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PEBP1 suppresses HIV transcription and induces latency by inactivating MAPK/NF-κB signaling

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Abstract

The latent HIV-1 reservoir is a major barrier to viral eradication. However, our understanding of how HIV-1 establishes latency is incomplete. Here, by performing a genome-wide CRISPR-Cas9 knockout library screen, we identify phosphatidylethanolamine-binding protein 1 (PEBP1), also known as Raf kinase inhibitor protein (RKIP), as a novel gene inducing HIV latency. Depletion of PEBP1 leads to the reactivation of HIV-1 in multiple models of latency. Mechanistically, PEBP1 de-phosphorylates Raf1/ERK/IκB and IKK/IκB signaling pathways to sequestrate NF-κB in the cytoplasm, which transcriptionally inactivates HIV-1 to induce latency. Importantly, the induction of PEBP1 expression by the green tea compound epigallocatechin-3-gallate (EGCG) prevents latency reversal by inhibiting nuclear translocation of NF-κB, thereby suppressing HIV-1 transcription in primary CD4$^+$ T cells isolated from patients receiving antiretroviral therapy (ART). These results suggest a critical role for PEBP1 in the regulation of upstream NF-κB signaling pathways governing HIV transcription. Targeting of this pathway could be an option to control HIV reservoirs in patients in the future.

Keywords CRISPR-Cas9; genome-wide screening; HIV latency; NF-κB; PEBP1
Subject Categories Immunology; Microbiology, Virology & Host Pathogen Interaction; Signal Transduction
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Introduction

Current antiretroviral therapy (ART) has succeeded in reducing human immunodeficiency virus type 1 (HIV-1) to undetectable levels in HIV-1-infected patients (Ruelas & Greene, 2013; Margolis *et al*, 2016). However, ART alone cannot cure AIDS. The viral reservoirs comprised of latently infected and long-lived resting CD4$^+$ T cells can survive for decades, thereby preventing our current efforts to cure HIV (Finzi *et al*, 1997; ;Chun *et al*, 1998; Richman *et al*, 2009; ;Barouch & Deeks, 2014). These latent HIV reservoirs are considered as the main barrier for viral eradication (Ho *et al*, 2013; Kim *et al*, 2018; Pitman *et al*, 2018; Rojas *et al*, 2019). In the last few decades, progress has been made to elucidate the molecular mechanisms underlying the establishment of HIV-1 latency, mostly acting at the level of transcriptional suppression of the viral promoter-long terminal repeats (LTR) (Verdin *et al*, 1993; Bieniasz *et al*, 1999; Blazkova *et al*, 2009; Archin *et al*, 2014; Kumar *et al*, 2015; Elsheikh *et al*, 2019). It has been shown that transcriptional blocks to productive HIV-1 replication are associated with multiple layers of regulation, including epigenetic modifications at the HIV-1 LTR, inadequate availability of transcription factors at the HIV LTR, such as NF-κB, positive transcription elongation factor b (P-TEFb or CDK9/CycinT1), HIV-1 Tat, and among others (Mancebo *et al*, 1997; Bieniasz *et al*, 1999; Fiume *et al*, 2012). Many small molecule compounds to target these signaling pathways have been tested to directly reactivate latent HIV-1 (Alexaki *et al*, 2007; Imai *et al*, 2010; Beans *et al*, 2013; Li *et al*, 2013; Spivak *et al*, 2014; Søgaard *et al*, 2015; Boehm *et al*, 2017; Wang *et al*, 2017). Unfortunately, none of these latency reversal agents (LRAs) can effectively reduce the reservoir size in patients although latent HIV-1 can be disrupted *in vivo* (Archin *et al*, 2012; Søgaard *et al*, 2015). While an opposing intervention strategy has been proposed to deeply silence the HIV reservoirs (Mousseau *et al*, 2015; Elsheikh *et al*, 2019), an effective prevention of viral rebound has not yet been achieved in a clinical or pre-clinical setting (Kessing *et al*, 2017), indicating that our current understanding of how HIV-1 establishes and maintains its latency remains limited.

In recent years, the methodology behind genome editing has greatly expanded with the emergence of the CRISPR/Cas9 system. In 2014, Dr. Zhang's laboratory constructed a lentivirus library to

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target human genome using array libraries (Shalem *et al*, 2014). By using the CRISPR/Cas9 library, researchers successfully identified the host factors necessary for viral infection, such as flavivirus, Zika virus, and HIV-1, to invade host cells for their own replication (Ma *et al*, 2015; Marceau *et al*, 2016; Savidis *et al*, 2016; Park *et al*, 2017; Jin *et al*, 2018; Huang *et al*, 2019; Li *et al*, 2019), which greatly helped us understand the host–pathogen interaction during viral infection.

Here, we carried out a CRISPR-based genetic screen in a latently HIV-infected $CD4^+$ T-cell model of latency using a high complexity and whole genome-wide sgRNA library. Among the enriched genes, we identified that *PEBP1 or RKIP* is associated with the suppression of HIV replication and promotes the establishment of HIV latency. The knockout of *PEBP1* gene reactivated latent HIV-1 by inducing Raf1/ERK/IκB and IKK/IκB/NF-κB signaling pathways in several HIV latency models, including a primary $CD4^+$ T-cell model of HIV latency. Importantly, PEBP1 can directly inhibit HIV-1 infection and induce HIV latency in primary $CD4^+$ T cells. When PEBP1 was induced by a small molecule extracted from Chinese green tea, epigallocatechin-3-gallate (EGCG), the reactivation of HIV latency was effectively blocked in the primary $CD4^+$ T cells isolated from HIV-positive individuals receiving suppressive ART. To our knowledge, this is the first report to elucidate how upstream signaling of the NF-κB pathway is controlled by PEBP1 or RKIP during the establishment of HIV latency. Our study has discovered mechanistically novel insights of HIV latency. The EGCG compound identified in this study could be further investigated as a new tool for therapeutic intervention of HIV latency in the future.

Results

Genome-wide CRISPR/Cas9 library screening enriches host factors associated with the establishment of HIV latency

To identify host factors associated with HIV-1 latency, we conducted a GECKO library screen (Shalem *et al*, 2014) in the $CD4^+$ T model of HIV latency, C11, which was previously established in our laboratory (Qu *et al*, 2013; Wang *et al*, 2017). This GECKO library contains over 120,000 gRNAs targeting 19,050 human genes. The C11 cell line of HIV latency model is derived from $CD4^+$ T cells (Jurkat), which harbors an HIV-1 proviral DNA with a reporter gene encoding green fluorescent protein (GFP). In the latent state, HIV-1 expression in C11 cells is silenced with an expression level of GFP below 2% (Qu *et al*, 2013; Wang *et al*, 2017). We prepared the GECKO lentivirus library to infect the C11 HIV latency model with a multiplicity of infection (MOI) of 0.2. The cell line was screened with puromycin (2 μg/ml) selection for 14 days (Fig 1A). Then, roughly 10% of GFP-positive C11 cells were enriched after two rounds of cell sorting (Fig 1A and B). For the positive knockout cells, the targeted sgRNA sequence was confirmed by PCR. Our data showed that the integrated sgRNA was found in both the unselected group and the positive cells sorted in the first or second round of screening (Fig 1C). Under fluorescence microscopy, we confirmed that the sorted C11 cells were GFP positive after gene knockout (Fig 1D). Following genomic DNA (gDNA) extraction from both sorted and unsorted control cells and PCR amplification of each sgRNA sequence, we performed Illumina sequencing to generate read counts for each gene-targeting GECKO construct. The gene of interest was compared with the distribution of the $\log_2$ enrichment values of the negative control sgRNAs and the initial control sgRNAs. We found that several genes such as *UBB*, *SERBP1*, *ZDHHC1,CNTNAP1,* and *PEBP1* (Beshir *et al*, 2010; Lin *et al*, 2010; Oh *et al*, 2013; Laquerriere *et al*, 2014; Bolger, 2017) are among the hotspot candidate genes with high abundance of sgRNAs, along with *BRD2* and *BRD4* genes which are known as HIV latency-associated genes (Boehm *et al*, 2013) (Fig 1E and Dataset EV1).

Validation of candidate HIV latency inducing genes

To validate whether these candidate genes are related to HIV-1 transcription, we directly infected HIV latency model of C11 cells with CRISPR/Cas9 lentiviruses containing sgRNAs which target these candidate genes, followed by clone selection with puromycin (2 μg/ml) for 14 days. Among these top-hit genes, we found that the knockout of *PEBP1* gene significantly induced the reactivation of latent HIV-1 (roughly 20%), which was higher than the cells with the known HIV latency-related gene *BRD2* or *BRD4* knockout (Fig 2A), supporting our idea that *PEBP1* gene is associated with HIV latency. A similar effect was observed after *PEBP1* was knocked out in two other HIV latency models, J-Lat 10.6, and ACH2 cells (Fig 2B and C). To prove that *PEBP1* gene was indeed knocked out in these latency disrupted C11 cells, we sequenced the genomic targeting sites in these *PEBP1* knockout cell clones by genomic DNA sequencing. We found that *PEBP1* gene was deleted in the target sites of *PEBP1* sgRNA1 with different forms of indels (Fig 2D). The PEBP1 protein was nearly undetectable after the knockout of *PEBP1* gene targeted with *PEBP1*-specific guide RNAs (Fig 2E). In order to further determine the effect of PEBP1 on HIV-1 latency, a random PEBP1 monoclonal cell line was obtained by flow sorting (Streaming data is not shown). We sequenced the genomic target sites of five different monoclonal cell lines by genomic DNA sequencing and found that *PEBP1* gene was successfully knocked out in all five monoclonal cell lines (Fig EV1A). With Western blot, we confirmed that PEBP1 protein expression significantly decreased (Fig EV1B). The levels of HIV-1 transcription were similar among these monoclonal cell lines and cells without clone screening after gene knockout (Figs 2A and EV1C). Cell proliferation and apoptosis were also evaluated in PEBP1-KO-C11 cells and mock C11 cells by CCK8 assay and TUNEL staining. We found the proliferation rate was slightly higher in PEBP1-KO-C11 cells than that of mock knockout C11; however, *PEBP1* knockout did not affect apoptosis (Fig EV2A and B). Taken together, our data suggest that *PEBP1* is a new HIV latency-associated gene.

