HIV reprograms host m\textsuperscript{6}Am RNA methylome by viral Vpr protein-mediated degradation of PCIF1

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$N^6,2\prime-O\text{-dimethyladenosine (m}^6\text{Am)$ is an abundant RNA modification located adjacent to the 5\prime-end of the mRNA 7-methylguanosine (m\textsuperscript{7}G) cap structure. m\textsuperscript{6}A methylation on 2\prime-O-methylated A at the 5\prime-ends of mRNAs is catalyzed by the methyltransferase Phosphorylated CTD Interacting Factor 1 (PCIF1). The role of m\textsuperscript{6}Am and the function of PCIF1 in regulating host-pathogens interactions are unknown. Here, we investigate the dynamics and reprogramming of the host m\textsuperscript{6}Am RNA methylome during HIV infection. We show that HIV infection induces a dramatic decrease in m\textsuperscript{6}Am of cellular mRNAs. By using PCIF1 depleted T cells, we identify 2237 m\textsuperscript{6}Am genes and 854 are affected by HIV infection. Strikingly, we find that PCIF1 methyltransferase function restricts HIV replication. Further mechanism studies show that HIV viral protein R (Vpr) interacts with PCIF1 and induces PCIF1 ubiquitination and degradation. Among the m\textsuperscript{6}Am genes, we find that PCIF1 inhibits HIV infection by enhancing a transcription factor ETS1 (ETS Proto-Oncogene 1, transcription factor) stability that binds HIV promoter to regulate viral transcription. Altogether, our study discovers the role of PCIF1 in HIV-host interactions, identifies m\textsuperscript{6}Am modified genes in T cells which are affected by viral infection, and reveals how HIV regulates host RNA epitranscriptomics through PCIF1 degradation.

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RNA contains more than 100 chemical modifications and recent studies on the structure and functions of these modifications have led to a new frontier in biology and medicine termed epitranscriptomics. One of these modifications, N6-methyladenosine (m6A) is the most prevalent RNA modification in many species, including mammals, and is found in 5′-UTR, 3′-UTRs, and stop codons1-3. The m6A modification is catalyzed by the RNA methyltransferase complex containing METTL3 that catalyzes the addition of a methyl group at the N6 position of adenosine which affects gene expression via regulation of RNA metabolism, function, and localization4-5. Another abundant RNA modification near the mRNA cap structure is dimethylated adenosine, N7,2′-O-dimethyladenosine (m6Am)6,7. Since m6Am is found at the first transcribed nucleotide in ~30% of the cellular mRNAs, m6Am can have a major influence on the gene expression of the transcript6. Recent studies have identified the Phosphorylated CTD Interacting Factor 1 (PCIF1) as the enzyme that catalyzes m6Am methylation on 2′-O-methylated A at the 5′-ends of mRNAs8-11. RNA modifications including m6A play important roles in modulating host–viral interactions12-14. Previous studies have found that diverse viral transcripts can be modified by m6A, thus affecting viral replication by a variety of mechanisms involving transcription, splicing, stability or export of viral RNA15-23. In addition, flavivirus infections such as Zika, West Nile, Dengue, and hepatitis C viruses alter the m6A in cellular mRNAs24,25, and m6A affects viral propagation by regulating host factors24,25. The SARS-CoV-2 virus also has m6A modifications, which are enriched in the 3′ end of the viral genome.26, m6A reduction in viral RNA, by depleting host cell m6A methyltransferase METTL3, increased RIG-I binding and subsequently enhanced the downstream innate immune signaling pathway and inflammatory gene expression.26. HIV genomic RNA can be modified by diverse RNA modifications, including Am, m6A, and m5C19,21,23,27,28. Recently, Tsi et al.14 reported that HIV RNA is acetylated to ac4C by cellular NAT10 and this modification enhances HIV gene expression through increased viral RNA stability.

So far, the role of m6Am RNA modification and the catalytic function of PCIF1 in biological and disease processes, especially in regulating viral infections and host–pathogen interactions have not to be determined. Here we report that PCIF1 restricts HIV replication and is degraded by viral protein Vpr, which leads to an impaired m6Am modification of cellular mRNA. PCIF1 inhibits HIV infection by enhancing a transcription factor ETS1 (ETS Proto-Oncogene 1, transcription factor) stability that binds to the HIV promoter to regulate viral transcription.

Results

m6Am modification of cellular mRNA is decreased by HIV infection and is mediated by Vpr-induced PCIF1 degradation. To investigate the mRNA methylation by HIV infection, we infected MT4 T cells with HIV and quantified m6Am and m6A modifications in cellular mRNA by LC–MS/MS26,29. We found that m6Am modification of cellular mRNAs, but not m6A, was significantly decreased upon HIV-1 infection in T cells (Fig. 1a and Supplementary Fig. 1a). Since PCIF1 is the only known enzyme that catalyzes m6Am RNA methylation8-10, we analyzed the RNA and protein expressions of PCIF1 in MT4 cells infected with either the HIV-1 LAI or NL4-3 strain. Our results showed that despite robust infection, neither of the two strains reduced the mRNA levels of PCIF1 (Fig. 1b and Supplementary Fig. 1b, Supplementary Data 1). On the other hand, protein levels of PCIF1 were significantly decreased by both of the two HIV strains in a dose-dependent manner in MT4 T cells (Fig. 1c). PCIF1 degradation was also observed in primary CD4+ T cells on days 2 and 3 post-infection (Fig. 1d). Further, PCIF1 degradation is not cell line restricted because HIV infection also dose-dependently downregulated PCIF1 protein in HeLa cells infected with an HIV pseudovirus with only one cycle of replication, suggesting that the degradation was dependent on HIV replication (Supplementary Fig. 1c). Altogether, these results demonstrate that m6Am RNA methylation was reduced due to HIV-mediated degradation of the methyltransferase protein PCIF1.

To reveal which degradation pathway is responsible for PCIF1 downregulation, MT4 cells were infected with HIV-LAI and then treated with proteasome or lysosome inhibitor. PCIF1 degradation induced by HIV infection was significantly rescued by the proteasome inhibitor, MG132, but not by lysosome inhibition. p62 is an autophagy substrate and was used as a reporter of proteasome and lysosome activity (Fig. 1e and Supplementary Fig. 2a). These results suggested that PCIF1 was degraded by HIV through a proteasome pathway. The HIV genome expresses six accessory proteins including Tat, Rev, Vpu, Vif, Nef, and Vpr, four of which (Vpu, Vif, Nef, and Vpr) are known to target host restriction proteins’ degradation30. To identify which of these protein(s) is involved in PCIF1 degradation, we overexpressed each of the four proteins (Vpu, Vif, Nef, and Vpr) in HeLa cells and analyzed PCIF1 levels. We found that Vpr from both HIV-LAI and HIVNL4-3 decreased PCIF1 expression (Fig. 1f). Furthermore, Vpr-induced PCIF1 degradation in a dose-dependent manner (Supplementary Fig. 2b). To confirm whether Vpr is the factor responsible for PCIF1 degradation, cells were inoculated with either the wild-type or Vpr deleted (ΔVpr) HIV. The expressions of p24 were comparable in both wild-type and ΔVpr viruses, while the Vpr protein band was abolished and PCIF1 was not decreased by ΔVpr HIV (Fig. 1g). To rule out the role of Vpu, we performed the same experiment as for ΔVpr by using wild-type and ΔVpu HIV. Our results showed that ΔVpu was not able to rescue PCIF1 degradation (Supplementary Fig. 2c). Together, these results indicate that PCIF is degraded by the proteasome and suggest that HIV Vpr is the key protein involved in PCIF1 degradation.

