N⁶-Methyadenosine–binding proteins suppress HIV-1 infectivity and viral production

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The internal N⁶-methyadenosine (m⁶A) modification of cellular mRNA regulates post-transcriptional gene expression. The YTH domain family proteins (YTHDF1–3 or Y1–3) bind to m⁶A-modified cellular mRNAs and modulate their metabolism and processing, thereby affecting cellular protein translation. We previously reported that HIV-1 RNA contains the m⁶A modification and that Y1–3 proteins inhibit HIV-1 infection by decreasing HIV-1 reverse transcription activity. Here, we investigated the mechanisms of Y1–3–mediated inhibition of HIV-1 infection in target cells and the effect of Y1–3 on viral production levels in virus-producing cells. We found that Y1–3 protein overexpression in HIV-1 target cells decreases viral genomic RNA (gRNA) levels and inhibits both early and late reverse transcription. Purified recombinant Y1–3 proteins preferentially bound to the m⁶A-modified 5′ leader sequence of gRNA compared with its unmodified RNA counterpart, consistent with the strong binding of Y1–3 proteins to HIV-1 gRNA in infected cells. HIV-1 mutants with two altered m⁶A modification sites in the 5′ leader sequence of gRNA exhibited significantly lower infectivity than WT, replication-competent HIV-1, confirming that these sites alter viral infection. HIV-1 produced from cells in which endogenous Y1, Y3, or Y1–3 proteins were knocked down singly or together had increased viral infectivity compared with HIV-1 produced in control cells. Interestingly, we found that Y1–3 proteins and HIV-1 Gag protein formed a complex with RNA in HIV-1–producing cells. Overall, these results indicate that Y1–3 proteins inhibit HIV-1 infection and provide new insights into the mechanisms by which the m⁶A modification of HIV-1 RNA affects viral replication.

Among the more than 100 distinct modifications identified in mRNAs in different organisms, N⁶-methyadenosine (m⁶A)³ methylation is the most prevalent internal modification, accounting for 0.1% of adenosines in mammalian mRNAs (1). The dynamic addition, removal, and recognition of m⁶A in cellular RNAs are coordinately regulated by three groups of host proteins, including methyltransferases (termed writers), demethylases (erasers), and m⁶A-binding proteins (readers). The writers include methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14), and Wilms tumor 1-associated protein (WTAP); and erasers include fat mass and obesity-associated protein (FTO) and alkB homologue 5 (ALKB5H) (2). The readers include YT521-B homology (YTH) domain family proteins (YTHDF1, YTHDF2, YTHDF3, and YTHDC1) that specifically recognize m⁶A modification via a conserved m⁶A-binding pocket in the YTH domain (3–5). In addition to YTH domain-containing proteins, the host proteins FMR1 and LRPPRC have also been identified as m⁶A readers (6, 7). These reader proteins modulate m⁶A-modified mRNA stability and translation, thereby playing an important role in regulating post-transcriptional gene expression (8–12).

Three recent studies highlighted the importance of m⁶A modifications of HIV-1 RNA in regulating viral replication and gene expression (13–15). Despite some consistent results, there are discrepancies in the locations, effects, and mechanisms of m⁶A modification of HIV-1 RNA in these studies (13–15). Our published study identified m⁶A modifications in the 5′ and 3′ untranslated regions (UTRs), as well as in the rev and gag genes of the HIV-1 genome (15). We previously reported that overexpression of Y1–3 proteins in cells inhibits HIV-1 infection by primarily decreasing HIV-1 reverse transcription, whereas knockdown of endogenous Y1–3 in Jurkat CD4⁺ T-cell line or primary CD4⁺ T-cells increases HIV-1 infection (15).

The abbreviations used are: m⁶A, N⁶-methyadenosine; IP, immunoprecipitation; gRNA, genomic RNA; m.o.i., multiplicity of infection; hpi, hours post-infection; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium, inner salt; qPCR, quantitative PCR; nt, nucleotide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DIS, dimer initiation sequence; PBS, primer-binding site; YTH, YT521-B homology; ZIKV, Zika virus; HCV, hepatitis C virus; KSHV, Kaposi’s sarcoma-associated herpesvirus; IAV, influenza A virus; DMMEM, Dulbecco’s modified Eagle’s medium; SIV, simian immunodeficiency virus; NC, nucleocapsid; VSV-G, vesicular stomatitis virus glycoprotein.

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ever, the underlying mechanisms of Y1–3–mediated inhibition of HIV-1 infection remain unclear.

Here, we report that Y1–3 inhibited HIV-1 infection in target cells by lowering incoming viral genome RNA (gRNA) levels and reverse transcription products. We demonstrate that the m6A-modified HIV-1 RNA fragment preferentially bound to Y1–3 proteins compared with an unmodified RNA counterpart, and mutations of two m6A sites in the 5′ UTR significantly decreased viral infectivity. Knockdown of endogenous Y1, Y3, or Y1–3 proteins together in virus-producing cells positively modulated HIV-1 infectivity. Furthermore, we found that endogenous Y1–3 proteins and HIV-1 Gag protein formed a complex with RNAs in HIV-1–producing cells. Together, these findings suggest new mechanisms by which Y1–3 proteins mediate HIV-1 inhibition during early steps of the viral life cycle.

