Molecular Therapy
Methods & Clinical Development
Original Article

Protein Delivery of Cell-Penetrating Zinc-Finger Activators Stimulates Latent HIV-1-Infected Cells

Pedro R.L. Perdigão,[1,2,3] Catarina Cunha-Santos,[1] Carlos F. Barbas III,[2,4] Mariana Santa-Marta,[1] and Joao Goncalves[1]

[1]Molecular Microbiology and Biotechnology Department, Research Institute for Medicines (iMed ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal; [2]Department of Chemistry, Department of Cell and Molecular Biology, The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA, USA

Despite efforts to develop effective treatments for eradicating HIV-1, a cure has not yet been achieved. Whereas antiretroviral drugs target an actively replicating virus, latent, nonreplicative forms persist during treatment. Pharmacological strategies that reactivate latent HIV-1 and expose cellular reservoirs to antiretroviral therapy and the host immune system have, so far, been unsuccessful, often triggering severe side effects, mainly due to systemic immune activation. Here, we present an alternative approach for stimulating latent HIV-1 expression via direct protein delivery of cell-penetrating zinc-finger activators (ZFAs). Cys_2-His_2 zinc-fingers, fused to a transcription activation domain, were engineered to recognize the HIV-1 promoter and induce targeted viral transcription. Following conjugation with multiple positively charged nuclear localization signal (NLS) repeats, protein delivery of a single ZFA (3NLS-PBS1-VP64) efficiently internalized HIV-1 latently infected T-lymphocytes and specifically stimulated viral expression. We show that short-term treatment with this ZFA protein induces higher levels of viral reactivation in cell line models of HIV-1 latency than those observed with gene delivery. Our work establishes protein delivery of ZFA as a novel and safe approach toward eradication of HIV-1 reservoirs.

INTRODUCTION

The HIV-1 pandemic remains a major public health concern. Antiretroviral drugs targeting key steps of the HIV-1 replicative cycle have improved the lifespan of infected patients. However, these do not provide a cure to HIV infection that requires continuous administration of drugs associated with severe side effects.[1] The inability of antiretroviral therapy to eradicate HIV is due, at least in part, to the existence of latent reservoirs, mostly found in resting cluster of differentiation 4^+ ($CD4^+$) T lymphocytes harboring a transcriptionally silent integrated provirus that persists in patients and reemerges if treatment is discontinued.[2] Latent reservoirs escape antiretroviral action and host immune response due to the lack of viral replication, representing a major barrier toward a cure for HIV infection.[3]

With the consideration of the underpinning mechanisms of HIV latency, a "shock-and-kill" strategy has been proposed aiming to activate latent HIV expression and expose the virus reservoir to the host immune response or to the HIV cell-specific cytopathic effects. The main route explored up to date to reverse HIV latency is based on pharmacological drugs that trigger viral transcription by stimulating endogenous signaling pathways or removing epigenetic repression markers associated with the HIV provirus.[3] Most prominent candidates include protein kinase C (PKC) pathway agonists[4,5] and inhibitors of histone deacetylases (HDACs).[6,7] Despite evidence that these agents can induce latent viral expression, clinical studies evidenced that none of these treatments has been able to dissociate the reduction of latent reservoir in patients from nontolerable toxic effects.[3] For instance, PKC agonists cause a striking increase in HIV transcription from aviremic patients at the cost of triggering toxic inflammatory responses derived from an undesirable global immune activation. On the other hand, patients treated with HDAC inhibitors manifested minimal adverse effects, but the increase in viral expression was rather modest, and no significant reduction of the reservoir size was observed.[3] This highlights the need for alternative strategies that could directly induce latent HIV expression without depending on the modulation of endogenous pathways.

Despite the absence of clear biomarkers identifying HIV reservoirs, the presence of an integrated provirus constitutes a permanent genetic element that can be explored to develop targeted antiviral strategies. Programmable DNA-binding domains can be engineered to target specific sites across the human genome to enable precise sequence modification or modulate the transcriptional environment.[8]

Received 19 March 2020; accepted 19 May 2020;
https://doi.org/10.1016/j.omtm.2020.05.016.

[3]Present address: Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

[4]Deceased

Correspondence: Mariana Santa-Marta, Molecular Microbiology and Biotechnology Department Research Institute for Medicines (iMed ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal.
E-mail: mariana.santamarta@research.fchampalimaud.org
Correspondence: Joao Goncalves, Molecular Microbiology and Biotechnology Department Research Institute for Medicines (iMed ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal.
E-mail: jgoncalv@ff.ulisboa.pt

Most relevant DNA-binding domains are composed of zinc fingers,[9] transcription activator-like effectors (TALEs)[10] or the more recent CRISPR-Cas9 system.[11] Engineered zinc-finger domains, in particular, were the first to be implemented successfully for genome engineering applications.[12] The Cys_2-His_2 motif is among the most well-studied zinc-finger scaffolds and is composed of approximately 30 amino acids arranged in a ββα conformation. Particular residues within the α-helix (i.e., positions −1, 3, and 6) make specific base contact with base pair (bp) triplets and are therefore critical to determine specificity toward the major groove of the DNA sequence.[9] Target specificity can also be affected by interactions from adjacent zinc-finger modules within a DNA-binding array.[13] Engineered polydactyl zinc-finger proteins with investigator-defined specificity can be assembled using a number of different methods and fused to certain effector domains for either targeted transcriptional activation/repression or epigenetic modulation,[14] site-directed DNA cleavage,[15] or recombination.[16] Such zinc-finger-based tools have also enabled the development of targeted antiviral strategies for treating HIV infection. More specifically, site-directed zinc-finger nucleases have been explored to inhibit or prevent viral infection by eliminating the integrated provirus from infected cells following targeted mutagenesis[17,18] or by engineering resistance to HIV infection via disruption of the major HIV coreceptors,[19,20] the latter culminating in clinical studies.[21] Design of zinc fingers targeting the HIV promoter[22] has also proven effective at inhibiting HIV replication by directly shutting down HIV transcription[23–25] or interfering with viral integration.[26,27]

Given the silent nature of the latent HIV reservoir, targeted transcription activators have gathered interest as tools for reversing virus latency. Specifically, we[28] and others[29–36] have demonstrated the potential of site-directed artificial transcription factors to recognize the HIV promoter and stimulate latent virus expression, thereby providing a targeted shock-and-kill approach. Interestingly, zinc fingers possess an intrinsic ability to cross the anionic cellular membrane, likely owing to the positive net charge of these DNA-binding domains.[9] This cell-penetrating activity grants zinc fingers the potential to serve as protein-based therapeutics,[37] capable of inducing precise genome perturbations.[38–40] Herein, we describe a novel approach for reactivating latent HIV by the direct delivery of cell-penetrating zinc-finger activators (ZFAs). We show that ZFA proteins engineered to recognize the HIV promoter can directly enter latently infected cells as a protein transduction domain and specifically induce viral expression. Our work demonstrates that engineered ZFAs are promising tools for targeting latent HIV infection and that their innate cell-penetrating activity could overcome some of the limitations associated with delivering artificial transcription factors to cells.

RESULTS

Design of Zinc-Finger Transcription Factors Targeting the HIV-1 Promoter

We initially investigated whether synthetic ZFAs could specifically activate HIV expression. Based on the Carlos Barbas library of zinc-finger modules,[9] we designed ZFAs to target the HIV 5′ long-terminal repeat (5′ LTR) promoter. Building on our previous knowledge using TALE-based activators,[28] we designed zinc fingers to target a "hot spot" section of the 5′ LTR covering the 200 bp upstream of the transcription start site (TSS). This region was submitted to the Zinc Finger Tools web server—an automated zinc-finger design tool[41] based on an experimentally characterized database—to obtain the most promising zinc-finger candidates to target this particular segment. To enforce binding selectivity, we selected target sites enriched for GNN[42] and ANN[43]nucleotides —given the superior specificity of zinc-finger domains targeting these triplets[44]—and restricted, at most, to one CNN.[45] We designed ZFAs comprising four-finger (ZLT4A, ZLT4B, ZLT4C, and ZLT4D) and six-finger (ZLT6A and ZLT6B) domains (Figure 1A; Table 1). Whereas shorter four-finger (12-bp targets) domains may facilitate binding to the DNA major groove,[46] six-finger (18-bp targets) domains should confer genome-wide specificity.[47] For this study, we also explored zinc fingers (six-finger) previously designed to target the 5′ LTR (HLTR1, HLTR3, and HLTR6) or the tRNA primer binding site (PBS), adjacent to the 3′ end of the 5′ LTR promoter and required for initiation of viral retrotranscription (PBS1, PBS1a, and PBS3) (Figure 1A; Table 1). These zinc fingers were reported to inhibit HIV replication when fused to a transcription repressor domain.[24,25] Each ZFA construct is composed of the zinc-finger DNA-binding domain fused to the VP64 transactivation domain[48]—a tetrameric repeat of the herpes simplex virus VP16—that recruits the transcription initiation complex to induce gene expression when directed to its regulatory region.[49] We incorporated into each ZFA construct a C-terminal hemagglutinin (HA) tag for protein detection, as well as an internal simian vacuolating virus (SV40)-derived nuclear localization signal (NLS) sequence between the DNA-binding and transactivation domains (Figure 1B, top) The amino acid sequence of each protein is presented in Table S1. Western blot analysis of ZFA expression in human embryonic kidney 293T (HEK293T) cells showed expression of all four-finger and six-finger ZFAs designed. However, some ZFAs show a reduced level of expression, particularly ZLT6A, ZLT6B, and HLTR1 (Figure 1B, bottom). Variations in intracellular levels of ZFA proteins could also be partially a result of potential fluctuations in transfection efficiency of ZFA constructs. Nevertheless, this result indicates that ZFAs are expressed at different levels, anticipating possible differences in their intrinsic stability.

