

HIV-1 Replication Benefits from the RNA Epitranscriptomic Code

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Abstract

The effects of RNA methylation on HIV-1 replication remain largely unknown. Recent studies have discovered new insights into the effect of 2'-O-methylation and 5-methylcytidine marks on the HIV-1 RNA genome. As so far, HIV-1 benefits from diverse RNA methylations through distinct mechanisms. In this review, we summarize the recent advances in this emerging field and discuss the role of RNA methylation writers and readers in HIV-1 infection, which may help to find alternative strategies to control HIV-1 infection.

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Introduction

Over 150 distinct chemical modifications have been identified in noncoding RNAs including tRNAs, rRNAs, and snRNAs [1], and more than 10 modifications have been found in mRNAs. Methylation of RNA is an important epitranscriptomic marker that influences gene expression and function. There are three abundant RNA modifications that have been extensively studied to date, namely N⁶-methyladenosine (m⁶A), 5-methylcytidine (m⁵C), and 2'-O-methylation (Nm). These RNA methylations were initially described in noncoding RNA, such as tRNAs and rRNAs, but have subsequently been found in mRNAs, and demonstrated to be crucial for mRNA stability, splicing, nucleocytoplasmic export, and translation efficiency [2,3]. Recently, RNA methylations have been identified on the HIV-1 RNAs and shown to play a key regulatory role in the viral life cycle. Capitalizing on this knowledge may help to develop the alternative strategies to control HIV-1 infection.

Methods of RNA Methylation Detection

Currently, a few biochemical methods have been established and used to identify the RNA methylation residues, which are well summarized in elsewhere [4–6]. These methods usually rely on deep sequencing. To specifically detect m⁶A methylation, four methods have been developed: Me-RIP-seq, advanced PA-m⁶A-seq, miCLIP, and PAR-CLIP. PAR-CLIP relies on the fact that m⁶A reader specifically binds with m⁶A RNA to map m⁶A sites. Compared with Me-RIP-seq, the other methods yield extremely low background and high resolution [4]. To measure the m⁵C RNA methylation, RNA bisulfite sequencing can be performed by treating RNA with bisulfite, which converts unmodified cytosine residues to uracil, while 5-methylcytosines are left intact [7]. However, this type of chemical conversion is harsh, destroying much of the RNA, and resulting in diminished signal. An alternative, PA-m⁵C-seq, borrows from the approach used to detect m⁶A and makes use of an efficient m⁵C antibody [5]. Another method, miCLIP, was developed to take advantage of a feature of m⁵C RNA methyltransferases in which mutagenesis of the cysteine of conserved releasing sites leads to RNA methyltransferases being

trapped on the methylated residues of RNAs [8]. AZA-IP was developed to detect the m5C residues by feeding the cells with 5-azacytidine, which inhibits the release of the methylated RNA from its enzyme [9]. There are several classical methods for RNA 2'-O-methylation analysis [6]. However, these traditional methods, such as enzymatic hydrolysis and RNA analytical chemistry, are time-consuming and have certain limitations including the use of radioactively labeled RNA. Deep sequencing-based methods have been proposed to detect the RNA 2'-O-methylation including RiboMethSeq, 2'-OMe-Seq, RibOxi-Seq, and Nm-Seq [10], which allow high-throughput analysis and the use of lower amounts of input RNA. Overall, these emerging methods generally satisfy the immense surge of the interest in the research community regarding the analysis of biological functions of RNA modifications.

