

RESEARCH ARTICLE

Reactivation of Latent HIV-1 Expression by Engineered TALE Transcription Factors

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Abstract

The presence of replication-competent HIV-1 –which resides mainly in resting CD4⁺ T cells–is a major hurdle to its eradication. While pharmacological approaches have been useful for inducing the expression of this latent population of virus, they have been unable to purge HIV-1 from all its reservoirs. Additionally, many of these strategies have been associated with adverse effects, underscoring the need for alternative approaches capable of reactivating viral expression. Here we show that engineered transcriptional modulators based on customizable transcription activator-like effector (TALE) proteins can induce gene expression from the HIV-1 long terminal repeat promoter, and that combinations of TALE transcription factors can synergistically reactivate latent viral expression in cell line models of HIV-1 latency. We further show that complementing TALE transcription factors with Vorinostat, a histone deacetylase inhibitor, enhances HIV-1 expression in latency models. Collectively, these findings demonstrate that TALE transcription factors are a potentially effective alternative to current pharmacological routes for reactivating latent virus and that combining synthetic transcriptional activators with histone deacetylase inhibitors could lead to the development of improved therapies for latent HIV-1 infection.

Introduction

Over the past two decades, numerous advances in the treatment of HIV/AIDS have significantly increased the lifespan—and quality of life—of individuals infected with HIV type 1 (HIV-1). Highly active antiretroviral therapy (HAART), in particular, has emerged as a powerful treatment option, capable of decreasing plasma viral loads to below the limit of detection of many clinical assays [1–3]. Yet despite its effectiveness, HAART does not cure patients of HIV-1 infection, due to the existence of residual latent and replication-competent virus hidden in

cellular reservoirs [4–8]. This population of cells, which consists mainly in resting memory CD4⁺ T cells, harbors integrated proviral DNA that re-emerges shortly after discontinuation of HAART. HIV-1 latency is typically established when activated CD4⁺ T cells become infected with the virus and revert back to a resting memory state [8]. These cells are thus non-permissive for viral gene expression and refractory to many treatments, including HAART. Although the mechanisms behind latency are complex [8,9], they likely involve: (i) the absence of key host transcription factors that drive transcriptional initiation [10,11] or elongation [12,13] in resting CD4⁺ T cells; (ii) low levels of the trans-activator of transcription (Tat) regulatory protein [14]; (iii) proviral integration into condensed chromatin regions [15,16] or expressed regions that become silenced by promoter occlusion [17–20]; and (iv) the induction of epigenetic modifications that can inhibit viral gene expression, including DNA methylation [21,22] and histone deacetylation [23].

Because the presence of latent HIV-1 represents an enormous barrier toward its eradication, numerous strategies have been developed to purge it from its cellular reservoirs. Chief among these has been activation of latently infected T cells via treatment with cytokines [24] or monoclonal antibodies [25], as well as NF- κ B stimulation via protein kinase C agonists [26,27]. Histone deacetylase (HDAC) inhibitors, such as valproic acid [28] and Vorinostat [29], have also proven capable of inducing viral gene expression by disrupting recruitment of HDAC proteins to the HIV long terminal repeat (LTR) promoter [30,31]. These approaches, however, have been unable to eradicate virus from all latent pools and have even been associated with adverse effects, including severe immune reactions [32–35]. As a result, new strategies capable of inducing viral gene expression are needed to enable the development of next-generation HIV-1 therapeutics.

The emergence of customizable DNA-binding platforms, including engineered zinc-finger [36] and transcription activator-like effector (TALE) [37] proteins, as well as CRISPR-Cas9 [38], has provided investigators with a set of tools capable of sequence-specific DNA recognition [39]. TALE proteins, in particular, have now been utilized to create a broad range of tools capable of gene modification and regulation, including transcriptional activators [40,41] and repressors [42], nucleases [40,43,44], site-specific recombinases [45] and epigenetic effectors [46–48]. The DNA binding domain of a TALE protein consists of a series of repeat domains, each ~34 amino acid residues in length, that coordinate to recognize a single base pair (bp) via two adjacent amino acid residues, termed repeat variable diresidues (RVDs) [49,50]. A variety of approaches have now been developed that enable rapid construction of custom TALE arrays capable of recognizing nearly any contiguous sequence [51,52]. As a result, TALEs have achieved widespread use throughout biotechnology, with the potential to impact future developments in human gene therapy.

Numerous studies have also demonstrated the utility of genome engineering for combating HIV/AIDS. Specifically, zinc-finger based transcriptional repressors [53–56], in addition to RNA interference [57–59], have proven effective at inhibiting HIV replication. Targeted nucleases have also demonstrated the capacity to excise integrated proviral DNA from infected cells [60–62] and confer HIV resistance to cells by inducing knockout of the primary co-receptors for HIV infection [44,63–65]. Targeted gene regulation technologies may also prove effective at reversing HIV-1 latency. Specifically, due to their versatility and ability to stimulate robust levels of gene expression in a highly specific manner [66], TALE transcription factors [40] could be used to stimulate viral gene expression within latent HIV-1 reservoirs, providing new means for enabling “shock and kill” therapy. Here we demonstrate that TALE transcription factors can be engineered to recognize the HIV-1 LTR promoter and induce viral gene expression in cell line models of HIV-1 latency. We also show that complementing TALE transcription factors with HDAC inhibitors can further enhance TALE-induced activation of latent HIV-1 expression. These findings indicate that TALE transcription factors are potentially effective

tools for reactivating latent virus and could contribute to the development of next-generation HIV-1 therapies.

