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ZBRK1 represses HIV-1 LTR-mediated transcription

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ABSTRACT

The identification of cellular proteins that interact with the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) provides a basic understanding of HIV-1 gene expression, which is the major determinant regulating virus replication. We show that ZBRK1 negatively regulates the HIV-1 LTR. Ectopic expression of ZBRK1 represses transcriptional activity of the HIV-1 LTR, whereas the depletion of endogenous ZBRK1 leads to activation of the HIV-1 LTR. The repressor activity of ZBRK1 is required for TRIM28 binding. Furthermore, ZBRK1 is bound to the HIV-1 LTR in vivo. These results indicate that ZBRK1 could be involved in a potent intrinsic antiretroviral defense.

Structured summary of protein interactions:
ZBRK1 physically interacts with **TRIM28** by anti tag coimmunoprecipitation (View interaction).

1. Introduction

The binding of host factors to long terminal repeats (LTRs) of proviral DNA tightly regulates retroviral gene expression. Recent reports have shown that TRIM28 (tripartite motif-containing protein 28; also known as KRAB-associated protein 1 (KAP1)), a well-characterized transcriptional repressor, suppresses endogenous retroviruses by recruiting the H3K9 methyltransferases ESET (also called SETDB1 or KMT1E) and heterochromatin protein 1 (HP1) in mouse ES cells [1–2]. Exogenous retroviruses, such as moloney murine leukemia virus (M-MLV), are also silenced by TRIM28 in ES cells [3]. TRIM28-mediated gene-specific transcriptional repression requires a Krüppel-associated box (KRAB)-zinc finger protein, such as ZFP809, to directly recognize integrated viral DNA [4]. Allouch et al. have proposed that TRIM28 inhibits HIV-1 replication by suppressing IN acetylation during integration [5].

KRAB-zinc finger proteins have the potential to regulate HIV-1 gene expression. Several groups have reported that artificially engineered KRAB domain-containing zinc finger proteins that bind to HIV-1 sequences also induce proviral silencing [6,7]. By contrast, endogenous OTK18, which contains 13 C_2H_2-type zinc finger motifs and a KRAB domain and was identified by differential display of mRNA from HIV type 1-infected macrophages, was shown to interact with and suppress the NRE within the HIV-1 LTR [8]. Thus, some KRAB-zinc finger proteins efficiently inhibit HIV-1 replication.

ZBRK1, which is also known as KRAB-zinc finger protein, binds to the GGGxxxCAGxxxTTT DNA recognition motif within intron 3 of the DNA damage-responsive gene *GADD45* following cellular DNA damage. Previously, breast cancer susceptibility gene1 (BRCA1), which has been shown to interact physiologically with ZBRK1 [9], was identified through large-scale screening as a host factor for HIV-1 replication [10].

ZBRK1 has been implicated in the tumorigenesis of several human cancers. BRCA1, CtIP, and ZBRK1 form a repressor complex at a ZBRK1 recognition site within the *ANG1* promoter, and a defect in the formation of this repressor complex de-represses ANG1 expression. This complex promotes the survival of neighboring endothelial cells [11]. Moreover, ZBRK1 and the ATXN2 complex regulate *SCA2* gene transcription and have been linked to cellular RNA metabolism and endocytotic processes.

We report here the molecular characterization of ZBRK1-mediated HIV-1 LTR repression. ZBRK1 acts as a transcriptional repressor of the HIV-1 LTR in a TRIM28-dependent manner. These data shed further light on the mechanistic role of ZBRK1 in HIV-1 gene expression.

2. Materials and methods

2.1. Plasmids

The details of the plasmid constructs used in this study are provided in the Supplementary Materials and methods section.

2.2. Preparation of lentiviral vectors

293T cells (1×10^6) were plated in 60-mm dishes and co-transfected with the appropriate lentiviral-shRNA expression

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vector (1.6 μg), the vesicular stomatitis virus G expression vector pMD.G (0.5 μg), the *rev* expression vector pRSV-Rev (0.5 μg), and the *gag-pol* expression vector pMDLg/pRRE (1.2 μg) using Lipofectamine 2000 (Invitrogen). At 48 h post-transfection, the culture supernatants were harvested and filtered through 0.45-μm pore size filters.