RNA Methylation of the HIV-1 Genome

All three of the common RNA methylation signatures—m6A, m5C, and 2'-O-methylation—have been identified in the HIV-1 genome. The m6A modification was first reported in HIV-1 infected CD4⁺ T cells by three independent groups [11–13]. HIV-specific methylation marks have been shown to distribute across the HIV genome, including the coding sequence (CDS), intronic sequences, and both the 5' and 3' untranslated regions (UTRs) [12,13]. Another study reported that m6A modification largely maps to the 3' UTR, especially located at the NF- κ B binding sites and TAR RNA hairpin [11]. In contrast, m5C residues have been identified across the HIV-1 genome and shown to be evenly distributed between 5'UTR, CDS and 3' UTR [14]. Recently, seventeen 2'-O-methylated residues were identified in the HIV-1 genome as well [15]. About 88% of internal methylation sites correspond to the adenosine residues, whereas internal methylation of uracil residues appears to be restricted to the 5' UTR and no 2'-O-methylated cytosine or guanine residues have been detected. Surprisingly, these RNA residues which are methylated by 2'-O-methylation methyltransferases in the NL4-3 HIV strain are conserved across major HIV-1 strains [15], suggesting that 2'-O-methylated adenosine residues may play an evolutionarily important role in HIV-1 replication. Notably, these modifications were identified by using RiboMethSeq, and the sequencing depth was lower than that of previous studies [16], so it may have been insufficient to completely identify all 2'-O-methylated residues. In contrast, Courtney et al. recently discovered that there are numerous 2'-O-methylated nucleosides on HIV-1 RNA, including on cytosine and guanine residues [14]. This discrepancy could be due to the use of different cell types and/or different biochemical methods. Nevertheless, these pioneering studies confirmed that HIV-1 RNA is highly modified with the distinct

methylation patterns: while 2'-O-methylations and m5C modifications are distributed across the HIV-1 genome, m6A modifications are enriched in the 3' UTR.

HIV-1 Replication Benefits from Diverse RNA Methylations

Previous studies have shown that RNA methylations contribute to RNA the stability, maturation, and translation efficiency of target RNAs. These RNA modifications can also be sensed by pattern recognition receptors (PRRs), a part of the innate immune system [17]. In terms of HIV-1, all studies to date have indicated that m6A editing increases viral replication through distinct mechanisms, such as increasing the steady state level of viral mRNA expression [11], affecting the binding affinity of Rev to Rev response element (RRE) to enhance viral RNA nuclear export [12], and increasing the expression level of Gag protein [13]. In addition, m5C editing enhances the alternative splicing of HIV-1 transcripts and promotes the stability of HIV-1 RNA, thereby affecting viral gene expression [14]. 2'-O-methylation editing has been shown to shield HIV-1 RNA from MDA-5 sensing, thus dampening induction of type I interferons [15]. So far, only 2'-O-methylation modifications have been linked to escape of HIV-1 RNA from immune sensors, while whether this function also applies to m5C or m6A signatures remains to be investigated. It was recently shown that following viral infection, knock-down of the m6A “writer” METTL3 or “reader” YTHDF2 led to the induction of interferon-stimulated genes, indicating that m6A editing is a negative regulator of innate immune signaling through control of the turnover of interferon mRNAs [18,19]. It was also found that durable neoantigen-specific immunity in dendritic cells (DC) is regulated by mRNA m6A modifications through the m6A-binding protein YTHDF1 [20]. Mechanistically, DC-specific YTHDF1 depletion promotes the cross-presentation of tumor antigens and the cross-priming of CD8⁺ T cells, thus inhibiting tumor development. Nevertheless, these findings implied that m6A methylation modulates the immune response. In summary, it is generally believed that methylations of the HIV-1 RNA facilitate viral propagation through distinct mechanisms that ultimately orchestrate and maximize the replication of the HIV-1 genome.

The Role of RNA Methylation Writers and Erasers During HIV Replication

The m6A modification is catalyzed by a multi-component methyltransferase complex that includes

the proteins METTL3, METTL14, RBM15, WTAP, KIAA1429, and ZC3H13 [21,22]. The two methyltransferases—METTL3 and METTL14—are the key factors in this complex and function as “writers” of m6A editing. They each have a conserved methyltransferase domain (MTD) and interact to form a heterodimer complex. Consistent with the positive effect of m6A editing on HIV-1 replication, depletion of METTL3 or METTL14 impairs HIV-1 replication [11–13]. However, an opposite effect has been shown for the Zika virus (ZIKV) and hepatitis C virus (HCV), both members of the Flaviviridae family, in which downregulation of the methyltransferases facilitates their replication by promoting the viral packaging [23,24]. These discrepancies may be attributed to either differences in ZIKV and HCV life cycle versus HIV-1, or as a result of differences in the cell types used for the studies.