Materials and Methods

Plasmid construction

The HIV-1 pNL4-3 plasmid [67] was obtained from the NIH AIDS Research and Reference Reagent Program. pTat expression plasmid was kindly provided by Dr. Maryanne Simurda (State University of New York, Buffalo). TALEs were generated as previously described [51,68] using the Golden Gate TALEN and TAL Effector Kit 2.0 (Addgene ID: 100000024) [52]. TALE arrays were cloned into the BsmBI restriction site of pcDNANT-T-VP64 [69] to generate pTLT-1 through 10. The pTALE-TF reporter vectors were constructed through PCR by amplifying the luciferase gene from pGL3-Basic (Promega, Madison, WI, USA) using the primers 5' TALE-Luc-TLT1 through 10, which contained four direct repeats of each TALE binding site and 3' Luc-Rev. PCR products were digested and cloned into the XhoI and SphI restriction sites of pGL3-Basic to generate pGL3-TLT-1 through 10. The HIV-1 LTR reporter plasmid was constructed by PCR amplifying the U3-R region of the LTR promoter from pNL4-3 using the primers 5' LTR-Fwd and 3' LTR-Rev. PCR product was digested and cloned into the XhoI and SphI restriction sites of pGL3-Basic to generate pGL3-LTR. Correct construction of each plasmid was verified by sequence analysis (S1 Table). Primer sequences are provided in S2 Table.

Cell culture

Human embryonic kidney 293T (HEK293T) (American Type Culture Collection; ATCC) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 2 mM L-glutamine and 1% (v/v) antibiotic-antimycotic (Anti-Anti; Gibco, Carlsbad, CA, USA). J-Lat clones (NIH AIDS Reagents) were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 1% (v/v) Anti-Anti. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Luciferase assays

Luciferase assays were performed as previously described [68]. Briefly, HEK293T cells were seeded onto 96-well plates at a density of 4×10^4 cells per well. At 16–24 h after seeding, cells were transfected with 200 ng of pTLT-1 through pTLT-10, 5 ng of pGL3-TLT-1 through pGL3-TLT-10 and 1 ng of pRL-CMV (Promega, Madison, WI, USA) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. At 48 h after transfection, cells were washed once with Dulbecco's PBS (DPBS; Life Technologies, Carlsbad, CA, USA) and lysed with Passive Lysis Buffer (Promega, Madison, WI, USA). Luciferase expression was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) using a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA) according to the manufacturer's instructions. Normalized luciferase activity was determined by dividing firefly luciferase activity by Renilla luciferase activity.

Western blots

HEK293T cells were seeded onto a 6-well plate at a density of 5×10^4 cells per well. At 16–24 h after seeding, cells were transfected with 5 µg of pTLT-1 through pTLT-10 or pcDNA backbone vector by the calcium phosphate method [70]. At 48 h after transfection, cells were harvested and lysed with RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium

deoxycholate and 0.1% SDS, pH 7.6) supplemented with EDTA-free Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland). The Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) was used to determine protein concentration according to the manufacturer's instructions. TALE transcription factor expression was analyzed by 4–12% SDS-PAGE (National Diagnostics, Atlanta, GA, USA). Samples were transferred onto a 0.2 μm nitrocellulose membrane as described [71] and detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) and Amersham HyperfilmECL (GE Healthcare, Little Chalfont, UK) chemiluminescence film. TALE transcription factors were detected by horseradish peroxidase-conjugated anti-HA monoclonal antibody (Roche, Basel, Switzerland). β -actin was used as an internal control and detected using a mouse anti- β -actin monoclonal antibody (Sigma, St. Louis, MO, USA) and horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Bio-Rad, Hercules, CA, USA) (kindly provided by Dr. Cecilia Rodrigues).

HIV-1 reactivation

J-Lat cells were seeded onto a 10 cm dish at a density of 1×10^5 cells per mL. At 48 h after seeding, 2×10^5 cells per transfection were centrifuged at $100 \times g$ for 10 min at room temperature and resuspended in Nucleofector Solution SE (Lonza, Basel, Switzerland) with 2 μg of pTLT-1 through pTLT-10 or pTat. Cells were transferred to 16-well Nucleocuvette Strips (Lonza, Basel, Switzerland) and electroporated by a 4D-Nucleofector System (Lonza, Basel, Switzerland) using the program CL-120, according to the manufacturer's instructions. J-Lat cells were either left untreated or incubated with 10 ng/ μL of TNF- α (R&D Systems, Minneapolis, MN, Canada). At 48 h after transfection, cells were washed twice with DPBS (Life Technologies, Carlsbad, CA, USA) and GFP expression was evaluated by flow cytometry (BD LSR II Flow Cytometer System; BD Biosciences, Franklin Lakes, NJ, USA). For each sample, 10,000 live events were collected, and data was analyzed using FlowJo (Tree Star, Inc., San Carlos, CA, USA).

HDAC inhibitor treatments

J-Lat cells were seeded onto a 10 cm dish at a density of 2×10^5 cells per mL. At 48 h after seeding, 1×10^6 cells per transfection were centrifuged at $100 \times g$ for 10 min at room temperature and resuspended in Nucleofector Solution V (Lonza, Basel, Switzerland) with 4 μg of pTLT-5 through pTLT-8. Cells were transferred to a Nucleocuvette (Lonza, Basel, Switzerland) and electroporated with an Amaxa Nucleofector II Device (Lonza, Basel, Switzerland) using the program X-001 according to the manufacturer's instructions. At 24 h after transfection, J-Lat cells were treated with DMSO 0.1% or 330 nM, 660 nM or 1 μM of SAHA (NIH AIDS Reagents) [72] for 24 h. After treatment, cells were washed twice with DPBS and GFP expression was evaluated by flow cytometry analysis (BD LSR II Flow Cytometer System; BD Biosciences, Franklin Lakes, NJ, USA). For each sample, 10,000 live events were collected, and data was analyzed using FlowJo (Tree Star, Inc., San Carlos, CA, USA).