Vpr interacts with host factors and reprograms Cullin4-VprBP (a Vpr binding protein) E3 ligase complexes to ubiquitinate and degrade host proteins31,32. To investigate the underlying mechanism of Vpr-induced degradation of PCIF1, we first performed co-immunoprecipitation (Co-IP) to determine Vpr and PCIF1 interactions. We transfected HeLa cells with plasmids expressing PCIF1 and Flag-tagged Vpr followed by Co-IP and western blotting. Our results showed that Vpr has the ability to interact with PCIF1 (Fig. 1h). To determine Vpr–PCIF1 interactions in T cells, we infected MT4 cells expressing Flag-tagged PCIF1 with HIV-LAI for 3 days followed by Flag-IP and immunoblotting. Our results showed a clear interaction between PCIF1 and Vpr (Fig. 1i). In addition, we found that both Vpr and PCIF1 were mainly in the nucleus and the degradation of PCIF1 was obvious in the nucleus (Supplementary Fig. 2d). To investigate whether Vpr reprograms Cullin4-VprBP complexes to ubiquitinate and degrade PCIF1, a Vpr mutant Q65R, which cannot bind to VprBP33, was constructed to determine its effect on PCIF1. The Q65R mutant abolished the ability of Vpr to induce PCIF1 degradation (Fig. 1j). Furthermore, MG132 treatment accumulated polyubiquitylated PCIF1 in cells expressing Vpr, but not the Vpr-Q65R mutant (Fig. 1k). Altogether, these results demonstrate that HIV-1 Vpr promotes PCIF1 ubiquitination and degradation by the proteasome in a VprBP E3 complex.

PCIF1 restricts HIV infection by repressing viral replication. To investigate whether PCIF1 is involved in the HIV life cycle, we designed four sgRNAs to knockout (KO) PCIF1 in MT4 cells (Fig. 2a). sgRNA #1 shares the same sequences as reported by
Boulias et al. 9. All four sgRNAs successfully abolished PCIF1’s expression (Fig. 2b). Compared to control cells, the expression of viral core protein p24 in PCIF1 KO cells was significantly increased (Fig. 2b). The RNA expressions of the HIV gp120 gene in both the infected cells and also in the released viral particles were also enhanced in KO cells (Supplementary Fig. 3a, b). These results suggest that PCIF1 restricts HIV infection. To further confirm these findings by another approach, we used two shRNAs constructs to knockdown PCIF1 expression. The PCIF1 mRNA and protein expressions were significantly interfered by shRNAs (Supplementary Fig. 3c). The released HIV particle, p24 protein expression, and gp120 RNA expressions were significantly enhanced by PCIF1 KD (Supplementary Fig. 3c, d).

PCIF1 consists of two core regions including methyltransferase and helicase domains. The active enzyme site with an NPPF motif in the methyltransferase is required for substrate recognition and catalysis8. To determine whether the methyltransferase activity of PCIF1 is responsible for HIV restriction, rescue experiments were

**Figure Legends:**

- **Fig. 2a** shows a graph of m6Am levels in mock and HIV-infected cells.
- **Fig. 2b** displays the expression of PCIF1 over time after HIV infection.
- **Fig. 2c** illustrates the relative expression of PCIF1 and viral core protein p24.
- **Fig. 2d** compares the expression of PCIF1 and p24 in HIV-infected Primary CD4+ T cells.
- **Fig. 2e** presents the expression of PCIF1 and p24 in HeLa cells with different treatments.
- **Fig. 2f** shows the release of HIV particle, p24 protein expression, and gp120 RNA expressions in HeLa cells with different treatments.
- **Fig. 2g** depicts the expression of PCIF1 and negative control proteins in MT4 cells.
- **Fig. 2h** illustrates the expression of PCIF1 and Vpr in HeLa cells expressing Flag-Vpr and PCIF1.
- **Fig. 2i** shows the expression of PCIF1 and Vpr in MT4 cells expressing Flag-PCIF1 infected with HIV.
- **Fig. 2j** displays the expression of PCIF1 and Vpr in HeLa cells expressing Flag-Vpr and PCIF1.
Fig. 1 HIV infection downregulates m6A modification of cellular mRNA by Vpr-induced degradation of the m6A methyltransferase PCIF1. a m6A modification of cellular mRNA is decreased by HIV infection. m6A-modified levels were quantified in MT4 cells infected with HIV-LAI (MOI = 0.4, 3 days) by LC-MS/MS. n = 3 biological independent experiments. Two-sided t-test. Mean ± SD. ***p = 0.0008. b PCIF1 mRNA levels are not changed by HIV infection. PCIF1 mRNA levels were quantified in MT4 cells infected with HIV-LAI (MOI = 0.4) or HIVNL4-3 (MOI = 2). n = 3 biological independent experiments. Two-sided t-test. Mean ± SD. c HIV infection decreases PCIF1 in MT4 cells. Immunoblotting of PCIF1 and p24 in MT4 cells infected with HIV-LAI or HIVNL4-3 for 3 days. d HIV infection decreases PCIF1 in primary CD4+ T cells. Immunoblotting of PCIF1 and p24 in activated primary CD4+ T cells infected with HIV-LAI (MOI = 1). e PCIF1 is downregulated by HIV through proteasome degradation. Immunoblotting of PCIF1, p62, and p24 in MT4 cells infected with HIV-LAI (MOI = 0.4, 3 days). Cells were incubated with DMSO or MG132 (0.25 µM) at 1 day before lysis. f PCIF1 is degraded by HIV viral protein Vpr. Immunoblotting of PCIF1 and the viral proteins in HeLa cells transfected with indicated expression vectors (E.V: empty vector). g Vpr deleted HIV does not degrade PCIF1 and ETS1 protein expression. Immunoblotting of indicated proteins in MT4 cells infected with HIV-LAI or HIVNL4-3 Vpr deleted virus (HIV-LAI-Vpr) (MOI = 0.4, 3 days). h Vpr interacts with PCIF1. Flag immunoprecipitation was performed in HeLa cells co-transfected with plasmids expressing Flag-PCIF1 and Flag-tagged Vpr for 2 days. PCIF1 and Vpr expression and enrichment were detected by western blotting. i Vpr interacts with PCIF1 in T cells. MT4 cells expressing Flag-PCIF1 was infected with HIV (MOI = 1) for 3 days followed by Flag immunoprecipitation. Flag-PCIF1 and Vpr expression and enrichment were detected by western blotting. j The E3 complex binding site of Vpr is necessary to decrease PCIF1. Immunoblotting of PCIF1 and Vpr in HeLa cells transfected with plasmids expressing empty vector (E.V.), Vpr, or Vpr-Q65R mutant vector for 2 days. k Vpr induces PCIF1 ubiquitination. HA or FLAG immunoprecipitation was performed in HeLa cells co-transfected with plasmids expressing FLAG-PCIF1, HA-Ub, either Vpr or Vpr-Q65R mutant for 2 days. HA-tagged ubiquitin, Flag-tagged PCIF1, and Vpr expression were detected by western blotting. Similar results were obtained from three independent experiments, and GAPDH expression was shown as a loading control (c–j).