2.3. CHIP assay

The CHIP assay was performed using the ChIP-IT Express kit according to the manufacturer's recommendations (Active Motif, Carlsbad, California, USA). Briefly, 293T LTR-Luc cells (5×10^6) were plated in 100-mm dishes, washed with phosphate-buffered saline, and treated with 1% formaldehyde for 10 min. After the reaction was quenched with 0.1 M glycine, the cross-linked material was sonicated for ten pulses of 20 s each, with a 30-s rest on ice between each pulse. Immunoprecipitations were performed with protein G magnetic beads and 5 μg of either the control antibody or the anti-ZBRK1 antibody. The chromatin solution was precleared by adding protein G magnetic beads for 2 h at 4 °C. The protein G magnetic beads were blocked with 1 μg/μl of salmon sperm DNA and 1 μg/μl of bovine serum albumin overnight at 4 °C and then incubated with the chromatin and antibody for 2 h. The immunoprecipitated material was washed 3 times with the wash buffer. The cross-linking was reversed by incubating the samples for 5 h at 65 °C in 200 mM NaCl with 10 μg of RNase A to eliminate any RNA. The recovered material was treated with proteinase K and extracted using the Wizard SV Gel PCR Clean-Up System (Promega). The DNA was analyzed by quantitative PCR with *StepOne* (Applied Biosystems) and the following primers: 5′-TGA CCT TTG GAT GGT GCT TC-3′ and 5′-TCC ACA CTA ATA CTT CTC CC-3′.

2.4. Immunoprecipitation

293T cells were transfected with 0.5 μg of pNFLAG-ZBRK1 or pNFLAG-ZBRK1-DV12,13AA using Lipofectamine 2000. At 48 h post-transfection, the transfected cells were harvested and suspended in 0.5 ml of lysis buffer (20 mM Tris–HCl, pH 7.5; 250 mM NaCl; 1 mM EDTA; 5% glycerol; 1% Triton X-100). The cell lysates were centrifuged at 15,000×g for 20 min at 4 °C. The supernatants were incubated with 1 μg of anti-TRIM28 antibody and 40 μl of protein G-magnetic beads for 2 h at 4 °C. The beads were washed with PBS containing 0.02% Triton X-100. The immunocomplex was eluted by boiling with 20 μl of 5× sample buffer and was analyzed by SDS–PAGE and Western blotting.

2.5. MAGI assay

MAGI cells were plated in 96-well plates at 1×10^4 cells per well in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The next day, the cells were infected with dilutions of the virus in a total volume of 50 μl in the presence of 20 μg/ml of DEAE-dextran for 2 h. At 2 days post-infection, the cells were fixed with 100 μl of fixative (1% formaldehyde/0.2% glutaraldehyde in PBS) at room temperature for 5 min and then washed twice with PBS. The cells were incubated with 100 μl of staining solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, and 0.4 mg/ml X-Gal) for 50 min at 37 °C. The reaction was halted by removing the staining solution, and the blue cells were then counted using a microscope.

2.6. Real-time RT-PCR

Total RNA was extracted from shControl- or shZBRK1-transduced cells using RNeasy Mini kits (Qiagen), and cDNA was prepared with Revatra Ace (Toyobo) using oligo(dT) primers. Quantitative real-time PCR was performed with the Power SYBR Green PCR Master Mix (Applied Biosystems), and fluorescent signals were analyzed with the StepOne RT-PCR system (Applied Biosystems). The PCR primer pairs used were as follows: ZBRK1-F, 5′-AGA AAC AAG AGG CAG CCA AG-3′; ZBRK1-R, 5′-GGC TGT CCC ACA AGG ACT AC-3′; beta-actin-F, 5′-GTA CCA CTG GCA TCG TGA TGG ACT-3′; and beta-actin-R, 5′-CCG CTC ATT GCC AAT GGT GAT-3′.

2.7. Luciferase assay

The details of the luciferase assay are provided in the Supplementary Materials and methods section.

2.8. Measurement of HIV-1 p24 antigen

The details of the measurement of the p24 antigen are provided in the Supplementary Materials and methods section.

2.9. Electrophoresis mobility shift assay (EMSA)

The details of the EMSA are provided in the Supplementary Materials and methods section.