Statistical analysis

Statistical analyses for all experiments were performed from three independent experimental replicates ($n = 3$) unless otherwise indicated. Two-tailed Student's t -test was used for paired and unpaired samples (Prism Software 5.0, GraphPad Software).

Results

Designing TALE transcription factors to target the HIV-1 promoter

We sought to reverse HIV-1 latency by inducing viral gene expression using engineered TALE transcription factors. We constructed ten TALE proteins designed to recognize distinct 16-bp

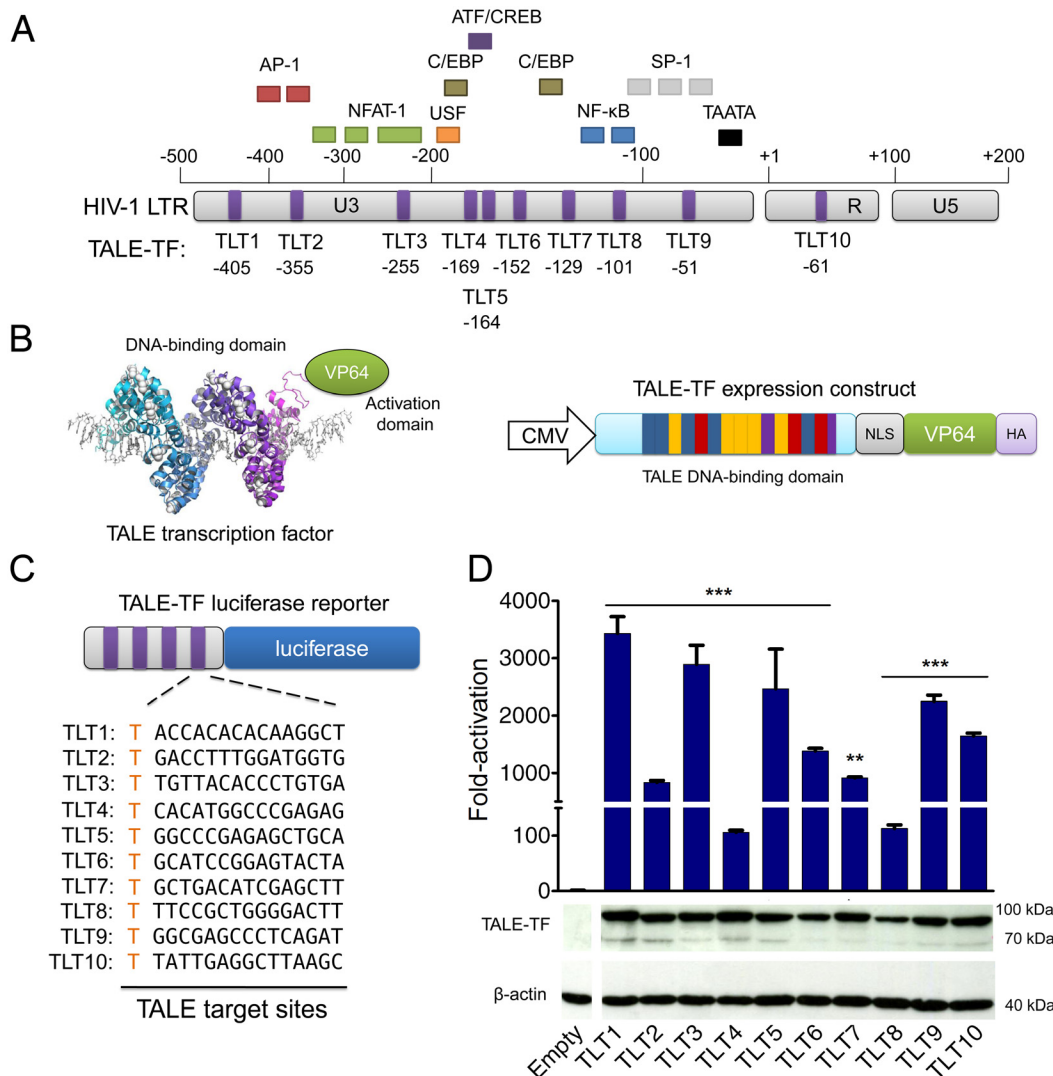


Fig 1. TALE transcription factors (TALE-TFs) designed to target the HIV-1 LTR promoter. (A) Schematic representation of the TALE transcription activator (TLT) binding sites within the HIV-1 long terminal repeat (LTR) promoter relative to the transcriptional start site (TSS) and main endogenous transcription factor binding sites. (B) (Left) Cartoon illustrating the structure of a TALE-TF, adapted from [109]. TALE repeats are colored cyan and purple, DNA shown as grey sticks. (Right) Schematic representation of the TLT expression construct used in this study. CMV indicates the cytomegalovirus promoter, TALE repeats are shown as individual bars (16 repeats total), VP64 denotes the tetrameric repeat of the herpes simplex virus VP16 transactivation domain, NLS stands for the nuclear localization signal derived from the simian virus (SV40) and HA indicates the hemagglutinin A tag. (C) Schematic representation of the luciferase reporter system containing four direct repeats of the TALE target sites for each TALE activator. Each TALE target site is shown. (D) (Top) Fold-activation of luciferase expression after co-transfection of TALE-TFs with luciferase reporter plasmid into HEK293T cells. Luciferase expression was normalized to cells transfected with reporter plasmid only. *Renilla* luciferase expression was used to normalize for transfection efficiency and cell number. Error bars indicate standard deviation of one experiment with three transfection replicates ($n = 3$; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; *t*-test sample vs control (4x TALE binding site vector only)). (Bottom) Western blot of lysate from HEK293T cells transfected with TALE-TFs. Samples were taken 48 h after transfection and probed with horseradish peroxidase-conjugated anti-HA and anti-β-actin (loading control) antibodies. Empty indicates lysate from HEK293T cells transfected with empty pcDNA vector only.

