

## FBXO34 promotes latent HIV-1 activation by post-transcriptional modulation

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### ABSTRACT

Acquired immunodeficiency syndrome (AIDS) cannot be completely cured, mainly due to the existence of a latent HIV-1 reservoir. However, our current understanding of the molecular mechanisms underlying the establishment and maintenance of HIV-1 latent reservoir is not comprehensive. Here, using a genome-wide CRISPR-Cas9 activation library screening, we identified E3 ubiquitin ligase F-box protein 34 (FBXO34) and the substrate of FBXO34, heterogeneous nuclear ribonucleoprotein U (hnRNP U) was identified by affinity purification mass spectrometry, as new host factors related to HIV-1 latent maintenance. Overexpression of FBXO34 or knockout of hnRNP U can activate latent HIV-1 in multiple latent cell lines. FBXO34 mainly promotes hnRNP U ubiquitination, which leads to hnRNP U degradation and abolishment of the interaction between hnRNP U and HIV-1 mRNA. In a latently infected cell line, hnRNP U interacts with the ReV region of HIV-1 mRNA through amino acids 1-339 to hinder HIV-1 translation, thereby, promoting HIV-1 latency. Importantly, we confirmed the role of the FBXO34/hnRNP U axis in the primary CD4<sup>+</sup> T lymphocyte model, and detected differences in hnRNP U expression levels in samples from patients treated with antiretroviral therapy (ART) and healthy people, which further suggests that the FBXO34/hnRNP U axis is a new pathway involved in HIV-1 latency. These results provide mechanistic insights into the critical role of ubiquitination and hnRNP U in HIV-1 latency. This novel FBXO34/hnRNP U axis in HIV transcription may be directly targeted to control HIV reservoirs in patients in the future.



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### Introduction

Acquired immunodeficiency syndrome (AIDS) is an infectious disease caused by human immunodeficiency virus (HIV) infection, which seriously endangers people's health and lives [1]. At present, the clinical treatment for AIDS is antiretroviral therapy (ART), which minimizes viral replication in patients and reduces plasma viral load to a level that cannot be detected by conventional testing methods. However, once the treatment is stopped, viral load will rebound to the pre-treatment level [2-4]. An important reason why HIV is difficult to eliminate from the body is the long-term existence of HIV latent reservoir [5-8]. The HIV latent virus reservoir composed of latently infected cells is a huge obstacle that cannot be eliminated by current clinical treatments [9-11]. It is not only a clinical therapeutic problem, but also a very challenging basic scientific problem.

To eliminate latent virus in infected cells, it is necessary to have a clear understanding of the mechanism of HIV-1 latency and maintenance. At present, the researchers have initially revealed the latent molecular mechanism of HIV-1 latency by HIV-1 latent infection cell lines and primary T-cell models [9,10,12], which is mainly related to the following factors: (1) the activity at the level of transcriptional suppression of the viral promoter-long terminal repeats (LTR) is correlated with HIV-1 latency [13-16]; (2) HIV-1 transcriptional blocks related to epigenetic modifications at the HIV-1 LTR, including EZH2 [17,18], HDAC1/2 [16-21] and histone methylase [22,23]; (3) inadequate availability of transcription factors at HIV LTR, such as NF- $\kappa$ B [24,25], positive transcription elongation factor b (P-TEFb or CDK9/CycinT1) [26,27], HIV-1 Tat [28,29], and others [30-32]. Recently, based on the above-mentioned

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understanding of the mechanism of HIV-1 latency and maintenance, a variety of drugs that interfere with HIV-1 latency have been tested in clinical trials. However, these drugs do not reduce the latent reservoir [33–35]. Therefore, it is necessary to conduct further research on the molecular mechanisms underlying HIV-1 latency.

Recently, with the continuous advancement of gene editing technology, especially CRISPR technology, it has become possible to achieve unbiased genetic screening of the whole genome [36–39]. Through CRISPR library screening, researchers have discovered many viral host factors that have not been previously reported to be involved in infection, replication, and latency of viruses, such as flavivirus [40], Zika virus [41], and HIV-1 [42]. Nevertheless, due to the differences in cellular expression profiles in different cell models, the possibility that CRISPR knocks out candidate genes to obtain the corresponding phenotype depends on whether the gene is expressed in the cell [43]. Therefore, different cell models used for the same phenotype may lead to differences in the screening results obtained [44–46]. The newly developed genome-wide CRISPR activation library with gain-of-function can, to a certain extent, overcome the limitations of the expression profile of cell models [47].

Here, we carried out a CRISPR-based gain-of-function genome-wide screening in a latent HIV-infected CD4<sup>+</sup> T cell model of latency. We found that the enriched gene FBXO34, an E3 ubiquitin ligase, is related to HIV-1 latency. Activating the expression of FBXO34 can activate latent HIV-1 in multiple latent infection cell lines and primary CD4<sup>+</sup> T lymphocyte latent models. Through mass spectrometry, we found that FBXO34 can ubiquitinate the downstream protein hnRNP U and target it for degradation. hnRNP U can hinder HIV-1 translation by interacting with HIV-1 mRNA, thereby promoting HIV-1 latency. To the best of our knowledge, this is the first study to determine the biological role of FBXO34, and we have also initially clarified the role of FBXO34 in the establishment and maintenance of HIV-1 latency.

## Materials and method

### Ethics statement

This study was approved by the Ethics Committee of School of Life Sciences, Fudan University and the methods were consistent with the relevant guidelines and regulations of that committee (BE2001). All patient samples were collected from the Shanghai Public Health Clinical Center, and samples of healthy people were collected from Shanghai Changhai Hospital. All patients and healthy donors gave informed consent for this experiment.

### HIV-1 latent cell lines

The HIV-1 latent infection model C11 cell line (constructed in our lab) [22,48] and J-Lat 10.6 cell line (obtained from NIH AIDS Reagent Program) contain a single integrated latent HIV-GFP reporter genome. ACH2 is a clone of HIV-1 latently infected CEM cells that contains a single copy of proviral DNA per cell (obtained from NIH AIDS Reagent Program). TZM-bl cells contain an integrated HIV LTR-luciferase construct (obtained from NIH AIDS Reagent Program).

### Cell culture

C11, J-Lat 10.6, ACH2, Jurkat and YA [22,48] cells were cultured in RPMI1640 (Gibco, C11875500BT) with 10% fetal bovine serum (FBS) (Gibco, 10110154) and 1% penicillin/streptomycin (Gibco, 15140-122) in a 37°C incubator containing 5% CO<sub>2</sub>. TZM-bl and 293T cells were cultured in DMEM (Gibco, C11995500BT) and supplemented with 10% fetal calf serum (ExCell Bio, FSP500), and 1% penicillin/streptomycin (Gibco) in a 37°C incubator containing 5% CO<sub>2</sub>.

### Antibody and reagents

The following antibodies were used throughout this study: from ABclonal(Wuhan, China), anti-FLAG (AE063; 1:2500 for Western blot), anti-Myc (AE070; 1:2500 for Western blot), anti-β-Actin (AC026; 1:5000 for Western blot). From Abcam (Cambridge, UK), anti-hnRNP U (ab264142; 1:1000 for Western blot). From Proteintech (Wuhan, China), HRP goat anti-mouse IgG (15014; 1:5000 for Western blot), HRP goat anti-rabbit IgG (15015; 1:5000 for Western blot). Anti-Flag Magnetic Beads (HY-K0207) was purchased from MCE. 2 × Taq Master Mix (P112), High fidelity PCR enzyme-2 × Phanta Max Master Mix (P515) were purchased from Vazyme (Nanjing, China). PMD18-T (6011) was purchased from Takara (Beijing, China). Cell Genome Extraction Kit (DP304), Plasmid Extraction Kit (DP103, DP108, DP117) were purchased from Tiangen (Beijing, China). Gel Extraction Kit (CW2302) was purchased from CWBIO (Nanjing, China). Luciferase detection kit (E6110) was purchased from Promega (Madison, USA). Cell Counting Kit (CCK-8) and TUNEL-Alexa Flour 640 apoptosis kit were purchased from Yeasen (Shanghai, China). Luciferase and nanoluc detection kit (E6110, N1110) was purchased from Promega (Madison, USA). Recombinant human IFN-α (11200-1), recombinant human IFN-β (8499-IF) and recombinant human IFN-γ (285-IF) were purchased from R&D Systems (UN).