Results

Overexpression of Y1–3 proteins in target cells inhibits post-entry HIV-1 infection by decreasing HIV-1 gRNA levels and inhibiting viral reverse transcription

Our previous study showed that Y1–3 proteins negatively regulate HIV-1 post-entry infection in target cells, including primary CD4+ T-cells (15). To better understand the underlying mechanisms, we compared incoming HIV-1 gRNA levels and early and late reverse transcription (RT) products in stable HeLa cell lines overexpressing individual Y1–3 proteins or vector control after infection with vesicular stomatitis virus glycoprotein (VSV-G)–pseudotyped single-cycle HIV-1 expressing firefly lucerase. Consistent with our previous results (15), Y1–3 overexpression (Fig. 1A) resulted in significantly lower levels of HIV-1 post-entry infection (26, 8.6, and 23% in HeLa/Y1, HeLa/Y2, and HeLa/Y3 cells, respectively) compared with HeLa/vector control cells (set as 100%, Fig. 1B).

To delineate at which stage of HIV-1 replication, post-entry, the YTHDF proteins might be acting on inhibition, we next examined whether Y1–3 proteins alter the levels of incoming HIV-1 gRNA at 1, 3, and 6 h post-infection. The levels of incoming HIV-1 gRNA were quantified using RT-qPCR (16) and were lower in HeLa/Y1–3 cells relative to those in HeLa/vector control cells. At each time point, the levels of both early and late RT products were significantly lower in HeLa/Y1–3 cells compared with vector control cells (Fig. 1, D and E). These results demonstrate that overexpression of Y1–3 proteins leads to reduced levels of incoming HIV-1 gRNA.

After HIV-1 infection, the capsid core disassembles in the cytoplasm, and viral reverse transcriptase generates early and late RT products. Late RT products translocate into the nucleus and integrate into the host genome (17). Our previous study showed that Y1–3 proteins inhibit accumulation of HIV-1 late RT products in infected cells (15); however, it is unclear whether Y1–3 proteins affect HIV-1 early RT efficiency. To address this question, HeLa/Y1–3 cells or HeLa/vector control cells infected by HIV-1–Luc/VSV-G were collected at 6, 12, and 24 hpi for quantification of HIV-1 early and late RT products by quantitative PCR (qPCR), as reported previously (18). At each time point, the levels of both early and late RT products were significantly lower in HeLa/Y1–3 cells compared with vector control cells (Fig. 1, D and E). At 24 hpi, the levels of early and late RT products were significantly lower in HeLa/Y1–3 cells compared with vector control cells (Fig. 1, D and E).
late RT products in HeLa/Y1–3 cells decreased to 37–54%, whereas HIV-1 infection decreased to 8.6–26%, compared with vector control cells (set to 100% in Fig. 1, B, D, and E). Given that Y1–3 overexpression decreased incoming HIV-1 gRNA levels, and further decreased early and late RT products (Fig. 1, C–E), this suggests that Y1–3 proteins expressed in target cells act predominantly by decreasing incoming viral gRNA stability but also concomitantly decreasing downstream early and late RT product synthesis.

**Overexpression of Y1–3 proteins in HeLa/CD4 target cells inhibits wildtype (WT) HIV-1 replication**

Because the firefly luciferase mRNA contains consensus sequences for m⁶A modifications and our previous study used HIV-1–Luc/VSV-G virus containing a luciferase reporter gene (15), it was possible that the HIV-1 inhibition we observed could be attributed to luciferase m⁶A modifications, as well as the inhibitory effects of Y1–3. Furthermore, the entry mechanisms of VSV-G–pseudotyped HIV-1 differ from fusion entry mediated by replication-competent WT HIV-1 through its CD4 receptor (19, 20) and could impact the effect of Y1–3 proteins on downstream HIV-1 replication. To demonstrate the importance of Y1–3 proteins in inhibition of HIV-1 infection mediated through its natural fusion entry, we used WT, replication-competent HIV-1NL4-3, to infect HeLa cells overexpressing CD4 and individual Y1–3 proteins (HeLa/CD4/Y1–3) or vector control. Using flow cytometry, we confirmed that the majority of these cells were double-positive for the HIV-1 primary receptor CD4 and co-receptor CXCR4 (71–81%), which would allow efficient fusion-mediated viral entry (Fig. 2A). We further demonstrated that stable overexpression of individual Y1–3 proteins in HeLa/CD4 cells did not significantly affect cell proliferation during 72 h in culture (Fig. 2B). Immunoblotting also confirmed that HeLa/CD4/Y1–3 cells stably expressed FLAG-tagged individual Y1–3 proteins (Fig. 2C).

To examine the effects of Y1–3 overexpression on WT HIV-1 replication, we measured cellular Gag protein and p24 release at 72 hpi. Consistent with the results from single-cycle HIV-1–Luc/VSV-G (Fig. 1B) (15), Y1–3 overexpression efficiently inhibited infection of WT HIV-1NL4-3 as Gag (Pr55) protein levels in cells and p24 levels in supernatants were significantly lower in HeLa/CD4/Y1–3 cells compared with vector control cells (Fig. 2, C and D). These results confirmed the inhibitory effects of Y1–3 proteins on HIV-1 infection and also suggested that potential m⁶A modification of the inserted luciferase gene in HIV-1–Luc/VSV-G does not contribute to Y1–3–mediated inhibition of HIV-1. Furthermore, infection of HeLa cells overexpressing Y1–3 proteins with WT HIV-1 also resulted in significantly lower early and late HIV-1 RT products (Fig. 2, E and F) and consequently reduced gag mRNA levels compared with vector control cells (Fig. 2G). These data suggest that Y1–3 proteins inhibit WT HIV-1NL4-3 infection before or during the RT stage.