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sites within the HIV-1 LTR, the region of the virus that serves as its promoter (Fig 1A). TALE binding sites were constrained only by the presence of a 5' thymidine (T₀) nucleotide [69]. We fused each synthetic TALE array to VP64 [73], a tetrameric repeat of the herpes simplex virus VP16 transactivation domain, to generate synthetic transcriptional activators. VP64 is a widely used transactivation domain [47,74–79] capable of recruiting host cellular transcription factors

to targeted genomic loci [80,81], but does not activate gene expression alone [73]. Each TALE transcription factor contained a C-terminal hemagglutinin (HA) tag and an internal nuclear localization signal (NLS) sequence between the DNA binding and transactivation domains (Fig 1A). The amino acid sequence of each protein is presented in S1 Table.

In order to determine whether each TALE could recognize its intended target site and induce gene expression, we adapted a previously described transient reporter assay [68] that correlates TALE-induced gene activation with increased luciferase expression. We inserted four direct repeats of each LTR binding site upstream of a luciferase reporter gene and co-transfected human embryonic kidney (HEK) 293T cells with reporter plasmid and expression vectors for each TALE activator (Fig 1C and 1D). This strategy was undertaken in order to increase reporter gene expression and more accurately evaluate TALE activity. Eight of the ten TALE activators (all but TLT4 and TLT8) induced a >800-fold increase in luciferase expression, with TLT1 (~3,400-fold), TLT3 (~2,900-fold), TLT7 (~2,500-fold) and TLT9 (~2,200-fold) inducing the highest levels of activation ($p < 0.001$) (Fig 1D). TLT4 and TLT8 achieved similarly high levels of absolute luciferase activity, but induced a modest ~100-fold increase in activation over mock-transfected cells. Even in the absence of a TALE activator, transfection of the TLT4 and TLT8 reporter plasmids led to a significant increase in luciferase expression ($p < 0.001$) (data not shown). Not surprisingly, however, the binding sites for TLT4 and TLT8 overlap with those recognized by the endogenous transcription factors C/EBP and NF- κ B [82] (Fig 1A), respectively, indicating that native proteins could have been contributing to reporter gene activation. Compared to reporter plasmid only though, increased luciferase expression was evident after co-transfection with the specific TALE activator, indicating that TALEs have the potential to outcompete endogenous transcription factors for LTR binding sites.

Western blot analysis of HEK293T lysates also revealed that each TALE activator was expressed (Fig 1D). Low levels of a non-specific band (~70 KDa), however, were detected in several samples, possibly due to translation of a second open-reading frame present within the TALE mRNA transcript or recombination within the TALE DNA-binding domain, a phenomenon that can occur within a highly repetitive motif [83].

TALE transcription factors activate gene expression from the HIV LTR

We next set out to test the ability of each TALE activator to stimulate transcription from the full-length U3 and R regions of the HIV-1 LTR using an episomal reporter assay. The U3-R regions of the LTR, in particular, contain the core promoter, enhancer and modulatory region, and regulate viral expression. Notably, unlike the transient reporter assay described above, which asked whether each TALE protein could bind its intended DNA target, this analysis aimed to evaluate the ability of each TALE activator to stimulate transcription from the full-length HIV-1 promoter.

HEK293T cells were co-transfected with TALE activator and a reporter vector that contained the sequence between -455 and +96 from the LTR transcriptional start site (TSS) upstream of a luciferase reporter gene (Fig 2). We separately co-transfected an expression vector encoding the HIV-1 Tat protein as a positive control. Multiple activators, including TLT4, 5, 6, 7 and 8, induced a 7.5- to 14-fold increase in luciferase activity ($p < 0.01$), while Tat yielded only a ~7-fold increase in activation (Fig 2), likely because it stimulates transcriptional elongation more efficiently than initiation [84].

Previous reports have demonstrated that co-delivery of combinations of TALE transcription factors can lead to a synergistic increase in gene expression via cooperative effects that could mimic those associated with natural transcriptional processes [77,78]. The most potent TALE