We evaluated the potential of each ZFA to specifically induce gene expression from their target sites using a luciferase (Luc) reporter assay in HEK293T cells that we previously employed to evaluate designer TALE activators.[28] This assay correlates luciferase activity with the ability of each ZFA to recognize its target and stimulate gene activity. We constructed Firefly luciferase reporter plasmids containing four direct repeats of each ZFA binding site to drive gene expression (Figure 1C, top). HEK293T cells were cotransfected with each ZFA construct and its corresponding reporter. Potential fluctuations in transfection efficiency and cell number between each sample were normalized by cotransfection of a Renilla luciferase expression vector. We observed increased luciferase activation for all ZFA constructs compared to its reporter alone (Figure 1C, bottom). From all designed ZFAs, ZLT4D (~1,300-fold), HLTR6 (~1,600-fold),

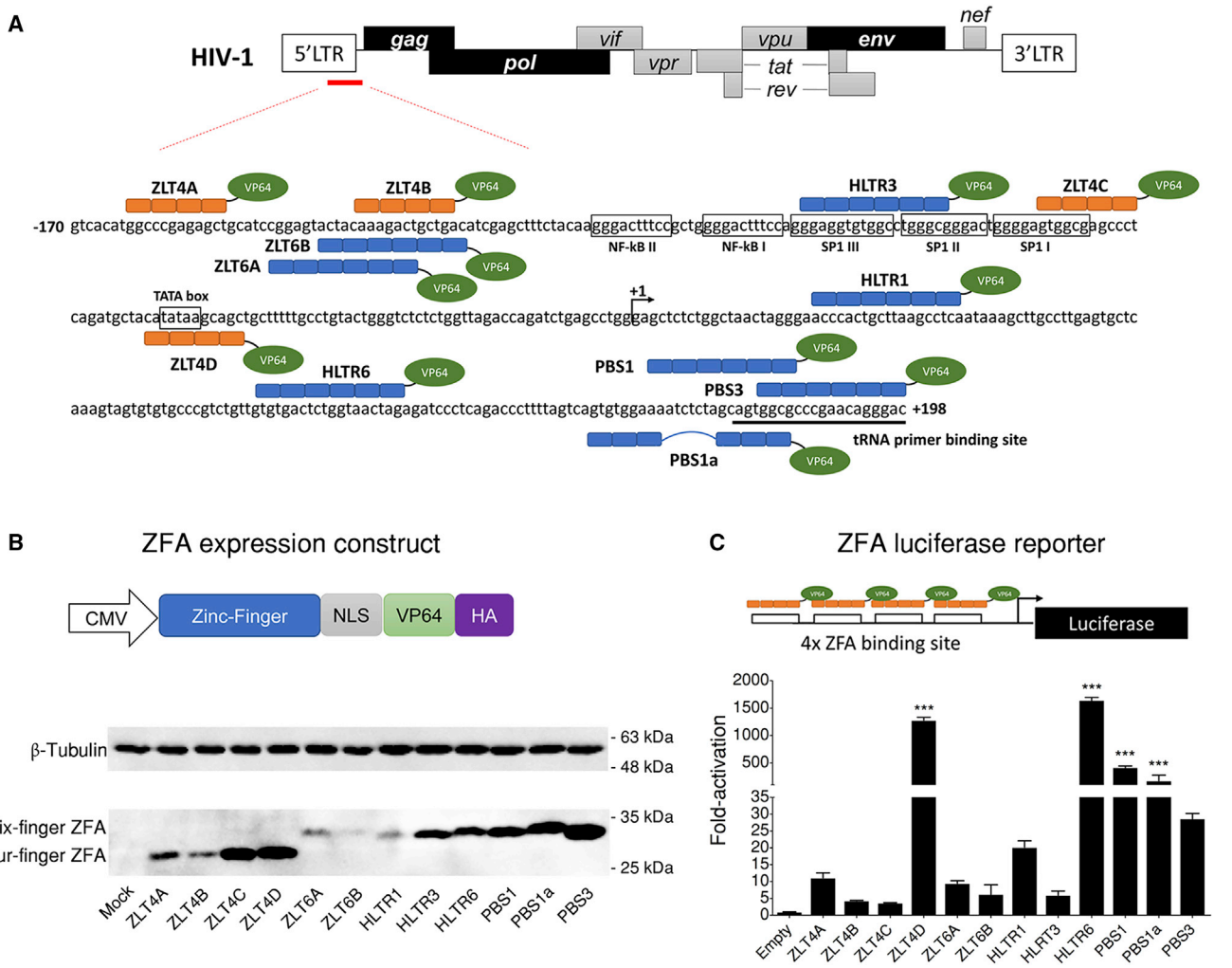

Figure 1. Zinc-Finger Activators (ZFAs) Designed to Target the HIV-1 Promoter

(A) Schematic illustration of ZFA target sites at the 5′ long-terminal repeat (5′ LTR) promoter and tRNA primer binding site (black solid line) within the HIV-1 genome. Most relevant endogenous transcription factor binding sites (nuclear factor κB [NF-κB] and SP1) are referenced. (B) Top: schematic representation of ZFA activator expression constructs. ZFA coding sequences are incorporated into a pcDNA backbone vector driven by the cytomegalovirus (CMV) promoter. 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. Bottom: western blot of 20 μg lysate of HEK293T transfected with indicated ZFA constructs. Samples were taken 48 h after transfection and probed with horseradish peroxidase-conjugated anti-HA and anti-β-tubulin (loading control) antibodies. "Mock" indicates lysate from HEK293T cells transfected with empty pcDNA vector only. (C) Top: schematic representation of the Firefly luciferase reporter system containing four direct repeats of the zinc-finger target sites for each ZFA construct. Bottom: fold activation of luciferase expression after cotransfection of indicated ZFA with the Firefly luciferase reporter plasmid into HEK293T cells. Firely luciferase expression was normalized to cells transfected with reporter plasmid only (Empty). Renilla luciferase expression vector was also cotransfected to normalize for transfection efficiency and cell number. Error bars indicate standard deviation of three independent experimental replicates (n = 3); ***$p < 0.001$; one-way ANOVA sample versus "Empty" (4× ZFA binding-site vector only).

PBS1 (~400-fold), and PBS1a (~200-fold) induced the highest level of gene expression from their target sites ($p < 0.001$). The remaining ZFA constructs induced moderate levels (~3- to 30-fold increase) of gene activation. We observe that the most effective ZFAs—ZLT4D, HLTR6, PBS1, and PBS1a—were also expressed at high levels following transient transfection in HEK293T cells (Figure 1B, bottom). These results indicate that target sequence composition, as well as intracellular protein stability, plays a major role in the design of potential ZFA for optimal gene activation.