3. Results and discussion

3.1. ZBRK1 suppresses HIV-1 LTR promoter activity

We initially determined whether ZBRK1 could inhibit HIV-1 LTR promoter activity. To do this, we co-transfected 293T cells with the ZBRK1 expression vector and the HIV-1 LTR-driven luciferase reporter plasmid. Ectopic expression of ZBRK1 reduced the luciferase activity (Fig. 1A). Additionally, ZBRK1 reduced the LTR-driven luciferase mRNA level (Fig. 1B). To investigate whether ZBRK1 could repress full-length HIV-1 transcription, the viral titer of an HIV-1 molecular clone (NL4-3) produced from 293T cells expressing ZBRK1 was determined in MAGI cells. We observed that expression of ZBRK1 significantly reduced the viral titer (Fig. 1C). Moreover, we determined whether ZBRK1 could affect p24 antigen expression, which would result in a loss of viral titer. The expression of ZBRK1 resulted in a decrease in HIV-1 p24 antigen production in intracellular and culture supernatants (Fig. 1D). These results suggest that ZBRK1 represses not only the HIV-1 LTR-driven luciferase reporter plasmid but also full-length HIV-1 transcription. Next, to evaluate the function of ZBRK1 in chromatin-mediated repression of the HIV-1 LTR, we ectopically expressed ZBRK1 in HeLa cells containing an integrated LTR-luciferase reporter gene (HeLa–LTR–Luc). We then expressed HIV-1 Tat in HeLa–LTR–Luc cells, as these cells have a low basal level of luciferase activity. Expression of ZBRK1 in HeLa–LTR–Luc cells reduced the level of luciferase activity (Fig. 1E, left). To ensure that the effect observed was not due to ZBRK1-mediated suppression of Tat gene transcription, we analyzed Tat expression by Western blotting and found that ZBRK1 had no effect on the Tat expression level (Fig. 1E, right). This result demonstrates that ZBRK1 plays a significant role in chromatin-mediated repression of the HIV-1 LTR. To investigate the function of endogenous ZBRK1 in transcriptional repression of the LTR, we utilized a lentiviral vector encoding an shRNA corresponding to the ZBRK1 sequence. The expression level of endogenous ZBRK1 was reduced upon introduction of the shZBRK1-expressing lentiviral vector in 293T cells, as measured by qRT-PCR (Fig. 1F). The knockdown of ZBRK1 expression enhanced the transcriptional activity of the LTR (Fig. 1G). To further confirm the effect of endogenous ZBRK1 in HIV-1-integrated cells, 293T cells were infected with an $HIV_{NL43\text{-}luc}$ pseudotyped virus and then transduced with the shControl or shZBRK1 vectors. ZBRK1 knockdown significantly