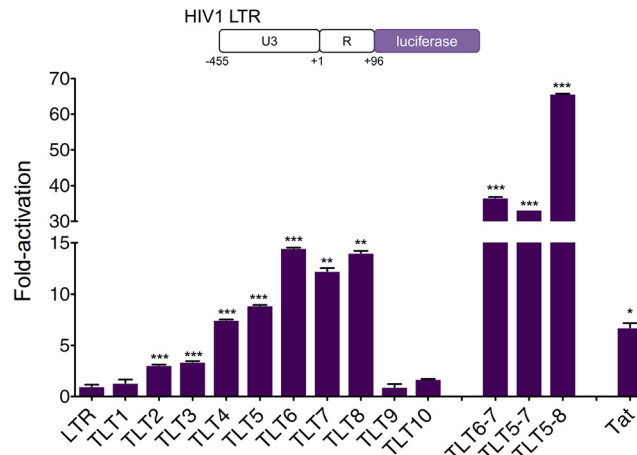


Fig 2. TALE-TF-mediated gene activation from the HIV-1 LTR promoter. (Top) Schematic representation of the luciferase reporter system used to evaluate TALE-TF activity from the HIV-1 LTR promoter. The U3 and R regions of the HIV LTR were placed upstream of the luciferase reporter. (Bottom) Fold-activation of luciferase expression in HEK293T cells co-transfected with reporter plasmid and TALE-TF or Tat expression vectors. Luciferase expression was normalized to cells transfected with reporter plasmid only. Error bars indicate standard deviation of one experiment with three transfection replicates ($n = 3$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; t-test sample vs. control (TLTNT)).

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activators, TLT5, 6, 7 and 8, were designed to recognize a small region of the LTR between -170 and -100 bp from the TSS (Fig 1A). Indeed, sequence analysis of different strains of HIV-1 subtype B (i.e. the most predominant subtype across Europe, America, Australia and Japan) revealed that the binding sites for these TALEs are generally well conserved (S3 Table). We thus co-transfected HEK293T cells with LTR reporter plasmid and different combinations of TALE activators to test whether these proteins could be used in tandem to further enhance gene expression. Increased gene activation was observed for each set tested, most notably with a ~70-fold increase in luciferase expression after co-transfection with TLT5, 6, 7 and 8 (hereafter referred to as TLT5-8) ($p < 0.001$) (Fig 2). Overall, these data demonstrate that TALE transcription factors designed to target the U3 and R regions of the HIV LTR promoter can induce efficient gene activation.

Reactivation of latent HIV-1 by TALE transcription factors

We next asked whether TALE transcription factors could reactivate viral expression in a cell line model of HIV-1 latency. To explore this, we used the Jurkat-derived J-Lat lymphocytic cell lines, which harbor a full-length integrated HIV-1 proviral genome containing a GFP gene that serves as a reporter for viral gene expression (HIV1- Δ Env-GFP) (Fig 3A). J-Lat cells poorly express the integrated proviruses under normal conditions, but viral gene expression can be efficiently induced by stimulation using tumor necrosis factor (TNF)- α [16]. Since each J-Lat clone is derived from a unique HIV-1 integration event, they display differential levels of gene and/or chromatin repression, as demonstrated by their distinct gene activation thresholds after TNF- α stimulation [85].

We nucleofected J-Lat 10.6 cells, which display a low viral gene activation threshold, with expression vectors encoding TALE transcription factors or Tat and evaluated HIV-1 expression by measuring the percentage of GFP-positive cells by flow cytometry (Fig 3A). As expected, cells treated with TNF- α or transfected with Tat showed robust reactivation, with upwards of 55% and 75% of cells producing GFP, respectively. Among all individual TALE

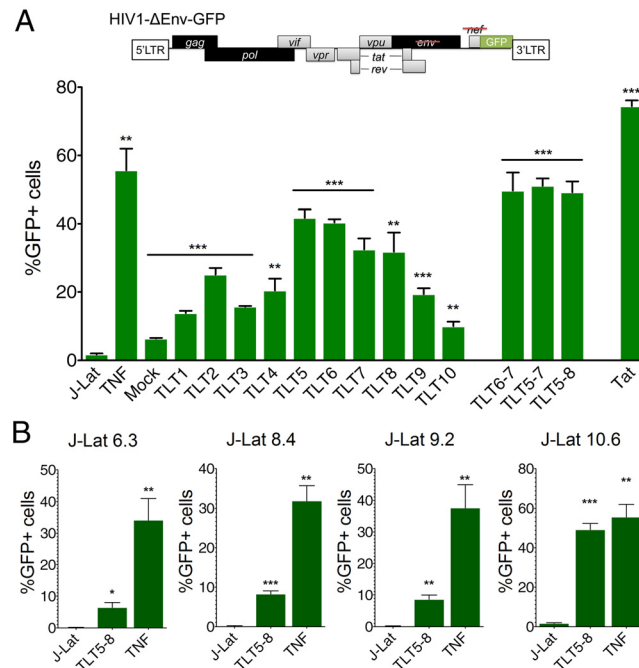


Fig 3. Reactivation of latent HIV-1 expression by TALE-TFs in multiple cell line models of HIV-1 latency. (A) (Top) Schematic representation of the HIV-1 proviral genome present in J-Lat cells. Full-length HIV-1 was derived from the molecular clone pNL4-3-ΔEnv-GFP and expresses a GFP gene from the LTR promoter. Structural viral genes are shown in black, auxiliary genes are shown in grey. The *nef* and *env* genes were inactivated to force a single infection cycle. (Bottom) Percentage of GFP positive J-Lat 10.6 cells after nucleofection with TALE-TF and Tat expression plasmids, or treatment with TNF-α (10 ng/μL). GFP positive cells were measured by flow cytometry 48 h after nucleofection. “J-Lat” indicates non-transfected J-Lat 10.6 cells. “Mock” indicates cells transfected with an empty pcDNA backbone. Error bars indicate standard deviation of three independent experiments (n = 3). (B) Percentage of GFP positive J-Lat 6.3, 8.4 and 9.2 cells after nucleofection with TLT5-8 and Tat expression plasmids, or treatment with TNF-α (10 ng/μL). GFP positive cells were measured by flow cytometry 48 h after nucleofection. “J-Lat” indicates non-nucleofected cells. Error bars indicate standard deviation (n = 3; *p < 0.05; **p < 0.01; ***p < 0.001; t-test sample vs control (J-Lat)).