ZFA Specifically Stimulates Latent HIV-1 Expression

In addition to its binding efficacy, the ability of synthetic transcription factors to induce gene expression is also connected to the target location relative to the TSS and chromatin accessibility,[50] as well as the presence of proximal regulatory elements.[51] To evaluate the potential of ZFAs to induce HIV transcription from the native promoter, we constructed a luciferase reporter containing the full-length 5′ LTR and PBS regions (−455 to +198 relative to TSS) (Figure 2A, top). This segment of HIV-1 contains all of the essential elements that regulate viral gene

Table 1. Representation of Zinc Fingers Designed in This Study to Target the HIV Promoter

ZF	Position[a]	Predicted Target Sequence (5′–3′)	ZF α-Helix Sequence[b]					
			F6	F5	F4	F3	F2	F1
ZLT4A	−163	GGC CCG AGA GCT			DPGHLVR	RNDTLTE	QLAHLRA	TSGELVR
ZLT4B	−134	AAG ACT GCT GAC			RKDNLKN	THLDLIR	TSGELVR	DPGNLVR
ZLT4C	−52	GTG GCG AGC CCT			RSDELVR	RSDDLVR	ERSHLRE	TKNSLTE
ZLT4D	−31	CAT ATA AGC AGC			TSGNLTE	QKSSLIA	ERSHLRE	ERSHLRE
ZLT6A	−146	CGG AGT ACT ACA AAG ACT	RSDKLTE	HRTTLTN	THLDLIR	SPADLTR	RKDNLKN	THLDLIR
ZLT6B	−140	ACT ACA AAG ACT GCT GAC	THLDLIR	SPADLTR	RKDNLKN	THLDLIR	TSGELVR	DPGNLVR
HLTR1[c]	+23	TGG GTG ACG AAT TCG GAG	RSDNLVR	TSGELVR	QSSNLAS	QSGDLRR	RSDVLVR	TSGHLVR
HLTR3[c]	−80	GGA GGC GTG GCC TGG GCG	QSSHLVR	DPGHLVR	RSDVLVR	DCRDLAR	RSDHLTT	RSDDLVR
HLTR6[c]	+118	ACA CTG AGA CCA TTG ATC	QRHSLTE	TSGSLVR	DKKDLTR	QLAHLRA	DPGALVR	SPADLTR
PBS1[c]	+167	AAA TCT CTA GCA GTG GCG	QRANLRA	RGGWLQA	QRHSLTE	QSGDLRR	RSDVLVR	RSDDLVR
PBS1a[c,d]	+160	GTG TGG AAA atctcta GCA GTG GCG	RSDVLVR	RSDHLTT	QRANLRA	QSGDLRR	RSDVLVR	RSDDLVR
PBS3[c]	+180	TGG CGC CCG AAC AGG GAC	RSDHLTT	HTGHLLE	RNDTLTE	DSGNLRV	RSDHLAE	DPGNLVR

[a]Position relative to transcription start site of the HIV-1 promoter.
[b]Amino acids from position −1 to +6 relative to zinc-finger α-helix domain are shown.
[c]Zinc fingers previously designed and reported.[24,25]
[d]PBS1a consists of two, three-finger modules separated by a long flexible linker (Gly3SerGly4). The F6 to F4 modules bind the first half site (GTG TGG AAA), and the F3 to F1 modules bind the second half site (GCA GTG GCG). Nonbound DNA sequence is indicated by lowercase letters.

expression, as well as the target sites for each designed ZFA. We cotransfected HEK293T cells with ZFA constructs and the LTR-PBS reporter. To control for nonspecific activation from six-finger activators, we created a ZFA based on Aart—herein named Aart6 (predicted target site: 5′-ATG TAG AGA AAA ACC AGG-3′)—a previously described zinc finger[43] that theoretically should not bind any sequence within the HIV provirus. Nonspecific activation from four-finger activators was accounted by designing a four-finger ZFA variant of Aart (Aart4; predicted target site: 5′-AGA AAA ACC AGG-3′). We observed that HLTR6-VP64 (~3-fold activation; $p < 0.001$) and PBS1-VP64 (~4-fold activation; $p < 0.001$) six-finger ZFAs increased activation from the LTR promoter at levels above the Aart6 control (Figure 2A, bottom), indicating these ZFAs as the most potent at inducing transcription from the HIV-1 promoter. None of the other six-finger and four-finger ZFAs could induce gene activation more efficiently than their nontargeting control (Aart6-VP64 and Aart4-VP64, respectively), which presented an ~2-fold activation background that could be attributed to nonspecific activity from the VP64 domain. The ZFAs ZLT4D and PBS1a were unable to induce activation from the full-length LTR in spite of their strong ability to induce gene expression from a promoter element consisting of only their binding site (Figure 1C). Interestingly, HLTR3-VP64 inhibited expression from the LTR compared to cells transfected with LTR-PBS reporter alone ($p < 0.001$). This effect could be owed to the overlap between HLTR3 and SP1 binding sites, causing the ZFA to outcompete the endogenous factor and block LTR activation,[24] highlighting the importance of zinc-finger location within the HIV promoter. Opposed to that previously observed with HIV-targeted TALE activators,[28] the combination of different ZFAs did not increase LTR activation (data not shown).

We next set out to evaluate whether ZFAs could reactivate latent HIV-1 expression. Latency reversal was assessed using the lymphocytic Jurkat-derived J-Lat cell line. This latency model harbors a full-length integrated HIV-1 proviral genome containing an *EGFP* gene that serves as a reporter for viral gene expression (HIV-1-ΔEnv-EGFP) (Figure 2B, top). These latently infected cells do not express an integrated provirus unless reactivated by a stimulus, such as the tumor necrosis factor α (TNF-α).[52] The J-Lat clone 10.6, which is particularly sensitive to reactivating stimuli, was nucleofected with expression vectors encoding either nonspecific Aart6-VP64 or HIV-targeted HLTR6-VP64 or PBS1-VP64, and EGFP expression was assessed by flow cytometry. PBS1-VP64 drove robust levels of reactivation in J-Lat 10.6 cells (~30% EGFP-positive cells) (Figure 2B, bottom), consistent with its ability to activate 5′ LTR transcription from the luciferase reporter in HEK293T (Figure 2A). On the other hand, HLTR6-VP64 led to minor levels of reactivation (~5% EGFP-positive cells), indicating that gene activation from the 5′ LTR promoter (Figure 2A) alone was not sufficient to enforce the reactivation of HIV expression in latent cells. In conclusion, these results demonstrate that the PBS1-VP64 activator promotes not only targeted stimulation of HIV-1 expression from the 5′ LTR viral promoter but also reactivation of viral expression in a model of HIV-1 latency. The PBS1 zinc finger targets the primer binding site region, the primary location for initiation of retro-transcription following binding of human $tRNA^{Lys}$.[53] Accordingly, the PBS1 binding site is extremely conserved across HIV-1 subtypes, with full target sequence homology in 80%–90% of strains among subtypes of the major (M) group (Figure S1). These include the most dominant subtype B (prevalent in the Americas, Western Europe, Japan, and Australia) and subtype C (prevalent in Southern and Eastern Africa, China, and India).

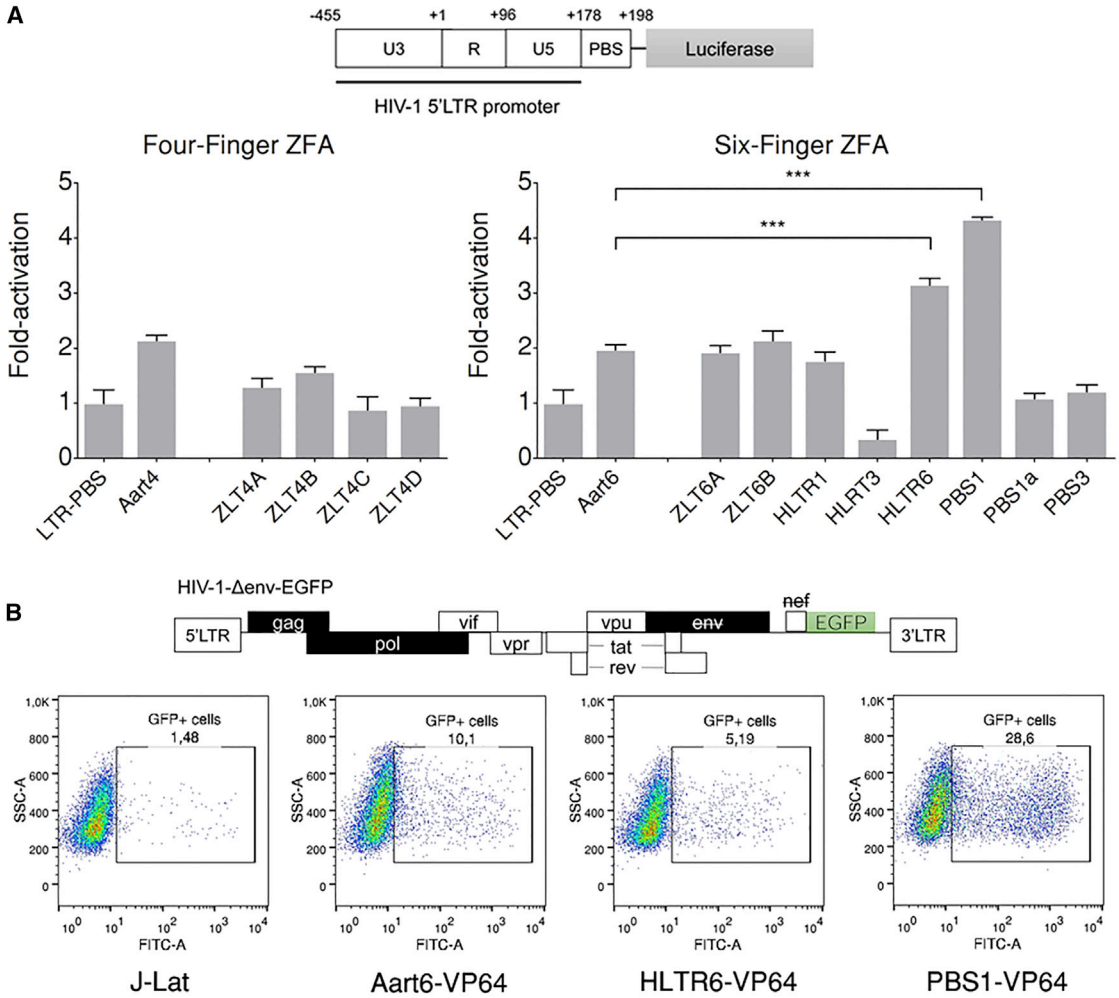

Figure 2. ZFA Proteins Target the LTR Promoter and Activate Latent HIV-1 Expression