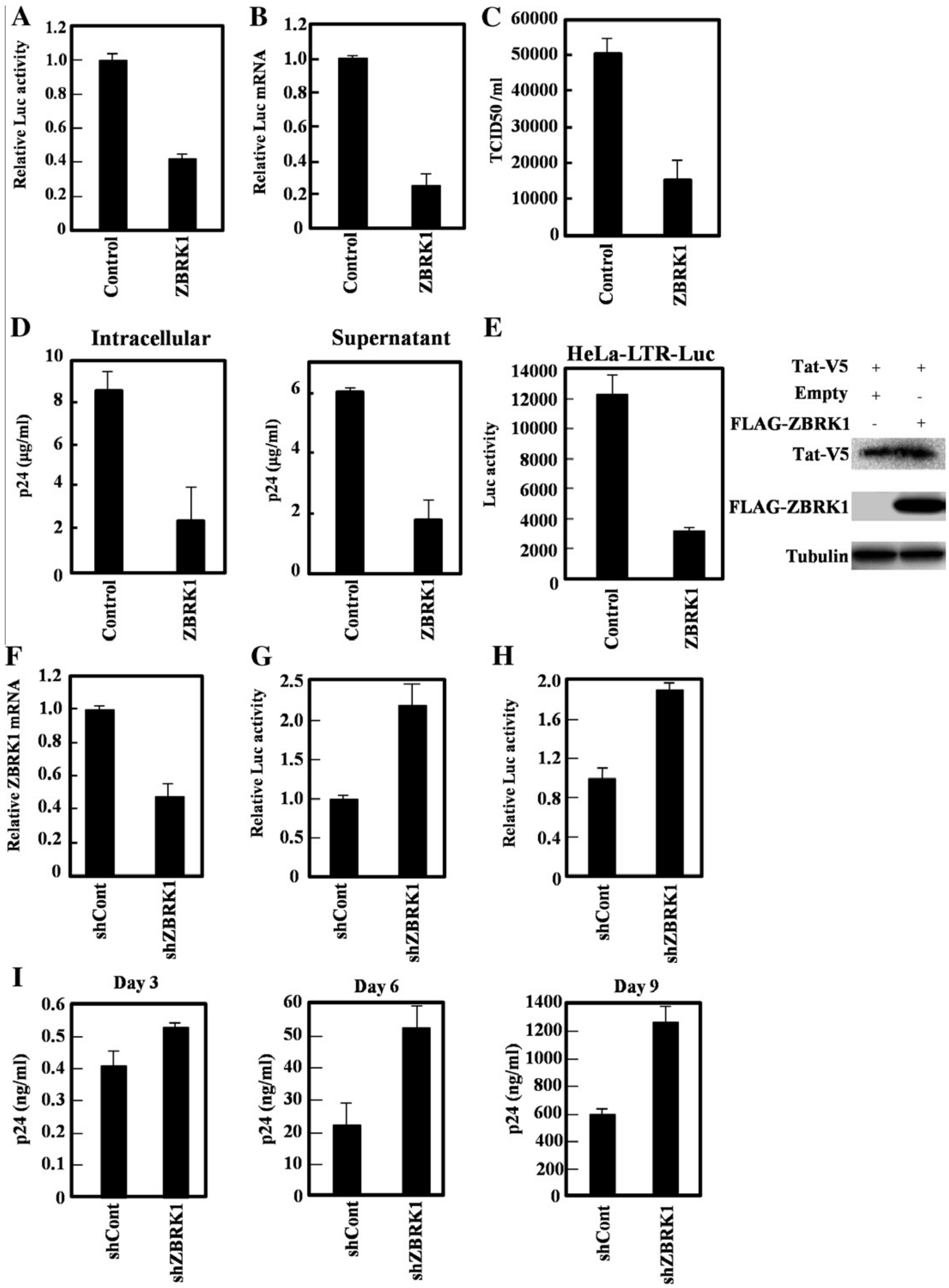

Fig. 1. ZBRK1 inhibits HIV-1 LTR-driven transcription. (A) 293T cells were transfected with 1 ng of pLTR-Luc, 1 ng of pCMV-Renilla-Luc and 200 ng of pNFLAG or pNFLAG-ZBRK1. At 48 h post-transfection, the levels of luciferase gene expression were determined by measuring the luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity. (B) 293T cells were transfected with 200 ng of pNFLAG or pNFLAG-ZBRK1 along with 5 ng of pLTR-Luc. At 48 h post-transfection, the levels of luciferase mRNA were determined by qRT-PCR. Quantification of the beta-actin gene was performed as an internal control to correct for sample-to-sample variations in mRNA levels. (C, D) 293T cells were transfected with 200 ng of pNL4–3 and 300 ng of pNFLAG or pNFLAG-ZBRK1. At 48 h post-transfection, the virus titer (as shown, TCID50/ml (C)) were measured by MAGI assay. The amounts of p24 antigen in the intracellular compartments (D, left panel) and culture supernatants (D, right panel) were measured by a chemiluminescent enzyme immunoassay (CLEIA). (E) HeLa-LTR-Luc cells were transfected with 500 ng of pCMV-Tat-V5 and 500 ng of pNFLAG or pNFLAG-ZBRK1. At 48 h post-transfection, the cell lysates were analyzed for luciferase activity (left panel), and Tat-V5, FLAG-ZBRK1 and tubulin expression by Western blotting (right panel). (F, G) 293T cells were infected with the indicated lentiviral vectors. At 48 h post-infection, the cells were transfected with 5 ng of pLTR-Luc. At 48 h post-transfection, the levels of ZBRK1 mRNA (F) and luciferase activity (G) were determined by quantitative RT-PCR and a luciferase assay, respectively. Quantification of the beta-actin gene was performed as an internal control to correct for sample-to-sample variations in mRNA levels. (H) The pseudotyped virus was generated by co-transfection of 293T cells with 1 μg of the pNLlucΔenv and 1 μg of pMD.G. The culture supernatants were harvested at 48 h post-transfection and filtered through 0.45-μm pore size filters. 293T cells were infected with VSV-G pseudotyped NL-Luc [12]. At 1 h post-infection, 293T cells were infected with the lentiviral vectors expressing shControl or shZBRK1. At 48 h post-shRNA transduction, the levels of luciferase gene expression were determined by measuring luciferase activity. (I) MT-4 cells were infected with the lentiviral vectors expressing shControl or shZBRK1. At 5 days post-infection, the cells were infected with 2 ng of NL4-3 p24. Virus replication was monitored every 3 days after infection by measuring p24 viral antigen in the culture supernatant. The results are representative of three independent experiments, and error bars show the standard deviation of the mean values.

induced HIV-1 gene expression in the HIV-1-integrated cells (Fig. 1H). To evaluate the effect of ZBRK1 in T cells, MT-4 cells were transduced with the control or ZBRK1-specific shRNA vectors and then infected with HIV-1. Virus replication was monitored by measuring the production of p24 in the supernatant every 3 days post-infection. The depletion of ZBRK1 in MT-4 cells conferred a 2-fold enhancement on HIV-1 replication at 6 and 9 days post-infection (Fig. 1I). These results suggest that ZBRK1 inhibits HIV-1 gene expression through transcriptional repression of the LTR.