### Construction and production of lentivirus

For production of lentivirus, HEK293T cells were seeded into 10 cm dishes one day before transfection. When the cells reached 80% confluence, plasmid and PEI (the ratio of the plasmid and PEI was 1:3) were added into the Opti-MEM (Gibco), mixed evenly, and left standing for 20 min. MPH or SAM library, psPAX2 and PMD2.G vector were co-transfected into HEK293T cells to produce the lentivirus. After incubation at 37°C and 5% CO<sub>2</sub> for 8–12 h, the culture medium was changed with DMEM with 2% FBS and 1% P/S. The supernatants containing lentivirus were harvested at 48 and 72 h after transfection and filtered by 0.45 µm pore size. The filtrates were centrifuged at 25,000 rpm and 4°C for 2 h. The supernatants were discarded, and the lentivirus stocks were dissolved in DMEM for storage at –80°C.

### Pooled genome-wide CRISPR screen

A total of  $1 \times 10^7$  C11 cells were infected with MPH lentivirus (MOI = 2). After 72 h, C11 cells were selected with 100 µg/ml Hygromycin B for 14 days. Then, a total of  $1 \times 10^9$  C11 cells which expressed MPH were infected at a low multiplicity of infection (MOI = 0.2), to ensure that most cells received only one viral construct. After 72 h, the cells were selected with 2 µg/ml Blasticidin for 14 days. 10<sup>8</sup> Blasticidin-resistant C11 cells were sorted by FACS to obtain GFP + C11 cells. Sorted C11 cells were cultured for 1 week and cell sorting was performed again. The gDNA was extracted by a genome extraction kit. PCR was performed with the indicated primers to confirm that the selected cell genome contains sgRNA targeting different genes.

### Screen analysis

Sequencing reads were aligned to the sgRNA library and the abundance of each sgRNA was calculated. SgRNAs with less than 25 counts in the initial set were removed from downstream analyses. The log<sub>2</sub> fold change in abundance of each sgRNA was calculated for the sorted and unsorted final population samples.

### Vector construction

Individual sgRNA constructs targeting C1ORF27, C20ORF27, TEX38, IGLL1, CXCL6, FBXO34, IGLA4, POLD3, AFF4 and CARM1 were cloned into lentiSAMv2 (addgene 75112). The primers were listed in Supplementary Table 3.

For cDNA expression vectors, a linearized lentiviral backbone was generated from pCMV (Youbio, Hunan, China). Protein-coding plasmids were gifts

from Professor Han Jiahui Laboratory. All the constructed plasmids were confirmed by restriction enzyme digestion and DNA sequencing.

### Cas9-mediated gene activation, knockout and cDNA overexpression

C11, J-Lat 10.6 and ACH2 cells were infected with lentivirus at an MOI of 1 and then selected with 2 µg/ml puromycin for 14 days. The knockout and activation were detected by Western blot (WB) analysis.

### Visualization of GFP and flow cytometry assay

Green fluorescent protein (GFP), a marker for the activation of HIV-1 in infected cells, was visualized by fluorescence microscopy after cell sorting. The cells were collected and washed with phosphate buffered saline (PBS). Cells were kept in PBS before analysis on a BD LSRII flow cytometer for enhanced GFP expression. FlowJo software (FlowJo LLC, Ashland, OR) was used to perform the flow cytometry analysis.

### ELISA detection of antigen p24 levels

ACH2 cells were each seeded at a density of  $1 \times 10^6$  on a 6-well plate. After 48 h of culture, HIV-1 production was measured via quantification of p24 in culture supernatant using p24 ELISA kit (R&D System, Minnesota, USA).

### Western blot

A total of  $1 \times 10^6$  cells were seeded in a 10-cm dish and cultured for 24 h. Then, the cells were harvested, lysed, and subjected to Western blot. Membranes were visualized using the Immuno-Star WesternC Chemiluminescence Kit (Bio-Rad) and images were captured using the ChemiDoc XRS + System and processed using ImageLab software (Bio-Rad).

### Isolation of primary CD4<sup>+</sup> T cells

Peripheral blood mononuclear cells (PBMCs) isolated from healthy donors were purchased from the Changhai Hospital of Naval Medical University (Shanghai, China). Naive CD4<sup>+</sup> T cells were further purified from peripheral blood mononuclear cells by negative selection according to the manufacturer's instructions (Thermo). The Naive CD4<sup>+</sup> T cells were maintained in serum-free medium supplemented with 1% penicillin–streptomycin and 5 ng/ml recombinant human IL-2 (R&D) and 10 ng/ml IL-7 (R&D) at 37°C in 5% CO<sub>2</sub>.

### Cell proliferation by CCK-8 assay

Cells differently treated were seeded at a density of  $0.5 \times 10^4$  on 96-well plate. 10% CCK-8 solution was added to fresh culture medium. The cells were incubated at 37°C for 1 h. The OD 450 nanometer value was measured to determine cell proliferation.

### Apoptosis detected by TUNEL staining

A total of  $1 \times 10^6$  cells were collected in a 1.5 ml tube and centrifuged at 300g for 5 min. The cells were washed twice with 500  $\mu$ l PBS and analyzed with TUNEL-Alexa Flour 640 apoptosis detection kit according to the manufacturer's instructions. The proportion of Alexa Flour 640-positive cells was assayed by flow cytometer and analyzed by FlowJo software (FlowJo LLC, Ashland, OR).

### RNA immunoprecipitation

$1 \times 10^7$  cells from different treatments were collected by centrifugation at 1500 rpm for 5 min at 4°C and washed with ice-cold PBS. After lysis in complete RIP lysis buffer, the cell lysate was centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatant was transferred to a new tube containing RIP immunoprecipitation buffer and magnetic beads bound with anti-Flag antibody or rabbit IgG. After incubation overnight at 4°C, the sample was placed on a magnetic separator (MCE, USA), and the supernatant was discarded. The beads were washed six times with cold PBS. Protein K buffer was added, and the mixture was incubated at 55°C for 30 min to digest the proteins. The purified RNA obtained was subsequently detected by qRT-PCR.

### Extraction of total RNA, reverse transcription, and qRT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions or obtained by RIP experiment. cDNA was synthesized by reverse transcription using a BeyoRT™ III cDNA Synthetic premix. (Beyotime, China). qRT-PCR was then performed on an Applied Biosystems 7500 Real Time PCR System (Thermo Fisher Scientific, USA) using a reaction mixture consisting of 5  $\mu$ l SYBR-Green Universal qPCR Master Mix (Vazyme, China). GAPDH was used as an internal reference for mRNA. Specific primers for qRT-PCR are designed according to PrimerBank and listed in Supplementary Table 3.

### Statistical analysis

Data are representative of three independent experiments, and error bars represent standard errors

(SD). Paired samples *t*-tests were performed with use of SPSS version 13.0 (SPSS Inc., Chicago), and statistical significance was indicated at \**p* < 0.05, \*\**p* < 0.01 or \*\*\**p* < 0.001.

## Results

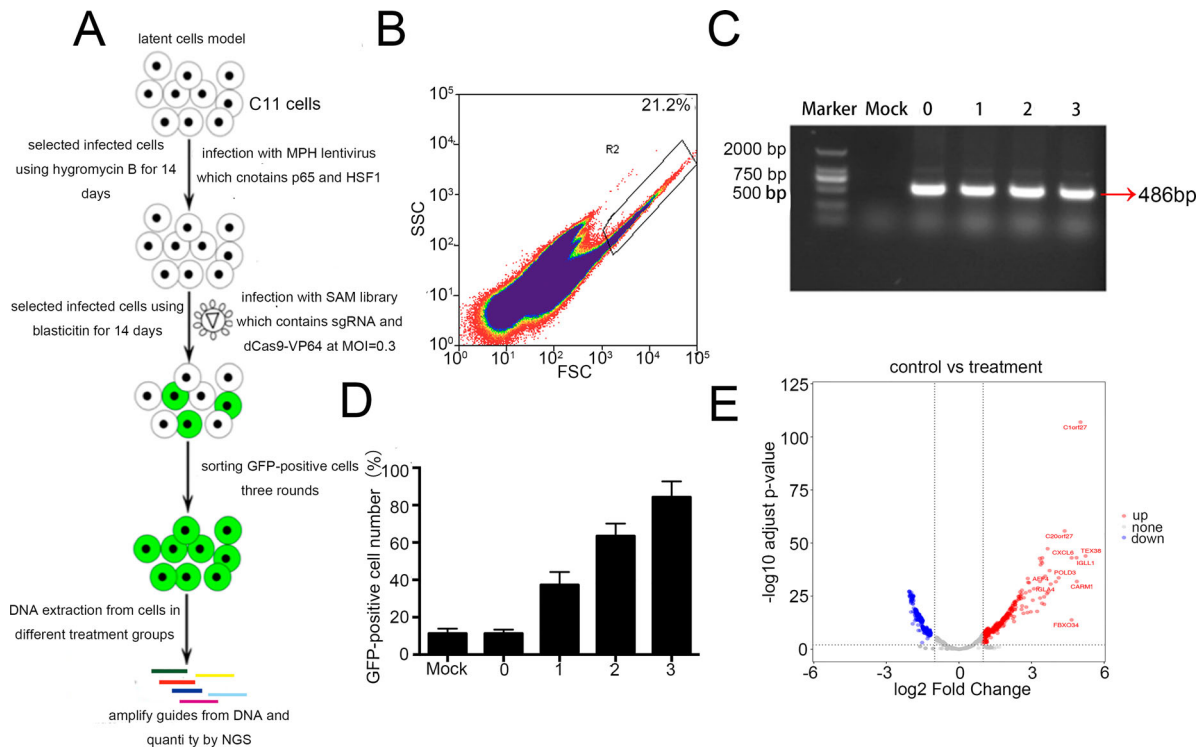
### Genome-wide CRISPR/Cas9 activation library screening reveals host factors associated with HIV latency