(A) Top: schematic representation of the LTR-PBS luciferase reporter used to evaluate ZFA activity from the HIV-1 LTR promoter. Full-length 5′ LTR (U3-R-U5) and primer binding site (PBS) regions were placed upstream of the Firefly luciferase reporter. Bottom: fold activation of luciferase expression in HEK293T cells cotransfected with indicated ZFA expression constructs and LTR-PBS luciferase reporter. Luciferase expression was normalized to that obtained with transfection of reporter plasmid alone (LTR-PBS). Renilla luciferase expression vector was also cotransfected to normalize for transfection efficiency and cell number. Error bars indicate standard deviation of three independent experimental replicates (n = 3); ***$p < 0.001$; one-way ANOVA. (B) Top: schematic representation of the HIV-1 proviral genome present in J-Lat 10.6 cells. Full-length HIV-1 was derived from the molecular clone pNL4-3-ΔEnv-EGFP and expresses an EGFP gene from the LTR promoter. Structural viral genes are shown in black; auxiliary genes are shown in gray. The *nef* and *env* genes were inactivated to force a single infection cycle. Bottom: percentage of EGFP-positive J-Lat 10.6 cells nucleofected with LTR-targeted HLTR6-VP64 or PBS1-VP64 or nonspecific Aart6-VP64 ZFAs. "J-Lat" indicates nontransfected J-Lat 10.6 cells. EGFP-positive cells were measured by flow cytometry at 48 h after nucleofection. Dot plots are representative of a single experiment from three independent replicates.

As a result, the PBS1-VP64 synthetic activator should be highly valuable to provide a broad therapeutic effect across patients carrying distinct strains of HIV-1.

Protein Delivery of Cell-Penetrating ZFA Reactivates Latent HIV-1 Expression

To explore novel delivery routes for treatment of HIV reservoirs, we capitalized on the innate ability of Cys_2-His_2 zinc fingers to cross the cell membrane[37] and evaluated whether protein delivery of cell-penetrating ZFAs could directly internalize HIV latently infected cells and specifically stimulate viral expression. To further enhance cell-penetrating activity, we fused three NLS repeats to the N-terminal region of the PBS1-VP64 activator, herein named 3NLS-PBS1-VP64, since the incorporation of positively charged NLS sequences has previously been shown to improve cell permeability of zinc-finger nucleases and enhance their gene-editing activity.[39] We cloned the genes encoding PBS1-VP64 and 3NLS-PBS1-VP64 activators together with an N-terminal histidine tag (polyhistidine [6×His]) into the pET28b vector for protein expression in *E. coli* and subsequent purification (Figure 3A). A positively charged polyhistidine tag is retained following

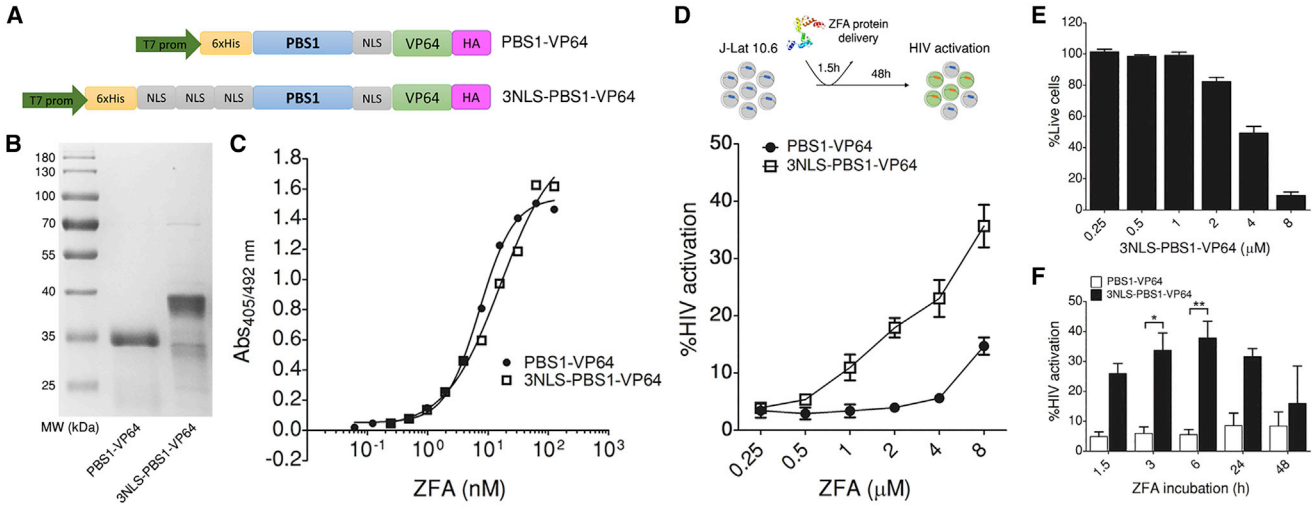

Figure 3. Reactivation of HIV Latent Cells by Protein Delivery of Engineered ZFA

(A) Schematic representation of ZFA expression vectors for protein production in *Escherichia coli*. PBS1-VP64 was genetically fused to the pET28b expression vector driven by the IPTG-inducible T7 promoter. PBS1-VP64 contains a NLS between PBS1 zinc-finger and VP64 activation domains. 3NLS-PBS1-VP64 contains three additional NLS repeats at the N-terminal of the DNA-binding domain to enhance cell permeability. 6×His indicates six histidine tags for protein purification. HA indicates the hemagglutinin A tag for protein detection. (B) SDS-PAGE of purified ZFA proteins. ZFAs were detected using BlueSafe protein staining. (C) ELISA analysis of PBS1-VP64 and 3NLS-PBS1-VP64 binding to an oligonucleotide incorporating PBS1 target sequence (5′-AAA TCT CTA GCA GTG GCG-3′). $Abs_{405/492\ nm}$ values are represented as the mean of two independent experiments. (D) Top: schematic representation of HIV latent cell treatment with ZFA proteins. J-Lat 10.6 cells were incubated with ZFA protein for 1.5 h under standard cell-culture conditions. HIV activation of the J-Lat 10.6 cell line was evaluated through detection of GFP-positive cells by flow cytometry at 48 h after protein incubation. Bottom: percentage of HIV activation in the J-Lat 10.6 cell line following treatment with 0.25 μM to 8 μM of PBS1-VP64 (filled circles) or 3NLS-PBS1-VP64 (open squares). (E) Percentage of live cells of J-Lat 10.6 cells treated with increasing concentrations (from 0.25 μM to 8 μM) of 3NLS-PBS1-VP64 for 90 min. Cell viability was assessed through flow cytometry at 48 h after protein treatment by gating the forward-scatter/side-scatter (FSC/SSC) live population. Percentage of live cells is normalized for J-Lat cells treated with ZF storage buffer alone. (F) Percentage of HIV activation in the J-Lat 10.6 population treated with 2 μM of PBS1-VP64 (white columns) or 3NLS-PBS1-VP64 (black columns) for different periods of protein incubation. HIV activation of the J-Lat 10.6 population was evaluated through detection of GFP-positive cells by flow cytometry at 48 h after protein incubation. Error bars indicate standard deviation of three independent experiments (n = 3); *$p < 0.05$; **$p < 0.01$; one-way ANOVA.

protein purification and thus, will contribute to increase the overall positive charge of ZFA and potentially increase its cell-penetration ability. SDS-PAGE analysis of purified ZFAs demonstrates over 90% of estimated purity of these proteins (Figure 3B). To confirm that purified ZFA proteins retained their functionality, we performed an enzyme-linked immunosorbent assay (ELISA) analysis to evaluate protein binding to synthetic DNA target site (Figure 3C). Both PBS1-VP64 and the 3NLS-PBS1-VP64 activators similarly recognized the PBS1 binding site in a concentration-dependent manner, indicating that the addition of NLS repeats does not influence ZFA efficacy to bind DNA.