The second major RNA methylation, m5C, is catalyzed by methyltransferases of the NOP/SUN (NSUN) family. The NSUN1, NSUN4, and NSUN5 proteins are responsible for rRNA methylation [25–27], whereas NSUN3 and NSUN6 are known to methylate tRNA [28,29]. DNMT2, originally identified as a DNA methyltransferase, also contributes to m5C modifications in tRNAs [30]. For mRNAs, however, NSUN2 is the main contributor of m5C modifications that facilitate their nuclear export [31]. NSUN2 also contributes to m5C methylation of tRNAs, protecting them from endonucleolytic cleavage, and regulating the cell metabolic state [32]. Interestingly, although DNMT2 has been reported to methylate the HIV-1 RNA *in vitro* [33], one recent study suggested that NSUN2 functions as the major m5C methyltransferase of HIV-1 RNA [14]. This claim was supported by the fact that depletion of NSUN2, but not DNMT2, resulted in a significant reduction of m5C modifications in the HIV-1 genome as well as viral protein expression, but had no effect on mRNA expression level [14], which indicated that DNMT2 may not be the m5C writer of HIV-1 RNA. Similarly, NSUN2 serves as an m5C writer to methylate the RNA of murine leukemia virus (MLV), and depletion of NSUN2 impairs MLV replication [34]. However, the effect of NSUN2 depletion on HIV-1 m5C modification was not absolute, suggesting that other methyltransferases may contribute to m5C methylation of HIV-1 RNA. It is important to note that NSUN1, similar as Tat and Rev, which possesses arginine-rich RNA-binding regions, was reported to bind with 5' UTR of HIV-1 [35,36] and thus may also serve as an m5C methyltransferase for HIV-1 RNA.

FTSJ1 was the first characterized human tRNA 2'-O-methyltransferase and belongs to the RRMJ/fibrillarin superfamily [37]. Two conserved homologs of FTSJ1—FTSJ2 and FTSJ3—are putative rRNA methyltransferases containing the KDKE conserved catalytic motif. The yeast methyltransferase

Spb1(FTSJ3) was identified to modify both rRNA and mRNA for ribosome assembly [38], indicating that human FTSJ3 may have a similar capability to methylate RNAs. Recently, FTSJ3, but not FTSJ2, was identified to be recruited by TRBP to methylate the HIV-1 RNA [15]. Although FTSJ1 may also methylate the HIV-1 RNA given to its 2'-O-methyltransferase activity, this has yet to be demonstrated experimentally. Other putative 2'-O-methyltransferases, such as TRMD44, have been predicted to methylate tRNA [37]. In addition, several enzymes, such as fibrillarin, CMTR1, and HENMT1, are known to catalyze the 2'-O-methylation of mRNA and other small RNAs as well [37]. However, their role in modulating HIV-1 infection remains to be elucidated.

There is a critical discovery for m6A modification, elucidating that two different enzymes, FTO and ALKBH5, serve as RNA demethylases to inhibit HIV-1 replication but enhances ZIKV replication [12,23,24]. It is interesting to speculate whether there may be similar erasers for the m5C and 2'-O-methylations.