HIV infection decreases PCIF1 in primary CD4 T cells. Primary CD4 T cells were activated by using CD3 and CD28 antibodies. Two specific shRNAs targeting PCIF1 were mixed as a pool to increase the knockdown efficiency. Knockdown cells were enriched by puromycin selection for 5 days. Two donors were used to rule out individual differences. PCIF1 mRNA levels were significantly reduced (Fig. 2j, k). Knockdown cells were then infected with HIV-LAI for 3 days. Consistent with results in MT4 and Jurkat T cells, knocking down PCIF1 increased p24 expression ~7-fold in both donors (Fig. 2i). Next, we asked whether PCIF1’s ability to inhibit HIV is strain-type dependent. To address this question, we depleted PCIF1 in THP1 cells and differentiated them into macrophages followed by infection using HIV-BaL, a CCR5-tropic strain. Our results show that the expression of gp120 mRNA was increased by PCIF1 depletion (Supplementary Fig. 3j, k). Taken together, these results show that PCIF1 is a broad HIV inhibitor that regulates CXCR4 and CXC5R-tropic viral replication in primary CD4 T cells and macrophages.

PCIF1 does not methylate HIV genomic RNA. There are two possibilities for the mechanism through which PCIF1 regulates HIV transcription: (1) HIV transcripts are methylated by PCIF1 and thus affect their stability. (2) m6A-modified host genes are changed by PCIF1 which then regulates the transcription of HIV. To investigate whether HIV transcripts are modified by PCIF1, we performed MeRIP-seq in control and PCIF1 KO Jurkat T cells infected with HIV. LC/MS–MS quantification of modified nucleotides in control and PCIF1 KO cells showed that m6A was undetectable in the PCIF1 KO cells as compared to control cells, while m6A levels were unchanged in PCIF1 KO cells (Supplementary Fig. 4a, b). In vertebrates, the m6A modification is typically near the 3′ stop codons of mRNAs13, while m6A modification is adjacent to the 5′ cap of mRNAs8–10. Three high-intensity m6A peaks were detected in the HIV genomic RNA. One was in the 5′UTR, the other two were near the 3′UTR. The peaks near the 3′UTR were predicted to be m6A peaks and were
also detected in the previous studies. Not surprisingly, the peaks near the 3′UTR were not changed by PCIF1 KO. Notably, the peak in the 5′UTR was also not changed in PCIF1 KO cells compared with control cells, suggesting that it was an m⁶A modification but not an m⁶Am modification (Supplementary Fig. 4c, d). These results suggest that PCIF1 does not modify HIV transcripts, and thus, rule out the first possibility for the mechanism through which PCIF1 regulates HIV transcription.

Identification of m⁶Am-modified host genes. We then investigated the second possible mechanism for PCIF1’s regulation of HIV transcription. To determine which host genes are modified by PCIF1 and regulate HIV inhibition, we performed an m⁶A-methylated RNA immunoprecipitation sequencing (MeRIP-Seq) using m⁶A antibodies in control and PCIF1 KO Jurkat cell lines as well as cells infected with HIV (Supplementary Fig 5). As m⁶A and m⁶Am are structurally similar and both can be recognized by m⁶A antibodies, previous reports suggested that peaks in the 5′ end of the mRNAs are expected to contain m⁶Am modifications. Consistent with previous observations, the global m⁶A distribution in the gene body and 3′UTR were not drastically different from control cells (Supplementary Fig. 5a, b), suggesting that PCIF1 did not affect genome-wide m⁶A
distribution. We observed a slight decrease in the 5′ end of mRNAs in the PCIF1 KO cells and HIV-infected cells (Supplementary Fig. 5a, b). We performed a motif analysis of the called peaks and all groups had DRACH (D = A, G, U; R = A, G; H = A, C, U) m^6^A consensus as the common motif, suggesting that most of the peaks were m^6^A peaks (Supplementary Fig. 5c). These results suggest that the m^6^A MeRIP-Seq may not be an ideal method to directly identify m^6^Am-modified transcripts.

To identify transcriptome-wide m^6^Am enriched RNAs, we used m^6^Am-Exo-Seq, a recently developed methodology by Sendinc et al.\(^a\), in control and PCIF1 KO Jurkat cells. In parallel, m^6^Am-Exo-Seq in cells infected with HIV was performed to identify m^6^Am-modified genes that were altered by HIV infection. The density of m^6^A peaks was drastically decreased in the 5′UTR when PCIF1 was depleted (Fig. 3a). Additionally, in HIV-infected cells, we observed a significant decrease in the 5′ UTR (Fig. 3b). To identify m^6^Am-modified genes in T cells, we compared the m^6^A peak in the 5′UTR in control and KO cells. The peaks that were depleted or decreased in the KO cells compared to control cells were considered to be m^6^Am peaks (Fig. 3c and Supplementary Data 2, 3). There were 4202 peaks in 2237 genes that were identified as m^6^Am peaks (Supplementary Data 2 and 3). A motif analysis of significant peaks showed the DRACH m^6^A consensus as to the most common motif in PCIF1 KO cells (Supplementary Fig. 6a). Previously, m^6^Am-Exo-Seq in the MEL624 melanoma cell line identified 1521 m^6^Am enriched genes. Boulias et al., performed miCLIP-Seq in control and PCIF1 KO HEK293T cells and identified 1850 genes enriched in m^6^Am modification. From our m^6^Am enriched genes in T cells, we found 425 and 539 genes common with m^6^Am-Exo-Seq and miCLIP-Seq results, respectively. There were 217 m^6^Am genes common in all three analyses, suggesting that these modifications may not be cell type-dependent. For example, PARP1, there was a significant peak near the annotated transcriptional starting site in the 5′UTR in control cells and was significantly depleted in KO cells (Supplementary Fig. 6b). Two of SMCI isoforms with distinct TSS were both m^6^Am modified (Supplementary Fig. 6c). These data are consistent with the notion that the m^6^Am modifications are predominantly in TSS-proximal peaks.