The PEBP1/Raf1 protein complex promotes HIV-1 latency through inactivation of MAPK and NF-κB signaling pathways in $CD4^+$ T cells

Previous studies indicated that PEBP1, also known as Raf1 kinase inhibitor protein RKIP, is involved in MAPK and NF-κB signaling pathways via interaction with Raf1 and IKK in cancer cells (Yeung *et al*, 2001; Lee *et al*, 2006; Tavel *et al*, 2012; Wei *et al*, 2015). When PEBP1 is defective, the MAPK and NF-κB signaling pathways are activated, leading to the translocation of NF-κB from the cytoplasm to the nucleus (Lin *et al*, 2010). It has been demonstrated that

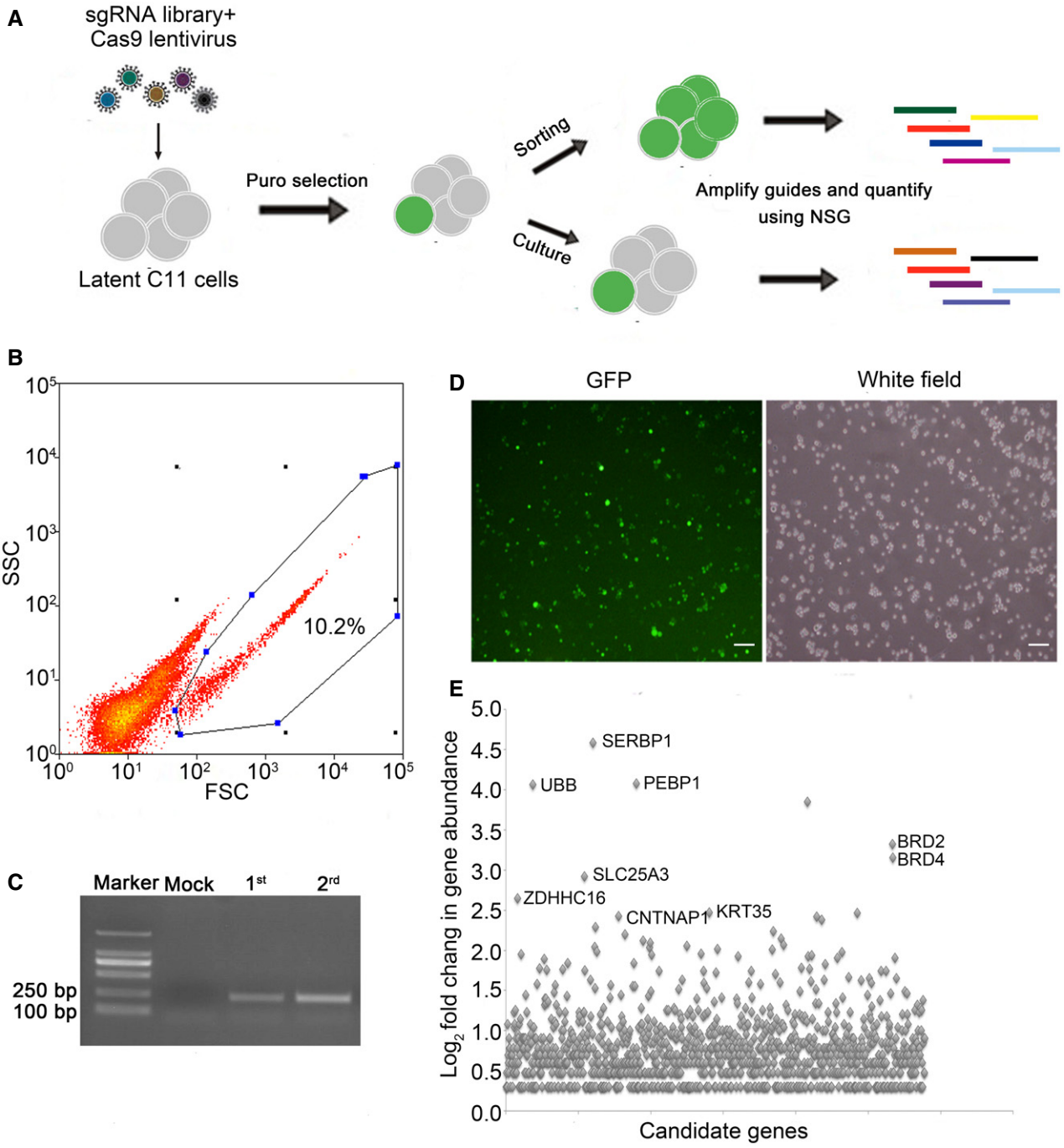

Figure 1. A pooled, genome-wide CRISPR screen for candidate genes involved in HIV-1 latency.

A The outline of the genome-wide CRISPR screen strategy. C11 cells were infected with a lentiviral library containing Cas9 proteins and sgRNAs that target 19,050 human genes. After fourteen days of puromycin selection, genomic DNA was extracted from GFP-positive cells after two rounds of sorting. The candidate genes were then identified by next generation sequencing.

B Flow cytometry of cells infected with the lentiCRISPR v2.0 library where the expression of GFP indicates latency reactivation. Target genes were enriched through two-round sorting. Continuously cultured C11 latent cells infected with the lentiCRISPRv2.0 served as control. Blue dots and the encircled area represent the GFP-positive cells for flow cytometry analysis.

C Validation of sgRNAs in unsorted and sorted C11 cells by PCR after lentiCRISPR v2.0 library infection.

D Before next generation sequencing, the GFP expression in two-round sorted C11 cells was confirmed by fluorescence microscopy. Scale bar, 100 μm.

E Fold change (Log_2) of the abundance of target genes in sorted C11 cells. Enriched sgRNA genes are highlighted.

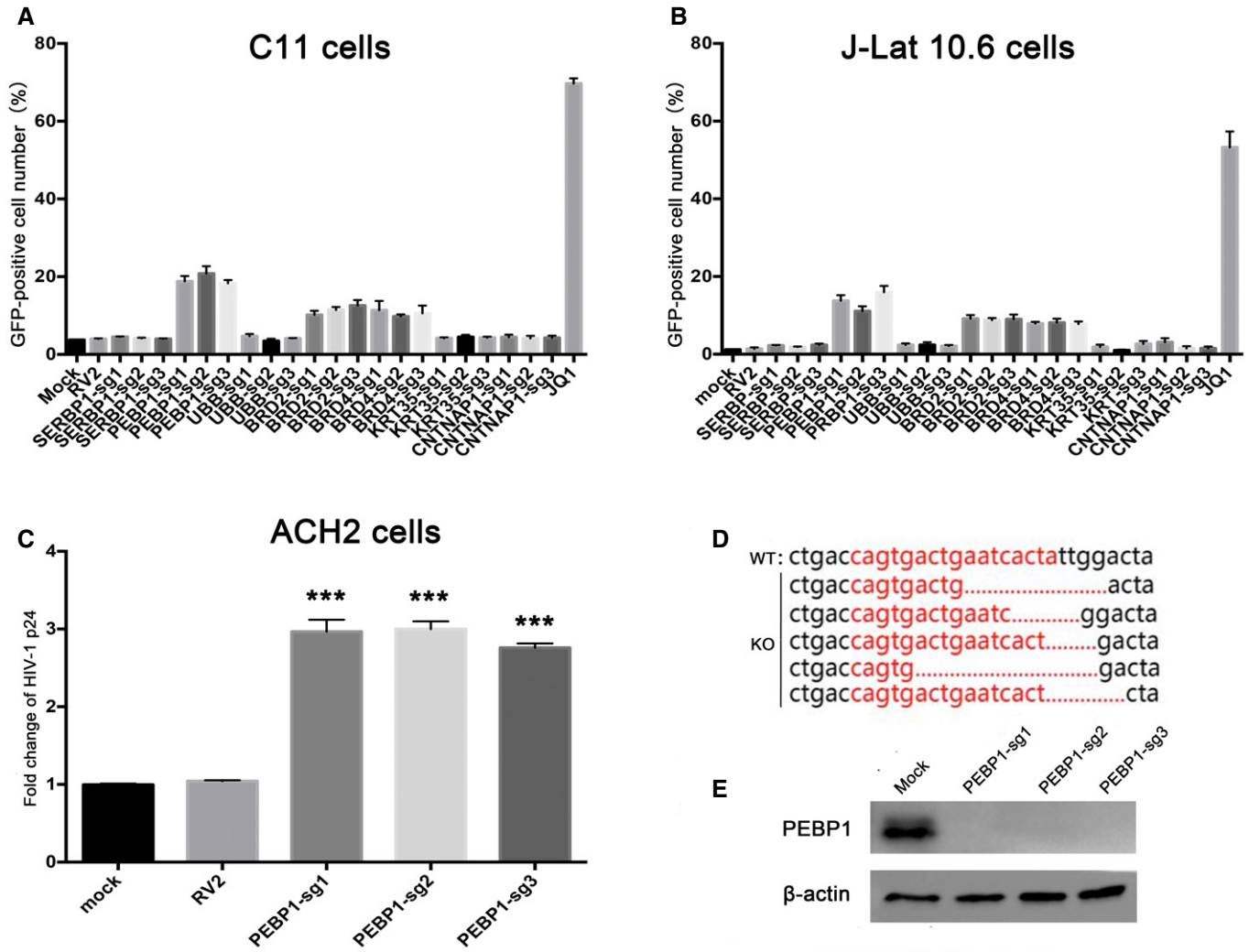

Figure 2. Validation of the candidate genes screened from the lentiCRISPR v2.0 library in HIV-1 latently infected cell lines.