To identify new host genes related to HIV-1 latency, we carried out CRISPR-based activation library screening in the latent infected CD4<sup>+</sup> T model-C11 [47], which is a HIV-1-infected latent cell lines with a green fluorescent protein (GFP) reporter gene established in our laboratory [22,48]. The SAM library that we used had 70290 sgRNAs that target 23430 genes [47]. In the latent state, the positive rate of GFP in C11 cells was low (<2%) [22,48]. In our study, we first packaged the helper plasmid MPH of the SAM library into a lentivirus to infect the C11 cell line and selected them using hygromycin B (100  $\mu$ g/ml) for 14 d incubation. Then, we infected C11-MPH cells with SAM library lentivirus at a multiplicity of infection (MOI) of 0.3 and we selected infected clones using blasticidin (20  $\mu$ g/ml) for 14 d incubation (Figure 1(A)). Approximately 20% of GFP-positive C11 cells were enriched after three rounds of cell sorting (Figure 1(A,B)). To confirm that the infected and sorted cells contained integrated sgRNA sequences, we extracted the cell genome and performed PCR verification. The results showed that C11 cells were infected and all cells with integrated sgRNAs were sorted (Figure 1(C)). We further confirmed by cytoflow. that the sorted C11 cells were GFP-positive (Figure 1(D)). Next, we extracted the genomic DNA of unsorted (as control) and sorted C11 cells, and then detected the changes in the abundance of targeted sgRNA by next-generation sequencing (NGS). The distribution of the gene of interest was compared to the distribution of log2 enrichment values of the unsorted control sgRNAs by volcano map. Among the enriched top ten genes, we found a gene related to HIV-1 latency-AFF4 [49], which further proves the reliability of our experimental results. In addition to *AFF4*, we observed enrichment of genes that have not been previously reported to be related to HIV-1 latency, including *C10RF27*, *C200RF27*, *IGLL1*, *CXCL6*, *FBXO34*, *CARM1*, *POLD3*, *IGLA4*, and *TEX38* (Figure 1(e), Supplementary Table 1). In short, through CRISPRa screening, we identified many new candidate genes related to HIV-1 latency.

### Validation of candidate HIV latency inhibiting genes

To examine whether the candidate genes enriched in the CRISPR activation library are related to HIV-1



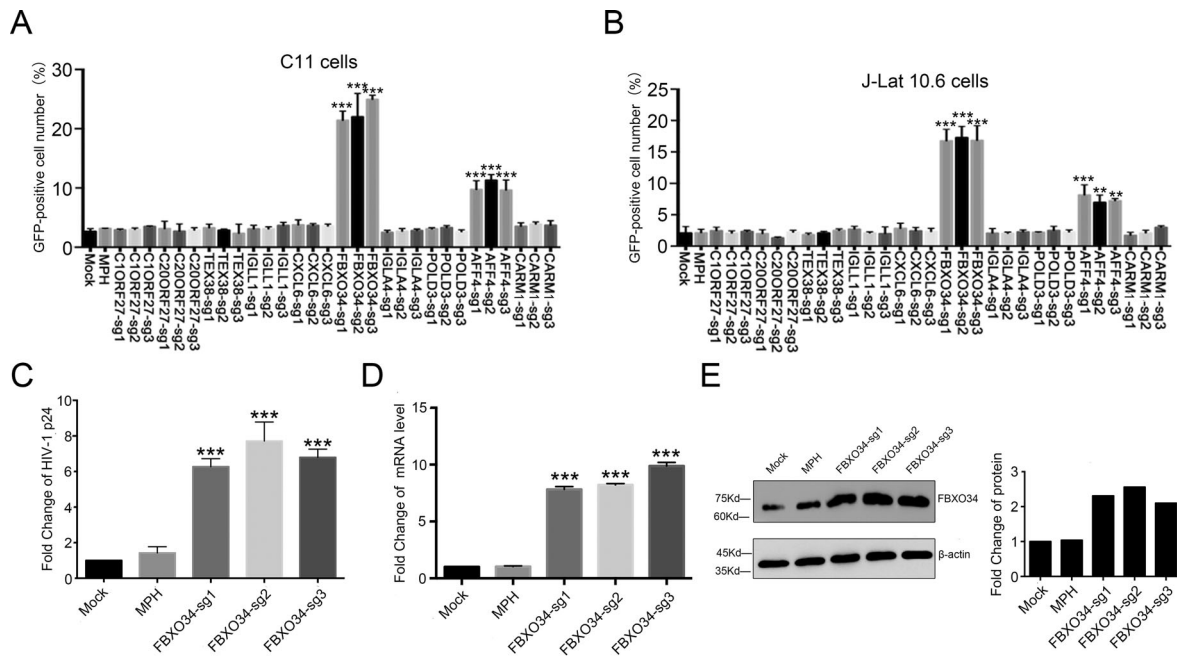


**Figure 1.** A pooled, genome-wide CRISPR screening for candidate genes involved in HIV-1 latency. (a), The outline of genome-wide activation CRISPR screen strategy. The C11 cells were infected with lentiviral containing MS2-p65-HSF1 proteins treated with Hygromycin B for fourteen days. Then, the C11 cells expressing MS2-p65-HSF1 infected with lentiviral dCas9 and sgRNA library that target 20234 human genes. Fourteen days post screening with blasticidin, genomic DNA was extracted from GFP-positive cells after three-round sorting. The candidate genes were identified by the next generation sequencing. (b), Flow cytometry of cells infected with the lentiSAM library where the expression of GFP is a reporter of latency reactivation. Target genes were enriched through three-round sorting. Continuously cultured C11 latent cells infected with the lentiSAM served as control. Blue dots and encircled area represent the GFP-positive cells for flow cytometry analysis. (c), Validation of sgRNAs in unsorted and sorted C11 cells with PCR after lentiSAM library infection. (d), Before next generation sequencing, the GFP expression in three-round sorted C11 cells was confirmed by flow cytoflow. (e), Fold change ( $\text{Log}_2$ ) of the abundance of target genes in sorted C11 cells where enriched sgRNA genes were highlighted in Volcano illustration.

latency, we infected C11-MPH cells with dCas9-sgRNA lentivirus targeting the candidate genes, and these cells were selected using blasticidin (20  $\mu\text{g}/\text{mL}$ ) for 14 d, and detected the expression of GFP by flow cytometry. We found that when FBXO34 was activated by dCas9-sgRNA, GFP expression levels in latently infected cells increased to approximately 25% (Figure 2(A)), indicating that overexpression of FBXO34 can promote activation of latent HIV-1. To further confirm the effect of FBXO34 on HIV-1 latency, we repeated the above experiment in two other latently infected cell lines, J-Lat 10.6, and ACH2. For FBXO34, we obtained similar results (Figure 2(B,C)). Then, we further verified the changes in the expression levels of FBXO34 in C11 cells latently infected by HIV-1 by RT-qPCR and western blotting, and the results showed that the expression levels of FBXO34 increased significantly (Figure 2(D, E)). As a potential drug target, we wanted to further understand whether FBXO34 influences cell proliferation and apoptosis. The results showed that activating the expression of FBXO34 did not affect the proliferation and apoptosis of C11 cells (Supplementary Figure 1). These data indicate that FBXO34 is a new gene associated with HIV-1 latency.