Identification of HIV-changed m^6^Am genes. Among 4202 m^6^Am genes, the m^6^A peaks of 854 genes were decreased by HIV, while the other genes retained their peaks (Supplementary Data 3). These results further confirmed that HIV infection led to a large change in m^6^Am modification in T cells (Fig. 3c and Supplementary Fig 7, Supplementary Data 3, 4). Molecular functional analyses indicate that 93 of the 854 HIV-changed m^6^Am genes are in the category of transcription regulatory activity (Fig. 3d). As we confirmed that HIV transcription was inhibited by PCIF1 (Fig. 2d–i), we decided to focus on transcription factors for further mechanistic studies. To narrow down the most significant targets of PCIF1 that may affect HIV transcription, we overlapped the 93 transcription regulatory genes with an HIV interaction database (Supplementary Data 4, https://www.ncbi.nlm.nih.gov/genome/viruses/retroviruses/hiv-1/interactions/) and selected the targets with annotated interaction with HIV. There were 18 HIV-altered m^6^Am targeted transcription factors: ETS1, EGR1, JUND, SREBF2, PLSCR1, FOS, MYC, YY1, DDIT3, RARA, SP4, CEBPA, IKZF1, CDC5L, ATF4, MLLT1, ROR1, and SMAD7, that were among the HIV interactors. For example, the peaks in the TSS of ETS1 (Fig. 3f), CDC5L (Supplementary Fig. 7a), DDIT3 (Supplementary Fig. 7b), CEBPA (Supplementary Fig. 7c), FOS (Supplementary Fig. 7d), and MYC (Supplementary Fig. 7e) were significantly abolished in HIV infected or PCIF1 KO group. Most of the genes are annotated to have TSS as adenosine except for EGR1, YY1, DDIT3, CDC5L, and ROR1, which could be due to incorrect TSS annotation, although we cannot exclude the possibility that they are not m^6^Am modified.

**PCIF1 increases ETS1 stability and restricts HIV replication.**

To investigate which genes are responsible for PCIF1-directed inhibition of HIV transcription, we first investigated whether the RNA levels of selected m^6^Am target genes were significantly changed by HIV. During HIV infection, the mRNA of ETS1 was decreased at day 3 post-infection (Fig. 3g), which corresponds to a similar trend observed for PCIF1 degradation (Fig. 1), indicating that ETS1 was regulated by PCIF1 during HIV replication. The RNA levels of FOS, and EGR1 were significantly increased as early as 1-day post-infection. Since PCIF1 was not significantly changed at day 1 post-infection, suggesting that PCIF1 may not be the only factor affecting FOS and EGR1 expressions during HIV infection (Supplementary Fig. 8a). In addition, an unbiased high throughput yeast one-hybrid (eY1H) screen identified ETS1 interaction with HIV-1 LTR. Therefore, we next investigated how ETS1 was regulated by PCIF1 and whether ETS1 can modulate HIV replication.

In PCIF1 KO cells, mRNA expression of ETS1 was decreased significantly compared to control cells, while in ectopic PCIF1 expressing T cells, mRNA expression of ETS1 was increased 2-fold compared to control cells (Fig. 4a). These data suggested that PCIF1 enhanced the mRNA expression of ETS1. To determine whether ETS1 is regulated by PCIF1 through its methyltransferase activity, wild-type PCIF1 or a catalytically inactive PCIF1...
mutant was expressed in PCIF1 KO cells. Importantly, down-regulation of ETS1 mRNA in PCIF1 KO cells can be rescued by expressing wild-type PCIF1, but not catalytically inactive PCIF1 mutant, indicating that PCIF1 regulates ETS1 expression and requires the methyltransferase activity of the enzyme (Fig. 4b).

RNA modifications could affect mRNA expression by changing its cytoplasmic export or stability. To investigate how m6Am regulates ETS1 mRNA expression, the distribution and half-life of ETS1 mRNA was quantified in control and PCIF1 KO cells. The localization of ETS1 transcripts was not significantly changed in PCIF1 KO cells (Supplementary Fig. 9a). The half-life of ETS1 mRNA in PCIF1 KO cells, however, was decreased from 3.2 to 2.4 h (Fig. 4c). Meanwhile, the expression and half-life of GAPDH, an un-modified gene, were not changed in PCIF1 KO cells (Supplementary Fig. 9b). In addition, crosslinking immunoprecipitation (CLIP) assays demonstrated the direct binding of PCIF1 to ETS1 transcripts (Fig. 4d). Altogether, these results suggest that ETS1 is m6Am modified by PCIF1 and the mRNA stability of ETS1 is enhanced by m6Am modification. The regulation of ETS1 by PCIF1 is in agreement with the findings of Boulias et al. that m6Am modification enhanced the stability of a subset of mRNAs9.

Next, we determined the protein expression of ETS1 by immunoblotting. Consistent with the mRNA expression data, the protein levels of ETS1 were decreased in PCIF1 KO cells (Fig. 4e). Similar to the decrease seen in mRNA (Fig. 3g), protein expression of ETS1 was also downregulated in HIV-infected cells (Fig. 4f). Additionally, in Vpr deficient HIV-infected cells, ETS1 mRNA was not changed (Fig. 4g and Supplementary Fig 9c), and its protein expression was rescued compared to wild-type HIV-infected cells (Fig. 1g). These data suggested PCIF1 and ETS1 are regulated by Vpr expressions. Altogether, our data strongly