A Validation of the top candidate genes in the C11 cell line. C11 cells were infected by lentiCRISPR v2.0 packaged lentiviruses with sgRNA following by screening for 14 days with 2 μg/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 candidate genes on HIV latency was further verified in J-Lat 10.6 (B) and ACH2 (C) models of HIV latency. GFP expression in J-Lat 10.6 cells and p24 in ACH2 cells was analyzed by flow cytometry and ELISA, respectively.

D *PEBP1* was deleted after CRISPR/Cas9 knockout. PCR products related to PEBP1 from control or PEBP1 knockout cells were cloned and then sequenced. PEBP1-sg1 target sites are shown in red letters. Dashes indicate the deleted bases relative to the wild-type PEBP1 gene sequence.

E PEBP1 protein levels were measured by Western blot after knock out in C11 cells by LvPEBP1-sg1. Mock C11 cells served as control.

Data information: Data represent the mean ± SD of three independent experiments ($n = 3$) and were analyzed with t-test. ***$P < 0.001$.
Source data are available online for this figure.

inactivation of NF-κB signaling is essential for the establishment of HIV latency while activation of NF-κB signaling pathway disrupts latent HIV-1 (Fiume *et al*, 2012). However, it is not clear how cytoplasmic sequestration of NF-κB is regulated during the establishment of HIV latency in $CD4^+$ T cells. We hypothesized that *PEBP1* gene is essential for the upstream signaling of NF-κB by preventing its translocation into the nucleus, therefore turning off the transcription of HIV for transcriptional silence. We found that both the expression of PEBP1 mRNA and protein in the C11 HIV latent cell line were significantly higher than its parental Jurkat cell line (Fig 3A). To see whether the expression of PEBP1 causes silencing of HIV by inactivating NF-κB signaling pathway and whether this is through its interacting with Raf1 and IKK, we performed a co-immunoprecipitation (Co-IP) assay and revealed that PEBP1 not only interacted with Raf1 but also with IKK in C11 HIV latency cell model (Fig 3B). After *PEBP1* was knocked out, the expression levels of MEK1/2, RSK, ERK1/2, IKKβ, and IKBα was marginally changed; however, their phosphorylation levels increased significantly, indicating that the Raf1/ERK/IκB and IKK/IκB/NF-κB signaling pathways were indeed activated when *PEBP1* gene was knocked out (Fig 3C). The level of NF-κB/p65 significantly increased in the nucleus when *PEBP1* gene was knocked out (Fig 3D), leading to a

significant binding of NF-κB/p65 to HIV-1 LTR in C11-PEBP1-KO cells but not in the control C11 cells (Fig 3E). In addition, more NF-κB/p65 entered the nucleus after *PEBP1* was knocked out in monoclonal knockout cell lines (Fig EV1D).

To see whether PEBP1 regulates HIV transcription through its interaction with NF-κB signaling, 293T cells were transiently transfected with wild-type or mutated LTR-driven luciferase reporter plasmids with deletion mutations in YY1-binding site, Sp1-binding site, Ap1-binding site, or NF-κB-binding site at the HIV LTR (Wang *et al*, 2005; Gary *et al*, 2008), after *PEBP* gene knockout. We found that there were significant differences in luciferase expression among cells transfected with all other deletion mutations except cells transfected with NF-κB-binding site-deleted HIV LTR-driven luciferase reporter plasmids when PEBP1 was knocked out (Fig 3F). When NF-κB nuclear entry was pharmacologically inhibited by NF-κB inhibitor SC75741, the GFP expression level of PEBP1-KO-C11 cells decreased in a dose or time-dependent manner (Fig EV2C and D). These observations further supported our hypothesis that the regulation of PEBP1 in HIV transcription is directly through NF-κB signaling.

The induction of PEBP1 inhibits HIV-1 replication during viral infection

Next, we wanted to determine whether PEBP1 can directly inhibit the viral transcription in the early stage of infection. To test this

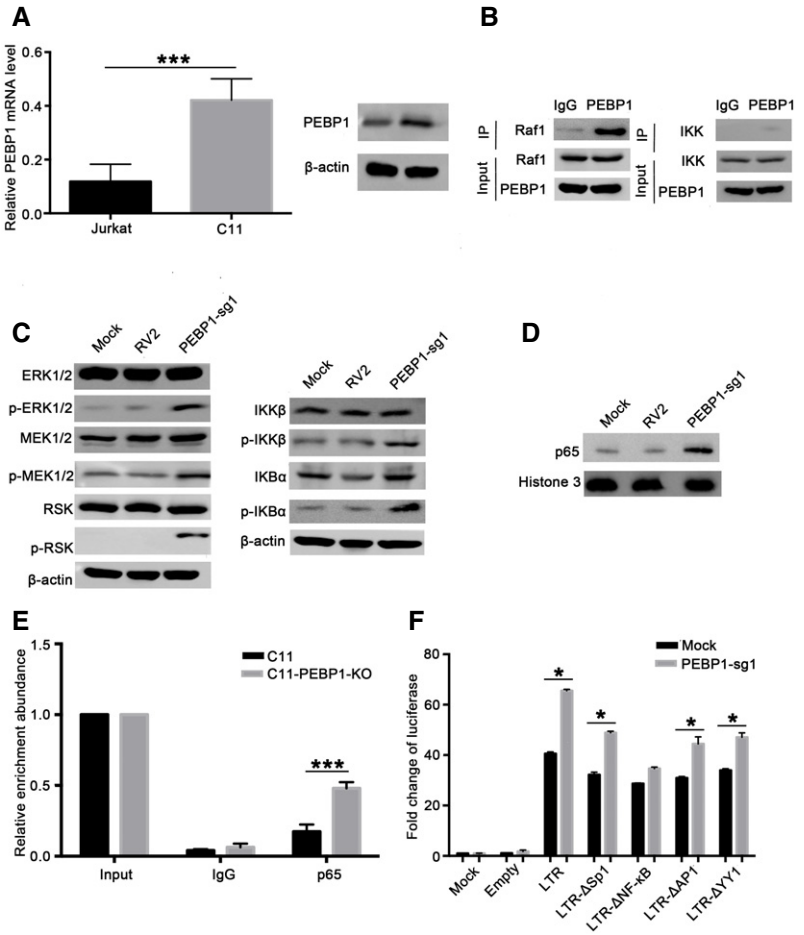

Figure 3. PEBP1 inhibits HIV-1 reactivation by inactivating Raf1/IKK/NF-κB signaling pathways.

A PEBP1 expression was measured by qPCR (left panel) and Western blot (right panel) in the latently infected C11 cells and the parental Jurkat cells.

B The immunoprecipitation assay was performed in C11 cell lysates with anti-PEBP1 antibody followed by Western blot with anti-Raf1 or anti-IKK antibodies.

C, D Effect of PEBP1 on the Raf1/ERK/IκB and IKK/IκB/NF-κB signaling pathways. The levels of indicated proteins in total protein lysates (C) or the levels of NF-κB/p65 protein in nucleus (D) were analyzed by Western blot in C11-PEBP1-KO cells and mock C11 cells.

E NF-κB/p65 protein recruitment into HIV LTR was analyzed by ChIP-qPCR with specific primers targeting the HIV LTR after normalization to the input.

F The impact of PEBP1 on the activation of the HIV LTR was explored by luciferase reporter assay in 293T cells. 293T cells were co-transfected with PEBP1-sg1 alone or PEBP1-sg1 with HIV-1 LTR-empty plasmids, HIV-1 wild-type LTR-luciferase plasmids, HIV-1 LTR-Δsp1-luciferase, HIV-1 LTR-ΔNF-κB-luciferase, HIV-1 LTR-ΔAp1-luciferase, or HIV-1 LTR-ΔYY1-luciferase. Transcription of HIV-1 was determined by luciferase reporter assay.

Data information: Data are normalized to the HIV LTR-empty plasmid transfection group. Data represent the mean ± SD of three independent experiments ($n = 3$) and were analyzed with *t*-test. *$P < 0.05$, ***$P < 0.001$.
Source data are available online for this figure.

idea, PEBP1 was overexpressed in TZM-bl cell line (Fig 4A). Then, the PEBP1 overexpressing TZM-bl cells were infected with HIV-1 in serum supernatants isolated from patient peripheral blood. Patient serum supernatants were collected by low-speed centrifugation from blood containing the virus with a viral load of 129 copies/ml. Both TZM-bl cells and supernatants were harvested 72 h post-infection where HIV-1 transcription was measured by luciferase activity and p24 expression, respectively. Our data showed that the overexpression of PEBP1 significantly inhibited the transcription and replication of HIV-1 (Fig 4B and C). It has been reported that EGCG and Dihydroartemisinin (DHA) are PEBP1 inducers (Kim & Kim, 2013; Hu *et al*, 2014). In HIV latency model of C11 cells, both EGCG and DHA effectively induced PEBP1 protein expression (Fig 4D). Importantly, EGCG or DHA can also induce PEBP1 in primary $CD4^+$ T cells (Fig 4E), which significantly reduced the level of p24 in the culture supernatants compared with mock cells (Fig 4F). These data indicate that the induction of PEBP1 expression directly restricts HIV-1 transcription during the early stage of viral infection.