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transcription factors tested, TLT5 and 6 (~40% GFP-positive cells each) and TLT7 and 8 (~30% GFP-positive cells each) yielded the highest levels of expression, with cells transfected with TLT5-8 also showing upwards of 50% GFP-positive cells ($p < 0.05$) (Fig 3A). Nucleofection of an empty vector (pcDNA) resulted in minor (~5%) reactivation, indicating that stress from the nucleofection process can also contribute to reactivation (Fig 3A).

The relative potencies of the TALE activators in J-Lat 10.6 cells correlated with their ability to stimulate transcription in the reporter assay used in Fig 2. Analysis of mean fluorescence intensity (MFI) in transfected J-Lat 10.6 cells further indicated that each TALE activator induced approximately a 10 to 15-fold increase in viral gene expression (S1A Fig). Interestingly, in contrast to the episomal studies presented in Fig 2, TLT5-8 yielded a similar number of GFP positive cells as the combinations TLT6-7 and TLT5-7 (Fig 3A). One possible explanation for this is that measuring the number of GFP positive J-Lat 10.6 cells may not necessarily afford the amount of sensitivity needed to distinguish between the potencies of specific combinations of activators. Indeed, analysis of MFI in J-Lat 10.6 cells revealed that TLT5-8 induced higher amounts of viral gene expression (~20-fold compared to the negative control) than TLT7 or the combinations TLT6-7 and TLT5-7 (~14-fold compared to the negative control) (S1B Fig).

To test the versatility of the TALE transcription factors, we next evaluated their ability to induce HIV-1 transcription in the J-Lat clones 6.3, 8.4 and 9.2, which each possess a higher gene activation threshold than J-Lat 10.6 cells [16]. Sequence mapping of these clones previously revealed that the HIV-1 provirus is integrated into actively transcribed genes, unfavorable to HIV transcription [18,86]. We observed significant reactivation in each cell line tested after co-transfection with TLT5-8 ($p < 0.05$) but at rates much lower than by TNF- α stimulation (Fig 3B), indicating that the level of repression within latently-infected cells can influence the ability of TALEs to mediate activation.

Combining TALE transcription factors with an HDAC inhibitor enhances latent HIV activation

We next explored the possibility of enhancing HIV-1 reactivation by combining TALE transcription factors with a histone deacetylase (HDAC) inhibitor. Because HIV-1 proviral integration in J-Lat clones favors heterochromatic regions, especially those near aliphoid DNA repeat elements [16], we hypothesized that chromatin remodeling by HDAC inhibition could enhance TALE binding to the HIV-1 LTR, thereby increasing viral gene expression. Indeed, previous reports have indicated that the LTR promoter is typically hypoacetylated and that treatment with HDAC inhibitors can lead to the recruitment of the transcriptional machinery to the HIV-1 promoter [23], as well as activation of the positive transcription elongation factor b (P-TEFb), which can stimulate viral transcriptional elongation [87]. Moreover, multiple studies have shown that combining HDAC inhibitors with other compounds also capable of reversing HIV latency can synergistically increase viral reactivation across a variety of repression states [88–91].

We transfected J-Lat 10.6 and 6.3 cells, which each display distinct activation thresholds, with TLT5-8 and treated each population with 0.33, 0.66 or 1.0 μM of Vorinostat (i.e., suberoylanilide hydroxamic acid or SAHA), an HDAC inhibitor used for the treatment of malignant cancers but also capable of inducing expression of latent HIV-1 [29–31,92,93]. An initial screen led us to identify this specific range of SAHA concentrations (i.e. those that stimulate minimal amounts of HIV-1 transcription and induce low cell death) (S2 Fig). SAHA, in particular, is an FDA-approved inhibitor of Class I HDAC isotypes that has been shown to induce viral transcription in latent CD4⁺ T cells from HIV-infected patients [30,31] (though it was unable to increase HIV-1 production [30]). Compared to cells transfected with TLT5-8 only, we observed a significant increase ($p < 0.05$) in HIV-1 expression upon co-treatment with 1 μM SAHA (Fig 4). Specifically, reactivation was evident in up 65% and 15% of J-Lat 10.6 and 6.3 cells, respectively, corresponding to a 1.5- and 2-fold increase in HIV-1 transcription (Fig 4). We also observed increased levels of HIV-1 transcription in J-Lat 8.4 and 9.2 cells co-treated with SAHA and TLT5-8, but these values were neither significant nor dose-dependent (data now shown). Analysis of MFI in treated J-Lat 10.6 cells also revealed a significant and dose-dependent increase in viral gene expression after co-treatment with TLT5-8 and SAHA (S3 Fig). Collectively, these results demonstrate that complementing TALE transcription factors with HDAC inhibitors can lead to enhanced reactivation of latent HIV-1 expression.

Discussion

HIV-1 latency is a substantial obstacle facing its eradication. Many approaches have been developed to indirectly activate HIV-1 from persistent cellular reservoirs, typically by altering the transcriptional landscape surrounding the integrated provirus [24–31]. While promising, these strategies have been unable to completely purge all virus from the reservoir and, in some cases, have even been associated with adverse effects, including immune reactions [32–35].