Fig. 3 Identification of m6Am-modified cellular genes altered by HIV infection. a m6Am-Exo-MeRIP peaks in the 5'UTR are decreased in PCIF1 KO cells. Metagene analysis of m6Am-Exo-MeRIP peaks near the transcription start site (TSS) of all expressed genes in control or PCIF1 KO Jurkat cells. b m6Am-Exo-MeRIP peaks in the 5'UTR are reduced in HIV-infected cells. Metagene analysis of m6Am-Exo-MeRIP peaks in Jurkat cell mock-infected or infected with HIVLAI at an MOI of 4. c m6Am peaks are altered by HIV infection. m6A-MeRIP peaks were aligned to the human genome. And peaks that are located in the 5'UTR and decreased in PCIF1 KO cells were considered as high-confidence m6Am peaks. Of them, peaks that are decreased by HIV infection were shown as potential m6Am peaks changed by HIV. All the peaks from a-c were conserved peaks called from two independent samples. d High-confidence m6Am genes are enriched in the category of transcriptional factors. Molecular function GO analysis was performed in the 854 m6Am genes changed by HIV using GSEA (http://www.gsea-msigdb.org/gsea/msigdb/annotate.jsp). e Venn plot shown the 18 HIV altered m6Am targeted transcription factors. The 854 HIV-changed m6Am genes were overlapped the 93 transcription regulatory genes and the HIV interaction database (listed in Supplementary Table 4, https://www.ncbi.nlm.nih.gov/genome/viruses/retroviruses/hiv-1/interactions/). f m6Am-Exo-MeRIP peak of ETS1 gene in the 5'UTR is decreased in PCIF1 KO cells and HIV-infected cells. Genome tracks of the ETS1 gene were plotted with called m6A peaks. The m6Am peaks were enlarged in the right panel. One representative of two experiments is shown. g Kinetics of ETS1 mRNA during HIV infection. ETS1 mRNA expression was quantified in MT4 cells infected with HIVLAI at MOI 0.4 for 1, 2, or 3 days. n = 3 independent experiments. Two-sided t-test. Mean ± SD. *p = 0.039.
suggest that PCIF1 inhibits HIV infection through the methylation of ETS1 mRNA.

To elucidate the role of ETS1 in the HIV life cycle, we edited the ETS1 gene using specific sgRNAs in MT4 cells, and then infected cells with HIV pseudovirus. ETS1 expression was successfully abolished in edited T cells (Fig. 5a) and the HIV infection was significantly increased in ETS1 edited cells (Fig. 5b), as observed in PCIF1 KO cells (Fig. 2d). Additionally, interfering ETS1 expression with two shRNAs significantly decreased ETS1 and increased HIV pseudovirus infection (Fig. 5c, d). The increase in HIV replication by ETS1 knockdown was enhanced when using multi-cycle HIV infection (Fig. 5e).

To confirm the binding of ETS1 to the HIV promoter, we analyzed ETS1 interactions with the HIV promoter by performing ChIP experiments in HIV-infected MT4 cells. As shown in Fig. 4m, ETS1 exhibited significant interactions with the HIV promoter. ETS1 interactions with c-myc and GAPDH were used as positive and negative controls, respectively (Fig. 5f). To investigate the function of ETS1 on the HIV life cycle, we prepared an HIV reporter construct where the 5′LTR (long terminal repeat) containing regulatory modules was cloned in front of a luciferase gene and the luciferase activity was quantified as described above. We observed that the activity of the promoter was enhanced in ETS1 knockdown cells, suggesting that ETS1 reduced HIV transcription (Fig. 5g). All in all, these results demonstrate that ETS1 binds to the HIV promoter to decrease HIV transcription.

Finally, we asked if ETS1 expression contributes to HIV pathogenesis in people living with HIV (PLWH). We analyzed the mRNA expression of ETS1 in HIV-infected donors (28,610 cells) and six healthy donors (15,121 cells) and six HIV-infected donors (28,610 cells). Our analysis showed that ETS1 mRNA expression was quantified in MT4 cells infected with HIV Vpr at MOI 0.4 for 2, 3, or 5 days. All data are represented as mean ± SD and analyzed by a two-sided t-test in Fig. 4a-d, g, n = 3 (a, c, d, g), or 4 (b) independent experiments. Similar results were obtained from three independent experiments in e and f.

Discussion

In this study, we demonstrate the role of m6Am methyltransferase, PCIF1, as an HIV restriction factor that is dependent on the methyltransferase activity of the enzyme. To counteract viral infection by PCIF1, HIV Vpr degrades PCIF1 proteins through a proteasome pathway. We identified 2237 m6Am genes
Fig. 5 ETS1 inhibits HIV infection. a, b Knockout of ETS1 promotes HIV infection. MT4 were edited with ETS1 sgRNAs for 7 days, and then control or edited cells were infected with HIVpp-luc for 2 days. ETS1 protein levels were determined using western blotting (g). HIV replication was detected using luciferase assays (h). **p = 0.0012, ***p = 0.00015, ***p = 0.00094. c-e Interfering ETS1 expression increases HIV replication. Levels of single-cycle HIV infection were determined using luciferase assays in control shRNA or two ETS1 knockdown shRNA transduced MT4 cells infected with HIVpp-luc at an MOI of 0.2 for 2 days (c). ETS1 (d) and gp120 (e) mRNA expression levels were quantified in control shRNA or ETS1 KD MT4 cells infected with HIVLAI at an MOI of 0.01 for 3 days. c (**p = 0.0017, ***p = 0.0021, d **p = 0.001, ***p = 0.002. e ***p = 2.99781E−06, ****p = 1.20386E−06. f ETS1 is recruited to the HIV promoter. HIV-infected MT4 cells were prepared for ETS1-CHIP analysis. RT-qPCR of the HIV promoter regions or c-myc or GAPDH region coimmunoprecipitated with ETS was performed. *p = 0.049, *p = 0.020, **p = 0.007, *p = 0.0309, ns not significant. g ETS1 inhibits HIV promoter activity. A plasmid containing HIV 5’ LTR and a luciferase reporter (HIV-LTR-luc) was used to quantify HIV promoter activity. The reporter plasmid was electroplated into control or ETS1 KD MT4 cells and then protein expression was quantified using luciferase assay after 2 days. ***p = 0.00017, **p = 0.007. h Model illustrating Vpr-PCIF1-ETS1 regulation axis in HIV replication. During HIV replication, PCIF1 restricts HIV transcription and is targeted by viral protein Vpr for proteasome-mediated degradation, which leads to an impaired m6Am modification in cellular mRNAs. PCIF1 inhibits HIV infection by stabilizing a transcription factor ETS1 (ETS Proto-Oncogene 1, transcription factor) mRNA that binds HIV promoter to regulate viral transcription. All data are represented as mean ± SD and analyzed by a two-sided t-test in b–g. n = 3 (c, d, g), or 4 (b, e, f) independent experiments. Similar results were obtained from three independent experiments in a.
in T cells, with most of their modifications abolished during HIV infection. We confirmed that PCIF1 enhanced the expression of ETS1, a transcriptional factor that was among the identified m6Am genes in T cells, which was decreased during HIV infection. We also found that ETS1 depletion promotes HIV transcription, suggesting that PCIF1 represses HIV replication by enhancing ETS1 stability.

HIV genomic RNA can be modified by diverse RNA modifications, including m6Am, m6A, and m5C. Recently, Tsai et al. reported that HIV RNA is acetylated at ac4C by cellular NAT10 and that this modification enhances HIV gene expression by increasing viral RNA stability. m6A modification of HIV and its effects on viral replication have been previously reported. With the most prominent m6A peaks are located in the 5′UTR and 3′UTR. m6A and m6Am are structurally similar and could not be distinguished by m6A-MeRIP-seq in previous studies. It is possible that m6A peaks in the 5′UTR also contain m6Am. To address this, we performed MeRIP-seq in control and PCIF1 KO cells infected with HIV. We observed three similar m6A peaks in both control and KO cells, indicating that the peaks represent m6A modification (Supplementary Fig. 4a–d). In addition, HIV genomic RNA lacks the typical cap-A structure for m6A. Therefore, we conclude that HIV genomic RNA does not contain any m6Am modifications.