The Role of RNA Methylation Readers in HIV-1 Replication

On addition of methyl groups to ribonucleotides, specific proteins can recognize the methylated RNA leading to post-transcriptional regulation of mRNA splicing, metabolism, and translation. The m6A-modified RNA residues can be selectively recognized by YTH family proteins, including the cytoplasmic m6A readers YTHDF1, YTHDF2, YTHDF3, and YTHDC2, as well as the nuclear m6A reader YTHDC1 [17,39]. Two new m6A readers, fragile X mental retardation protein (FMRP) and PRRC2A, were recently identified to preferentially bind with m6A-modified mRNAs to facilitate nuclear export through CRM1 and control oligodendroglial specification, respectively [40,41]. There are also several putative m6A readers from the hnRNP family in nuclei, such as hnRNPA2B1, hnRNPC, and hnRNPG [42–44]. The role of YTHDF1-3 in HIV-1 replication remains controversial. One study suggested that m6A-interacting proteins bind HIV-1 RNA and enhance HIV-1 replication [11] while another report claimed that YTHDF1-3 can inhibit HIV-1 replication by lowering incoming viral genome RNA levels and reducing the products of reverse transcription [13,45]. This discrepancy could be attributed to different cell types, lentiviral vectors, or virus strains used in each study. Furthermore, three different models have been proposed to describe the events following cellular entry—immediate uncoating, cytoplasmic uncoating, and uncoating at nuclear pores [46]—and these may differentially affect the opportunity for m6A readers to access the RNA. In the model of uncoating at nuclear pores, viral RNA is

likely protected by viral capsid and barely has the chance to bind with YTHDF1-3 before it enters the nucleus, which would limit its ability to inhibit HIV-1 reverse transcription. Also, according to the model, the viral RNA and reverse-transcribed DNA would not be detected by cytoplasmic sensors. Support for this model includes the observation that the single-cycle HIV-1 virus (lacking the *env*, *nef*, and *vpr* genes) induces a weak innate immune response, including type I interferons, and is able to escape from cellular sensors [47]. On the other hand, the data from Tirumuru et al. lend support to the models of immediate uncoating and cytoplasmic uncoating [46]. In these models, YTHDF1-3 would have access to HIV-1 RNA after it is released from the capsid core, and could thus block reverse transcription. In addition, the completion of reverse transcription can affect the kinetics of uncoating [48], which may further affect the binding of YTHDF1-3 to the products of reverse transcription. The actual process of HIV-1 uncoating may also depend on the type of cells used for HIV-1 infection and its status of activation. These readers have also been shown to

negatively regulate the replication of ZIKV and HCV [23]. FMRP, another m6A reader, contains a nuclear export signal (NES) that is similar to the NES identified in the Rev regulatory protein of HIV-1 [49]. FMRP also interacts with HIV-1 Gag and can be packaged into viral particles [50]. Taken together, these data support a model in which FMRP may bind m6A-labeled HIV-1 RNA to regulate viral infectivity. In line with this hypothesis, overexpression of FMRP inhibits HIV-1 replication [50]. Although we do not know whether PRRC2A can bind m6A HIV-1 RNA, it is interesting to note that PRRC2A is upregulated during early HIV-1 infection [51]. Lastly, hnRNP proteins have been implicated in trafficking and splicing of HIV-1 RNAs [52–57] and subcellular distribution of hnRNP proteins is also affected by HIV-1 infection. Various hnRNP D isoforms were shown to have different effects on expression of Gag protein [57], and a few hnRNP proteins interact with the HIV-1 Rev protein [58]. Thus, it is possible that hnRNP proteins may mediate the binding of Rev to the m6A-containing RRE element present in HIV-1 RNA. The effect of other potential m6A readers on