**Y1–3 proteins bind to HIV-1 gRNA in infected HeLa/CD4 cells**

Our previous study showed that incoming gRNA from HIV-1–Luc/VSV-G binds to YTHDF proteins during early infection (15). To confirm that Y1–3 proteins bind to incoming HIV-1 gRNA in infected cells where entry was mediated through viral envelope and cellular receptor fusion, we performed immuno-precipitation (IP) of Y1–3 in HeLa/CD4/Y1–3 cells lines and vector control cells infected with WT HIV-1NL4-3 (Fig. 3A). To validate the specificity of Y1–3 binding to HIV-1 RNA, we also included an unrelated cellular protein MAL (MyD88 adapter-like, also known as Toll/IL-1 receptor (TIR) domain-containing adapter protein, or TIRAP) (21, 22) as an additional negative control (Fig. 3A). We then quantified the amounts of Y1–3–bound HIV-1 gRNA using RT-qPCR assays as reported previously (15). We observed that Y1–3 efficiently bound to incoming WT HIV-1 gRNA in HIV-1NL4-3–infected HeLa/CD4/Y1–3 cells compared with vector control or MAL-expressing cells (Fig. 3B). The levels of incoming HIV-1 gRNA bound to Y1 appeared higher than those bound to Y2 and Y3 (Fig. 3B), which could be due to the higher expression of Y1 in HeLa/CD4 cells relative to Y2 and Y3 proteins (Fig. 3A) and consequently a higher level of IP products (Fig. 3B). These data confirm that Y1–3 proteins bind to incoming WT HIV-1NL4-3 gRNA in infected cells.

**Purified recombinant Y1–3 proteins preferentially bind to an m⁶A-modified HIV-1 RNA fragment in vitro**

Our previous study showed that HIV-1 RNA contains m⁶A modifications at both the 5’ and 3’ UTR (15). Given the critical role of the 5’ UTR in initiation of HIV-1 reverse transcription (23), as well as additional roles in TAR-dependent transcriptional activation, dimerization, and packaging of the RNA genome (24), we focused on the m⁶A sites in the 5’ UTR of HIV-1 gRNA. The GGACU motif is the most predominant sequence that undergoes m⁶A modification (25, 26). The m⁶A peak detected by high-throughput RNA-sequencing in the 5’ UTR of HIV-1 gRNA harbors two GGACU motifs (15). The first motif is located in the primer-binding site (PBS), and the second is in a region upstream of the dimer initiation sequence (DIS) (Fig. 4A), and both are located proximal to the Y1–3 protein–binding peaks identified in the 5’ UTR of HIV-1 gRNA (15).

To study the binding properties of Y1–3 proteins to HIV-1 RNA containing m⁶A modifications, we synthesized two RNA fragments corresponding to nucleotides 235–281 of HIV-1NL4-3 gRNA containing either m⁶A modification in the second GGACU motif located in the 5’ UTR (Fig. 4B). These RNA fragments were inserted luciferase gene in HIV-1–Luc/VSV-G does not contribute to Y1–3–mediated inhibition of HIV-1. Furthermore, infection of HeLa cells overexpressing Y1–3 proteins with WT HIV-1 also resulted in significantly lower early and late HIV-1 RT products (Fig. 2, E and F) and consequently reduced gag mRNA levels compared with vector control cells (Fig. 2G). These data suggest that Y1–3 proteins inhibit WT HIV-1NL4-3 infection before or during the RT stage.
although both RNA fragments had detectable binding to Y1–3 proteins at 125 and 625 nM (Fig. 4D). The immunoblotting results of two independent pulldown experiments were quantified and normalized to 1, based on protein pulldown levels by m6A RNA fragment at 625 nM protein input (Fig. 4E). To compare affinity of RNA fragments to Y1–3 proteins, we calculated the concentrations of each Y1–3 protein (ranging from 1 to 625 nM) required for 50% pulldown levels based on the regression curves (Fig. 4, E and F). The 50% pulldown efficiencies indicated that Y1–3 proteins bound to m6A RNA fragment 7-, 13-, and >20-fold higher than control RNA, respectively (Fig. 4F). These results demonstrate that Y1–3 proteins exhibit substantially higher affinity for m6A-modified HIV-1 RNA in vitro, which may contribute to Y1–3–mediated inhibition of HIV-1 infection in cells.