We next determined whether ZFA proteins could penetrate cells latently infected with HIV-1 and activate viral expression. J-Lat 10.6 cells were incubated with increasing amounts of PBS1-VP64 or 3NLS-PBS1-VP64 for 1.5 h before analyzing HIV reactivation by flow cytometry 48 h post-treatment (Figure 3D, top). We observed a strong reactivation in J-Lat cells following a single treatment with 3NLS-PBS1-VP64. Reactivation was concentration dependent and reached a maximum level (~40% GFP-positive cells) after treatment with 8 μM of ZFA (Figure 3D, bottom). We detected relatively modest levels of reactivation using 8 μM of PBS1-VP64 (~15% GFP-positive cells), indicating the importance of NLS repeats for promoting ZFA-mediated HIV activation by protein delivery. However, an analysis of treated J-Lat 10.6 cells following treatment with 3NLS-PBS1-VP64 indicated that cell viability was reduced to 80% with 2 μM of ZFA and drastically decreased after treatment with >2 μM of protein (Figure 3E). Potential protein aggregation resulting from high levels of ZFA concentration could be a source of cell toxicity. We therefore performed our subsequent protein treatments with a maximum of 2 μM of ZFA. To further maximize the activity of ZFA protein treatment, we observed that extending the incubation period to 6 h increased reactivation of the latent HIV population up to ~40% of cells with 2 μM of 3NLS-PBS1-VP64, similar to reactivation levels observed with 8 μM of ZFA (Figure 3D, bottom) and surpassing those observed with ZFA nucleofection (Figure 2B, bottom), while having minimal effect over PBS1-VP64 activity (Figure 3F). The supplementation of cell medium with $ZnCl_2$ or L-arginine, previously used for assisting activity of cell-penetrating zinc-finger nucleases,[39] had no influence on ZFA activity (data not shown). Overall, these data show that engineered cell-penetrating ZFAs can reactivate latent HIV-1 expression following protein delivery.

Incorporation of Multiple NLS Repeats Enhances ZFA Cell Permeability

According to a reported protein delivery of multi-NLS-conjugated zinc-finger nucleases,[39] we assessed whether incorporation of NLS repeats is necessary to promote efficient ZFA protein uptake. We compared

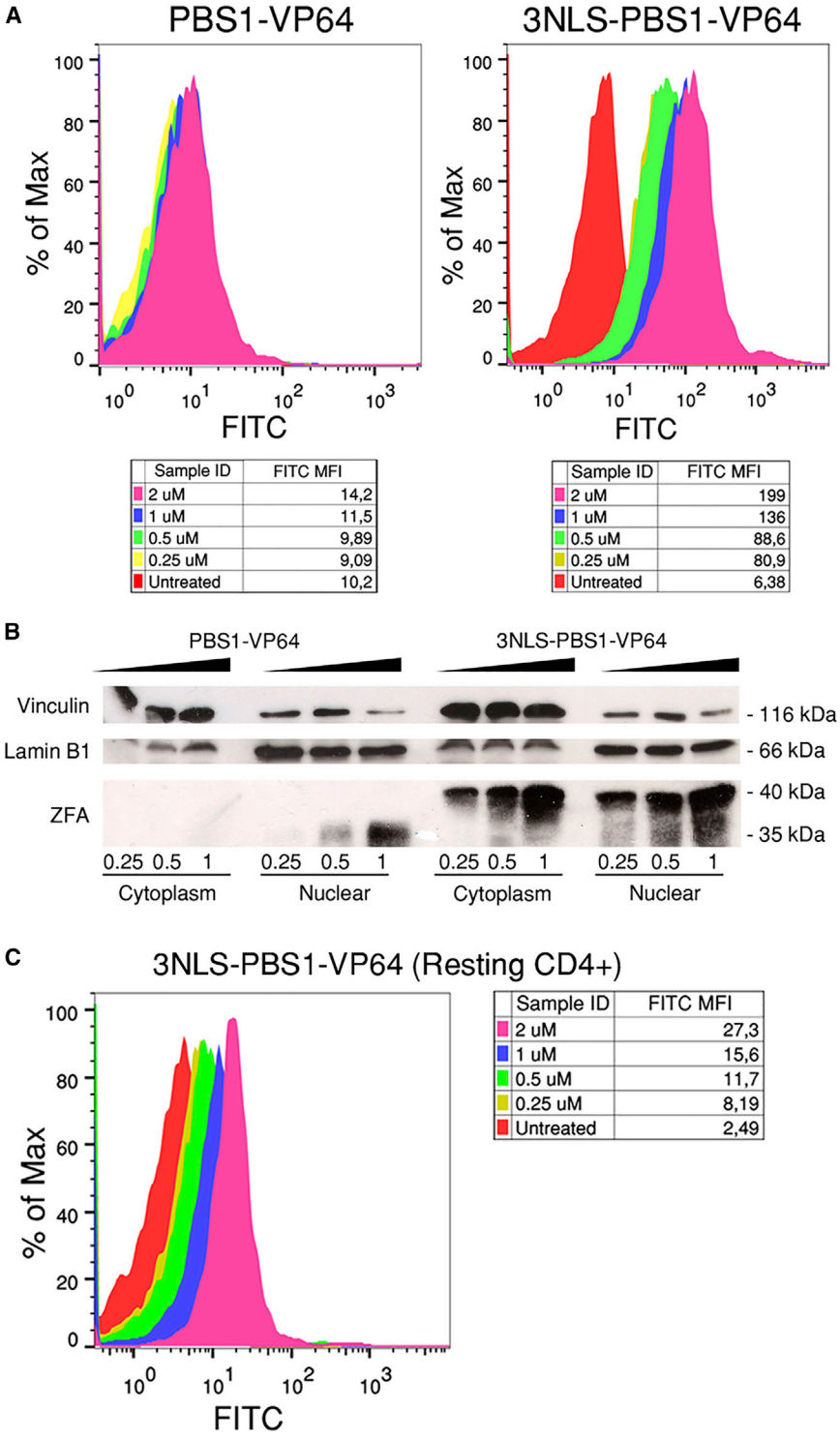

Figure 4. Incorporation of Multiple NLS Repeats Improves ZFA Cell-Penetrating Ability

(A) Mean fluorescence intensity (MFI) analysis determined by flow cytometry of Jurkat cells, untreated or treated with increasing concentrations (from 0.25 μM to 2 μM) of FITC-stained PBS1-VP64 or 3NLS-PBS1-VP64 for 6 h. Histograms are representative of a single experiment from three independent replicates. (B) Western blot analysis of cytoplasmic (40 μg lysate) and nuclear (20 μg lysate) fractions of J-Lat 10.6 cells treated with indicated ZFA protein concentrations (in micromoles) of PBS1-VP64 or 3NLS-PBS1-VP64 for 6 h. Vinculin and lamin B1 were detected as loading controls for cytoplasmic and nuclear fractions, respectively. (C) MFI analysis, determined by flow cytometry, of resting $CD4^+$ T cells, untreated or treated with increasing concentrations (from 0.25 μM to 2 μM) of FITC-stained 3NLS-PBS1-VP64 for 6 h. Histograms are representative of a single experiment from three independent replicates.

PBS1-VP64 and 3NLS-PBS1-VP64 cell-penetration efficacy through detection of fluorescein isothiocyanate (FITC)-conjugated ZFA in Jurkat T lymphocytes. Cells were washed twice with trypsin/EDTA prior to analysis to remove any surface-bound ZFA. We observed that only cells treated with 3NLS-PBS1-VP64 efficiently internalized FITC-conjugated ZFA (Figure 4A), presenting a 31-fold increase in FITC mean fluorescence intensity (MFI) compared to untreated cells and a 14-fold increase compared to cells treated with PBS1-VP64 following treatment with 2 μM of protein. Western blot analysis of nuclear and cytoplasmic fractions of J-Lat 10.6 cells treated with ZFA proteins further supports the improved cell permeability of 3NLS-PBS1-VP64 and shows that both PBS1-VP64 and 3NLS-PBS1-VP64 are efficiently targeted to the nucleus in a concentration-dependent manner (Figure 4B). These results demonstrate that superior HIV reactivation observed with 3NLS-PBS1-VP64 is a result of its increased cell-penetration ability in general and not associated with improved nuclear import.