PEBP1 prevents HIV latency reversal in primary $CD4^+$ T cells isolated from HIV-positive individuals receiving ART

When tested in primary $CD4^+$ T cells purified from PBMCs from patients under ART (Shan *et al*, 2013), we found that EGCG, the PEBP1 agonist, significantly prevented the reactivation of latent HIV under α-CD3/CD28 stimulation in four out of five patients examined (Fig 5A; Table EV1). In addition, the gene expression of PEBP1 from reactivation-restricted samples was significantly induced (Fig 5B). As an example, EGCG significantly induced protein expression of PEBP1 in cells from patient 1 compared with control treatment (Fig 5C), along with a reduction of nuclear protein NF-κB/p65 induced by α-CD3/CD28 stimulation (Fig 5D). Taken together, these data further validate that PEBP1 suppresses HIV transcription and prevents the reactivation of latent HIV by inactivating the NF-κB signaling pathway *ex vivo*.

PEBP1 induces HIV latency in primary $CD4^+$ T cells

The data above supports our hypothesis that *PEBP1* is a latency inducing gene. To directly test whether PEBP1 promotes HIV-1 latency in the primary human $CD4^+$ T lymphocyte, we established a primary HIV-1 latent infection model as described before (Bosque & Planelles, 2011; Kim *et al*, 2014; Pandeló José *et al*, 2014) (Fig 6A). Nanoluc-luciferase expression was detected 2 days post-HIV-1 infection to indicate active HIV replication. Twelve days post-gradual decreasing of IL-2, a significant reduction of nanoluc-luciferase was observed (Fig 6B), indicating that HIV-1 was in a latent state. Importantly, *PEBP1* gene expression decreased during early active viral infection (day 3 post-infection). However, during the latency stage induced by a continuous decrease of IL-2 concentration, PEBP1 gene expression significantly increased until latency was established at day 12 post-infection (Fig 6C). These data indicates that *PEBP1* gene expression is positively related to latency induction in primary $CD4^+$ T-cell model of latency.

It has been shown that many factors involved with the restriction of HIV-1 transcription are related to interferon signaling (Hotter *et al*, 2019; Liu *et al*, 2019). We hypothesize that PEBP1 induction is related to interferon signaling during HIV-1 infection of primary T lymphocytes. We found that both mRNA and protein levels of PEBP1 were increased significantly after treatment of Jurkat $CD4^+$ T cells with IFN-β, but not IFN-α or IFN-γ, for 48 h (Fig 6D and E). These observations indicate HIV-1 infection-triggered innate immunity in T lymphocytes may promote the expression of PEBP1 through IFN-β. Enhanced PEBP1 expression inhibits HIV-1 transcription and promotes HIV latency. In contrast, when *PEBP1* was

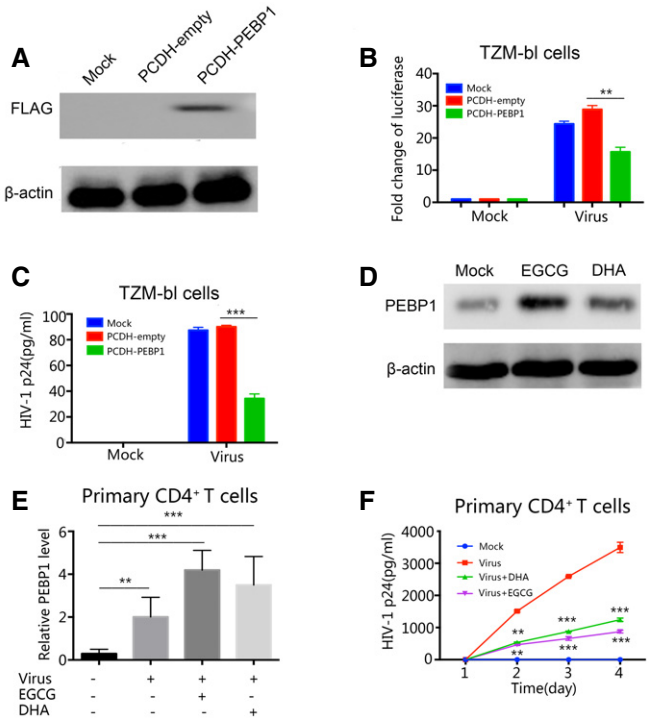

Figure 4. PEBP1 restricts HIV-1 transcription to induce HIV latency.

A The expression of PEBP1 was analyzed by Western blot in TZM-bl cells infected with mock, Lv-PCDH-empty, or Lv-PCDH-PEBP1 plasmids.

B, C Overexpression of PEBP1 suppressed HIV-1 replication. TZM-bl cells were transfected with Lv-PCDH-empty or Lv-PCDH-PEBP1 followed by infection of HIV derived from patient plasma whose viral load is 129 copies/ml. The transcription of HIV-1 was evaluated 72 h post-infection by luciferase activity (B) and levels of p24 in the supernatants (C).

D The expression level of PEBP1 was detected by Western blot using the whole cell lysate of C11 cells treated with 10 μM EGCG or DHA for 24 h.

E Induction of PEBP1 by EGCG or DHA in HIV-1 infected primary $CD4^+$ T cells. Primary $CD4^+$ T cells from healthy donors were treated with 10 μM EGCG or DHA during HIV-1 infection. Similar to Panel B, HIV-1 was isolated from the blood supernatants of patients receiving ART with a viral load of 129 copies/ml. Seventy-two hours post-treatment with 10 μM EGCG or DHA, the expression of PEBP1 was detected by qPCR.

F The induction of PEBP1 by EGCG or DHA suppressed HIV replication in the primary $CD4^+$ T cells. The primary $CD4^+$ T cells from healthy donors were treated with 10 μM EGCG or DHA during HIV-1 infection. Similar to Panel B and E, HIV-1 was isolated from the blood supernatants of patients receiving ART with a viral load of 129 copies/ml. The supernatants from HIV-1-infected $CD4^+$ T cell were collected 1, 2, 3 or 4 days post-infection. Replication of HIV-1 was analyzed by p24 ELISA.

Data information: Data represent the mean ± SD of three independent experiments ($n = 3$) and were analyzed with *t*-test and compared with TZM-bl cells infected with Lv-PCDH-empty vector or mock. $^{**}P < 0.01$; $^{***}P < 0.001$. Source data are available online for this figure.

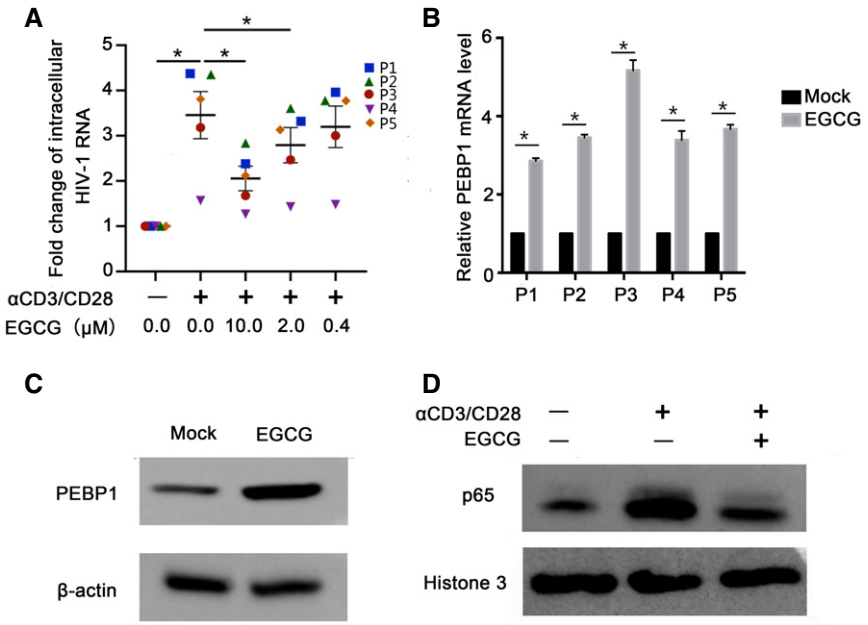

Figure 5. PEBP1 induction by EGCG inhibits the reactivation of latent HIV in primary CD4$^+$ T cells isolated from patients receiving ART.

A EGCG inhibited α-CD3/CD28 reactivation of latent HIV-1. Primary CD4$^+$ T cells were isolated from HIV-1-positive patients on antiretroviral therapy (n = 5, P1–P5). Primary CD4$^+$ T cells were treated with α-CD3/CD28 alone or α-CD3/CD28 and 10 μM EGCG. Cell-associated RNA was extracted 7 days post-treatment. The transcription of HIV-1 was determined by real-time qPCR.

B PEBP1 mRNA was induced by EGCG in patient primary CD4$^+$ T cells. Primary CD4$^+$ T cells were treated with 10 μM EGCG. The expression of PEBP1 in the cells was measured by qPCR.

C PEBP1 protein was induced by EGCG in patient primary CD4$^+$ T cells, which was determined by Western blot of whole cell lysate from primary CD4$^+$ T cells.

D EGCG-induced PEBP1 suppressed nuclear entry of NF-κB/p65. This was measured by Western blot of nuclear protein of primary CD4$^+$ T cells treated with α-CD3/CD28 or α-CD3/CD28 plus 10 μM EGCG.

Data information: Data represent the mean ± SD of three independent experiments (n = 3) and were analyzed with t-test. *P < 0.05; **P < 0.01, compared with mock treatment.
Source data are available online for this figure.

knocked out, the expression of HIV-1 was increased (Fig 6F and G), further supporting our hypothesis that PEBP1 is involved in HIV latency.