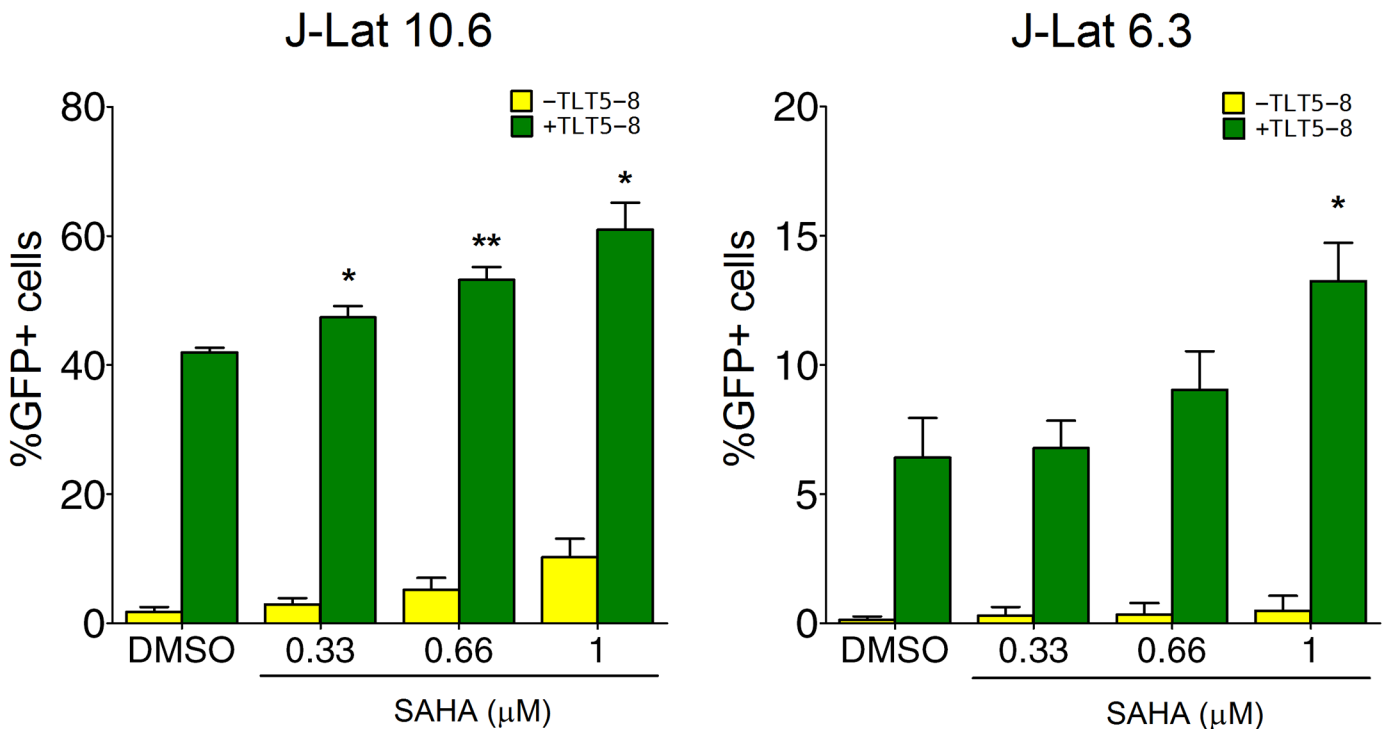


Fig 4. Enhanced reactivation of latent HIV-1 expression by combining TALE-TFs with a histone deacetylase inhibitor. Percentage of GFP-positive J-Lat 10.6 and 6.3 cells after nucleofection with TLT5-8 expression plasmids and treatment with increasing concentrations of SAHA or DMSO (0.1%) for 24 h. GFP-positive cells were measured by flow cytometry 48 h after nucleofection. Error bars indicate standard error of the mean of three independent experiments ($n = 3$; * $p < 0.05$; ** $p < 0.01$; t -test).

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While targeted nucleases and recombinases have the capacity to excise integrated proviral DNA from infected cells [60–62,94], these tools also have the potential to induce unwanted non-specific DNA breaks, and thus carry a substantial risk of genotoxicity [39]. Conversely, synthetic transcription factors, which can be designed to induce transcription from the native viral promoter, represent a potentially safe and effective genetic alternative for reactivating latent virus in cells.

Here, we designed ten TALE proteins that spanned nearly the entire length of the HIV-1 LTR promoter in order to create activators capable of stimulating HIV-1 transcription. We identified four proteins (TLT5, TLT6, TLT7 and TLT8) that induced viral gene expression in cell line models of HIV latency. Interestingly, we observed that the effectiveness of individual TALEs correlated with their proximity to the TSS, as they targeted a conserved segment of the HIV LTR modulatory region, located upstream of the NF- κ B and Sp1 *cis*-regulatory sites, and nearby regulatory elements that contribute to viral transcriptional initiation [82,95]. This data indicate that cooperation between endogenous transcription factors and engineered TALE activators may be an important factor for efficient reactivation of viral gene expression. These TALEs might thus promote transcription in a manner that mimics the natural activity of enhancer-like regulatory proteins, potentially serving as “molecular switches” for reactivation.

We showed that co-transfection of combinations of TALE transcription factors can further increase gene expression, indicating that strategies for mimicking the natural complexity of gene regulation [77,78] are also effective for inducing viral gene expression. Specifically, co-

transfection of J-Lat 10.6 cells with TLT5-8 led to similar amounts of HIV-1 expression as those previously reported for compounds such as phytohemagglutinin (PHA), phorbolmyristate acetate (PMA) and prostratin [96]. Moreover, the specificity of our TALE activators could be improved in the future by incorporating recently described chemical- [68,97] or light-inducible [47,79] features that enable spatial and temporal control of HIV-1 expression.