Furthermore, we assessed whether 3NLS-PBS1-VP64 could penetrate primary resting $CD4^+$ T lymphocytes, the major cell population harboring the HIV reservoir[2] and highly refractory to gene transfer.[54] We observed a substantial dose-dependent increase in FITC MFI in resting $CD4^+$ T cells, up to ~11-fold, following treatment with 2 μM of 3NLS-PBS1-VP64 compared with untreated cells (Figure 4C), in which approximately 75% of the cell population was internalized with FITC-conjugated ZFA (Figure S2). We did not observe any evident cell toxicity of resting CD4 T cells in the presence of ZFA (Figure S2). Still, internalization was not so efficient in resting $CD4^+$ T cells as in Jurkat cell line (Figure 4A), indicating that the cell-activation

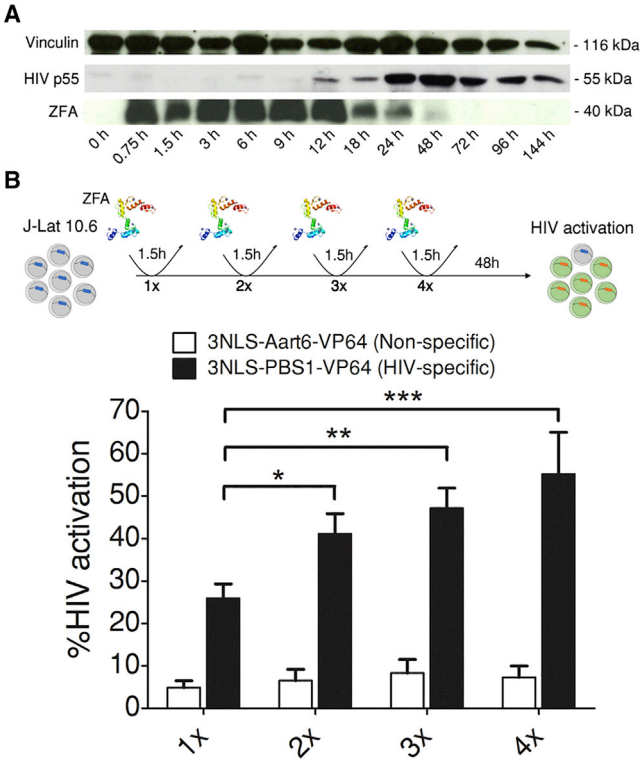

Figure 5. Increased Reactivation of Latent HIV Expression Following Multiple ZFA Short-Term Protein Treatment

(A) Western blot (30 μg lysate) analysis of zinc-finger protein delivery (ZFA) and HIV expression (HIV p55) in J-Lat 10.6 cells treated with 2 μM of 3NLS-PBS1-VP64 for 6 h. Samples were collected for analysis at the indicated time points. Vinculin was detected as loading control. (B) Top: schematic representation of HIV latent cell multiple treatment with ZFA protein. J-Lat 10.6 cells were incubated with ZFA protein for 1.5 h per each treatment under standard cell-culture conditions. HIV activation of the J-Lat 10.6 cell line was evaluated through detection of GFP-positive cells by flow cytometry at 48 h after protein incubation. Bottom: percentage of HIV activation in the J-Lat 10.6 cell line following multiple treatments with 2 μM of 3NLS-Aart6-VP64 (white columns) or 3NLS-PBS1-VP64 (black columns) proteins. HIV activation of the J-Lat 10.6 cell line was evaluated through detection of GFP-positive cells by flow cytometry at 48 h after protein incubation. Error bars indicate standard deviation of three independent experiments (n = 3); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; one-way ANOVA.

state might influence its permeability to these cell-penetrating proteins. Still, these results demonstrate the high intrinsic ability of multiple NLS-conjugated ZFAs for protein delivery into hard-to-transfect quiescent cells as those associated with the establishment of HIV reservoirs.

Short-Term ZFA Protein Treatments Induce High and Sustained Levels of HIV-1 Activation

To further evaluate ZFA internalization and activity kinetics following protein delivery, we incubated J-Lat 10.6 cells with 3NLS-PBS1-VP64 for 6 h and then monitored both ZFA cell entry and HIV expression for a period of 6 days (144 h) by western blot. We observed that 3NLS-PBS1-VP64 protein is immediately internalized as shortly as 45 min (0.75 h) after incubation, reaching intracellular peak levels between 3 h and 12 h, being almost fully degraded by 48 h post-treatment (Figure 5A). In turn, HIV stimulation—manifested by expression of viral capsid p55—is first detected at 12 h post-treatment and persists up to 6 days, the maximum time point analyzed, at which ZFA protein turnover has already occurred (Figure 5A). With the consideration of the short-term persistence of intracellular Gag p55,[55] these data indicate that protein delivery of ZFA remains in the latent cells for a short period but sufficient to induce HIV activation for longer periods.

With the consideration that a substantial fraction of ZFA protein enters the cells within the first hour, we evaluated whether multiple short-term (1.5 h) protein treatments would further improve ZFA-mediated HIV reactivation. J-Lat 10.6 cells were incubated with ZFA protein up to a total of four short-term treatments of 90 min, and HIV activation was assessed 48 h post-treatment (Figure 5B, top). Flow cytometry analysis of treated J-Lat cells shows a dramatic increase in the fraction of the reactivated population (~55% HIV activation with 4× ZFA treatments; $p < 0.001$) as the number of ZFA treatments increases (Figure 5B, bottom), considerably improving HIV activation overextending ZFA incubation to 6 h (Figure 3F). Stimulation of the J-Lat 10.6 population with 4× ZFA treatments draws reactivation levels closer to the maximum threshold observed in the presence of proinflammatory cytokine TNF-α.[52] For all conditions tested, we did not observe any significant changes in J-Lat 10.6 reactivation when cells were treated with a nonspecific 3NLS-Aart6-VP64 protein (Figure 5B, bottom), indicating that viral gene expression is not induced by the presence of a nonspecific ZFA. This result is not due to differences in cell-penetrating capacity between 3NLS-PBS1-VP64 and 3NLS-Aart6-VP64, as observed by MFI analysis of Jurkat cells treated with FITC-conjugated ZFA, despite the histogram showing less homogeneous internalization of the nonspecific ZFA (Figure S3). Comparison of HIV expression among latently infected cell lines displaying different contexts of gene repression—J-Lat 10.6 and 9.2,[52] ACH-2,[56] and U1[57]—showed a discrepancy in the response of each model to ZFA-mediated reactivation. Multiple treatments with 3NLS-PBS1-VP64 promoted a strong induction of HIV transcription in J-Lat 10.6 (84-fold activation) and ACH-2 (49-fold) latency models; however, treatment of U1 and J-Lat 9.2 cell lines did not affect viral expression (Figure S4). Overall, these results demonstrate that multiple short-term ZFA treatments can induce high levels of HIV latent expression in a targeted manner, yet the context of HIV repression within latently infected cells can affect the ability of ZFA protein delivery to stimulate viral expression.

DISCUSSION

The emergence of genome-engineering tools has greatly expanded the possibilities to directly target the HIV genome and promote an antiviral effect.[58] Site-directed nucleases[17,18] and recombinases[59] can recognize and excise the integrated HIV proviral genome from infected cells. Nevertheless, genotoxicity caused by unwanted cuts at the human genome[8] or the emergence of resistant HIV strains generated by double-strand breaks at the viral genome[60,61] raises concerns about the safety of this approach. In contrast to programmable nucleases, engineered transcription activators have been shown to

modulate gene expression[14] without causing disruptive DNA breaks and generally with the absence of off-target effects.[62] Indeed, genome-wide studies have shown that synthetic activators induce targeted gene activation in a precise manner with minimal alterations in global gene expression,[33,62,63] highlighting their safety for therapeutic applications. As a result, several studies[28–36] have explored the design of synthetic activators to induce latent HIV expression and demonstrated the potential of this approach to target HIV reservoirs. Among these, Cys_2-His_2 zinc fingers possess the innate ability to penetrate the anionic cell membrane,[37] which could be explored as a novel therapeutic route to target HIV reservoirs by protein delivery of engineered activators.