We want to know whether the activation of MAPK and IKK signaling pathways was different in CD4$^+$ T cells with or without infection. While both of the pathways were highly active in HIV-1 replicating YA CD4$^+$ T cells, MAPK or IKK signaling was mostly inactive in the HIV latently infected C11 cell line (Fig EV3A and B). Interestingly, the nuclear level of p65 in HIV-1 latently infected C11 cells was slightly lower than Jurkat cells but significantly lower than YA CD4$^+$ T cells where HIV-1 was highly replicated (Fig EV3C). Considering PEBP1 was highly expressed in C11 latent cells (Fig 3A) and knockout of *PEBP1* enhanced the activation of MAPK and IKK signaling pathways by phosphorylation of ERK1/2, MEK1/2, RSK, IKKβ, and IκBα (Fig 3C), these data indicate that PEBP1 inhibits the activation of MAPK and IKK signaling pathways and prevents NF-κB from entering the nucleus to induce HIV latency in CD4$^+$ T cells.

Discussion

In this study, we conducted a CRISPR genome-wide knockout library screen in an HIV latently infected cell line model. We discovered a previously unrecognized PEBP1/IKK/IκB/NF-κB signaling and PEBP1/Raf1/ERK/IκB signaling in the suppression of HIV transcription to induce the establishment of HIV latency. PEBP1 prevents NF-κB from translocation into the nucleus to induce HIV latency in resting CD4$^+$ T cells by directly regulating the upstream signaling of NF-κB. Accordingly, we proposed a working model that PEBP1 restricts HIV-1 transcription and is associated with HIV-1 latency (Fig 7). In the transcription initiation stage, protein level of PEBP1 is low; therefore, PEBP1 is relieved from its inhibitory function of Raf1 or IKK, leading to the phosphorylation of IKKβ and IκBα, the degradation of IKBα, and the translocation of NF-κB into the nucleus followed by its recruitment into the HIV LTR to activate HIV transcriptional machinery. On the contrary, when HIV-1 enters the latency state, PEBP1 protein is induced. A high level of PEBP1 protein interacts with both Raf1 and IKK to inhibit the NF-κB signaling cascade, resulting in the sequestration of p65/p50 heterodimer in the cytoplasm, thereby silencing HIV-1 transcription. It has been reported that PEBP1 forms a protein complex with Raf1 or IKKβ (Yeung *et al*, 2001; Granovsky *et al*, 2009). Our data also supported that PEBP1 formed a protein complex with Raf1 and/or IKK to suppress NF-κB signaling and induce HIV latency in CD4$^+$ T cells. However, it is not clear how the Raf1 or IKK phosphorylation signaling is blocked. It could be that within a protein complex with

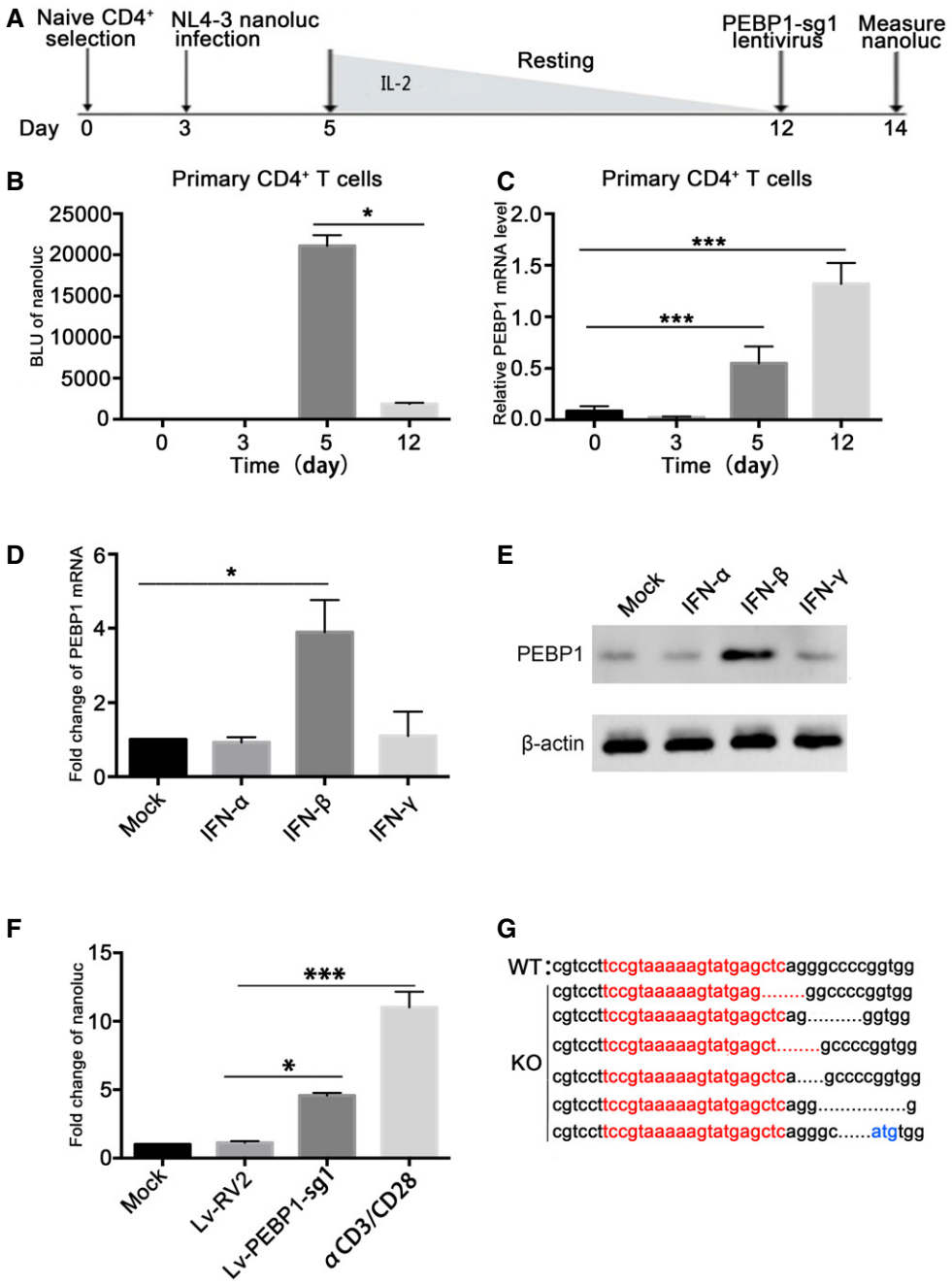

Figure 6. PEBP1 reactivates latent HIV-1 in the primary CD4⁺ T model of latency.

A Outline of latency establishment in the primary CD4⁺ T cells. Human primary CD4⁺ T cells were activated and expanded with α-CD3/CD28 beads at day 1. The α-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 infected with CRISPR/Cas9 and PEBP1-sgRNA lentivirus.

B Transcription of HIV-1 in the primary CD4⁺ T cells was determined by nanoluc-luciferase assays during HIV-1 infection.

C After infection with VSVG pseudotyped HIV-1 NL4.3-nanoluc, the mRNA expression of PEBP1 was measured by qPCR.

D, E Jurkat CD4⁺ T cells were treated with IFN-α, IFN-β, and IFN-γ for 24 h. The mRNA (A) or protein (B) expression levels of PEBP1 were measured by qPCR or Western blot.

F PEBP1 knockout enhanced HIV-1 transcription in the primary CD4⁺ T-cell model of latency. The expression of HIV-1 was measured by nanoluc after gene knockout where α-CD3/CD28 stimulation served as a positive control.

G PEBP1 gene deletion after Lv-PEBP1-sg1 knockout in the primary CD4⁺ T cells. The PCR products of PEBP1 were cloned and then sequenced. PEBP1-sg1 target gene sequences are shown in red letters. Dashes indicate deleted bases relative to the wild-type sequence.

Data information: Data represent the mean ± SD of three independent experiments ($n = 3$) and were analyzed with t-test. *$P < 0.05$; ***$P < 0.001$.
Source data are available online for this figure.

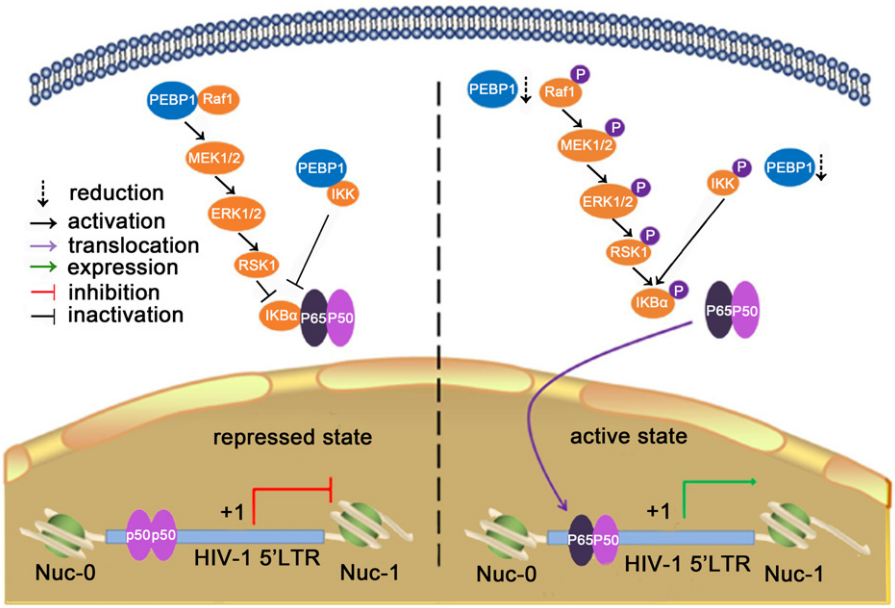

Figure 7. A working model of the role of PEBP1 in the establishment of HIV latency.