Epitranscriptomic changes to host transcripts during viral infection suggest that cellular mRNA modifications play important roles in host–pathogen interactions. We observed that after HIV infection, m6Am modifications of many cellular mRNAs were decreased (Fig. 1a). Among the 2237 m6Am genes, the m6Am peaks of 854 genes were decreased by HIV, indicating that the m6Am modification in T cells was drastically affected by HIV infection (Fig. 3c). By focusing on genes in the DNA-binding transcription factor category of molecular functions, we identified ETS1, which we found to be regulated by PCIF1 enzymatic activity and to play an important role in HIV replication and potentially in immune cell physiology during infection. ETS1 belongs to the large ETS family of transcription factors (TFs); the family members have highly similar DNA-binding domains (DBDs) with the core binding site consisting of the GGAA/T motif. Each member of the ETS family can bind many target genes with varying selectivity in vivo. Although we show that ETS1 directly binds to the HIV promoter and regulates viral transcription (Fig. 5f, g), our study cannot rule out the possibility of indirect effects of ETS1 on cellular genes which could affect host–virus interactions. ETS1 is preferentially expressed in T and B cells and is necessary for T-cell activation and survival. Based on the single-cell RNA seq data analysis related to HIV infection and immune cell exhaustion (Supplementary Fig. 10), we could infer that the decrease of ETS1 in HIV-infected individuals induces an impairment in CD4 T-cell function and contributes to HIV pathogenesis. We predict that by focusing on other molecular functions and genes affected by PCIF1 (Fig. 3), new proteins that regulate host–virus interactions will be discovered.

Vpr overcomes restriction to virus replication in non-cycling myeloid cell populations because HIV-1 replication is reduced in the absence of Vpr in myeloid cells such as monocyte-derived dendritic cells (MDDCs) and macrophages. Several previous studies have reported the role of Vpr in modulating HIV replication in T cells. Vpr drives systems-level proteomic remodeling by directly or indirectly targeting multiple proteins for degradation. Recently, Bubby et al. provided a new perspective on Vpr function in primary CD4+ T cells by showing that Vpr-induced widespread transcription changes during early infection stages in T cells. Furthermore, two reports demonstrate the functional significance of Vpr in HIV infection in T cells: (1) Vpr and Vpr overcome transcriptional repression of proviruses by the HUSH complex in Jurkat T cells; and (2) Vpr inhibits exonuclease 1-mediated restriction of HIV-1 Infection in T cells. In our experiments using MT4 cells, we observed that replication of HIVΔVpr was increased to only 2-fold in 24 h, which is significantly lower than the 5-fold increase seen in the WT virus under the same time points of infection (Supplementary Figs. 1b and 9c). Altogether, these results suggest that Vpr promotes HIV replication in T cells, but its effects could be more moderate than those observed in myeloid cells. Since PCIF1 expression was comparable in macrophages and T cells, PCIF1 inhibits HIV in both THP1 derived macrophages, MT4 T cells, Jurkat T cells, and importantly primary CD4+ T cells (Fig. 2 and Supplementary Fig. 3). Thus, PCIF1 is a broad inhibitor of HIV replication in macrophages and T cells that is counteracted by Vpr.

Methods

Cell culture and virus preparation. The procedures were approved by the Institutional Review Board. MT4, THP1, and Jurkat cells were cultured in RPMI containing 10% fetal bovine serum (FBS) and 50 μM β-mercaptoethanol (Sigma). HeLa and 293FT cells were cultured in DMEM (Invitrogen) with 10% FBS. To produce various viruses, HIV-LAI (pLAI.2, Cat# 2532, NIH AIDS Reagent Program) or HIVNL4-3 (A kind gift from Mario Morado, pNL4-3, Cat# 114, NIH AIDS Reagent Program), HIVNL4-3ΔVpr (pNL-U35, Cat# 968, NIH AIDS Reagent Program), HIVΔVpr, gNL4-3.Luc.R-E-, Cat# 3418, NIH AIDS Reagent Program), and HIVΔVpr-GFP (Cat# 11100, NIH AIDS Reagent Program) virus, 293FT cells were seeded in a 10-cm plate at 6 × 105 cells/ml. The viral vectors (15 μg) were transfected into 293FT cells using Lipofectamine 3000 (Invitrogen) and supernatants were collected after 2 days and centrifuged at 2000 g for 10 min to and filtered through 0.22 μm filters to remove debris. DMSO (100 μM, NEB) and RNase A (100 μM, Qiagen) were added and digested the free DNA and RNA in the supernatant at 37 °C for 1 h. Ultra-centrifuge (14,000 × g, 4 h) was applied to enrich the viral particles. TRizol was added into the precipitation and viral genomic RNA was extracted for LC-MS/MS analysis for RNA modifications.

Primary CD4+ T-cell isolation and activation. Human peripheral blood mononuclear cells (PBMCs) were isolated from blood samples (purchased from San Diego Blood Bank) using Ficoll density centrifugation (Invitrogen) according to the manufacturer’s recommendations. The PBMC-containing interface layer was collected and washed with phosphate-buffered saline (PBS) three times. Then, primary CD4+ T cells were isolated using the EasySep Human CD4+ T-cell Enrichment kit (StemCell) following the manufacturer’s instructions. Isolated cells were cultured in a complete RPMI-1640 medium, which consisted of RPMI-1640 supplemented with 10% FBS, 20 U/ml IL-2, and 1% penicillin–streptomycin. To activate primary T cells, 10 µl of HIVNL4-3ΔVpr and 20 T cells were added to 1 million cells for 1 week. To ensure robust infection, cells were re-activated with 10 µl activator per million cells for 24 h and then infected with HIV. To knockdown PCIF1 in primary T cells, activated Primary CD4+ T cells from Donor 1 and 2 were transduced with PCIF1 shRNA (shPCIF1) or control shRNA (shNC) and selected with puromycin (1.5 μg/ml) for 5 days.

CRISPR mediated knockout. The 4 guide RNAs targeting PCIF1 are depicted in Fig. 2a. Knockout and characterization were performed according to previously described methods. To knock out PCIF1 and ETS1, guide sequences (Supplementary Data 1) were synthesized and annealed for cloning into lentivector. Isolated cells were cultured in a complete RPMI-1640 medium, which consisted of RPMI-1640 supplemented with 10% FBS, 20 U/ml IL-2, and 1% penicillin–streptomycin. To activate primary T cells, 10 µl of HIVNL4-3ΔVpr and 20 T cells were added to 1 million cells for 1 week. To ensure robust infection, cells were re-activated with 10 µl activator per million cells for 24 h and then infected with HIV. To knockdown PCIF1 in primary T cells, activated Primary CD4+ T cells from Donor 1 and 2 were transduced with PCIF1 shRNA (shPCIF1) or control shRNA (shNC) and selected with puromycin (1.5 μg/ml) for 5 days.