3.2. Identification of potential ZBRK1 response elements in the HIV-1 LTR

To determine the LTR sequence responsible for the suppressive function of ZBRK1, we generated deletion mutants of the LTR (Fig. 2A). The expression of ZBRK1 repressed transcription from the −335 to +282 LTR segment (Fig. 2C), the −245 to +282 LTR segment (Fig. 2D), and the full-length LTR (Fig. 2B). By contrast, repression of the LTR by ZBRK1 was abolished by the deletion of LTR sequences corresponding to −454 to −107 (Fig. 2E). These results suggest that the −454 to −107 region within the LTR contains essential elements for transcriptional repression by ZBRK1. To further define the ZBRK1-responsive region of the LTR, we constructed several more precise deletion mutants of the LTR (Fig. S1A). Similar to the wild type LTR, transcription from the LTR deletion corresponding to −205 to −146 was decreased by expression of ZBRK1 (Fig. S1B). However, deletion of the LTR corresponding to −145 to −126 partially impaired the suppressive effect of ZBRK1. These results indicate that the −145 to −126 rejoin of the LTR is a candidate for transcriptional repression by the ZBRK1.

A previous study has shown that transcriptional silencing of the HIV-1 LTR by HP1-gamma requires Sp-1, P-TEFb (which leads to the phosphorylation of RNA polymerase IICTD by recruiting HIV-1 Tat to the TAR) and PCAF (which is known to possess histone acetyl transferase activity) [13].

To further determine the role of *cis*-elements of the LTR in ZBRK1 repression, we introduced mutations into the NF-κB or Sp-1 binding sites of the LTR. However, mutations of the NF-κB and Sp-1 binding sites within the LTR did not affect ZBRK1 repression (Fig. 2F and G). In addition, HIV-1 Tat was also not required for the repressive activity of ZBRK1 (Fig. 2H). These results suggest that ZBRK1 directly represses transcriptional activity of the HIV-1 LTR.