### FBXO34 could target hnRNP U degradation through ubiquitination

Members of the F-Box protein family are core elements of the E3 ubiquitin ligase complex, that recognize and recruit downstream proteins to the ubiquitin ligase complex to be ubiquitinated, thereby regulating cell function [50]. FBXO34 belongs to the Cull1 family of E3 ubiquitin ligases. Although, there has been considerable research on the functions of F-box protein family members [51,52], there are no reports about FBXO34. Nevertheless, based on the basic properties of FBXO34, we infer that FBXO34 may promote the degradation of a protein that inhibits HIV-1 activation through ubiquitination, thereby, promoting activation of latent HIV-1. To verify our hypothesis, we first constructed 293T cells overexpressing FLAG-FBXO34 (Figure 3(A)). Then, we carried out co-immunoprecipitation experiments with anti-FLAG antibody, obtained proteins potentially interacting with FBXO34, and identified them by mass spectrometry (Figure 3(B,C)). To identify the top ten most abundant potentially interacting proteins by mass spectrometry, we transfected FLAG-FBXO34



**Figure 2.** Validation of the candidate genes screened from the lentiSAM library in HIV-1 latently infected cell lines. (a), Validation of the top candidate genes in C11 cell line. C11 cell line expressing MS2-p65-HSF1 was infected by lentiSAM packaged lentiviruses with sgRNA following by screening for 14 days with 20  $\mu$ g/ml blasticidin. The percentage of GFP-positive cells was measured by flow cytometry to determine the level of HIV-1 reactivation. (b,c), The effect of candidate genes on HIV latency were further verified in J-Lat 10.6 (b) and ACH2 (c) models of HIV latency. The GFP expression in J-Lat 10.6 cells and p24 in ACH2 cells was analyzed by flow cytometry and ELISA, respectively. (d,e), FBXO34 expression was measured by qPCR (d) and Western blot (e) after activation in the latently infected C11 cells. Mock C11 cells served as control. Data information: Each data represented the mean  $\pm$  SD of three independent experiments ( $n = 3$ ) and were analyzed with  $T$ -test.  $**p < 0.01$ ,  $***p < 0.001$ .

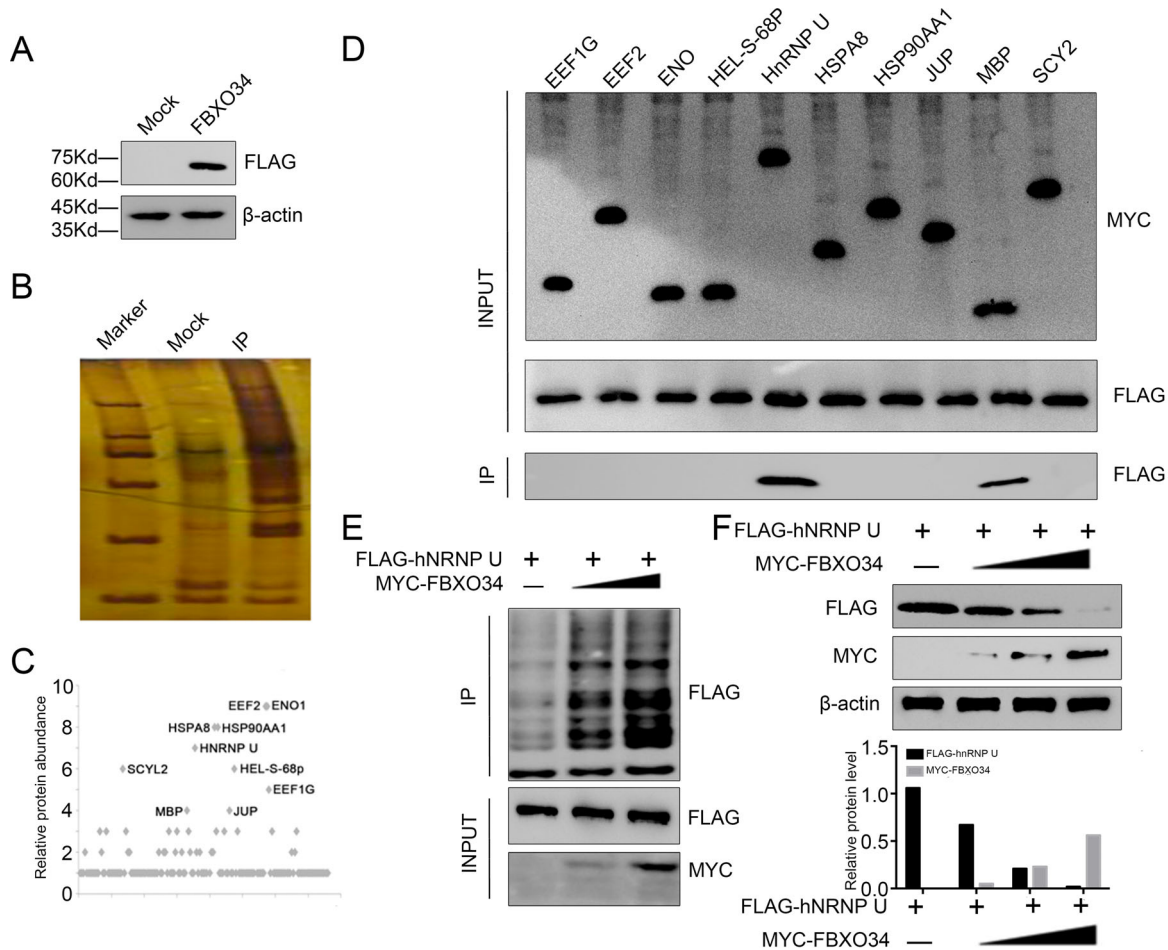
and MYC-EEF1G, MYC-EEF2, MYC-ENO, MYC-HEL-S-68P, MYC-hnRNP U, MYC-HSPA8, MYC-HSP90AA1, MYC-JUP, MYC-MBP, MYC-SCYL2 plasmids into 293T cells, and identified whether there was an interaction by immunoprecipitation with anti-MYC beads. The results showed that FLAG-FBXO34 interacted with MYC-MBP and MYC-hnRNP U (Figure 3(D)). In order to explore whether MBP and hnRNP U are related to HIV-1 transcription latency, we transiently transfected 293T cells with NL4-3-luciferase alone, MYC-MBP, or MYC-hnRNP U. Overexpression of hnRNP U was found to inhibit HIV-1 expression (Supplementary Figure 2(A)). Subsequently, we repeated the above experiment in the TZLbl cell line, which was integrated with the LTR-driven luciferase reporter gene and Ya cells, which constitutively express HIV-1 mRNA. Interestingly, we overexpressed hnRNP U in Ya cells and obtained similar results with 293T cells. In TZMbl cells, we found that overexpression of hnRNP U had no significant effect on the expression of LTR-driven luciferase (Supplementary Figure 2(B) and 2(C)). This suggests to us that hnRNP U may not promote HIV-1 latency by interacting with HIV-1 LTR.

Therefore, we consider that the activation effect of FBXO34 on latent HIV-1 may be achieved through hnRNP U. Therefore, our follow-up research focused on hnRNP U. Considering the E3 ubiquitin ligase

properties of FBXO34, we first explored whether FBXO34 mediates ubiquitination of hnRNP U. By chromatin immunoprecipitation in 293T cells, we showed that FBXO34 can promote the ubiquitination of hnRNP U (Figure 3(E)). Besides, we transiently transfected FLAG-FBXO34 and Myc-hnRNP U expressing plasmids simultaneously or separately in 293T cells, and found that as the expression of FLAG-FBXO34 protein increased, the expression levels of Myc-hnRNP U gradually decreased, which suggested that FBXO34 caused hnRNP U ubiquitination, which in turn led to hnRNP U degradation (Figure 3(E)). These results indicate that FBXO34 may affect HIV-1 latency by degrading hnRNP U.

### Knockout of hnRNP U can promote the activation of latent HIV-1

Given that hnRNP U can inhibit HIV-1 transcription and FBXO34 could activate latent HIV-1 by targeting hnRNP U for degradation, we further explored whether knocking out hnRNP U in latently infected cell lines would also cause latent HIV-1 activation. We infected C11 with Cas9/sgrNA lentivirus targeting hnRNP U and selected the cells using puromycin (2  $\mu$ g/mL) post 14 d incubation, and then detected the expression of GFP by flow cytometry. We found that when hnRNP U was knocked out by Cas9/sgrNA, GFP expression in latently infected cells