Vectors construction and HIV accessory protein expression. PCIF1 was cloned into plvx vector using in-fusion kit (Takara). PCIF1 knockout resistant vector and APPA mutated PCIF1 vector were generated using Q5 mutation kit (NEB). Vpr knockout resistant vector was cloned into pFLAG-CMV plasmid. Q65 APPA mutated PCIF1 vector were generated using Q5 mutation kit (NEB). Vpr vectors construction and HIV accessory protein expression

Supplementary Data 1. To express HIV accessory proteins in HeLa cells, the following vectors were obtained from the NIH AIDS Reagent Program: (1) HIV-1 NL-Δ3 ΔVpr (pcDNA-HV61, Cat #10076), (2) HIV-1 NL-Δ3 Vpr and (pcDNA-HV, Cat #11076), (3) HIV-1 R-U6 GFP-VPv (Cat #12478), (4) HIV-1 S2 Nef (pcDNA3.1 S2 Nef, Cat #11431), and (5) HIV-1 NL-Δ3 pEGFP-Vpr (Cat #11386). 1 μg of each vector was transfected into 0.3 million HeLa cells using lipofectamine

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m6A-MeRIP and m6Am-Exo-MeRIP-seq analysis. Ten million control or PCIF1 KO Jurkat cells were mock-treated or infected with HIV LAI strain at an MOI of 4 for 3 days. Total RNA was extracted using TRIzol. About 200 μg RNA were then used to isolate poly(A) RNA using two rounds of oligo(T) Dynabeads capture. For one sample, 10 μg poly(A) RNA was de-capped with 1U Cap-Clip enzyme (CellScript) at 37 °C for 1 h. RNA modification levels were quantified using LC-MS/MS. First, 50 ng m6A-modified or unmodified unit of nucleotides (P1) (NP1) for 2 h at 37 °C. Following the addition of 0.25 unit alkaline phosphatase (CLIP) and 3 μl of 1 M NH4HCO3 Buffer and incubated at 37 °C for 2 h. The digestion mixture was dried by speed vacuuming and reconstituted in 10 μl of ddH2O. For profiling and quantification of modified ribonucleoside by tandem mass spectrometry, 10 ng of digested RNA was added to labeled RNA elution buffer and then subjected to paired-end sequencing in the HT sequencing IGM Genomics. m6A-Am-Exo-Seq. In order to effectively map m6A enrichment, we used a technique called m6A-Am-Exo-Seq developed by the Shi group with minor modifications. m6A-Am-Exo-Seq was performed in control or PCIF1 KO Jurkat cells, as well as in Jurkat cells infected with HIV at an MOI of 4. For one sample, 100 μg poly(A) RNA was treated with 1U of T4 polynucleotide kinase and used for mapping. Fragmentation of the treated RNA was stopped by the addition of EDTA to a final concentration of 70 μM. RNA is purified with the RNA Clean and Concentrator-5 kit (Zymo Research). Uncapped and fragmented mRNAs are then phosphorylated by treatment with 200 U of T4 PNK (NEB) in T4 ligation buffer for 90 min at 37 °C. Then, 20 U of T4 ligase (T4 ligase) added to the mixture. Phosphate-Dependent Exonuclease (Lucigen) was added into the reaction supplied with 1× Exonuclease buffer A for 3 h at 30 °C to remove phosphorylated transcripts. The RNA enriched for 5′-capped transcripts is then purified with the RNA clean and concentrator-5 kit (Zymo Research). 30 U of Cap-Clip (CellScript) are added into the capped RNAs and incubated for 2 h at 37 °C to remove the cap. RNA is again purified with the RNA clean and Concentrator-5 kit. 10% of the treated RNA is saved as input, and 5 μg treated RNA is digested and incubated with 5 μg of anti-m6A antibody (Abcam, ab151230) at 4 °C for 2 h. 100 μl protein G magnetic beads were added to pull down the m6A immunoprecipitation complex. After washing for three times, the bound RNA was eluted in 100 μl elution buffer and purified using RNA clean and concentrator-5 kit (Zymo Research). Final libraries were amplified and were subjected to paired-end sequencing in the HT sequencing core facilities.

m6A-Am-MeRIP and m6Am-Exo-MeRIP-seq analysis. Fastqc was used to perform quality control on sequencing data. And then, cutadapt was applied to remove adapters and trim reads. The pre-processed reads were aligned to human genome (built with hg19) and gencode v12 genomic annotations using the software MACS2 (v2.1.1.4, q value: 0.05, call-summit mode) was based on its paired m6A-MeRIP/input data. The default peak range of m6A viewer is 200 nt and above 80% of peaks form MACS2 has range around 200 nt. To find the collection of common peaks for each group, peaks from biological replicates are filtered using an adjusted method. And then, peaks were randomly published to the common peaks, they should appear in at least two biological replicates, and peaks within 200 nt from each other are defined as one peak. These keep peaks together build the collection of common peaks for each group. To generate the peak distribution across chromosome region (including intron, CDS, intergenic region) and across gene expression, the peaks were mapped to RNA using BEDTools. The gene annotation used here is from GENCODE. For further m6Am peak analysis, we extracted common peaks whose summit is in the 5′UTR region. The data visualization of these peaks is realized using bedtools and IGV. To find potential in MeRIP, peaks in 5′UTR region in the control group were compared with PCIF1 KO groups. In MeRIP seq, peaks in controlled groups but not in two PCIF1 KO groups (HIV− and HIV+) were considered as potential m6Am peaks. These potential m6Am peaks were then compared with peaks in HIV-infected groups to identify HIV-changed peaks. Potential peaks which have decreased peaks in control and HIV-infected groups are considered as m6Am peaks changed by HIV infection.