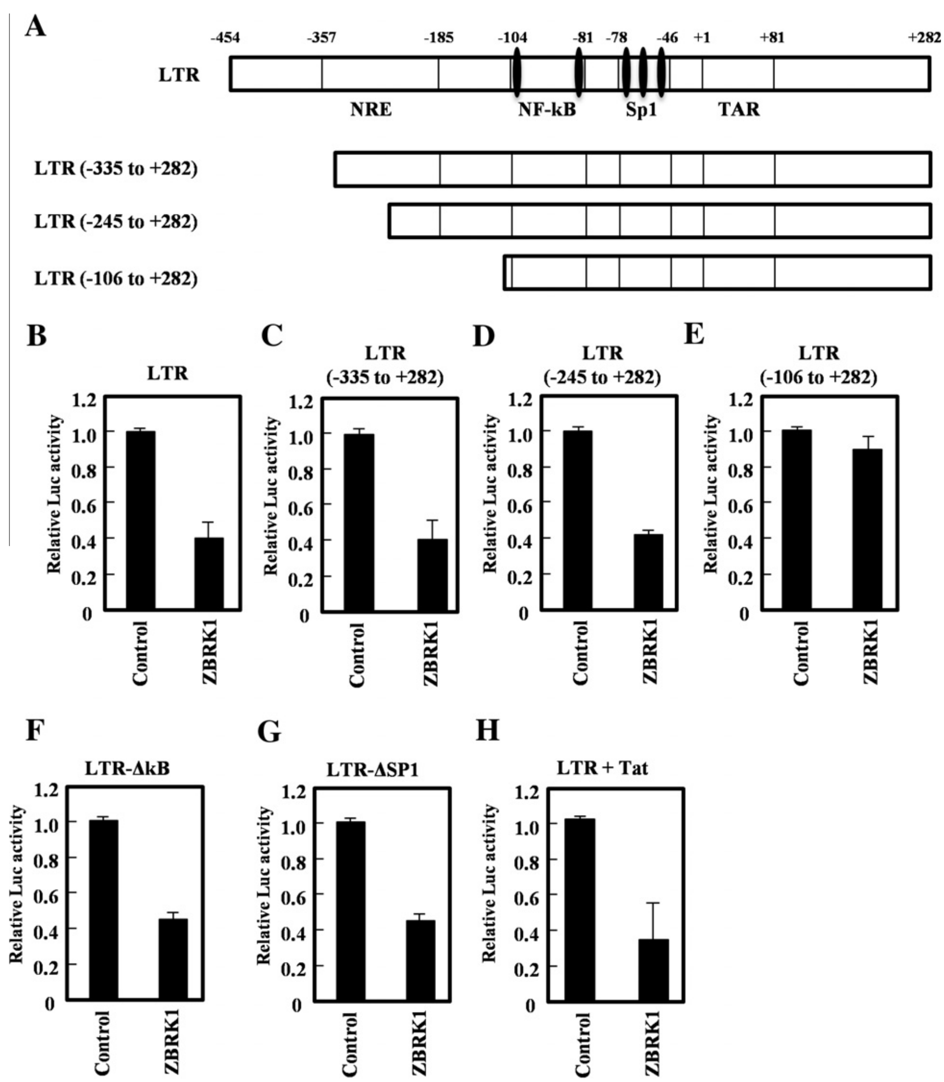

Fig. 2. The region of ZBRK1 responsible for transcriptional repression of the HIV-1 LTR. (A) A schematic representation of HIV-1 LTR-driven expression of firefly luciferase. The numbers are relative to the transcription start site nucleotide +1. (B–G) 293T cells were transfected with 1 ng of pCMV-Renilla-Luc and 200 ng of pNFLAG or pNFLAG-ZBRK1 along with 5 ng of pLTR-Luc (B) or its corresponding mutants (C–G). The luciferase assay was performed as described in Fig. 1A. (H) 293T cells were transfected with 1 ng of pCMV-Renilla-Luc, 5 ng of pLTR-Luc and 100 ng of pCMV-Tat along with 200 ng of pNFLAG or pNFLAG-ZBRK1. The luciferase assay was performed as described in Fig. 1A. The results are representative of three independent experiments, and error bars show the standard deviation of the mean values.

3.3. Transcriptional repression of the HIV-1 LTR by ZBRK1 requires TRIM28

KRAB-zinc finger proteins interact with TRIM28 through their KRAB box [14]. This interaction represses the target gene by recruiting the histone methyltransferases SETDB1 and HP1 [15]. To investigate the mechanism involved in ZBRK1-mediated repression of the LTR, we used siRNAs specific for TRIM28, HP1-gamma or SETDB1 in either empty plasmid-transfected or ZBRK1 expression plasmid-transfected cells. The introduction of each specific siRNA induced the efficient knockdown of that particular protein (Fig. 3A). The expression of ZBRK1 in siControl-transduced cells conferred a 2.4-fold repression of LTR-driven transcription (Fig. 3B). The depletion of HP1-gamma or SETDB1 had no significant effect on the ZBRK1-mediated repression of LTR transcription (Fig. 3C and D, 2.5-fold and 2.7-fold, respectively). By contrast, no repressive activity of ZBRK1 was observed in the siTRIM28-transduced cells (Fig. 3E). To further verify the TRIM28 requirement for the repressive activity of ZBRK1, we introduced two substitution mutations (DV to AA) within the KRAB domain that have previously been shown to disrupt the repressive function of KRAB domain-containing proteins. Relative to wild-type ZBRK1, ZBRK1-DV12,13AA was significantly defective in repression of LTR activity (Fig. 3F). Furthermore, FLAG-ZBRK1 was found to interact with endogenous TRIM28, but this interaction was less efficient with FLAG-ZBRK1-DV12,13AA (Fig. 3G). These results indicate that TRIM28 plays an essential role in the repressive function of ZBRK1.