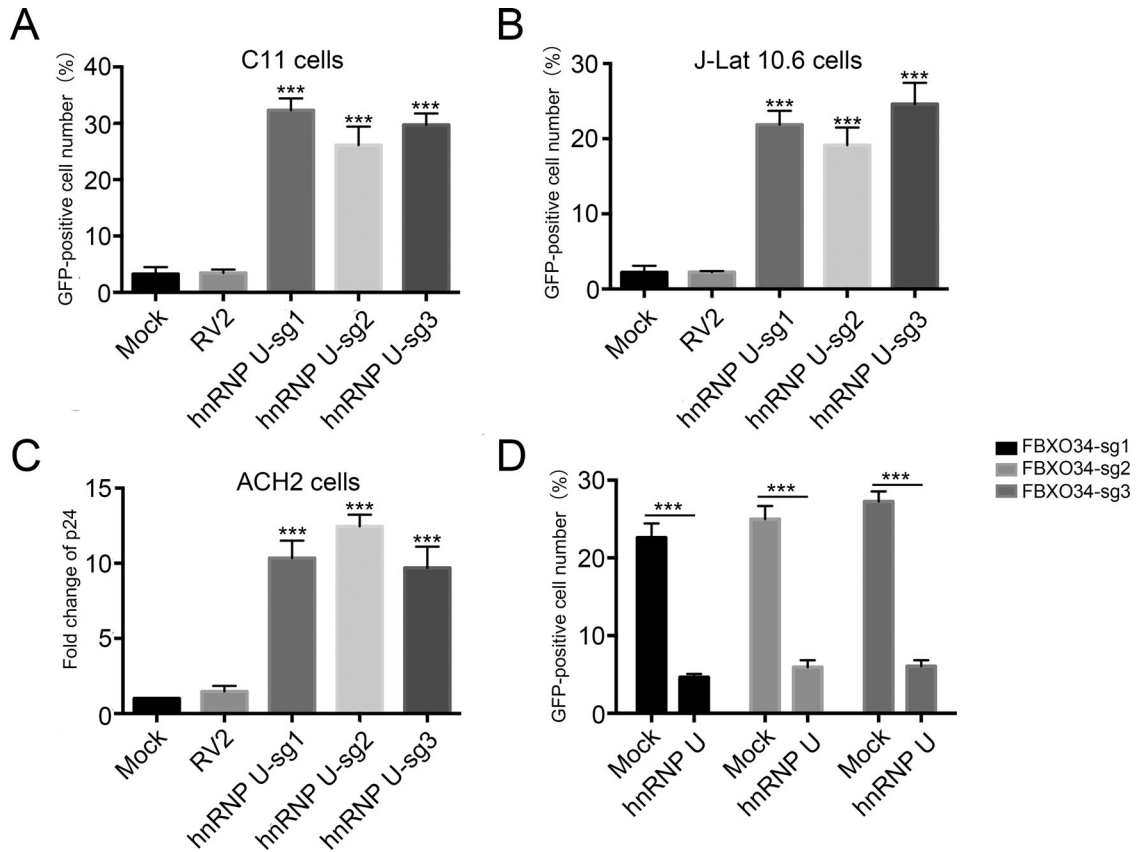
**Figure 3.** FBXO34 can promote the ubiquitination and degradation of hnRNP U. (a), Western blot of cell lysate of 293T and 293T infected with lenti-FLAG-FBXO34. (b), Silver stain SDS-PAGE of co-IP samples of IgG or anti-FLAG antibodies from cell lysate of 293T infected with lenti-FLAG-FBXO34. (c), The number of total peptides identified by mass spectrometry analysis with FLAG-FBXO34 as bait. (d), Western blot of co-IP samples of IgG or anti-FLAG antibodies from cell lysate of 293T cells infected with lenti-FLAG-FBXO34 and transfected with pCMV-MYC-EEF1G, pCMV-MYC-EEF2, pCMV-MYC-ENO, pCMV-MYC-HEL-S68P, pCMV-MYC-hnRNP U, pCMV-MYC-HSPA8, pCMV-MYC-HSP90AA1, pCMV-MYC-JUP, pCMV-MYC-MBP or pCMV-MYC-SCY1. (e), Western blot of co-IP samples of IgG or anti-FLAG antibodies from cell lysate of 293T cells transfected with pCMV-FLAG-hnRNP U and MYC-FBXO34. (f), Western blot of cell lysate of 293T cells transfected with pCMV-FLAG-hnRNP U and MYC-FBXO34.

increased to approximately 30% (Figure 4(A)), indicating that hnRNP U knockout promotes activation of latent HIV-1. To further confirm the effect of hnRNP U on HIV-1 latency, we repeated the above experiment in two other latently infected cell lines, J-Lat 10.6, and ACH2. We obtained results similar to those obtained using C11 cell lines (Figure 4(B,C)). To further explore whether FBXO34 affects HIV-1 latency through hnRNP U, we overexpressed hnRNP U in C11 cells that activate FBXO34 expression through sgRNA/dCas9. When hnRNP U was overexpressed, the expression levels of GFP in C11 cells with activated FBXO34 expression decreased significantly (Figure 4(D)), which further confirms that FBXO34 affects HIV-1 latent activation via hnRNP U. Finally, we also evaluated the effect of hnRNP U knockout on cell proliferation and apoptosis, and found that hnRNP U knockout had no significant effect on cell proliferation, but it slightly promoted cell apoptosis (Supplementary Figure 1). Based on the above results, we

preliminarily concluded that FBXO34 affects HIV-1 latency through hnRNP U.

#### **hnRNP U promotes HIV-1 latency at the post-transcriptional level by interacting with HIV-1 Rev element**

Considering that overexpression of hnRNP U has no effect on LTR-driven luciferase (Supplementary Figure 2(B)), we hypothesized that hnRNP U may not affect HIV-1 transcription. We first determined whether there was any change in the expression level of HIV-1 mRNA through GFP mRNA level in C11 cells overexpressing FBXO34 or in hnRNP U knockouts. Compared with the control group, the expression levels of HIV-1 mRNA in the experimental group did not change significantly (Figure 5(A)). Previous reports have shown that hnRNP U is an RNA-binding protein. Therefore, we explored whether hnRNP U can interact with HIV-1 mRNA. It was found that the HIV-1 mRNA could be obtained by RNA



**Figure 4.** hnRNP U can promote HIV-1 latency. (a), Validation of hnRNP U in C11 cell line. C11 cell line was infected by lentiCRISPR V2.0 packaged lentiviruses with sgRNA targeting hnRNP U following by screening for 14 days with 2  $\mu$ g/ml puromycin. The percentage of GFP-positive cells was measured by flow cytometry to determine the level of HIV-1 reactivation. (b,c), The effect of hnRNP U on HIV latency were further verified in J-Lat 10.6 (b) and ACH2 (c) models of HIV latency. The GFP expression in J-Lat 10.6 cells and p24 in ACH2 cells was analyzed by flow cytometry and ELISA, respectively. (d), The effect of overexpression of hnRNP U on the C11 cells which was enhanced FBXO34 expression by dCas9/sgRNA. The GFP expression in C11 cells which was enhanced FBXO34 expression by dCas9/sgRNA was analyzed by flow cytometry. Data information: Each data represented the mean  $\pm$  SD of three independent experiments ( $n = 3$ ) and were analyzed with  $T$ -test. \*\*\* $p < 0.001$ .

immunoprecipitation (RIP) using an anti-hnRNP U antibody (Figure 5(B)). This indicates that hnRNP U may interact with HIV-1 mRNA to hinder its post-transcriptional translation and promote HIV-1 latency.