Late RT-qPCR and Alu qPCR. To analyze the levels of late RT and integrated HIV DNA in control and PCIF1 KO cells, we used the methods developed by Bushman group. Control or PCIF1 KO Jurkat cells were infected with HIVpp-luc at 10 or 48 h, genomic DNA was extracted and used for late RT using the primary antibodies were applied at 4 °C overnight or at RT for 2 h. After washing, membranes were incubated with HRP-conjugated secondary antibodies for 20 min (Pierce Fast Western Blotting Kit). Antibodies and dilutions were used as follows: anti-Vpr antibody at 1:1000 (Proteintech, 16082-1), anti-p24 antibody at 1:1000 (Abcam, ab9071), anti-p62 antibody at 1:1000 (Abcam, ab56416), anti-Vpr antibody at 1:1000 (Proteintech, 51143-1-AP), anti-Nef antibody at 1:1000 (NIH AIDS Reagent Program, Cat# 969), anti-IVF antibody at 1:1000 (NIH AIDS Reagent Program, Cat# 1539), anti-Vif antibody at 1:1000 (NIH AIDS Reagent Program, Cat# 6460), anti GAPDH-HRP antibody at 1:5000 (Proteintech, HRP-60004), mouse anti-FLAG M2 antibody at 1:1000 (Sigma-Aldrich, F1804), anti-HDAC1 antibody at 1:1000 (Abcam, ab7028), anti-ETSI antibody at 1:2000 (Proteintech, 12118-1-AP), anti-GFP-tag antibody at 1:1000 (Proteintech, 30430-2-AP), HA antibody at 1:1000 (Santa Cruz, sc-809). The density of western bands was quantified by ImageJ (https://imagej.nih.gov/ij). GAPDH was used as a loading control.

Quantitative real-time PCR (RT-qPCR). Total RNA was extracted with TRIzol reagents (Invitrogen) according to the manufacturer’s instructions. 1 μg RNA was reverse transcribed using Script cDNA Synthesis Kit (Bio-Rad). RT-qPCR was performed using SYBR Green 2X SYBR Green Master Mix (Bio-Rad) and run on LightCycler480. PCR cycling conditions were 95 °C for 5 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. All RT-qPCR primer sequences are listed in Supplementary Data 1.

HIV detection using p24 ELISA, luciferase assay, or flow cytometry. For MT4 infected with LAI, supernatants were collected and viral p24 levels were detected using HIV-1 p24 Antigen ELISA Kit (RETROtek) following the manufacturers’ protocols. For cells infected with HIVpp-luc, cells were lysed and luciferase levels were measured using luciferase kit (Promega). For cells infected with HIVpp-GFP, cells were collected and fixed with 4% formaldehyde (Biolegend) at RT for 10 min. After washing with PBS 2 times, cells were separated into single cell using cell strainer. About 1 million cells were applied onto flow cytometry to detect GFP expression.

Immunoprecipitation. To identify Vpr interactions with PCIF1, 3 million Hela cells were seeded into a dish one day before transfection. 3 μg plvs-PCIF1 and 9 μg Flag-Vpr vectors were co-transfected into cells using lipofectamine 3000 according to its instruction. To identify whether PCIF1 could be ubiquitinated by Vpr, Vpr and Q65R stably expressed Hela cell line was created. 3 million Vpr, Vpr-Q65R, or control Hela cells were seeded into a dish one day before transfection. 3 μg plvs-PCIF1 and 5 μg HA-UB vectors were co-transfected into cells using lipofectamine 3000 according to its instruction. Two days later, cells were lysed in IP lysis buffer (ThermoFisher) supplemented with protease inhibitor and pre-cleaned with 20 μl magnetic protein G beads (CST) at 4 °C for 2 h. Then 1 mg cell lysate was incubated with 4 μg mouse IgG antibody (Invitrogen, 02-6502) or anti-Flag M2 antibody (Sigma-Aldrich, F1804) at 4 °C overnight. The next day, 40 μl BSA pre-blocked magnetic protein G beads were added and incubated for 2 h at 4 °C. After washing three times, 10 μl protein loading buffer (Biolegend) were directly added into the beads and protein was denatured at 95 °C for 10 min. Following the procedure, the washed beads were separated by a western blotting blot to detect the expression of target proteins. The reciprocal HA-IP was also performed in the same samples to determine the HA ubiquitin protein is
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PCIF1-cross-linking and RNA immunoprecipitation (CLIP). RNA immunoprecipitation was performed in Flag-PCIF1 expressing MT4 cells. 40 million cells were collected, and then washed with PBS. Cells were suspended with 10 ml of cold PBS and irradiated once with 400 ml/cm2 at 254 nm. MT4 cells were then harvested and nuclear extracts were isolated following the protocol described above. Nuclear extracts were sonicated and 1 mg of nuclei extract was incubated with 4 μg of IgG antibody (Invitrogen, 02-6502) or anti-Flag M2 antibody (Sigma-Aldrich, F1804) at 4°C overnight. The following day, 40 μl BSA pre-blocked magnetic protein G beads were added into the protein-antibody complex and incubated for 2 h at 4°C. After washing three times, beads were suspended in 100 μl PBS, followed by 20 U DNase I (New England Biolabs) digestion at 37°C for 15 min. Protein was digested by incubation with 50 μg of Proteinase K (New England Biolabs) at 37°C for 15 min. Finally, RNAs were recovered by TRizol, reverse transcribed, and detected by RT-qPCR.

RNA half-life measurement. To determine RNA stability of the m6Am genes, one million control and PCIF1 KO cells were treated with 5 μg Actinomycin D (ActD) to block gene transcription. Cells were collected at 0, 2, 4, 8, 16, and 24 h after actinomycin D (ActD) treatment. RNA was extracted using TRizol. 1 μg RNA was reverse transcribed and RNA expressions were detected by RT-qPCR and shown as percentage relative to mock treatment. GAPDH expression was not changed in PCIF1 cells and was shown as a negative control. Linear regression analysis was carried out to calculate the half-life of mRNA.

Statistical analysis. Details of the statistical analysis were shown in the figure legend. The comparisons were performed using GraphPad 8. The difference between two groups was calculated by Student’s t-test. Statistically, significance results were determined when p < 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Data are presented as the mean with standard deviations as indicated.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The accession number for the m6A-MeRIP-seq sequencing data reported in this paper is NCBI GEO: GSE171800. The raw data for LC/MS quantification curves of modified RNA and uncropped versions of western blots are provided in the Source Data file. Source data are provided with this paper.

Code availability
The coding for m6A-MeRIP and m6A-Am-Exo-Seq analysis is deposited at Github: https://github.com/KylinKang-bme/m6A_am_code, which is also available at zenodo: https://doi.org/10.5281/zenodo.5173217.
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Author contributions
Q.Z. designed and performed the experiments, analyzed the data, and wrote the manuscript; S.W. designed and performed experiments, and analyzed the data; W.L. prepared vectors and participated in experiments; Y.K. and H.H. performed the bioinformatics analyses; G.M.G. performed LC/M.S. experiments; Y.W. analyzed the data; T.M.R. conceived and planned the project, and participated in experimental design, data analysis, data interpretation, and manuscript writing. T.M.R. obtained funding for the project.

Competing interests
T.M.R. is a founder of ViRx Pharmaceuticals and has an equity interest in the company. The terms of this arrangement have been reviewed and approved by the University of California San Diego in accordance with its conflict of interest policies. The remaining authors declare no competing interests.

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