Two reports initially demonstrated that engineered zinc-finger and TALE transcription factors can induce latent HIV expression, albeit with relatively modest efficiencies [98,99]. More recently, several other studies have shown that TALE [100] and CRISPR-Cas9 [101–103] activators can induce robust activation of latent viral expression. Our data correlates with these most recent reports and further indicates the potential of artificial transcription factor technology for eliminating the latent reservoir.

Although the TALE activators used in our study induced efficient HIV-1 expression in J-Lat 10.6 cells, reduced levels of viral gene expression were observed in J-Lat clones containing more repressive transcriptional backgrounds. Specifically, integration of the HIV-1 provirus into condensed regions of heterochromatin can negatively affect viral gene expression by hindering DNA accessibility to key host transcription factors [8,9]. Because histone deacetylases (HDACs) play a central role in maintaining HIV latency by promoting compact chromatin structures around integrated proviral DNA [8], we hypothesized that treating cells with HDAC inhibitors could increase binding site accessibility and further enhance HIV-1 reactivation. We found that combining TALE transcription factors with SAHA led to a significant increase in viral gene expression in J-Lat 6.3 and 10.6 cells comparable to those previously described for other latency-reversing compounds, including PHA and Bryostatins [96]. This indicates the broad utility of this concept for HIV-1 reactivation, and supports further investigation into the effects of combining HDAC inhibition with TALE-mediated activation in primary cell models of HIV latency [96]. Combining synthetic transcription factors and HDAC inhibition with HAART or nuclease-induced knockout of HIV co-receptors CCR5 and CXCR4 may also prove effective for combating HIV infection.

While TALE transcription factors have the capacity to activate latent HIV-1 transcription, several barriers must be overcome in order for this technology to be implemented for therapeutic purposes. In particular, due to their highly repetitive nature, lentiviral vector-mediated delivery of TALEs into cells has proven challenging [39,104,105]. Methods for overcoming this limitation are rapidly emerging, including those based on adenoviral [106,107], mRNA [108] and protein-based delivery systems [109,110]. Additionally, recent work has indicated that TALE nucleases could be introduced into cells as mRNA using lentivirus particles containing inactivated reverse transcriptase [111]. However, it remains unknown whether such systems can support *in vivo* delivery to latent resting CD4⁺ T cells. Although our current study indicates that a specific combination of four TALE activators is optimal for inducing HIV-1 transcriptional activation, current evidence demonstrates that the potency of these activators can be further enhanced to promote single TALE systems [112].

In summary, we demonstrate that TALE transcription factors are effective tools for activating latent HIV expression and their use, alone or in combination with HDAC inhibitors, could pave the way for improved HIV therapies.

Supporting Information

S1 Fig. Analysis of GFP mean fluorescence intensity in J-Lat 10.6 cells nucleofected with TALE-TFs. Mean fluorescence intensity (MFI) of GFP expression in J-Lat 10.6 cells after nucleofection with a (A) single TALE activator (TLT5, TLT6, TLT7, and TLT8) or (B) a combination of TALE activators (TLT7, TLT6-7, TLT5-7, and TLT5-8). MFI was measured by flow

cytometry 48 h after nucleofection. “J-Lat” indicates non-transfected J-Lat 10.6 cells. Histograms are representative of a single experiment from three independent replicates.
(TIF)

S2 Fig. Cell viability of J-Lat 10.6 cells treated with SAHA. Cell viability and viral gene expression profiles of J-Lat 10.6 cells treated with increasing concentrations of SAHA or DMSO after 24 only. Cell viability was measured as percentage of viable cells as determined by forward and side scatter (FSS/SCC) gating during flow cytometry. Viral gene expression was determined by measuring the percentage of GFP positive cells by flow cytometry.
(TIF)

S3 Fig. SAHA increases mean fluorescence intensity in J-Lat cells nucleofected with combinations of TALE transcription factors. Mean fluorescence intensity (MFI) of GFP expression in J-Lat 10.6 cells nucleofected with TALE-TF and co-treated with SAHA. J-Lat 10.6 cells were nucleofected with TLT5-8 expression plasmids and treated with increasing concentrations of SAHA or DMSO only for 24 h. MFI was measured by flow cytometry 48 h after nucleofection. Histograms are representative of a single experiment from three independent replicates.
(TIF)

S1 Table. TALE proteins sequences used in this study. TALE N-terminal domain is colored orange. TALE DNA-binding domain is colored blue. RVD residues are shown in red. Nuclear localization signal (NLS) sequence is highlighted grey. VP64 domain is colored green. HA tag is colored purple.
(DOCX)

S2 Table. Primer sequences for the construction of the luciferase reporter plasmids used in this study. TALE binding sites are underlined. Restriction sites are in bold.
(DOCX)

S3 Table. Sequence conservation of the TALE transcription factor binding sites across HIV-1 subtype B strains. Data based on 2014 edition of the HIV Sequence Database (<http://hiv-web.janl.gov>). Dashes indicate sequence identity between subtype strains. Dots indicate gaps in the HIV genome sequence.
(DOCX)

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Dedicated to the memory of Carlos F. Barbas III.

Author Contributions

Conceived and designed the experiments: PP TG CFB MSM JG. Performed the experiments: PP. Analyzed the data: PP TG MSM CFB JG. Contributed reagents/materials/analysis tools: MSM JG CFB. Wrote the paper: PP TG MSM JG.

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