PEBP1 interacts and inhibits the activity of Raf1 and IKK kinases. This blocks the phosphorylation of downstream proteins ERK1, IKK, and IκBα, resulting in the sequestration of p65/p50 heterodimer in the cytoplasm, thereby silencing HIV-1 transcription. When PEBP1 is knocked out, Raf1 and IKK signaling pathways are activated to phosphorylate IκBα, thereby releasing p65/p50 heterodimer to translocate into the nucleus to drive HIV-1 transcription.

PEBP1, the kinase enzyme sites in these two proteins are blocked; therefore, the sites are inaccessible by their downstream target proteins. Importantly, we found that PEBP1 protein level can be induced in the primary $CD4^+$ T cells by pharmacologic small molecule EGCG, a compound discovered in Chinese green tea, and DHA. The induction of PEBP1 indeed prevents the reactivation of latent HIV-1 in resting $CD4^+$ T cells isolated from ART-suppressed patients. It has been reported that PEBP1 is also essential for type I interferon production in its anti-viral innate immunity (Gu *et al*, 2016). Our data are consistent with these observations and showed that IFN-β innate immune response may induce PEBP1 early in HIV-1 infection. Our study uncovered a previously unrecognized novel molecular mechanism of how the upstream signaling of canonical NF-κB pathway is finely regulated by PEBP1/Raf1/IKK signaling during the establishment of HIV latency. Our findings fill the knowledge gap regarding how NF-κB is sequestered into cytoplasm to inactivate initiation of HIV-1 transcription during the establishment of HIV latency in resting $CD4^+$ T cells, which has not been understood for many years. Interestingly, it has been reported that PEBP1 interacts with Nef by a yeast two-hybrid screen assay (Kammula *et al*, 2012). Whether HIV-1 Nef directly impacts PEBP1/Raf1 signaling for its own replication and whether PEBP1 affects other steps of HIV replication warrants further investigation in the future.

ShRNA libraries have been previously used to screen host genes related to HIV latency. Unfortunately, among the genes with the most significant changes in abundance, no HIV latency-related genes were discovered (Besnard *et al*, 2016). Compared with shRNA knockout libraries, CRISPR technology can completely disrupt gene expression while shRNAs can only temporarily reduce gene expression levels. Therefore, CRISPR technology was proposed as a better alternative for the identification of genes that play a functional role in a low abundance (Shalem *et al*, 2014). With this technology, tyrosylprotein sulfotransferase 2 (*TPST2*), solute carrier family 35 member B2 (*SLC35B2*) and activated leukocyte cell adhesion molecule (*ALCAM*) were identified as genes related to HIV-1 entry into $CD4^+$ T cells (Park *et al*, 2017). Similarly, histone demethylase *MINA53* and proteasome signaling pathways were recently reported as potential novel HIV-1 latency-promoting genes (Huang *et al*, 2019; Li *et al*, 2019). While it is not known whether these newly discovered HIV latency-related genes have any functional links with each other, these studies demonstrate that CRISPR/Cas9 library screening is a powerful tool for HIV latency study.

In summary, by high-throughput CRIPR/Cas9 library screening, we have discovered that PEBP1 suppresses HIV-1 transcription and is essential for the establishment of HIV latency in $CD4^+$ T cells by regulating upstream signaling of the NF-κB pathway. NF-κB signaling is among one of the most important targets currently investigated in the development of HIV cure strategies. The finding that the induction of PEBP1 by EGCG or DHA inhibits NF-κB function to suppress latency reversal indicates that upstream signaling of NF-κB pathways could be exploited to enforce HIV latency for a functional HIV cure.

Materials and Methods

HIV-1 latent cell lines

The HIV-1 latent infection model C11 cell line (constructed in our laboratory) (Qu *et al*, 2013; Wang *et al*, 2017) and J-Lat 10.6 cell

line (obtained from NIH AIDS Reagent Program; Jordan *et al*, 2001, 2003) contain a single integrated latent HIV-GFP reporter genome. ACH2 is a clone of HIV-1 latently infected $CD4^+$ CEM cells that contains a single copy of proviral DNA per cell (obtained from NIH AIDS Reagent Program). HeLa-based 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 (Qu *et al*, 2013) 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_2. TZM-bl and 293T cells were cultured in DMEM (Gibco, C11995500BT) and supplemented with 10% fetal calf serum (Lonsera, S711-001S), and 1% penicillin/streptomycin (Gibco) in a 37 °C incubator containing 5% CO_2.

Antibodies and reagents

The following antibodies were used throughout this study: Anti-PEBP1 (ab76582) and anti-Histone 3 (ab1791), anti-NF-κB p65 (ab16502), and anti-IKK (ab178870) were purchased from Abcam (Cambridge, UK). Anti-IKKβ (8943), anti-Phospho-IKKβ (Ser176/180) (14938), anti-IκBα (9242), anti-Phospho-IκBα (Ser32) (2859), anti-RSK (9533), anti-Phospho-RSK (Thr359/Ser363) (9341), anti-MEK (9126), anti-Phospho-MEK1/2 (Ser217/221) (9154), anti-ERK1/2 (4695), anti-Phospho-ERK1/2 (Thr202/Tyr204) (9101), and anti-β-Actin (4970) were purchased from Cell Signaling Technology (MA, US). 2 × Taq Master Mix (P112) and 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). FastFire qPCR PreMix (SYBR Green) (FP208), Cell Genome Extraction Kit (DP304), and Plasmid Extraction Kit (DP103, DP108, DP117) were purchased from Tiangen (Beijing, China). Gel Extraction Kit (CW2302) was purchased from CWBIO (Nanjing, China). Luciferase and nanoluc detection kit (E6110, N1110) was purchased from Promega (Madison, USA). Recombinant human IFN-alpha (11200-1), recombinant human IFN-beta (8499-IF), and recombinant human IFN-gamma (285-IF) were purchased from R&D Systems (UN). Cell Counting Kit-8 (CCK-8) and TUNEL Apoptosis Detection Kit (FITC) were purchased from Yeasen Biotechnology Co., Ltd (Shanghai, China). NF-κB inhibitor SC74751 (HY-10496) was purchased from MCE (NJ, USA).

Pooled genome-wide CRISPR screen

The lentivirus library was produced by co-transfection of GECKO library plasmid, Δ8.91 and VSVG plasmid into HEK293T cells at a 1000-fold concentration to increase the viral titer. A total of 1×10^7 C11 cells were infected at a low multiplicity of infection (MOI = 0.2), to ensure that most cells received only one viral construct. After 72 h, C11 cells were selected with 2 μg/ml puromycin for 14 days. After that, 10^8 puromycin-resistant C11 cells were sorting 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 $\log_2$ fold change in abundance of each sgRNA was calculated for the sorted and unsorted final population samples.

Vector construction

Individual sgRNA constructs targeting SERBP1, PEBP1, UBB, BRD2, BRD4, KRT35, and CNTNAP1 were cloned into lentiCRISPR v2.0 (addgene 52961). For all other experiments, PEBP1-sg1 was used.

For cDNA expression vectors, a linearized lentiviral backbone was generated from PCDH (YouBio, Hunan, China). Protein-coding plasmids were gifts from Professor Han Jiahuai laboratory. All the constructed plasmids were confirmed by restriction enzyme digestion and DNA sequencing.

Cas9-mediated gene knockout and cDNA overexpression

C11, J-Lat 10.6, ACH2, and TZM-bl cells were infected with lentivirus at an MOI of 1 and then selected with 2 μg/ml puromycin for 14 days. The knockout efficiency was analyzed using Sanger DNA sequencing. Single knockout cells were sorted by flow cytometry, and knockout efficiency was 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

TZM-bl and ACH2 cells were each seeded at a density of 1×10^6 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).

ChIP experiments

ChIP experiments were performed according to protocol provided by EZ-ChIP chromatin immunoprecipitation kit (Millipore). Briefly, C11 and C11-PEBP1-KO cells were cross-linked with 1% formaldehyde for 10 min at room temperature and quenched with 0.125 M glycine for 5 min. After lysis, nuclear extracts were separated and chromatin was sheared by sonicator (Bioruptor UCD-200; Diagenode) for 10 min (10 s on and 10 s off) on ice to obtain DNA fragments of 200–1,000 bp in length. One percent of total sheared chromatin DNA was used as the input. Nuclear extracts were incubated with the indicated antibodies at

4°C overnight. Protein G/A-labeled Dynabeads were added to each sample at 4°C for 2 h for immunoprecipitation. The immunoprecipitated DNA was analyzed by real-time PCR with Thunderbird SYBR qPCR mix (Toyobo).The NF-κB-binding sites of HIV LTR was amplified using the following PCR primer pairs: 5′-AGGTTTGACAGCCGCCTA-3′ and 5′-AGAGACCCAGTACAGGCAAAA-3′.

Luciferase reporter assay

PEBP1-sg1 (600 ng) was co-transfected with LTR-driven luciferase reporter plasmids containing the deletion of YY1-binding site, Sp1-binding site, Ap1-binding site or NF-κB-binding site (100 ng) and internal mock pcDNA3.1 (100 ng) (as empty) using Hieff Trans™ Liposomal Transfection Reagent (Yeasen Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The cells were harvested and lysed 72 h post-transfection where luciferase activity of the lysates was then measured by Dual-Luciferase Reporter Assay System (Promega). Each experiment was performed in triplicate. The genome of the TZM-bl cells is integrated with a luciferase reporter driven by the HIV-1 5′-LTR promoter. Cells were harvested at 72 h post-infection, and the lysate was assayed for luciferase activity. Triplicate cultures were measured for each experiment.

