impact cellular processes and immune responses during infection. Beyond recognition and regulation of m^{6}A-containing viral RNA by RNA-binding proteins, it is likely that we will discover additional means by which m^{6}A affects viral RNA. m^{6}A has the potential to impact

RNA structure, and it is becoming clear that viral RNA structures have a profound effect on viral replication (46, 81, 82, 145–147). Therefore, m^6A could affect viral infection by altering viral RNA structures. Indeed, m^6A sites that have been shown to regulate HIV-1 and HBV are found within viral RNA structures (113, 118). m^6A could also contribute to viral-induced changes to structures in host mRNAs to affect gene expression (148). Therefore, future studies should be aimed at interrogating the role of m^6A in altering secondary and tertiary structures in viral and host RNA and defining how these structural changes regulate infection.

Currently, m^6A-containing regions on RNA from several families of viruses have been mapped; however, we lack information regarding m^6A site occupancy at single-nucleotide resolution in these RNAs. The next steps in determining the m^6A landscape during infection will be assessing the phasing and stoichiometry of m^6A in viral RNAs. Phasing will reveal if m^6A modifications at distinct sites of a particular mRNA species reside in the same or different RNA molecules, while m^6A stoichiometry could be used to define both the fraction of each site and RNA species modified by m^6A. Determining the phasing and stoichiometry of m^6A in viral RNA will be of particular interest, as pools of viral RNAs operate in different stages of viral life cycles, and m^6A might allow for the temporal discrimination of such species. Importantly, advances in long-read direct RNA sequencing via nanopores capable of accurately discriminating between unmodified and modified bases will greatly enhance our understanding of both phasing and stoichiometry of m^6A in RNA. In the meantime, existing biochemical techniques that measure m^6A occupancy and stoichiometry at single sites will be valuable tools for validating m^6A sites in viral RNA (149–151).

These evolving methods for identifying m^6A sites in different RNA species will also be invaluable for studying the effect of m^6A in post-transcriptional regulation of the host response following infection. It is likely that the role of m^6A in the host response to infection extends beyond its recently described functions in the antiviral innate immune response. During infection, the host transcriptome is broadly remodeled, and m^6A could shape the output of this process (1). One example of m^6A regulation during a host transcriptional response is seen during heat shock. Altered m^6A patterns on stress-associated cellular mRNAs promote the heat shock response by affecting the localization and translation of these RNAs (95, 98). Similarly, changes in the m^6A epitranscriptome could exert a regulatory function on individual transcripts to determine the outcome of infection. It is unknown how these changes in the host epitranscriptome would occur. However, there is evidence that viruses such as EV71 and HCMV increase the expression of METTL3 and METTL14, suggesting that the cellular m^6A machinery might be altered during viral infection to catalyze these changes (107, 124, 125). Alternatively, during viral infection the m^6A machinery could interact with a new complement of RNA-binding proteins that could target them to specific RNAs to modulate their m^6A status. Recently, the m^6A methyltransferase complex has been shown to be recruited to the histone modification H3 trimethylation at lysine 36 during active transcription to increase m^6A in specific transcripts, which suggests that viral alteration of this epigenetic mark may result in differential m^6A modification of host transcripts during infection. Ultimately, it will be important to understand how and which specific transcripts gain or lose m^6A in response to infection, what effects m^6A has on these RNAs, and ultimately how these changes affect viral

infection. However, existing sequencing-based methods for m^{6}A detection are limited in their ability to computationally distinguish between differences in m^{6}A abundance, as opposed to transcript abundance or isoform usage. Better bioinformatic tools, including those that account for variability between samples, to accurately identify differential m^{6}A modification of RNA under diverse conditions will therefore be critical for discovering how m^{6}A regulates gene expression during infection. Indeed, using viral infection as a tool to perturb m^{6}A modification of host transcripts might also allow us to uncover new molecular mechanisms that control the specificity of the m^{6}A methyltransferase complex.