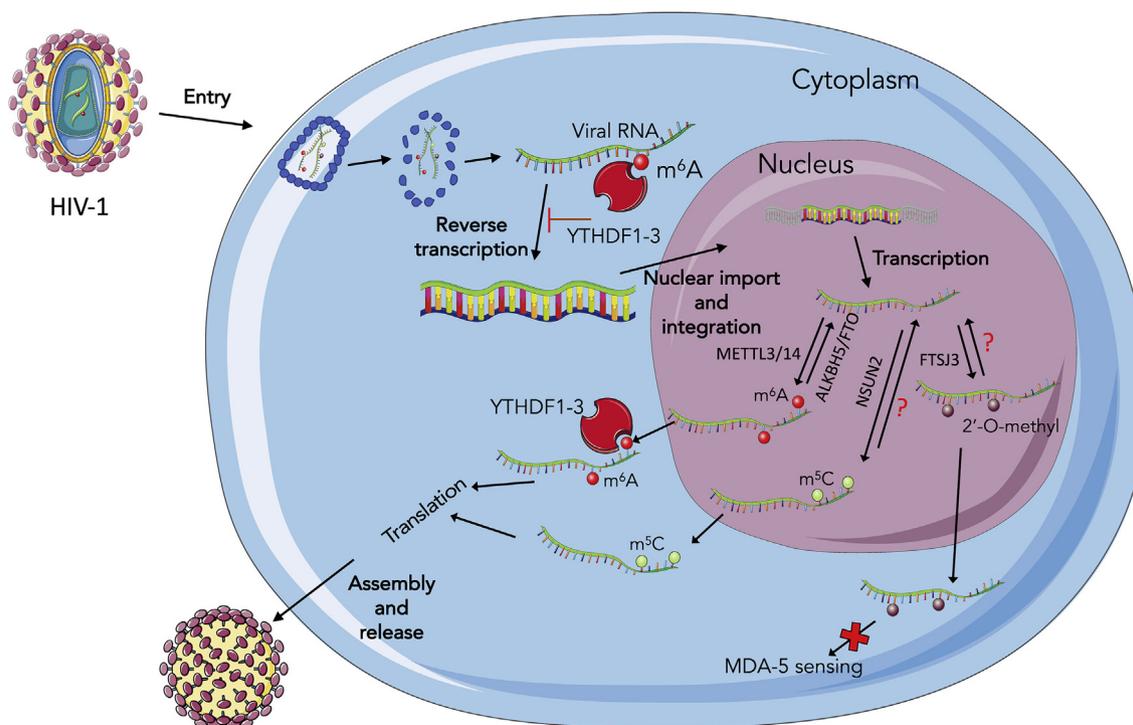


Fig. 1. A schematic presentation of the impact of RNA methylations on HIV-1 life cycle. The m6A writers (METTL3/14) add the methyl group to the HIV-1 RNA at N6-adenosine, while the m6A methylation erasers (ALKBH5/FTO) remove such methyl groups. m6A methylation readers (YTHDF1-3) bind to m6A-modified HIV-1 RNA to either enhance viral protein expression or inhibit reverse transcription. The m5C writer (NSUN2) adds the methyl group to the HIV-1 RNA at the C5 position of cytosine, which can be recognized by potential m5C readers to facilitate alternative splicing of HIV-1 transcripts and enhance viral protein expression. FTSJ3 methylates the HIV-1 RNA at the 2' hydroxyl (2'-O) of the ribose moiety and helps HIV-1 to escape from innate immune recognition. The question marks (?) indicate that the erasers of m5C and 2'-O-methylation remain to be discovered.

HIV-1 replication is still under investigation. Surprisingly, HIV-1 Rev protein was shown to accelerate nuclear export of viral RNA in an m6A-dependent manner [12]. Recently, ALYREF was reported to be a potential reader of m5C modification, which may facilitate the nuclear export of m5C-modified mRNA [31]. Although the data supporting a direct interaction between ALYREF and m5C-methylated HIV-1 RNA remains lacking, a bioinformatics study showed that ALYREF is likely involved in the early stage of HIV-1 infection [51]. YBX1 was identified as a new m5C reader recognizing m5C-modified mRNAs through its cold-shock domain [59]. Also, YBX1 has been shown to support different steps of HIV-1 replication through stabilizing HIV-1 genomic RNA [60,61], which indicated that YBX1 is highly likely the m5C reader for HIV-1 RNA. Additional m5C and 2'-O-methylated RNA readers may exist, and given the important role they play in the life cycle of virus, their discovery is a worthwhile pursuit.

Concluding Remarks

In summary, the most prevalent RNA methylations (m6A, m5C, and 2'-O-methylation) have all been identified in HIV-1 RNA. These RNA methylations in general facilitate HIV-1 replication through multiple mechanisms (Fig. 1). As we learn more about the epitranscriptome of HIV-1 and the host proteins that participate in these modifications during HIV-1 infection, we are getting closer to a full understanding of how they modulate the outcome of HIV-1 infection, interplay with the antiviral immune system, and regulate viral latency. These findings may also help us to develop new strategies targeting methylations of HIV-1 RNA to control HIV-1 infection.

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Conflicts of Interest

None declared.

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