A to G mutations in GGACU motifs of the 5¢ UTR of HIV-1 gRNA reduce viral infectivity

We previously identified enriched m6A modifications in the 5¢ and 3¢ UTRs of the HIV-1 genomic RNA. The 5¢ UTR is critical for HIV-1 reverse transcription, genome packaging, and viral infectivity (23, 28), and we demonstrated that m6A modifications in this region were important for Y1–3 binding to HIV-1 RNA and that overexpression of Y1–3 proteins reduces HIV-1 infection by inhibition of reverse transcription (15). We sought to focus on these m6A-modified regions on the HIV-1
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Previously published m^6A mapping results indicate that there are multiple m^6A sites in different regions of HIV-1 gRNA, in addition to the 5’ UTR (13–15), which could potentially contribute to regulating viral infectivity through interactions with Y1–3 proteins. To assess the potential contribution of other m^6A-modified regions, we examined whether silencing Y1–3 in target cells could restore the infectivity of the 5’ UTR mutant viruses that we generated. Endogenous Y1–3 proteins were simultaneously knocked down in TZM-bl cells by combined Y1–3–specific siRNA, and cells were then infected with the same p24 amount of WT or mutant viruses generated from normal HEK293T cells. We obtained partial knockdown of Y1–3 proteins in TZM-bl cells (Fig. 5F), and all three mutant viruses showed 40–50% lower infectivity relative to WT HIV-1 (Fig. 5G). These results suggest that m^6A modification of the GGACU motifs in the 5’ UTR is important for HIV-1 infection; however, because Y1–3 knockdown did not fully rescue HIV-1 mutants, additional m^6A modifications or mechanisms likely play a role in determining HIV-1 infectivity in TZM-bl target cells.

Given the important role of the PBS and DIS in structure and function of HIV-1 gRNA (30), we predicted the secondary structures of the RNA segments containing the PBS and DIS of WT and mutant viruses. Compared with the structure of WT HIV-1, the A to G mutation in the first GGACU motif (Mut1) resulted in a longer stem structure in the PBS sequence region, and Mut1 folding has a lower ΔG value (WT and Mut1 are −4.3 and −7.9 kcal/mol, respectively) (Fig. 5H). Mutation in the second GGACU motif did not change RNA structure containing the DIS, and WT and Mut2 have comparable ΔG values (−12.8 and −12.5 kcal/mol, respectively) (Fig. 5H). Thus, elimination of m^6A modification through Mut1 and Mut2 may account for reduced viral infectivity. RNA structural changes caused by the mutations may also affect tRNA\textsubscript{Lys}^3 binding, dimerization, or nucleocapsid–RNA interaction and therefore contribute to the decreased infectivity.

Y1–3 protein knockdown in virus-producing cells decreases HIV-1 Gag expression and alters viral production and infectivity

Our published results (15) and new data showed that Y1–3 proteins in target cells negatively regulate single-cycle and replication-competent HIV-1 infection (Figs. 1B and 2, B and C). To further elucidate the role of Y1–3 in HIV-1 post-integrative protein expression and infectivity, endogenous Y1–3 gene expression in virus-producing HEK293T cells was knocked down using specific siRNAs and transfected with HIV-1 NL4-3 plasmid DNA. Compared with nonspecific control siRNA, single knockdown of each Y1–3 significantly decreased HIV-1 Gag (Pr55) protein expression and correspondingly reduced the levels of processed p24 protein in cells (Fig. 6, A and B). Consistently, p24 levels in the supernatants of cells with single or combined Y1–3 knockdown also reduced ~2-fold compared with those of control cells, and there was no synergistic effect of combined knockdown (Fig. 6C), which might be due to different functions of Y1–3 in cells (2, 5).

To compare the infectivity of HIV-1 generated from cells with Y1–3 knockdown, viruses with equal amounts of p24
were used to infect TZM-bl indicator cells. As shown in Fig. 6D, HIV-1 generated from individual Y1 or Y3 knockdown cells had significantly higher infectivity compared with virus from control cells (p < 0.005), suggesting that Y1 and Y3 proteins in cells negatively affect infectivity of progeny HIV-1. In contrast, efficient Y2 knockdown in virus-producing cells resulted in a 25% decrease in HIV-1 infectivity (Fig. 6, A and D), suggesting a different mechanism of Y2-mediated inhibitory effect on viral infection. Moreover, combined Y1–3 knockdown efficiently reduced endogenous levels of Y1–3 proteins in virus-producing cells (Fig. 6A, last lane), but it only modestly increased HIV-1 infectivity (Fig. 6D), which were likely due to the different effects resulting from Y1/3 and Y2 knockdown.

Y1–3 proteins and HIV-1 Gag form a complex with RNAs in HIV-1-producing cells

To examine whether Y1–3 could interact with any HIV-1 proteins in cells, we performed IP of overexpressed Y1–3 in HEK293T cells co-transfected with pNL4-3 and then detected HIV-1 proteins in the input and IP products by immunoblotting using human anti-HIV-1 immunoglobulin (31). Interestingly, we found that Y1–3 proteins co-precipitated with HIV-1 Gag (Pr55) and intermediate Gag products but not with HIV-1 p24 (Fig. 7, lanes labeled with IP). The vector cells were used as a negative control for IP and showed a background band of Gag (Pr55) in the IP product (Fig. 7, 2nd lane from left). Immunoblotting of FLAG confirmed expression and IP of FLAG-tagged Y1–3 proteins in the transfected cells. Because both Y1–3 pro-
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Proteins and HIV-1 Gag can bind cellular and HIV-1 RNAs (10, 12, 15, 32), to examine whether RNAs mediate the association between Gag and Y1–3 proteins, cell lysates were treated with RNase A before IP. Interestingly, RNase A treatment completely eliminated HIV-1 Gag signal in Y1–3 precipitation (Fig. 7, lanes labeled with RNase + IP). These results suggest that Y1–3 proteins and HIV-1 Gag form a complex with viral and cellular RNAs in cells.