In this study, we report the construction of a cell-penetrating ZFA for direct protein delivery and activation of latent HIV-infected cells. We designed several ZFAs to target the HIV-1 5′ LTR promoter and induce viral gene expression. Opposed to TALE activators previously designed to target the HIV-1 promoter,[28] gene-activation efficiency was highly variable among designed ZFA, which could reflect fluctuations in DNA-binding affinity. Moving forward, optimization of the zinc-finger framework to enhance binding affinity could be accomplished by intercalate addition of longer linkers to support optimal placement of long zinc-finger arrays around the helix conformation of the DNA target.[64,65] PBS1-VP64 was identified as the single ZFA able to strongly stimulate latent HIV expression, attaining reactivation levels comparable to those reported in our previous study using TALE activators.[28] Despite the HLTR6-VP64 ability to activate gene expression from the 5′ LTR promoter within an episomal DNA context, this zinc finger may not access its target site within chromatin,[24] as indicated by its inability to stimulate latent HIV expression in the J-Lat 10.6 model. The PBS1 target site overlaps the tRNA primer binding site and flanking sequences located at the 3′ end of the LTR promoter, which is unbound to a nucleosome;[25] therefore, this region should be accessible to binding of this ZFA. More importantly, this region is described as the most conserved segment of the HIV-1 genome,[66] being required for initiation of viral retrotranscription following binding of human $tRNA^{Lys54}$. Although gene activation is generally stronger when engineered transcription factors are targeted upstream of the TSS, these can also be efficient from downstream regions,[14] as demonstrated by the PBS1-VP64 capacity to stimulate HIV transcription.

We further demonstrated that the PBS1-VP64 activator can be directly delivered to latent cells as a cell-penetrating protein. Significant stimulation of latent HIV expression was only obtained with the inclusion of multiple NLS repeats at the N-terminal region of PBS1-VP64. Despite previous reports demonstrating that protein delivery of cell-penetrating zinc-finger nucleases alone mediates efficient gene knockout,[38,39] the presence of acidic and negatively charged residues within transactivation domains, such as VP64,[49] might challenge their translocation through the anionic cell membrane. In fact, the PBS1-VP64 activator alone presents a neutral charge at the physiological pH even in the presence of a positively charged polyhistidine tag. Incorporation of 3×NLS repeats confers a high positive charge to this ZFA (+15.2), which was shown crucial for its superior cell permeability and activity. This result is consistent with a previous report by Liu et al.[39] that demonstrates the potential of NLS peptides to increase overall positive net charge and enhance protein delivery of zinc-finger nucleases. Strikingly, we show that the performance of multiple short-term ZFA treatments can enhance activation potency over that observed with gene delivery. However, we observed that reactivation of HIV expression was not consistent among distinct models of latent infection, indicating that certain repression mechanisms may not be reversed by ZFA alone. Interestingly, a combination of synthetic activators with other latency-reversing agents[28,34] has proven to alleviate suppression of the latent HIV provirus within such unfavorable contexts and may therefore expand the activity of ZFA within the heterogenous latent HIV population.

Successful therapeutic application of synthetic activators *in vivo* will be conditioned, in most cases, by the delivery method to ensure efficient bioavailability and broad distribution of these platforms in the target population. Relevant to this, direct protein delivery of ZFA could overcome limitations derived from standard gene-delivery methods. Although gene delivery by viral vectors can provide higher and sustained production of DNA-targeted activators,[67] protein delivery is generally less toxic while avoiding insertion-related mutagenesis. On the other hand, its small size promotes higher biodistribution and cellular uptake compared to nonviral liposome or nanoparticle-based delivery methods.[68] More importantly, protein delivery improves temporal control of engineered activators over gene-delivery methods. These so-called "hit-and-run" strategies are suitable for latent HIV activation, for which engineered activators remain in the cell shortly enough to stimulate HIV transcription but without causing potentially harmful off-target gene modulation from prolonged exposure.[58] In fact, we observed that the short-term presence of ZFA within latent HIV cells was sufficient to promote viral expression for days following ZFA degradation. Of note, ZFA-mediated production of *cis*-acting viral transactivator Tat could create a positive-feedback circuit that supports reactivation for longer periods.[69] Future experiments should investigate precisely the long-term persistence of HIV reactivation and whether it is enough to trigger the elimination of these latent reservoirs.

Despite the exponential growth of TALE- or CRISPR/Cas9-based therapeutic applications,[8] the biochemical properties of zinc-finger proteins combine both the cell-penetrating ability and site-specific targeting in a single molecule, which may overcome limitations associated with gene-delivery methods[70] and prove relevant in the context of clinical translation. In addition, the existence of zinc-finger domains in humans should potentially reduce risks of immunogenicity emerging from *in vivo* genome-engineering therapeutics.[65] Future studies should address the potential of ZFA protein delivery in a clinically more relevant setting, namely, in cells extracted from aviremic patients,[71] as well as primary cell[72] and animal[73] models of HIV latency that recapitulate the biological properties of HIV reservoirs that occur *in vivo*. In this context, further optimizations of the ZFA framework could be performed to enhance the efficacy and safety

of this approach. Gene-activation potency of ZFA could be enhanced by incorporating upgraded transactivation domains that recruit complementary transcription factors.[74] Additionally, previous reports have shown improved penetration of ZFA through conjugation with protein-transduction domains,[75,76] leaving room to further optimize the cell-penetrating capacity of ZFAs. One of these studies demonstrated *in vivo* localization of ZFA in the brain following intraperitoneal or subcutaneous injection into a mouse model of Angelman syndrome,[76] demonstrating its capacity to cross the blood-brain barrier and modulate gene expression—a desirable feature to target potential HIV reservoirs in the central nervous system.[77] Moreover, translation of this technology into infected patients might require a receptor-targeted approach[78,79] through ZFA conjugation with ligands that direct these into the relevant cell populations, particularly, resting $CD4^+$ T lymphocytes. Receptor-mediated delivery of zinc-finger nucleases has been shown to enhance cell internalization,[80] further supporting the potential of this approach for *in vivo* applications. The identification of potential biomarkers associated with HIV latent infection in $CD4^+$ T cells[81] may open new perspectives for targeted protein delivery of synthetic ZFAs directly to the HIV reservoir.

In conclusion, we provide evidence that cell-penetrating ZFAs engineered to target the HIV promoter can be directly delivered to latently infected cells without any carrier and strongly stimulate viral expression. This study is an important step to approach novel shock-and-kill strategies to eradicate latent HIV reservoirs.

MATERIALS AND METHODS

DNA Plasmid Constructs

Design of HIV-targeted polydactyl ZFAs was performed at Zinc Finger Tools web server (https://www.scripps.edu/barbas/zfdesign/zfdesignhome.php).[41] Zinc-finger arrays were generated by modular assembly.[82] ANN,[43] GNN,[42] or CNN[45] binding zinc-finger modules were isolated through XmaI/SpeI digestion and cloned into AgeI/SpeI restriction sites of the pSCV vector until generation of 4-finger or 6-finger arrays.[82] ZFAs were generated by cloning of the zinc-finger arrays into XhoI/SpeI restriction sites of pAart-VP64 (pAart6)[43] to generate ZFA constructs pZLT4A through pZLT6B and pAart4. Previously designed HIV-targeted zinc-finger repressors[24,25] were digested and cloned into XhoI/SpeI restriction sites of pAart6 to generate ZFA expression constructs pHLTR1 through pPBS3. Correct construction of each plasmid was verified by Sanger sequencing analysis (Table S1).

pET28b-PBS1-VP64 was generated by PCR amplifying PBS1-VP64 from pPBS1 using primers 5′ zinc finger (ZF)-forward (Fwd) and 3′ VP64-reverse (Rev) and cloning into NdeI/SacI restriction sites of pET28b-CCR5R-1NLS.[39] pET28b-3NLS-PBS1-VP64 and pET28b-3NLS-Aart6-VP64 were generated by, respectively, PCR amplifying PBS1-VP64 from pPBS1 and Aart6-VP64 from pAart6 using primers 5′ 3NLS-ZF-Fwd and 3′ VP64-Rev. PCR products were digested with SalI and SacI and cloned into XhoI/SacI restriction sites of pET28b-CCR5R-1NLS. Correct construction of each plasmid was verified by sequence analysis (Table S2). Primer sequences are provided in Table S3.

The pZFA luciferase reporter vectors were constructed through PCR by amplifying the luciferase gene from pGL3-Basic (Promega, Madison, WI, USA) using the primers 5′ ZF-Luc-ZLT4A through PBS3, which contained four direct repeats of each zinc-finger binding site and 3′ Luc-Rev. PCR products were digested and cloned into the XhoI/SphI restriction sites of pGL3-Basic to generate pGL3-ZF-ZLT4A through PBS3. The HIV-1 LTR-PBS reporter plasmid was constructed by PCR amplifying the 5′ LTR (U3-R-U5) promoter and the PBS region from pNL4-3 (NIH AIDS Reagents; NIH, Bethesda, MD, USA) using the primers 5′ LTR-PBS-Fwd and 3′ LTR-PBS-Rev. PCR product was digested and cloned into the MluI/NheI restriction sites of pGL3-Basic to generate pGL3-LTR-PBS. Primer sequences are provided in Table S3. Correct construction of each plasmid was verified by Sanger sequencing analysis.