7. CONCLUDING REMARKS

Recent developments in mapping m^{6}A on RNA, coupled with the ability to manipulate the enzymes involved in metabolism of m^{6}A, have shed light on how this modification regulates RNA function to influence biological processes, including viral infection. Here, we have summarized advances in understanding the many roles of m^{6}A in viral infection and immunity. For many viruses that contain m^{6}A in their RNA, depletion of the m^{6}A writer, eraser, or reader proteins affects diverse facets of viral replication that are often mediated by RNA-protein interactions. We note that such phenotypes might derive from the direct function of m^{6}A on viral RNA or from the function of m^{6}A on host transcripts. Overall, it is clear that m^{6}A is neither uniformly proviral nor antiviral but instead regulates many aspects of viral replication by modulating specific RNAs sometimes dependent on tissue or cell type (113–117, 122, 123).

Virus families that employ intrinsically different life cycle strategies can be regulated by m^{6}A and its machinery in diverse ways. Future experiments studying m^{6}A during viral infection should focus on the role of specific modification sites in individual transcripts, temporal dynamics of modification, and the functions of newly identified m^{6}A writers and readers; they should also continue to push these experiments beyond cell culture–based experimental approaches and into animal models. So far, we have only scratched the surface regarding understanding the role of m^{6}A in viral infection. We expect that future work in this field will enable us to learn more about viral replication as well as fundamental functions of m^{6}A in biology.

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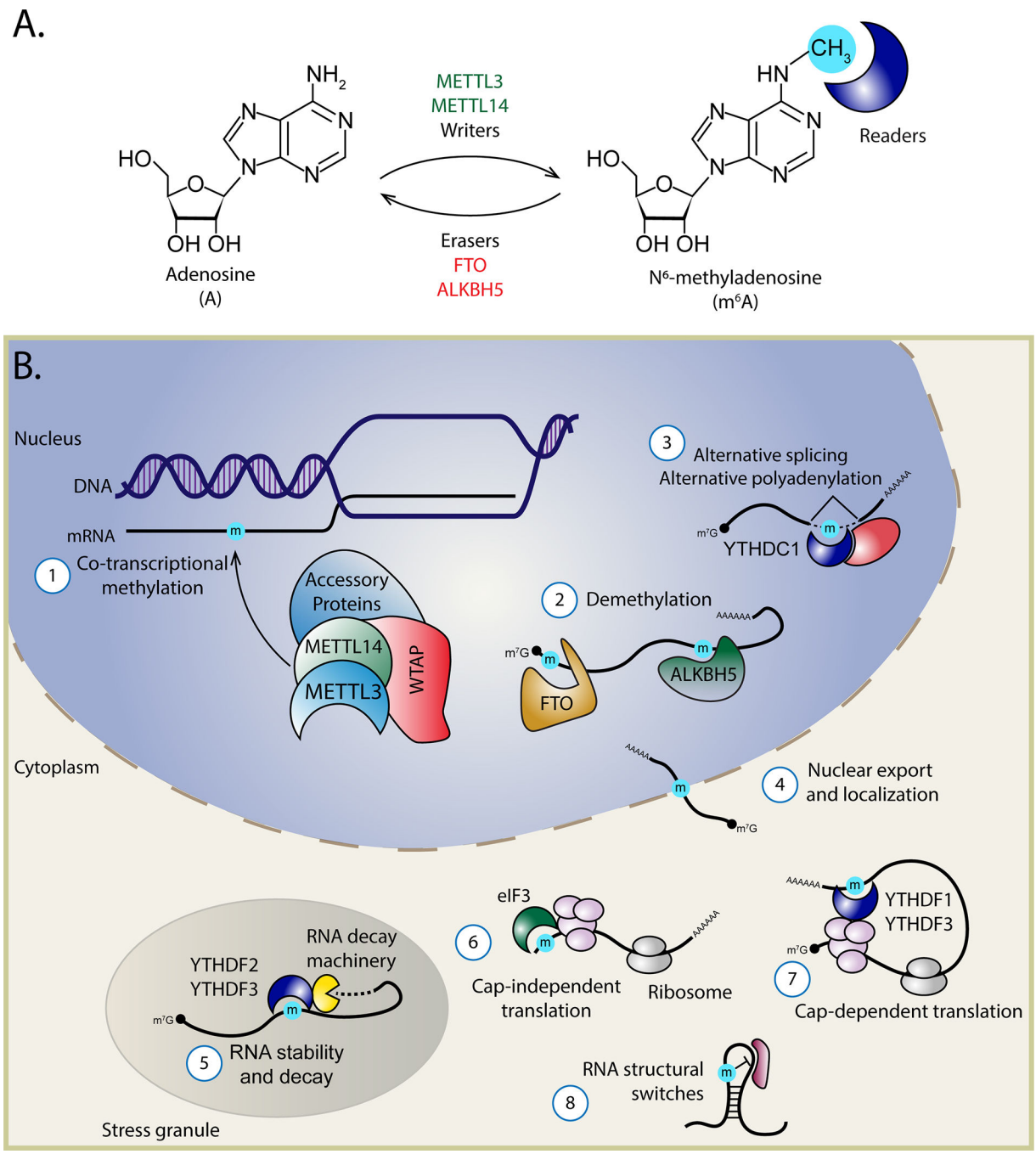