Given that our data demonstrated that Y1–3 proteins associated with Gag protein, we speculated whether this association in virus-producing cells could result in packaging of Y1–3 proteins into HIV-1 particles. We purified HIV-1 particles gener-
ated from HEK293T cells individually overexpressing Y1–3 proteins, and we then assessed the presence of each protein in purified HIV-1 virions by immunoblotting. We confirmed the presence of HIV-1 Gag protein in both cell lysates and virions. Although FLAG-tagged Y1–3 proteins had high-level expression in transfected cells, Y1–3 proteins were not detectable in highly purified virions (Fig. 7B), suggesting that Y1–3 proteins are not incorporated into HIV-1 virions.

**Discussion**

Reversible m^6^A modification is the most prevalent mRNA modification in eukaryotic organisms and plays critical roles in...
m^6A-binding proteins suppress HIV-1 infectivity

Figure 7. Y1–3 proteins and HIV-1 Gag form a complex with RNAs in HIV-1–producing cells. A, HEK293T cells were co-transfected with pNL4-3 and individual plasmids expressing HA- and FLAG-tagged Y1–3 or empty vector. Cell lysates were treated with or without RNase A (100 μg/ml) before IP of Y1–3 proteins using anti-HA–agarose beads. The input or IP products of HIV-1 Gag or CA (p24) were detected using HIV immunoglobulin. Expression and IP of Y1–3 were confirmed with anti-FLAG immunoblotting. Results presented are representative of three independent experiments. B, HEK293T cells were co-transfected with pNL4-3, empty vector, or FLAG-tagged Y1–3–expressing plasmids. Cells and supernatants were collected 46 h later. Virions in the supernatants were purified and concentrated for immunoblotting together with cell lysates using anti-p24, anti-FLAG (for Y1–3), and anti-GAPDH antibodies. GAPDH was used a loading control.

regulation of gene expression (5). Although m^6A modifications were previously identified in different viruses (33–35), the role in regulation of HIV-1 gene expression was recently recognized (13–15). Although these studies identified specific m^6A sites in HIV-1 gRNA, they disagreed on the extent and locations of m^6A modifications along the HIV-1 genome and their effects on viral replication (13–15). Lichinchi et al. (13) reported 14 peaks of m^6A modification in HIV-1 RNA, in which the m^6A modification in the Rev response-element region increased binding to Rev and facilitated nuclear export of viral RNA, thereby enhancing HIV-1 replication. In contrast, Kennedy et al. (14) found four clusters of m^6A modification in the 3′ UTR region of HIV-1 RNA. Our study showed that HIV-1 gRNA has m^6A modifications in the 5′ and 3′ UTRs as well as gag and rev genes (15).

Of note, m^6A modification is a dynamic, reversible process that can vary in different cellular backgrounds (2). It should not be too surprising that three studies reported a different number of m^6A modifications in different locations on HIV-1 RNA. In addition, there are discrepancies regarding the roles of Y1–3 proteins in HIV-1 replication. In the presence of Y1–3 overexpression, Kennedy et al. (14) observed increased HIV-1 replication, although our data showed decreased HIV-1 replication (15). These discrepancies could be influenced by different cell lines, methods used, and time course of infection. The purpose of this study was to determine the importance of Y1–3 proteins during HIV-1 infection by using replication-competent HIV-1, systematically determine at which point during the pre-integrative early events of HIV-1 infection Y1–3 proteins act, and compare the effects of m^6A modification on generation of HIV-1 particles in producer and infectivity in target cells.

In this study, using a time course analysis of HIV-1 pre-integration stages, we demonstrated that Y1–3 protein overexpression led to significantly lower levels of incoming HIV-1 gRNA, early and late RT products, and further decreased HIV-1 infection, suggesting that Y1–3 proteins may act by predominantly affecting incoming HIV-1 gRNA levels. These results further support our previous study that demonstrated that Y1–3 proteins specifically bind to the incoming genomic HIV-1 RNA, although it is not mechanistically clear how Y1–3 proteins would access gRNA protected by the core.

The GGACU is the predominant sequence in RRACH (R = G or A; H = A, C, or U) motif recognized by the m^6A writers (36). Our in vitro biochemical experiments reveal that Y1–3 proteins exhibit clear preference for the m^6A-modified HIV-1 RNA fragment over its unmodified RNA counterpart. The preferential binding of m^6A sites in the HIV-1 genome to Y1–3 proteins may contribute to decreased HIV-1 infection in HeLa/Y1–3 cells. There are two GGACU motifs in the 5′ UTR of HIV-1NL4-3 gRNA. The first GGACU motif is in the PBS region and is conserved in HIV-1 and simian immunodeficiency virus (SIV) (37), suggesting critical roles of the motif in HIV-1 and SIV life cycles. The second GGACU motif is in the DIS stem. This motif is conserved in HIV-1 subtype B isolates, but A to G mutations can be found in subtype C and group O isolates (https://www.hiv.lanl.gov/).