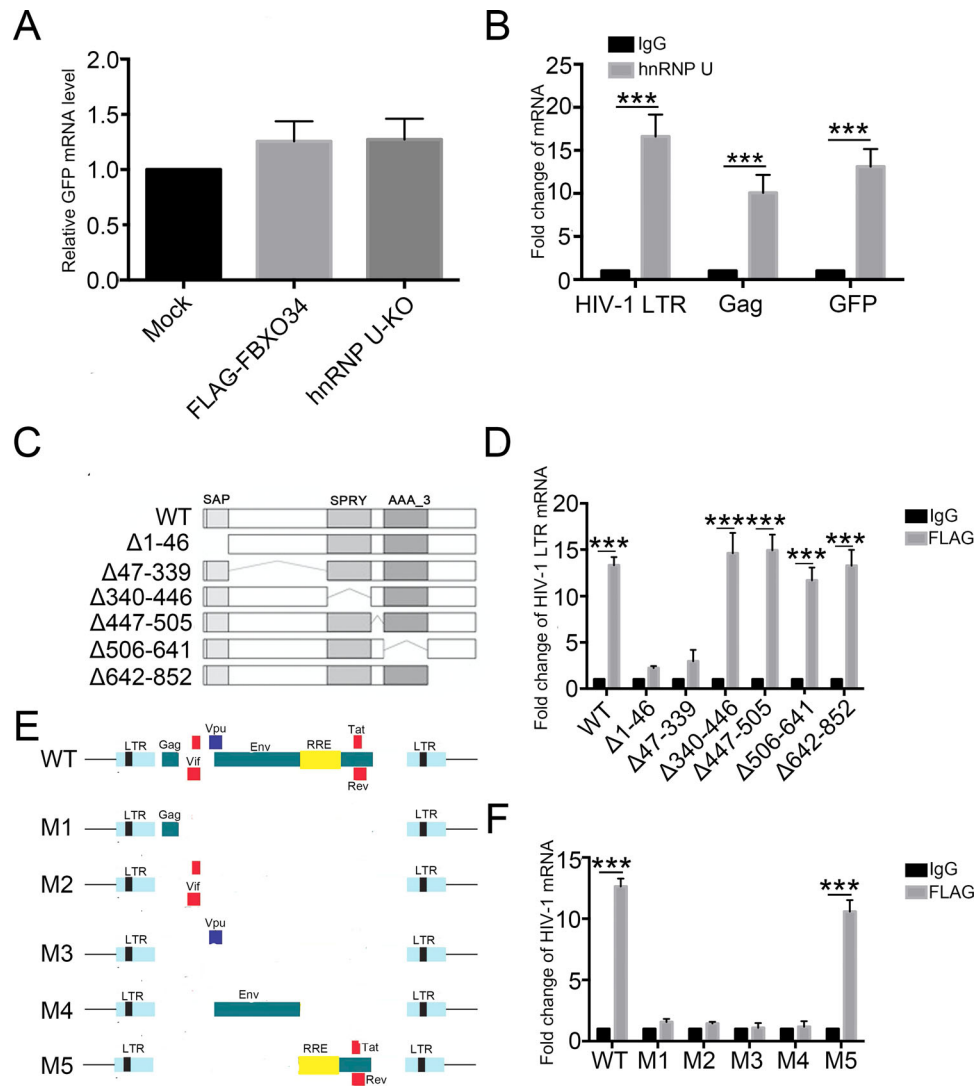
To explore how hnRNP U interacts with HIV-1 mRNA, we further examined which region of hnRNP U interacts with the HIV-1 mRNA. We first constructed six different deletion mutants according to the functional domain of hnRNP U (Figure 5(C)). Next, we co-transfected 293T cells with wild-type or each of the six deletion mutants with the NL4-3-luciferase plasmid and tested their interactions using RIP experiments and RT-qPCR. The results showed that when amino acids 1-339 were deleted, hnRNP U lost its ability to bind to HIV-1 mRNA (Figure 5(D)), which suggests us that amino acids 1-339 are critical for hnRNP U binding to HIV-1 mRNA. On the other hand, we also constructed a NL4-3-Luciferase plasmid carrying deletions of different HIV-1 elements (Figure 5(E)) and co-expressed them with WT hnRNP. RIP and RT-qPCR experiments indicated that when the REV element was deleted from the

NL4-3-Luciferase plasmid, hnRNP U could not interact with HIV-1 mRNA (Figure 5(F)). The above results indicate that hnRNP U interacts with the Rev region of HIV-1 mRNA to promote HIV-1 latency at the post-transcriptional level.

#### The FBXO34/hnRNP U axis promotes HIV-1 latency in primary CD4<sup>+</sup> T model cells

Previous data showed that the FBXO34/hnRNP U axis can promote HIV-1 latency. To directly test whether the FBXO34/hnRNP U axis promotes HIV-1 latency in primary human CD4<sup>+</sup> T lymphocytes, we established a primary HIV-1 latent infection model as described in previous studies (Figure 6(A)) [53–55]. On the third day of the experiment, the HIV-1 pseudovirus packaged with NL4-3-nanoluciferase was used to infect primary T lymphocytes. During the 5–12 d period, the concentration of IL-2 was gradually reduced in the primary T lymphocyte culture. On the 5th day and the 12th day, the expression levels of nanoluciferase in primary T lymphocytes was detected respectively. The results showed that the expression





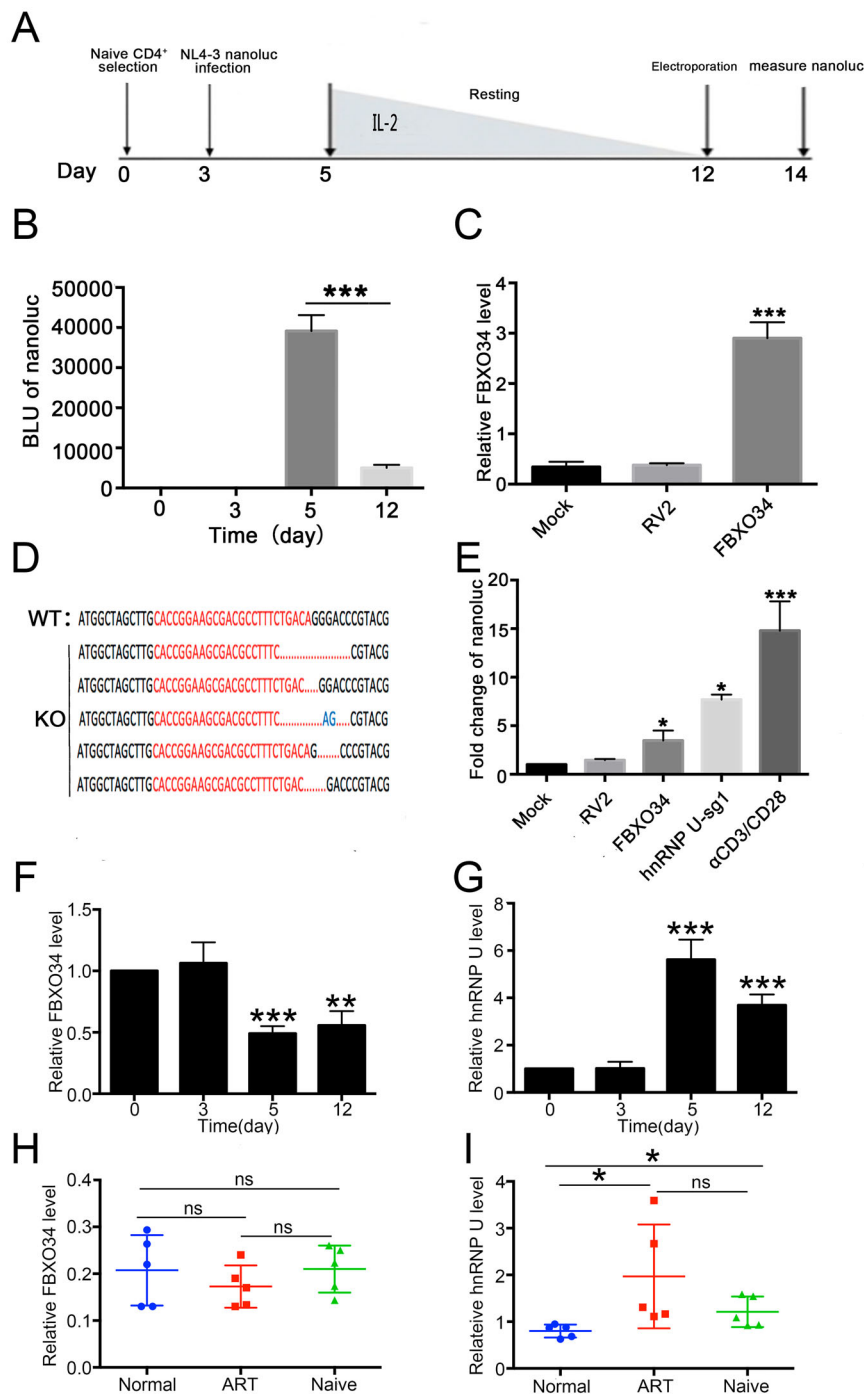
**Figure 5.** hnRNP U interacts with the Rev region of HIV-1 mRNA through amino acids 1-339 to promote HIV-1 latency at the post-transcriptional level. (a), HIV-1 mRNA expression was measured by qPCR in the latently infected C11 cells and C11 cells that stably expressing FLAG-FBXO34 or knock out hnRNP U. (b), hnRNP U interacts with HIV-1 mRNA. qPCR of RIP samples of IgG or anti-FLAG antibodies from cell lysate of 293T cells transfected with pCMV-FLAG-hnRNP U and NL4-3-luciferase. (c), Diagram showing the full length, truncated hnRNP U. (d), qPCR of RIP samples of IgG or anti-FLAG antibodies from cell lysate of 293T cells transfected with pCMV-FLAG-hnRNP U, different truncated pCMV-FLAG-hnRNP U and NL4-3-luciferase. (e), Diagram showing the full length, truncated HIV-1 genome. (f), qPCR of RIP samples of IgG or anti-FLAG antibodies from cell lysate of 293T cells transfected with pCMV-FLAG-hnRNP U, pcDNA3.1-WT-HIV-1 genome and different truncated pcDNA3.1-HIV-1 genome. Data information: Each data represented the mean  $\pm$  SD of three independent experiments ( $n = 3$ ) and were analyzed with  $T$ -test. \*\*\* $p < 0.001$ .

levels of nanoluciferase in the cells was significantly reduced on the 12th day compared to the 5th day (Figure 6(B)), and *tat/rev* of HIV-1 also has the same trend (Supplementary Figure 3(A)), indicating that HIV-1 was in a latent state. On the 12th day, we transfected FLAG-FBXO34 or Cas9/sgRNA plasmid targeting hnRNP U by electrotransduction, or used  $\alpha$ CD3/CD28 magnetic beads to treat the primary T lymphocyte latent cell model. Forty-eight hours after treatment, we detected the expression levels of nanoluciferase in the cells. The results showed that enhanced FBXO34 expression or *hnRNP U* knockout resulted in activation of HIV-1 (Figure 6(C-E)), and during the activation process, the mRNA level of *tat/rev* was not significantly increased, which further

demonstrated that FBXO34/hnRNP U promoted HIV-1 latent activation mainly at the post-transcriptional level (Supplementary Figure 3(B)). These results further supporting our hypothesis that the FBXO34/hnRNP U axis is involved in HIV latency.