Figure 1.

The cellular m6A machinery and functions of writers, erasers, and readers. (*a*) Structures of A and m6A. The methyl group is colored blue. METTL3 and METTL14 are the writer proteins that catalyze the covalent conversion of A to m6A on target RNAs. FTO and ALKBH5 are demethylases capable of removing the methylation. The function of m6A bearing RNAs is influenced by interaction with reader proteins. (*b*) ① m6A is co-transcriptionally added to RNA by a writer complex of proteins, which consists of METTL3, METTL14, and WTAP, as well as accessory factors that can determine RNA targeting. ②

m6A can be removed from RNA by the demethylases FTO and ALKBH5. m6A reader proteins, such as the YTHDF proteins, mediate diverse post-transcriptional processes on m6A containing RNA including ③ alternative splicing and polyadenylation, ④ nuclear export and RNA localization, ⑤ alteration of RNA stability, ⑥ cap-independent translation, ⑦ cap-dependent translation, and ⑧ modulation of protein-RNA interactions via structural switches. Abbreviations: A, adenosine; mRNA, messenger RNA; m6A, *N6*-methyladenosine, m^7G, 7-Methylguanosine.

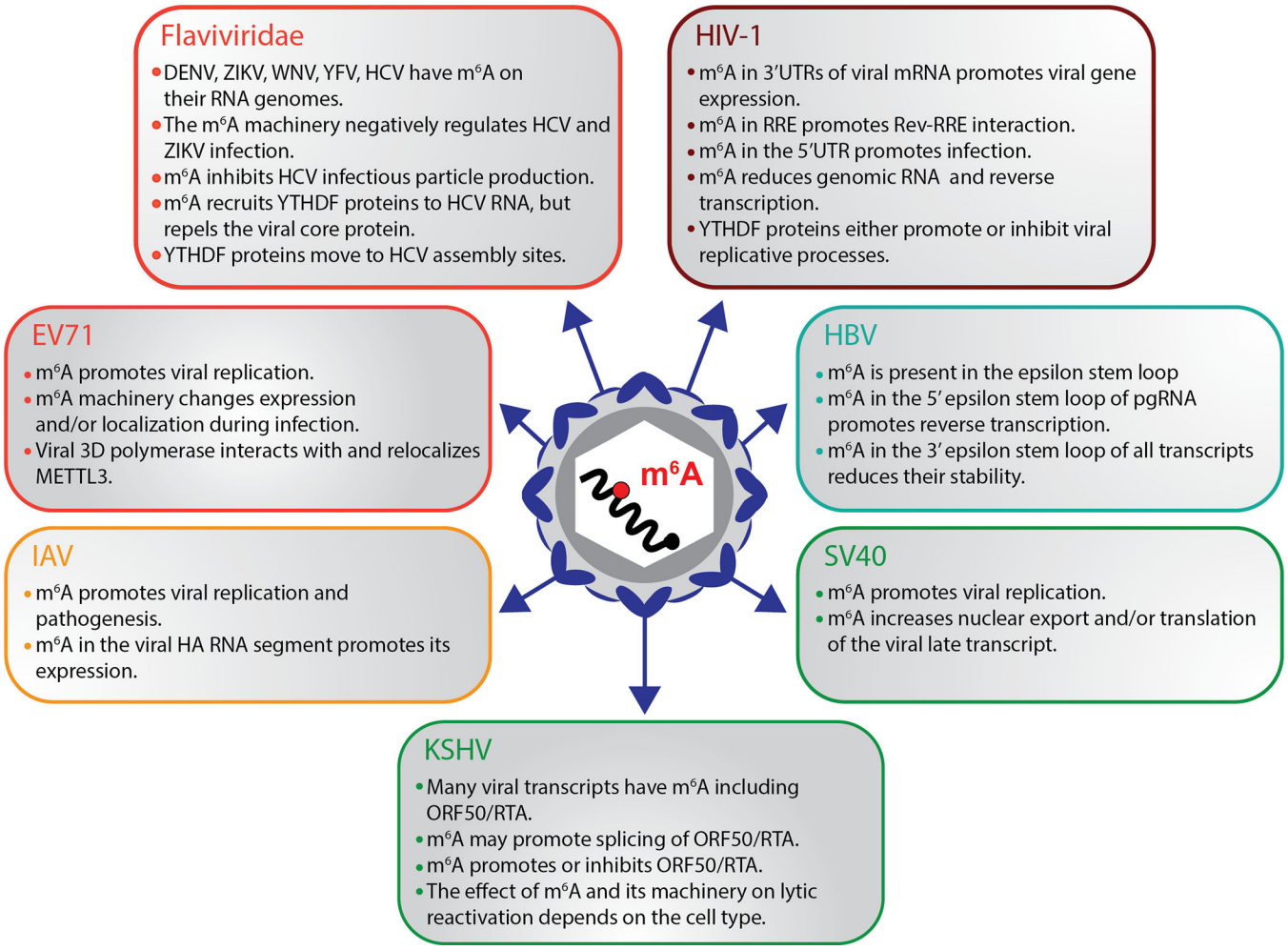

Figure 2.

The cellular m6A machinery impacts the replication of viruses from diverse families. Here we describe the main findings of how m6A on viral RNA regulates infection. Manipulation of cellular writers, erasers, and readers of m6A reshapes virus infection with differential outcomes depending on virus studied and cell type used for experiments. Viruses in the *Flaviviridae* family (DENV, ZIKV, WNV, YFV, and HCV) are negatively regulated by m6A writers while replication of enterovirus 71 and influenza A virus is promoted by m6A. m6A modification of viral transcripts derived from retroviruses and DNA viruses also bear m6A. The impact of m6A on these viruses is dependent on the stage of the viral replication cycle examined, host tissue, and viral strain studied. Color legend: Orange, positive-sense RNA viruses; Yellow, negative-sense RNA virus; Red, retrovirus; Blue, partially double-stranded DNA virus; Green, double-stranded DNA viruses. Abbreviations: DENV, dengue virus; EV71, enterovirus 71; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus-1; IAV, influenza A virus; KSHV, Kaposi's sarcoma-associated herpesvirus; m6A, *N6*-methyladenosine; pgRNA, pregenomic RNA; RRE, Rev response element; SV40, simian virus 40; WNV, West Nile virus; YFV, yellow fever virus; ZIKV, Zika virus.