Compared with WT viruses, mutant viruses harboring A to G mutations have significantly reduced infectivity. We noted that the first GGACU motif is in the PBS, and mutation at this site in Mut 1/3 viruses (A to G at nt 197) introduces a mismatch with tRNA^1^5^3^ primer (38), which may affect primer annealing and therefore impair viral infectivity. Recent studies showed that the second GGACU motif interacts with HIV-1 nucleocapsid (NC) protein in viral particles (38) and that nucleotides GGA in this motif strongly bind Gag precursor for viral genome packaging (39). Mutation at this site may also impair interactions between HIV-1 gRNA and NC protein, contributing to decreased infectivity in mutant viruses. Thus, the decreased infectivity of Mut2 and Mut3 viruses (A to G at nt 241) may be due to eliminated m^6A modification at the second GGACU motif and/or decreased interactions between HIV-1 gRNA and Gag/NC. The effects of these mutations on primer binding and
of endogenous Y1 or Y3 proteins in have no effect on IAV infection, whereas Y2 knockdown can significantly enhances IAV gene expression and replication (47). Although different approaches in these studies may account for discrepancies, these results suggest that m6A modification and Y1–3 proteins have distinct effects on various viruses.

In summary, we found that m6A reader proteins Y1–3 inhibit HIV-1 infection by decreasing viral gRNA and early reverse transcription products. We demonstrate that Y1–3 proteins preferentially bind to m6A-modified HIV-1 RNA. Mutation of m6A sites in the 5' UTR resulted in decreased viral infectivity, suggesting important roles of these sites for HIV-1 infection. Y1–3 proteins and HIV-1 Gag form a complex with RNAs in virus-producing cells (Fig. 8). Together, these results help better understand the roles and mechanisms of m6A modification of HIV-1 RNA in regulating viral replication.

In addition to HIV-1, recent studies have identified viral RNA m6A modifications and its roles in regulating replication of Flaviviridae viruses, including hepatitis C virus (HCV), dengue, Zika virus (ZIKV), yellow fever virus, and West Nile virus (43, 44), and influenza A virus (45, 46), and influenza A virus (IAV) (47). The m6A modification in viral RNA increases RNA expression of IAV (47), promotes KSHV lytic replication (45), but negatively regulates HCV and ZIKV production (43, 44). A more recent study reported that Y2 protein negatively regulates the KSHV lytic replication by enhancing decay of KSHV transcripts (46).

Experimental procedures

Cell culture

HEK293T cell line (a kind gift from Dr. Vineet KewalRamani, NCI, Frederick, MD) and TZM-bl cells ((29) obtained through the AIDS Reagent Program (National Institutes of Health), catalogue no. 8129) were maintained in complete DMEM as described (17). HeLa or HeLa/CD4 cell lines (kind gifts from Dr. Vineet KewalRamani, NCI, Frederick, MD) overexpressing empty vector (pPB-CAG), individual FLAG- and HA-tagged Y1, Y2, or Y3 protein were maintained in complete DMEM containing 2 μg/ml puromycin as described (15).

Generating HeLa cell lines stably express CD4 and Y1–3 proteins

HeLa/CD4 cells were generated by transduction of HeLa cells with a pMX retroviral vector expressing human CD4 (19). HeLa/CD4 cells were transfected separately with pPB-CAG vector-based Y1–3–expressing constructs or pPB-CAG empty vector, and then selected with puromycin (2 μg/ml). HeLa/CD4 cells stably expressing individual Y1–3 proteins were cultured as described (15).

Cell proliferation assay

Cell proliferation of HeLa/CD4 stably expressing individual Y1–3 proteins were determined by the CellTiter 96® AQueous One Solution cell proliferation assay (Promega, Madison, WI, catalogue number G3581, MTS assay) according to the manufacturer's instructions. Cells (2 × 10³) were plated in quadruplicate in a 96-well plate and cultured for 3 days, and the absorbance was read at 490 nm at the indicated times as described (15).
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**Table 1**

Sequences of siRNAs used for Y1–3 knockdown

| Targets     | Catalog no. (Qiagen) | Target sequences (5’–3’) |
|-------------|----------------------|-------------------------|
| YTHDF1      | SI00764715           | CGCCTCTGATGGTGTTCGAA    |
| YTHDF1      | SI04200117           | TTCCCTCCACAATGGACCTAA   |
| YTHDF1      | SI04204117           | TACGAGACTCTGGTACAGGA    |
| YTHDF2      | SI02879121           | CAGGTCGGAGAATAGCCAGCA   |
| YTHDF3      | SI06764757           | AAAGCCAGCTTCAATGACCAA   |
| YTHDF3      | SI0279777            | TACC TTCCTGATGGTGTTCGAA |
| YTHDF3      | SI06764778           | AAGTTTTAAATACGTTTCGAA   |
| YTHDF3      | SI04133339           | TAAGTCTCCAGGACTCTTATA   |
| Negative control | SI03650325        | TATCTCCAGGACTCTTATA    |

Mutagenesis in HIV-1 proviral DNA plasmid (pNL4-3)

Mutations in pNL4-3 were introduced using Agilent QuikChange Lightning multisite-directed mutagenesis kit (catalogue no. 210515–5) according to the instructions. Primers mut1 (5′-CCCCGACACGGGCTTGGAAAGC-3′) and mut2 (5′-TCGACGCAAGGCTCGGGTTT-3′) were used (mutation sites are underlined) to generate A to G mutation at the first (nt 197), the second (nt 241), or both GGACU motifs between HIV-1 gRNA U5 and gag gene. The mutations were confirmed by DNA sequencing.

siRNA knockdown of Y1–3 proteins

Y1–3 expressions were knocked down by two rounds of siRNA transfection using specific siRNAs (Qiagen) and Lipofectamine RNAiMax (Invitrogen) according to instructions (siRNA sequences and catalogue numbers are listed in Table 1). Briefly, HEK293T cells (1.5 × 10^6 per well) in 24-well plates were transfected with gene-specific siRNAs or control siRNA (80 nM). Twenty four h after transfection, cell culture media were replaced, and the second-round siRNA transfection was conducted. At 6 h after the second round of siRNA transfection, pNL4-3 (0.5 μg per well) was transfected to HEK293T cells. Cells and culture media were collected at 36 h after pNL4-3 transfection for immunoblotting, p24 quantification, and infection assays.