#### The differences in the expression of FBXO34/hnRNP U in the primary CD4<sup>+</sup> T cell model and patient samples

In the primary CD4<sup>+</sup> T cell model of latency by a continuous decrease in IL-2 concentration, we found that the expression levels of FBXO34 was significantly reduced on the 5th and 12th day, and the expression levels of hnRNP U gradually increased



**Figure 6.** FBXO34 reactivates latent HIV-1 in the primary CD4<sup>+</sup> T cell model of latency and there are differences in expression between the primary and patient samples. (a), The outline of latency establishment in the primary CD4<sup>+</sup> T cells. Human primary CD4<sup>+</sup> T cells were activated and expanded with  $\alpha$ -CD3/CD28 beads at day 1. The  $\alpha$ -CD3/CD28 beads were removed at day 3. Cells were then infected with HIV-1 NL4.3-nanoluc at 3rd day after expansion and maintained over 7 days with a decreasing concentration of IL-2 to establish latency until day 12. At day 12, cells were transfected with pCMV-FLAG-FBXO34 or CRISPR/Cas9 and hnRNP U-sgRNA by electrotransfection. (b), The transcription of HIV-1 in the primary CD4<sup>+</sup> T cells was determined by nanoluciferase assays during HIV-1 infection. (c), The mRNA level of FBXO34 was measured by qPCR after transfected with pCMV-FLAG-FBXO34. (d), hnRNP U gene deletion after hnRNP U-sg1 knockout in the primary CD4<sup>+</sup> T cells. The PCR products of hnRNP U were cloned and then sequenced. hnRNP U-sg1 target gene sequences were shown in red letters. Dashes indicate deleted bases relative to the wild-type sequence. (e), Overexpression of FBXO34 or knockout hnRNP U enhanced HIV-1 transcription in the primary CD4<sup>+</sup> T cell model of latency by electrotransfection. The expression of HIV-1 was measured by nanoluciferase where  $\alpha$ -CD3/CD28 stimulation served as a positive control. (f,g), After infection with VSV-G pseudotyped HIV-1 NL4.3-nanoluc in the primary CD4<sup>+</sup> T cells, the mRNA expression of FBXO34 (f) and hnRNP U (g) were measured by qPCR. (h,i), The mRNA expression of FBXO34, hnRNP U in primary CD4<sup>+</sup> T cells isolated from normal ( $n = 5$ ), naive-ART patients ( $n = 5$ ) and ART patients ( $n = 5$ ) were measured by qPCR. Data information: Each data represented the mean  $\pm$  SD of three independent experiments ( $n = 3$ ) and were analyzed with *T*-test. \* $p < 0.05$ ; \*\*\* $p < 0.001$ .

(Figure 6(F,G)). We also compared the expression levels of FBXO34 and hnRNP U in CD4<sup>+</sup> T lymphocytes in peripheral blood among five Naive-ART patients, ART treated patients and five healthy donors (Supplementary Table 2). The results showed that the expression levels of FBXO34 in patients receiving ART was lower than that in healthy people, however, the statistical difference was not significant, which may be due to individual differences (Figure 6(H)). Besides, the expression level of hnRNP U was significantly higher than that in healthy donors in both naive-ART treatment and ART treatment patients, and there was a statistical difference (Figure 6(I)). It is worth noting that in two patients treated with ART, the expression levels of hnRNP U were significantly increased (Figure 6(I)). These results suggest that the FBXO34/hnRNP U axis may also inhibit HIV-1 transcription and promote its latency in HIV-1 patients.

### **FBXO34/hnRNP U axis responds to IFN- $\gamma$ stimulation**

It has been shown that many factors involved in the inhibition of HIV-1 activation are related to interferon signalling [56,57]. Therefore, we hypothesized that the FBXO34/hnRNP U axis is related to interferon signalling during HIV-1 infection of primary T lymphocytes. We found that when Jurkat cells were treated with interferon gamma (IFN- $\gamma$ ), the mRNA levels of FBXO34 decreased significantly, while the expression levels of hnRNP U increased significantly, IFN- $\alpha$  and IFN- $\beta$  had no effect on the FBXO34/hnRNP U axis (Figure 7(A)). Since IFN- $\gamma$  can promote the FBXO34/hnRNP U axis, can it promote HIV-1 latency? To answer the above question, we treated Ya cell line that continuously expresses HIV-1 containing a GFP reporter gene with different interferon. It was found that the expression level of the GFP reporter gene in the HIV-1 genome in Ya cells was significantly decreased after IFN- $\gamma$  treatment, however, IFN- $\alpha$  and IFN- $\beta$  had no effect on the expression of HIV-1 in Ya cells (Figure 7(B)), suggesting that IFN- $\gamma$  may promote HIV-1 latency through the FBXO34/hnRNP U axis.

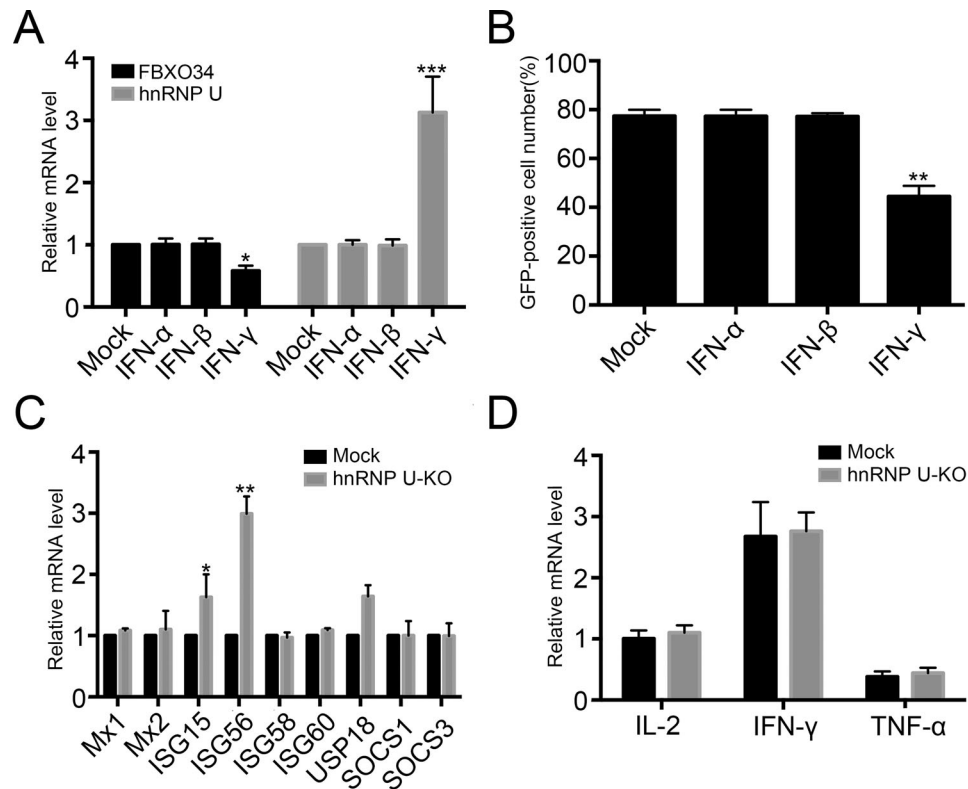
To explore whether the FBXO34/hnRNP U axis could regulate the expression of interferon-responsive genes (ISGs), we examined the expression of interferon-responsive genes and common cytokines, including IL-2, IFN- $\gamma$ , and TNF- $\alpha$  in C11 cells in hnRNP U knockout cells. We found that, except the mRNA levels of ISG56 were increased to a certain extent, the FBXO34/hnRNP U axis had no significant effect on the other ISGs tested (Figure 7(C)). In addition, we also found that knocking out hnRNP U has no significant effect on the transcription level of IL-2, IFN- $\gamma$ , and TNF- $\alpha$  (Figure 7(D)).