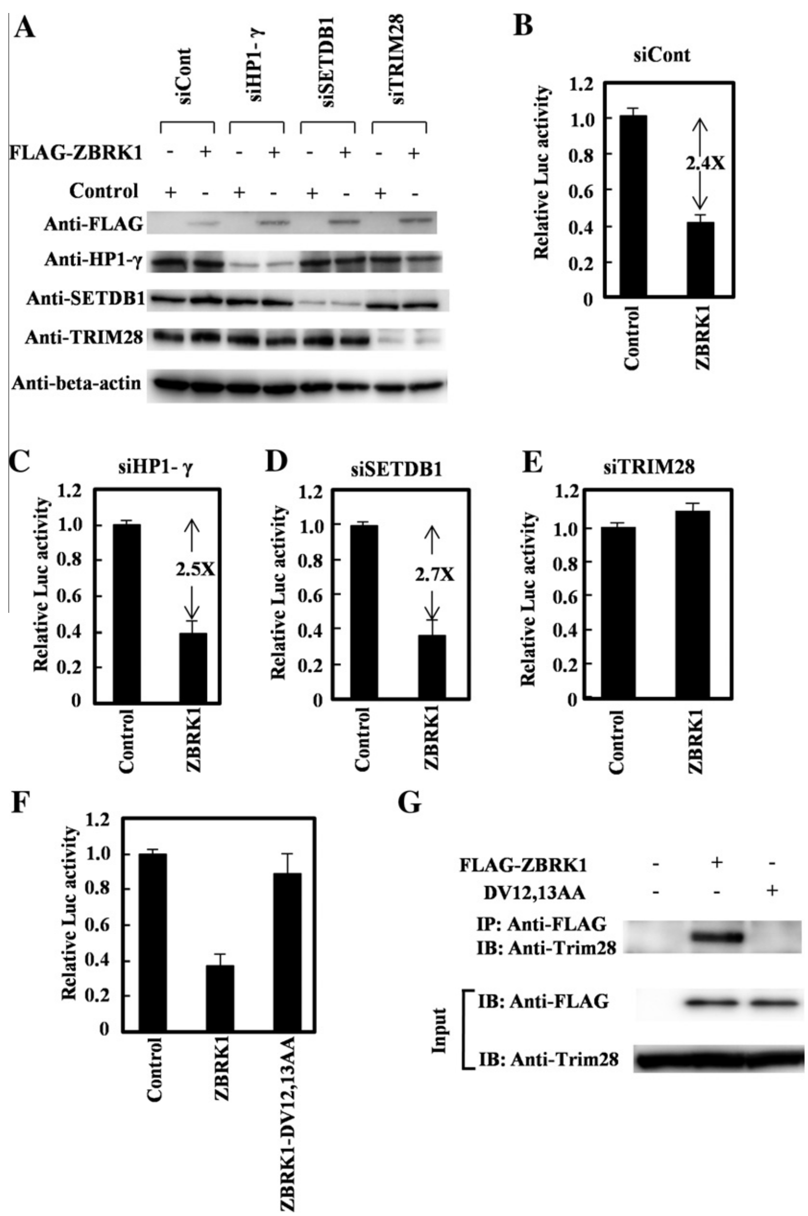

Fig. 3. TRIM28 is required for the repressive activity of ZBRK1. (A) HeLa cells were treated with 70 nM of the indicated siRNA for 24 h, prior to transfection with 5 ng of pLTR-Luc, 1 ng of pCMV-Renilla-Luc and 300 ng of pNFLAG or pNFLAG-ZBRK1. At 48 h post-transfection, the protein lysates were run on SDS–PAGE gels and probed for the indicated protein. (B–E) The protein lysates in Fig. 3A were analyzed for luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity. The values within the graph represent the fold change of luciferase activity. (F) 293T cells were transfected with 1 ng of pCMV-Renilla-Luc and 5 ng of pLTR-Luc along with 200 ng of pNFLAG, pNFLAG-ZBRK1 or pNFLAG-ZBRK1-DV12,13AA. The luciferase assay was performed as described in Fig. 1A. (G) 293T cells were transfected with 500 ng of pNFLAG-ZBRK1 or pNFLAG-ZBRK1-DV12,13AA. At 48 h post-transfection, the cell lysates were immunoprecipitated using an anti-FLAG antibody, followed by Western blotting analysis with an anti-TRIM28 antibody. The results are representative of three independent experiments, and error bars show the standard deviation of the mean values.