HIV-1 stocks and infection assays

Single-cycle HIV-luc/VSV-G was generated as described previously (15). Replication-competent WT and mutant HIV-1 stocks were generated by transfection of HEK293T cells with pNL4-3 or mutant proviral DNA plasmids using Lipofectamine 2000 (Invitrogen) according to instructions. For Y1–3 and pNL4-3 co-expression experiments, PB-CAG vector or PB-PB-CAG–expressing individual FLAG- and HA-tagged Y1–3 protein was transfected into HEK293T cells, and pNL4-3 proviral DNA was transfected 12 h later. At 48 h (WT and Mut1–3 plasmids) or 36 h (Y1–3 co-expression) after proviral DNA transfection, cells were collected for immunoblotting, and HIV-1 capsid p24 levels in viral stocks were quantified by an enzyme-linked immunosorbent assay (ELISA) using anti-p24–coated plates (AIDS and Cancer Virus Program, NCI-Frederick, MD) as described (15, 19). To compare infectivity of WT HIV-1 in HeLa/Y1–3 cells, HIV-luc/VSV-G was used for infection at multiplicity of infection (m.o.i.) at 1 as described (15). To compare the infectivity of WT HIV-1NL4-3 and mutant viruses, viruses with equal amounts of p24 (400 pg) were used to infect TZM-bl cells in 24-well plates. At 48 hpi, TZM-bl cells were washed twice with PBS and lysed for luciferase assay (Promega) according to the manufacturer’s instructions. Cell protein concentrations were quantified using a bicinchoninic acid assay (Pierce), and all luciferase results were normalized based on total protein input.

Flow cytometry measures cell-surface levels of CD4 and CXCR4

HeLa/C4D cells were treated with the nonenzymatic cell dissociation solution (C5914, Sigma) and double-stained with FITC-conjugated CD4 (MHCD0401-4, ThermoFisher Scientific) and phycoerythrin–conjugated CXCR4 (555974, Pharmingen) antibodies as described (15). Cells stained with a mouse IgG2a isotype antibody (555574, Pharmingen) were used as a negative control. Flow cytometry was analyzed using a Guava EasyCyte Mini, and data analysis was performed using FlowJo (FlowJo, LLC) software as described (48).

IP of Y1–3 proteins and RT-qPCR detection of HIV-1 gRNA

HeLa/C4D cells expressing MAL were generated by transfection with pEF-Bos MAL Flag (plasmid no. 41554 from Addgene) (22). HeLa/C4D cells expressing pPB-CAG vector, MAL, or Y1–3 proteins (3 × 10^6 cells) were seeded in a 60-mm diameter culture plate 1 day before HIV-1 infection. Cells were infected with HIV-1NL4-3 at m.o.i. of infection of 5 for 3 h. Cells were UV cross-linked and lysed in cell lysis buffer (Sigma). Co-immunoprecipitated RNA was isolated, and RT-qPCR quantification of HIV-1 gRNA was performed as described (15).

Antibodies and immunoblotting

Antibodies used in this study were as follows: anti-GAPDH (clone 4G5, AbD Serotec, Atlanta, GA); anti-FLAG (F-3165, Sigma); anti-YTHDF1 (ab99080, Abcam); anti-YTHDF2 (ABE542, EMD Millipore, Billerica, MA); anti-YTHDF3 (ab103328, Abcam); and anti-HIV-1 p24 (AIDS Reagent Program, catalogue no. 6458). Cells were harvested and lysed in cell lysis buffer (Cell Signaling, Beverly, MA) supplemented with the protease inhibitor mixture (Sigma). Immunoblotting was performed, and ImageJ software (National Institutes of Health) was used to calculate the densitometry of immunoblotting bands as described (15).

AlphaScreen assay

Recombinant GSH–S–transferase (GST)-tagged Y1–3 proteins were purified as described (10). Two RNA fragments, control RNA and m^6^A-modified RNA (m^6^A RNA), corresponding to 235–281 nt containing the second GGACU consensus sequence, were synthesized (Integrated DNA Technologies), and the RNA fragments were modified with biotin at both 5’ and 3’ ends. Control RNA has no m^6^A modification, whereas the m^6^A RNA fragment has m^6^A modification in GGACU motif. To eliminate RNA dimerization, the DIS sequence (AAGCGCGC) was substituted with nucleotides GAG as described in a previous study (49). AlphaScreen assays were conducted as reported previously with minor modifications (27). Proteins and RNA fragments were diluted with AlphaScreen buffer (100 mM NaCl, 1 mM MgCl_2, 1 mM DTT, 1 mg/ml BSA, 25 mM Tris, pH 7.5). For the binding of proteins and
acceptor beads, Y1–3 proteins (25 nM) and acceptor beads (catalogue no. AL110C, 1:100 dilution; PerkinElmer Life Sciences) were mixed and adjusted to 30 μl and incubated at 4 °C for 2 h. For RNA and donor bead binding, control or m6A RNA (50 nM) was mixed with donor beads (catalogue no. 6760002S, 1:100 dilution; PerkinElmer Life Sciences) in a 10-μl volume and incubated at 4 °C for 2 h. After the incubation, the protein and RNA were mixed and incubated at 4 °C for 1 h. Samples (25 μl) were added to the microplate and read with EnSpire multimode plate reader (PerkinElmer Life Sciences).