Cell proliferation by CCK-8 assay

Control KO C11 and PEBP1-KO-C11 cells were seeded at a density of 0.5×10^4 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 1×10^6 cells were collected in a 1.5-ml tube and centrifuged at 300 g for 5 min. The cells were washed twice with 500 μl PBS and analyzed with TUNEL-FITC apoptosis detection kit according to the manufacturer's instructions. The proportion of FITC-positive cells was assayed by flow cytometer and analyzed by FlowJo software (FlowJo LLC, Ashland, OR).

Western blot

A total of 1×10^6 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 Immun-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$^+$ T cells

Peripheral blood mononuclear cells (PBMCs) isolated from healthy donors were purchased from the Shanghai Blood Center (Shanghai, China). Naive CD4$^+$ T cells were further purified from peripheral blood mononuclear cells by negative selection according to the manufacturer's instructions (Thermo). The Naive CD4$^+$ T cells were maintained in serum-free medium supplemented with 1% penicillin-streptomycin and 5 ng/ml recombinant human interleukin-2 (R&D) and 10 ng/ml recombinant human interleukin-7 (R&D) at 37°C in 5% CO_2.

Treatment of patient CD4$^+$ T cells with EGCG

Shanghai Public Health Clinical Center approved this study, and the methods were carried out in accordance with the guidelines of Bullen CK' laboratory (Kim *et al*, 2014). All research participants in this study gave written informed consent. HIV-1-infected individuals were enrolled under the criteria of suppression of viremia to undetectable levels (< 50 copies/ml) on ART for at least six months. Peripheral blood mononuclear cells (PBMCs) were purified using density gradient centrifugation from whole blood. CD4$^+$ T lymphocytes were enriched by negative depletion (CD4$^+$ T-cell Isolation Kit, Miltenyi Biotec). EGCG was added at the time of α-CD3/CD28 stimulation. Cells were treated with EGCG and stimulated with α-CD3/CD28 for 72 h. HIV-specific qPCR was conducted as described (Shan *et al*, 2013).

Statistical analyses

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 $^{***}P < 0.001$.

Data availability

No primary datasets have been generated or deposited.

Expanded View for this article is available online.

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Author contributions
HZ conceived and designed the experiments. XY carried out most experiments. YW, PL, YS, XZ, YZhu, ZJ, HY, HP, LZ, YZho, JW, ZL, and XS participated in some of the experiments. HZ, GJ, HL, HW, JX, SJ, and DL directed and supervised the experiments and interpretation of data. HZ and GJ generated the initial concepts of this study. XY, GJ, and HZ wrote the paper. All authors approved the publication.

Conflict of Interest
The authors declare that they have no conflict of interest.

References

Alexaki A, Quiterio SJ, Liu Y, Irish B, Kilareski E, Nonnemacher MR, Wigdahl B (2007) PMA-induced differentiation of a bone marrow progenitor cell line activates HIV-1 LTR-driven transcription. *DNA Cell Biol* 26: 387 – 394

Archin NM, Liberty AL, Kashuba AD, Choudhary SK, Kuruc JD, Crooks AM, Parker DC, Anderson EM, Kearney MF, Strain MC *et al* (2012) Administration of vorinostat disrupts hiv-1 latency in patients on antiretroviral therapy. *Nature* 487: 482 – 485

Archin NM, Sung JM, Garrido C, Soriano-Sarabia N, Margolis DM (2014) Eradicating HIV-1 infection: seeking to clear a persistent pathogen. *Nat Rev Microbiol* 12: 750 – 764

Barouch DH, Deeks SG (2014) Immunologic strategies for HIV-1 remission and eradication. *Science* 345: 169 – 174

Beans EJ, Fournogerakis D, Gauntlett C, Heumann LV, Kramer R, Marsden MD, Murray D, Chun TW, Zack JA, Wender PA (2013) Highly potent, synthetically accessible prostratin analogs induce latent HIV expression *in vitro* and *ex vivo*. *Proc Natl Acad Sci USA* 110: 11698 – 11703

Beshir AB, Ren G, Magpusao AN, Barone LM, Yeung KC, Fenteany G (2010) Raf kinase inhibitor protein suppresses nuclear factor-kappaB-dependent cancer cell invasion through negative regulation of matrix metalloproteinase expression. *Cancer Lett* 299: 137 – 149

Besnard E, Hakre S, Kampmann M, Lim HW, Hosmane NN, Martin A, Bassik MC, Verschueren E, Battivelli E, Chan J *et al* (2016) The mTOR complex controls HIV latency. *Cell Host Microbe* 20: 785 – 797

Bieniasz PD, Grdina TA, Bogerd HP, Cullen BR (1999) Recruitment of cyclin T1/P-TEFb to an HIV type 1 long terminal repeat promoter proximal RNA target is both necessary and sufficient for full activation of transcription. *Proc Natl Acad Sci USA* 96: 7791 – 7796

Blazkova J, Trejbalova K, Gondois-Rey F, Halfon P, Philibert P, Guiguen A, Verdin E, Olive D, Van Lint C, Hejnar J *et al* (2009) CpG methylation controls reactivation of HIV from latency. *PLoS Pathog* 5: e1000554

Boehm D, Calvanese V, Dar RD, Xing S, Schroeder S, Martins L, Aull K, Li P-C, Planelles V, Bradner JE *et al* (2013) BET bromodomain-targeting compounds reactivate HIV from latency via a tat-independent mechanism. *Cell Cycle* 12: 452 – 462

Boehm D, Jeng M, Camus G, Gramatica A, Schwarzer R, Johnson JR, Hull PA, Montano M, Sakane N, Pagans S *et al* (2017) SMYD2-mediated histone methylation contributes to HIV-1 latency. *Cell Host Microbe* 21: 569 – 579.e566

Bolger GB (2017) The RNA-binding protein SERBP1 interacts selectively with the signaling protein RACK1. *Cell Signal* 35: 256 – 263

Bosque A, Planelles V (2011) Studies of HIV-1 latency in an *ex vivo* model that uses primary central memory T cells. *Methods* 53: 54 – 61

Chun TW, Engel D, Berrey MM, Shea T, Corey L, Fauci AS (1998) Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. *Proc Natl Acad Sci USA* 95: 8869 – 8873

Elsheikh MM, Tang Y, Li D, Jiang G (2019) Deep latency: a new insight into a functional HIV cure. *EBioMedicine* 45: 624 – 629

Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, Chaisson RE, Quinn TC, Chadwick K, Margolick J, Brookmeyer R *et al* (1997) Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* 278: 1295 – 1300

Fiume G, Vecchio E, De Laurentiis A, Trimboli F, Palmieri C, Pisano A, Falcone C, Pontoriero M, Rossi A, Scialdone A *et al* (2012) Human immunodeficiency virus-1 Tat activates NF-kappaB via physical interaction with IkappaB-alpha and p65. *Nucleic Acids Res* 40: 3548 – 3562

Gary DB, Joe W, Nathan C, Brett S, Stacey AR, Sergey AT, Andrew DB (2008) Infected cell killing by HIV-1 protease promotes NF-kB dependent HIV-1 replication. *PLoS ONE* 3: e2112

Granovsky AE, Clark MC, McElheny D, Heil G, Hong J, Liu XD, Kim Y, Joachimiak G, Joachimiak A, Koide S *et al* (2009) Raf kinase inhibitory protein function is regulated via a flexible pocket and novel phosphorylation-dependent mechanism. *Mol Cell Biol* 29: 1306 – 1320

Gu M, Liu Z, Lai R, Liu S, Lin W, Ouyang C, Ye S, Huang H, Wang X (2016) RKIP and TBK1 form a positive feedback loop to promote type I interferon production in innate immunity. *EMBO J* 35: 2553 – 2565

Ho YC, Shan L, Hosmane NN, Wang J, Laskey SB, Rosenbloom DI, Lai J, Blankson JN, Siliciano JD, Siliciano RF (2013) Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. *Cell* 155: 540 – 551

Hotter D, Bosso M, Jønsson KL, Krapp C, Stürzel CM, Das A, Littwitz-Salomon E, Berkhout B, Russ A, Wittmann S *et al* (2019) IFI16 targets the transcription factor Sp1 to suppress HIV-1 transcription and latency reactivation. *Cell Host Microbe* 25: 858 – 872.e13

Hu CJ, Zhou L, Cai Y (2014) Dihydroartemisinin induces apoptosis of cervical cancer cells via upregulation of RKIP and downregulation of bcl-2. *Cancer Biol Ther* 15: 279 – 288

Huang H, Kong W, Jean M, Fiches G, Zhou D, Hayashi T, Que J, Santoso N, Zhu J (2019) A CRISPR/Cas9 screen identifies the histone demethylase MINA53 as a novel HIV-1 latency-promoting gene (LPG). *Nucleic Acids Res* 47: 7333 – 7347

Imai K, Togami H, Okamoto T (2010) Involvement of histone H3 lysine 9 (H3K9) methyltransferase G9a in the maintenance of HIV-1 latency and its reactivation by BIX01294. *J Biol Chem* 285: 16538 – 16545

Jin S, Liao Q, Chen J, Zhang L, He Q, Zhu H, Zhang X, Xu J (2018) TSC1 and DEPDC5 regulate HIV-1 latency through the mTOR signaling pathway. *Emerg Microbes Infect* 7: 138

Jordan A, Defechereux P, Verdin E (2001) The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat, transactivation. *EMBO J* 20: 1726 – 1738