### **Summary and outlook**

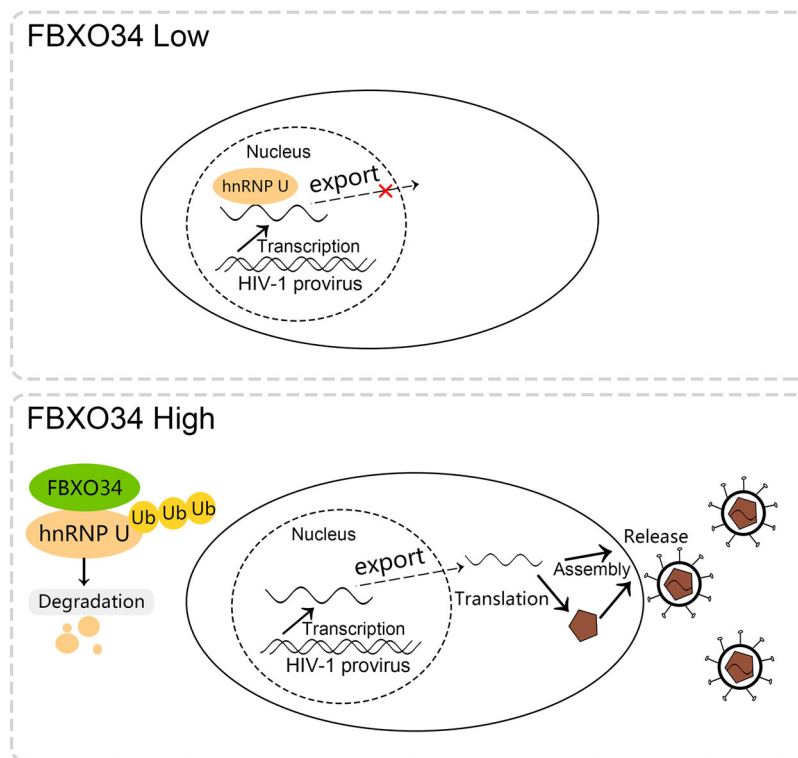
Although there have been many studies on the molecular mechanism of HIV-1 latency, latency activators developed based on the related mechanisms do not reduce the viral reservoirs in clinical treatment, which suggests that our understanding of the molecular mechanism of HIV-1 latency is not comprehensive [33–35]. Genome-wide gain-of-function screening based on a cDNA library is an efficient and rapid screening strategy for host factor related to HIV-1 latency [58]. However, due to the different lengths of cDNAs, there is a certain difference in plasmid size and transfection efficiency resulting in screening errors, which reduces the success rate of cDNA library-based gain-of-function screening [59,60]. The emergence of Cas9 technology, in particular the dCas9-sgRNA activation method system, makes it possible to screen the whole genome for differentiated screening [61]. Subsequently, the researchers used the genome-wide activated CRISPR library to screen a variety of restriction factors [62,63]. Therefore, using a CRISPR-activated library to screen HIV-1 latency-related host factors is a feasible strategy.

In this study, we used the CRISPR genome-wide activation library to screen host genes related to HIV-1 latency in an HIV-1 latent cell model. For the first time, we found that the FBXO34/hnRNP U axis is involved in HIV-1 latency. In the latent state, FBXO34 is at the low expression level, but when cells are stimulated to increase the expression of FBXO34, FBXO34 interacts with hnRNP U, which in turn leads to hnRNP U ubiquitination and degradation. In the presence of hnRNP U, hnRNP U interacts with HIV-1 mRNA, inhibits HIV-1 translation, and promotes HIV-1 latency at the post-transcriptional level (Figure 8). Therefore, in multiple latent infection cell lines and primary latent CD4<sup>+</sup> T lymphocyte models, overexpression of FBXO34 or knockout of hnRNP U gene causes activation of latent HIV-1 infection. In addition, we found that the FBXO34/hnRNP U axis acts downstream of IFN- $\gamma$ , thus playing the role of antiviral agent and promoting HIV-1 latency, which further shows the correlation between HIV-1 latency and innate immunity.

Prior to this study, researchers discovered that ubiquitination plays an important role in the establishment and maintenance of HIV-1 latency [64–66]. In 2017, Ali et al. found that USP7 stabilizes tat protein through deubiquitination, thereby promoting HIV-1 transcriptional activation [64]. In 2019, Pan et al. found that USP49 can stabilize the HIV-1 inhibitor APOBEC3G through deubiquitination, thereby promoting HIV-1 latency [65]. Recently, Liang et al. also found that UHRF1 mediates the ubiquitination of tat protein and targets it for degradation through the proteasome pathway, thereby promoting HIV-1



**Figure 7.** The FBXO34/hnRNP U axis is downstream of IFN- $\gamma$  and does not affect the expression of ISG in cells. (a), After treatment of IFN- $\alpha$ , IFN- $\beta$  and IFN- $\gamma$  in Jurkat CD4<sup>+</sup> T cells for 24 h, the mRNA expression levels of FBXO34 and hnRNP U were detected by qPCR. (b), The effect of interferon on HIV-1 were further verified in Ya cells which can continuously express HIV-1 and GFP reporter gene. The GFP expression in Ya cells was analyzed by flow cytometry. (c) ISGs expression were measured by qPCR in C11 and C11-hnRNP U-KO cells. (d), The mRNA level of IL-2, TNF- $\alpha$  and IFN- $\gamma$  were evaluated by qPCR in C11 and C11-hnRNP U-KO cells. Data information: Each data represented the mean  $\pm$  SD of three independent experiments ( $n = 3$ ) and were analyzed with *T*-test. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Figure 8.** A working model of the role of FBXO34/hnRNP U axis in the establishment of HIV latency. In latent state, hnRNP U interacts with HIV-1 mRNA and blocks HIV-1 mRNA translation. When FBXO34 up-regulation of expression, FBXO34 interacts with hnRNP U and promotes the ubiquitination of hnRNP U, which in turn leads to hnRNP U degradation. When hnRNP U is degraded, the translation of HIV-1 mRNA is enhanced, and HIV-1 turns from a latent state to an activated state.



latency [66]. However, the ubiquitination substrates found in the above studies are all proteins involved in HIV-1 latency and have been widely reported.

In this study, we report for the first time the physiological role and the substrate of FBXO34. This undoubtedly lays a preliminary foundation for a follow-up study of other functions of FBXO34. On the other hand, hnRNP U, the substrate of FBXO34, belongs to the hnRNP protein family, and several hnRNP family proteins have been reported to be involved in the transcription and replication of HIV-1 [67–69]. In 2006, Valente et al. reported that through cDNA library screening, fragments of hnRNP U were found to inhibit the transcription and translation of HIV-1 [70]. However, the upstream and downstream regions of hnRNP U were not clearly studied at that time. In contrast to Valente's article, we confirmed the effect of hnRNP U on HIV-1 latency in the latent infection T lymphocyte model and the primary latent cell model. In addition, we screened the upstream protein of hnRNP U through a CRISPR-activated library and biochemical experiments, which confirmed that hnRNP U interacts with HIV-1 mRNA through amino acids 1-339 to promote HIV-1 latency at the post-transcriptional level.

At the same time, we detected the expression of FBXO34/hnRNP U signalling axis between healthy control, latent HIV patients and acute HIV patients. However, due to the individual genetic variability and the complex molecular mechanisms that contribute to maintaining HIV latency, we noticed that some patients had significant changes in the expression level of FBXO34/hnRNP U-axis, in particular, two of the patients who received ART had significantly higher expression levels of hnRNP U than the others, which supports the results of our cell line and primary latent cell model. However, there are also some patients with no change in the FBXO34/hnRNP U axis, which further suggests the complexity of HIV-1 latency. Above result indicated that a cocktail of stimulatory compounds targeting distinct cellular and HIV gene regulatory pathways would be most effective to activate the latent reservoir in HIV-1-infected patients.

In summary, by high-throughput CRISPR/Cas9 activation library screening, we have determined that the FBXO34/hnRNP U axis is involved in the establishment and maintenance of HIV-1 latency in CD4<sup>+</sup> T cells at the post-transcriptional level. This novel FBXO34/hnRNP U axis in HIV activation may be directly targeted to control HIV reservoirs in patients in the future.

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HZ conceived and designed the experiments. XY carried out most experiments. XZ, YZ, JX, QW, HP, JY, JW, ZL, XS, YL, QL, JL and ML participated in some of the experiments. HZ

directed and supervised the experiments and interpretation of data. XY and HZ wrote the paper and approved the publication.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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