In vitro pulldown assays for RNA and Y1–3 protein binding

Streptavidin Dynabeads M-280 (Invitrogen, catalogue no. 11205D) were used in this experiment. Beads were washed and incubated with biotin-labeled m6A RNA or control RNA at 4 °C for 60 min according to the manufacturer’s instructions, and purified Y1–3 proteins were added at concentrations of 1, 5, 25, and 625 nM and incubated for 60 min. After washing, proteins bound to beads were eluted for immunoblotting using specific antibodies to Y1, Y2, or Y3.

qPCR assays

To quantify HIV-1 gRNA after infection of HeLa/Y1–3 cells, at 1, 3, and 6 h after viral infection, cells were collected, and total RNAs were extracted with an RNeasy Mini kit (Qiagen). Reverse transcription was conducted with first-strand synthesis (Invitrogen). qPCR was used to measure HIV-1 gRNA as described (50). To quantify HIV-1 early and late RT products after viral infection, cellular DNA was extracted using a QIAamp DNA Blood Mini kit (Qiagen). Because HIV-1 reverse transcription initiates from PBS, early RT products were quantified using primers ert2f (5′-GTGCCCCGTCTTGGTGTGAC-3′) and ert2r (5′-GGCCGCACTGCTAGAGATT-3′), which amplify a fragment overlapping with PBS and R regions with 74 and 7 nt, respectively. Late RT products were quantified as described (15). The late RT products were amplified with an LTR R-specific primer (forward, 5′-GGAGCTCTTCTGCTAACACT-3′) and a gag-specific primer (reverse, 5′-GGATTAACGCGAATCGTTC-3′). GAPDH levels were also quantified to normalize early and late RT data (15).

IP of Y1–3 proteins to detect the interactions with HIV-1 proteins

HEK293T cells (1 × 10⁶) were co-transfected with 2 μg of pNL4-3 and 2 μg of empty vector (pPB-CAG) or constructs expressing individual HA-tagged Y1–3 proteins as described previously (15). At 48 h post-transfection, cells were harvested and lysed in 1% digitonin, and total protein concentration was quantified. To elucidate the effects of RNAs in Gag and Y1–3 interactions, 1 aliquot of each cell lysate was treated with RNase A (100 μg/ml) for 1 h at room temperature, and IP was conducted as described (51). Proteins bound to anti-HA–agarose beads were eluted by boiling in sample buffer for immunoblotting. HIV immunoglobulin (HIV-IG, AIDS Reagent Program (National Institutes of Health), catalogue no. 3957) (31) was used to detect HIV-1 proteins precipitated by Y1–3 proteins.

HIV-1 virion purification

HIV-1 virions were purified according to published studies (52, 53). HEK293T cells were co-transfected with pNL4-3, vector, or Y1–3–expressing plasmids. Cells and supernatants were collected 48 h later. The cell culture media containing viruses were pelleted through 25% sucrose at 141,000 × g for 90 min. After resuspension in PBS, virions were layered on a 6–18% OptiPrep (Sigma, catalogue no. D1556) in an SW55 Ti rotor tube and ultra-centrifuged at 250,000 × g for 90 min. Fractions 14.4–18% containing virions were pooled, mixed with PBS, and ultracentrifuged at 250,000 × g for 1 h using the SW55Ti rotor to pellet purified virions. The purified virions were resuspended in the lysis buffer (Cell Signaling) supplemented with protease inhibitor mixture (Sigma) and used for immunoblotting.

Prediction of the secondary structure of HIV-1 RNA segments

The secondary RNA structures of HIV-1 5′ UTR segments from WT HIV-1 or mutants were predicted, and the theoretical ΔG values were calculated using the on-line mfold program (version 3.6) according to the user’s instruction (54).

qPCR assays

To quantify HIV-1 gRNA after infection of HeLa/Y1–3 cells, at 1, 3, and 6 h after viral infection, cells were collected, and total RNAs were extracted with an RNeasy Mini kit (Qiagen). Reverse transcription was conducted with first-strand synthesis (Invitrogen). qPCR was used to measure HIV-1 gRNA as described (50). To quantify HIV-1 early and late RT products after viral infection, cellular DNA was extracted using a QIAamp DNA Blood Mini kit (Qiagen). Because HIV-1 reverse transcription initiates from PBS, early RT products were quantified using primers ert2f (5′-GTGCCCCGTCTTGGTGTGAC-3′) and ert2r (5′-GGCCGCACTGCTAGAGATT-3′), which amplify a fragment overlapping with PBS and R regions with 74 and 7 nt, respectively. Late RT products were quantified as described (15). The late RT products were amplified with an LTR R-specific primer (forward, 5′-GGAGCTCTTCTGCTAACACT-3′) and a gag-specific primer (reverse, 5′-GGATTAACGCGAATCGTTC-3′). GAPDH levels were also quantified to normalize early and late RT data (15).

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