Jordan A, Bisgrove D, Verdin E (2003) HIV reproducibly establishes a latent infection after acute infection of T cells *in vitro*. *EMBO J* 22: 1868 – 1877

Kammula EC, Mötter J, Gorgels A, Jonas E, Hoffmann S, Willbold D (2012) Brain transcriptome-wide screen for HIV-1 Nef protein interaction partners reveals various membrane-associated proteins. *PLoS ONE* 7: e51578

Kessing CF, Nixon CC, Li C, Tsai P, Takata H, Mousseau G, Ho PT, Honeycutt JB, Fallahi M, Trautmann L (2017) *In vivo* suppression of HIV rebound by Didehydro-Cortistatin A, a "Block-and-Lock" strategy for HIV-1 treatment. *Cell Rep* 21: 600 – 611

Kim SO, Kim MR (2013) (-)-Epigallocatechin 3-gallate inhibits invasion by inducing the expression of Raf kinase inhibitor protein in AsPC1 human pancreatic adenocarcinoma cells through the modulation of histone deacetylase activity. *Int J Oncol* 42: 349 – 358

Kim M, Hosmane NN, Bullen CK, Capoferri A, Yang HC, Siliciano JD, Siliciano RF (2014) A primary CD4(+) T cell model of HIV-1 latency established after activation through the T cell receptor and subsequent return to quiescence. *Nat Protoc* 9: 2755 – 2770

Kim Y, Anderson JL, Lewin SR (2018) Getting the "Kill" into "Shock and Kill": strategies to eliminate latent HIV. *Cell Host Microbe* 23:14 – 26

Kumar A, Darcis G, Van Lint C, Herbein G (2015) Epigenetic control of HIV-1 post integration latency: implications for therapy. *Clin Epigenetics* 7: 103

Laquerriere A, Maluenda J, Camus A, Fontenas L, Dieterich K, Nolent F, Zhou J, Monnier N, Latour P, Gentil D *et al* (2014) Mutations in

CNTNAP1 and ADCY6 are responsible for severe arthrogryposis multiplex congenita with axoglial defects. *Hum Mol Genet* 23: 2279 – 2289

Lee HC, Tian B, Sedivy JM, Wands JR, Kim M (2006) Loss of Raf kinase inhibitor protein promotes cell proliferation and migration of human hepatoma cells. *Gastroenterology* 131: 1208 – 1217

Li Z, Guo J, Wu Y, Zhou Q (2013) The BET bromodomain inhibitor JQ1 activates HIV latency through antagonizing Brd4 inhibition of Tat-transactivation. *Nucleic Acids Res* 41: 277 – 287

Li Z, Wu J, Chavez L, Hoh R, Deeks SG, Pillai SK, Zhou Q (2019) Reiterative enrichment and authentication of CRISPRi targets (REACT) identifies the proteasome as a key contributor to HIV-1 latency. *PLoS Pathog* 15: e1007498

Lin K, Baritaki S, Militello L, Malaponte G, Bevelacqua Y, Bonavida B (2010) The role of B-RAF mutations in melanoma and the induction of EMT via dysregulation of the NF-kappaB/Snail/RKIP/PTEN circuit. *Genes Cancer* 1: 409 – 420

Liu Y, Fu Y, Wang Q, Li M, Zhou Z, Dabbagh D, Fu C, Zhang H, Li S, Zhang T *et al* (2019) Proteomic profiling of HIV-1 infection of human $CD4^+$ T cells identifies PSGL-1 as an HIV restriction factor. *Nat Microbiol* 4: 813 – 825

Ma H, Dang Y, Wu Y, Jia G, Anaya E, Zhang J, Abraham S, Choi JG, Shi G, Qi L *et al* (2015) A CRISPR-based screen identifies genes essential for west-nile-virus-induced cell death. *Cell Rep* 12: 673 – 683

Mancebo HS, Lee G, Flygare J, Tomassini J, Luu P, Zhu Y, Peng J, Blau C, Hazuda D, Price D *et al* (1997) P-TEFb kinase is required for HIV Tat transcriptional activation *in vivo* and *in vitro*. *Genes Dev* 11: 2633 – 2644

Marceau CD, Puschnik AS, Majzoub K, Ooi YS, Brewer SM, Fuchs G, Swaminathan K, Mata MA, Elias JE, Sarnow P *et al* (2016) Genetic dissection of Flaviviridae host factors through genome-scale CRISPR screens. *Nature* 535: 159 – 163

Margolis DM, Garcia JV, Hazuda DJ, Haynes BF (2016) Latency reversal and viral clearance to cure HIV-1. *Science* 353: aaf6517

Mousseau G, Kessing CF, Fromentin R, Trautmann L, Chomont N, Valente ST (2015) The tat inhibitor didehydro-cortistatin a prevents hiv-1 reactivation from latency. *mBio* 6: e00465-15

Oh C, Park S, Lee EK, Yoo YJ (2013) Downregulation of ubiquitin level via knockdown of polyubiquitin gene Ubb as potential cancer therapeutic intervention. *Sci Rep* 3: 2623

Pandeló José D, Bartholomeeusen K, da Cunha RD, Abreu CM, Glinski J, da Costa TB, Bacchi Rabay AF, Pianowski Filho LF, Dudycz LW, Ranga U *et al* (2014) Reactivation of latent HIV-1 by new semi-synthetic ingenol esters. *Virology* 462–463: 328 – 339

Park RJ, Wang T, Koundakjian D, Hultquist JF, Lamothe-Molina P, Monel B, Schumann K, Yu H, Krupzcak KM, Garcia-Beltran W *et al* (2017) A genome-wide CRISPR screen identifies a restricted set of HIV host dependency factors. *Nat Genet* 49: 193 – 203

Pitman MC, Lau JSY, McMahon JH, Lewin SR (2018) Barriers and strategies to achieve a cure for HIV. *Lancet HIV* 5: e317 – e328

Qu X, Wang P, Ding D, Li L, Wang H, Ma L, Zhou X, Liu S, Lin S, Wang X *et al* (2013) Zinc-finger-nucleases mediate specific and efficient excision of HIV-1 proviral DNA from infected and latently infected human T cells. *Nucleic Acids Res* 41: 7771 – 7782

Richman DD, Margolis DM, Delaney M, Greene WC, Hazuda D, Pomerantz RJ (2009) The challenge of finding a cure for HIV infection. *Science* 323: 1304 – 1307

Rojas CV, Valiente-EF Soto RR, Toro AD (2019) New challenges of HIV-1 infection: how HIV-1 attacks and resides in the central nervous system. *Cell* 8: 1245

Ruelas DS, Greene WC (2013) An integrated overview of HIV-1 latency. *Cell* 155: 519 – 529

Savidis G, McDougall WM, Meraner P, Perreira JM, Portmann JM, Trincucci G, John SP, Aker AM, Renzette N, Robbins DR *et al* (2016) Identification of Zika virus and dengue virus dependency factors using functional genomics. *Cell Rep* 16: 232 – 246

Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelson T, Heckl D, Ebert BL, Root DE, Doench JG *et al* (2014) Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 343: 84 – 87

Shan L, Rabi SA, Laird GM, Eisele EE, Zhang H, Margolick JB, Siliciano RF (2013) A novel PCR assay for quantification of HIV-1 RNA. *J Virol* 87: 6521 – 6525

Søgaard OS, Graversen ME, Steffen L, Rikke O, Brinkmann CR, Nissen SK, Kjaer AS, Schleimann MH, Denton PW, Hey-Cunningham WJ *et al* (2015) The depsipeptide romidepsin reverses hiv-1 latency *in vivo*. *PLoS Pathog* 11: e1005142

Spivak AM, Andrade A, Eisele E, Hoh R, Bacchetti P, Bumpus NN, Emad F, Buckheit R III, McCance-Katz EF, Lai J *et al* (2014) A pilot study assessing the safety and latency-reversing activity of disulfiram in HIV-1-infected adults on antiretroviral therapy. *Clin Infect Dis* 58: 883 – 890

Tavel L, Jaquillard L, Karsisiotis AI, Saab F, Jouvensal L, Brans A, Delmas AF, Schoentgen F, Cadene M, Damblon C (2012) Ligand binding study of human PEBP1/RKIP: interaction with nucleotides and Raf-1 peptides evidenced by NMR and mass spectrometry. *PLoS ONE* 7: e36187

Verdin E, Paras P Jr, Van Lint C (1993) Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. *EMBO J* 12: 3249 – 3259

Wang FX, Xu Y, Sullivan J, Souder E, Argyris EG, Acheampong EA, Fisher J, Sierra M, Thomson MM, Najera R *et al* (2005) IL-7 is a potent and proviral strain-specific inducer of latent HIV-1 cellular reservoirs of infected individuals on virally suppressive HAART. *J Clin Invest* 115: 128 – 137

Wang P, Lu P, Qu X, Shen Y, Zeng H, Zhu X, Zhu Y, Li X, Wu H, Xu J *et al* (2017) Reactivation of HIV-1 from latency by an ingenol derivative from euphorbia kansui. *Sci Rep* 7: 9451

Wei H, Gao HQ, Li HB, Qi SJ, Liu WL, Xu L, Li H, Liu JX, Dong ZM (2015) Correlation among RKIP expression, NF-κB p65 levels, and T-lymphocyte subsets in gastric cardia adenocarcinoma. *Genet Mol Res* 14: 16491 – 16496

Yeung KC, Rose DW, Dhillon AS, Yaros D, Gustafsson M, Chatterjee D, McFerran B, Wyche J, Kolch W, Sedivy JM (2001) Raf kinase inhibitor protein interacts with NF-kappaB-inducing kinase and TAK1 and inhibits NF-kappaB activation. *Mol Cell Biol* 21: 7207 – 7217