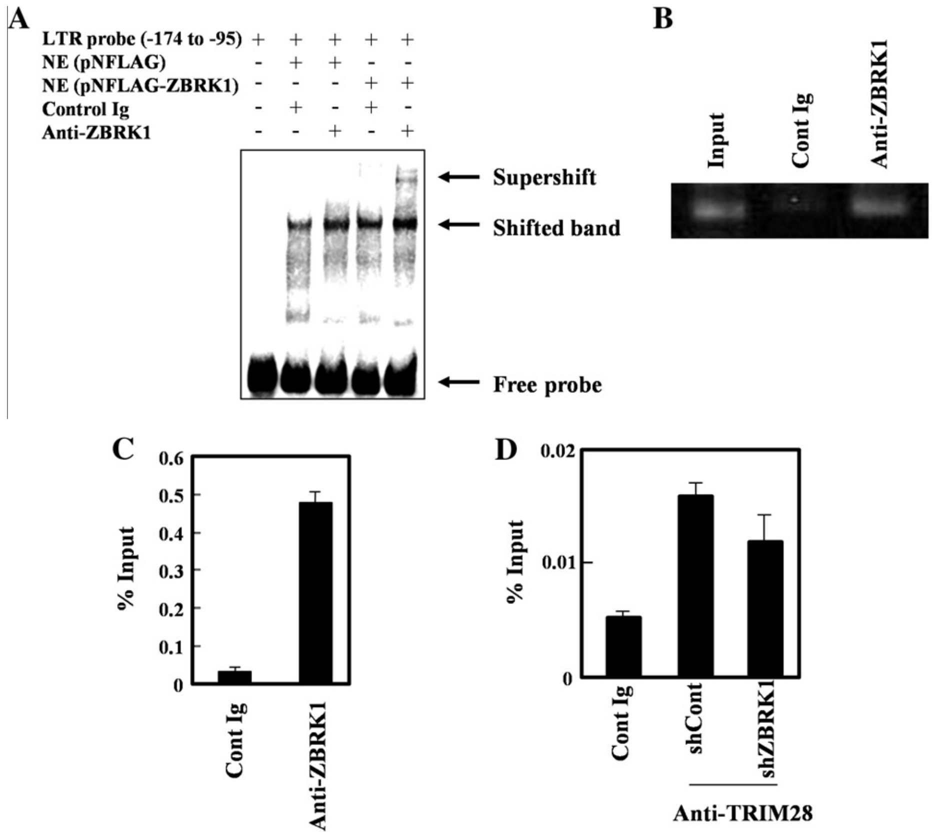

Fig. 4. ZBRK1 binds to the HIV-1 LTR in vivo. (A) An EMSA was performed by using a biotin-labeled probe corresponding to the LTR sequence (−174 to −95) and nuclear extracts from 293T cells transfected with empty plasmid or pNFLAG-ZBRK1 in the presence of control antibody or anti-ZBRK1 antibody. (B) Chromatin immunoprecipitations using anti-ZBRK1 and control Ig. PCR was performed using primers specific to the U3 region of the HIV-1 LTR. The PCR products were separated on 1.5% agarose gels and stained with SYBER Green. (C) Immunoprecipitated DNA was subjected to real-time PCR using primers specific to the U3 region of the HIV-1 LTR, as described in the materials and methods. The amount of immunoprecipitated DNA was normalized to the input DNA. The data are representative of three independent experiments. (D) The level of TRIM28 at the LTR in ZBRK1 knockdown cells.

3.4. ZBRK1 repression is histone deacetylase-dependent

As previously described, TRIM28 interacts with Mi-2alpha and other components of the NuRD complex. Additionally, TRIM28-mediated silencing requires association with NuRD and HDAC activity. By contrast, trimethylation of histone H3 on lysine 9 by SETDB1 creates high-affinity genomic binding sites for the TRIM28–HP1 complex, suggesting that SETDB1 may play an important role in TRIM28-mediated repression. However, our data indicated that SETDB1 and HP1-gamma did not affect ZBRK1-induced repression of LTR transcription (Fig. 3C and D).

To test whether histone deacetylases play a role in the repression of LTR activity by ZBRK1 in HeLa–LTR–Luc cells, we used the pan-HDAC inhibitor trichostatin A (TSA). Treatment with 400 nM of TSA significantly reversed ZBRK1-mediated repression in HeLa–LTR–Luc cells (Fig. S2A). Moreover, the repressive activity of ZBRK1 decreased in HDAC2-depleted cells (Fig. S2B). These results indicate that ZBRK1-mediated suppression of LTR activity requires HDAC2 activity.

3.5. ZBRK1 is enriched at the HIV-1 LTR

To investigate whether ZBRK1 could bind to the LTR, we performed a gel mobility shift assay using nuclear extracts from 293T cells ectopically expressing FLAG-ZBRK1. A shift of the LTR probe corresponding to LTR sequence (−175 to −95) was observed after incubation with nuclear extracts prepared from 293T cells transfected with empty plasmid or pNFLAG-ZBRK1 (Fig. 4A). However, a supershift of the labeled probe could not detect in the presence of anti-ZBRK1 antibody. This can be attributed to low levels of ZBRK1 expression in 293T cells (data not shown). In contrast, coincubation of the nuclear extracts from 293T cells expressing FLAG-ZBRK1 with anti-ZBRK1 antibody but not control antibody caused a supershift of the labeled probe. These results indicated that ZBRK1 can form a complex with HIV-1 LTR. If ZBRK1 is indeed critical for transcriptional repression of the HIV-1 LTR, it should be bound to the HIV-1 LTR in vivo. To test whether the HIV-1 LTR is bound by endogenous ZBRK1, LTR-integrated cells were analyzed by chromatin immunoprecipitation assay (CHIP assay) using an anti-ZBRK1 antibody. ZBRK1 enrichment was detected in the LTR (Fig. 4B). The level of CHIP enrichment was determined by CHIP-qPCR (Fig. 4C). In parallel, the level of binding to the housekeeping GAPDH gene was not detected by ZBRK1 enrichment (data not shown). To investigate whether ZBRK1 is required for recruitment of TRIM28 to the LTR, ZBRK1-depleted cells were analyzed by the CHIP assay using an anti-TRIM28 antibody. TRIM28 can bind to the LTR (Fig. 4D). Moreover, the depletion of ZBRK1 results in a 25% reduction in the level of TRIM28 at the LTR. These results indicate that ZBRK1 can bind to the HIV-1 LTR and partially influence recruitment of TRIM28 to the LTR. In conclusion, our data demonstrate that ZBRK1, in conjunction with TRIM28 and HDAC2, suppresses HIV-1 LTR-driven gene expression. The results of this study suggest that stimulation of KRAB-zinc finger proteins may aid in the development of antiviral therapies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.08.010.

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