Cell Culture

HEK293T (American Type Culture Collection [ATCC], Manassas, VA, USA) 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). The Jurkat E6-1 cell line and J-Lat 10.6 clone (NIH AIDS Reagents; NIH, Bethesda, MD, USA) 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% CO_2.

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy anonymous donors through the Instituto Português do Sangue e Transplantação (IPST; Lisboa, Portugal) as described.[83] Briefly, PBMCs were isolated using density gradient centrifugation and Ficoll-Paque PLUS (GE Healthcare, Chicago, IL, USA), according to the manufacturer's instructions. Resting $CD4^+$ T cells were purified from PBMCs by negative selection using the EasySep custom kit for Human Resting $CD4^+$ T Cell Enrichment Cocktail (Stem Cell Technologies, Vancouver, Canada). Unwanted cells were removed using anti-CD8, CD14, CD16, CD19, CD20, CD36, CD123, TCT γ/δ, GlyA, CD66b, CD25, HLA-DR, and CD69 magnetic-coated beads. Resting $CD4^+$ T cells were cultured in RPMI-1640 medium, supplemented with 10% (v/v) FBS, 2 mM L-glutamine, and 1% (v/v) Anti-Anti and maintained at 37°C in a humidified atmosphere of 5% CO_2.

Luciferase Assays

Luciferase assays were performed as previously described.[28] 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 pZFA constructs, 5 ng of pGL3-ZFA binding-site reporters, and 1 ng of pRL-cytomegalovirus (CMV) (Promega, Madison, WI, USA) using Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions. At 48 h after transfection, cells were washed once with Dulbecco's PBS (DPBS; Life Technologies)

and lysed with Passive Lysis Buffer (Promega). Luciferase expression was measured with the Dual-Luciferase Reporter Assay System (Promega) 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.

ZFA Transient Transfection

For expression analysis of designed ZFAs, we seeded HEK293T cells onto a 24-well plate at a density of 2×10^5 cells per well. 24 h after seeding, cells were transfected with 500 ng of indicated ZFA expression constructs or the pcDNA3.1 (Life Technologies) backbone vector using the TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA), according to the manufacturer's instructions. At 48 h after transfection, ZFA expression was analyzed by western blot as described below.

Episomal transfection of ZFAs in J-Lat 10.6 cells was performed by plasmid nucleofection as previously described.[28] Briefly, we seeded J-Lat cells onto a 10-cm dish at a density of 1×10^5 cells per mL. After 48 h, 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 indicated pcDNA ZFA construct. Cells were transferred to a 16-well Nucleocuvette (Lonza) and electroporated with a 4D-Nucleofector System (Lonza), selecting the program CL-120. At 48 h after transfection, cells were analyzed by western blot or flow cytometry as described below.

ZFA Protein Expression and Purification

Expression and purification of cell-penetrating ZFAs were adapted from a previously optimized protocol.[39] Zinc-finger pET28b expression constructs were transformed into a chemically competent *E. coli* BL21(DE3) strain (Merck KGaA, Darmstadt, Germany). Overnight culture from a single colony was inoculated into 500 mL of Luria-Bertani (LB) media supplemented with 200 mM NaCl, 50 μg/mL kanamycin, 100 μM $ZnCl_2$, and 0.2% glucose. Cell culture was grown at 37°C with agitation (220 rpm) until optical density 600 (OD_{600}) reached 0.5 and then at room temperature until OD_{600} reached 0.8. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Thermo Fisher Scientific, Waltham, MA, USA) for 4 h at room temperature. Cells were pelleted by centrifugation at $12{,}000 \times g$ for 5 min and stored at −20°C until purification protocol.

Purification of ZFAs was carried out by resuspending the cell pellet in 20 mL of ZF binding buffer (20 mM HEPES, pH 8.0, 2 M NaCl, 1 mM $MgCl_2$, 100 μM $ZnCl_2$, and 10% glycerol). This solution was supplemented with 1 mM β-mercaptoethanol, protease inhibitors (Roche, Basel, Switzerland), and 0.1% Triton X-100. Cells were lysed by sonication (10 min, 50% output, pulse on) and centrifuged at $12{,}000 \times g$ for 60 min at 4°C. The supernatant was cleared by running through a 0.45-μm low protein binding filter. Cell lysate was transferred to a His GraviTrap column (GE Healthcare). The column was washed with 10 mL of 5 mM imidazole (Merck KGaA) and with 5 mL of 35 mM imidazole in ZF binding buffer. ZF protein elution was performed with 300 mM imidazole in ZF binding buffer. Ten fractions of 0.5 mL eluted protein were collected and supplemented with 100 mM L-arginine. Fractions containing a majority of eluted protein were selected, combined, and buffer exchanged to ZF storage buffer (20 mM HEPES, pH 8.0, 500 mM NaCl, 1 mM $MgCl_2$, 100 μM $ZnCl_2$, 10% glycerol, and 100 mM L-Arg) using PD-10 desalting columns (GE Healthcare). ZF proteins were then concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (Merck KGaA) and stored at −80°C. Purified ZFA batches were analyzed by 4%–12% SDS-PAGE (National Diagnostics, Atlanta, GA, USA) and stained using BlueSafe reagent (NZYTech, Lisboa, Portugal).

ELISA

Assessment of ZFA proteins binding to target sites was performed by ELISA. Briefly, ELISA plates were coated with 400 ng streptavidin per well overnight at 4°C. After rinsing with double-distilled (dd)H_2O, wells were incubated with 25 ng biotin-marked oligonucleotides containing the ZFA target site for 1 h at 37°C. Plates were rinsed with ddH_2O and blocked with 3% bovine serum albumin (BSA) in ZNBA buffer (20 mM Tris-HCl, pH 7.4, 90 mM KCl, 1 mM $MgCl_2$, and 100 μM $ZnCl_2$) for 1 h at 37°C. Serial dilutions of ZFA protein were prepared, starting from 125 nM in ZF storage buffer, supplemented with 1% BSA and 3 μg herring sperm DNA (Promega). Plates were incubated with protein dilutions for 2 h at room temperature. Following washing with DPBS-Tween 20 0.1%, wells were probed with anti-HA monoclonal antibody (clone 3F10; Roche) in 1% BSA/ZF storage buffer for 30 min at room temperature. Following washing with ddH_2O, plates were developed with ABTS (Calbiochem; Merck KGaA), conjugated with H_2O_2 for 15 min, and analyzed by measuring absorbance (Abs) at 405/492 nm on a Tecan Infinite M-200 (Tecan Group, Mannedorf, Switzerland) plate reader.

ZFA Protein Treatment

For protein delivery of cell-penetrating ZFAs, cells were seeded 24 h before protein treatment in 24-well plates at a density of 2×10^5 cells per well. After 24 h, cells were centrifuged at $300 \times g$ for 5 min at room temperature. ZFA proteins were diluted in supplemented RPMI medium at the final concentration indicated. Cells were resuspended in zinc-finger protein solution and incubated at 37°C and 5% CO_2 atmosphere for 90 min, unless otherwise indicated. After protein treatment, cells were washed twice with supplemented RPMI media and then moved to 37°C and 5% CO_2 atmosphere. For each experiment, cells were also incubated with ZF storage buffer as a mock control.

Flow Cytometry

For analysis of HIV activation from J-Lat 10.6 cells, cells were collected 48 h after protein treatment and washed twice with DPBS (Life Technologies), and EGFP expression was evaluated by flow cytometry (Guava easyCyte 5HT; Merck Millipore, Burlington, MA, USA).

Evaluation of ZFA internalization by flow cytometry was performed through protein treatment of Jurkat cells with FITC-conjugated

ZFA. ZFA proteins were stained using the FluoReporter FITC Protein Labeling Kit (Thermo Fisher), according to the manufacturer's instructions. Cells were treated with FITC-conjugated ZFA proteins as described above, collected, washed twice with trypsin 0.25%-EDTA (GE Healthcare), and resuspended in DPBS (Life Technologies, Carlsbad, CA, USA). FITC-positive cells were evaluated by flow cytometry (Guava easyCyte 5HT; Merck Millipore).

For each sample, 10,000 live events were collected, and data were analyzed with FlowJo software (Tree Star, San Carlos, CA, USA).

Western Blot

For detection of intracellular ZFAs by western blot, cells were harvested and lysed with radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40 [NP-40], 1% sodium deoxycholate, and 0.1% SDS), supplemented with EDTA-free Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland). For the analysis of ZFA internalization and localization in the nuclear and cytoplasmic fractions, cells were washed twice with trypsin 0.25%-EDTA (GE Healthcare) and lysed using the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific), according to the manufacturer's protocol. The Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) was used to determine protein concentration, according to the manufacturer's instructions. ZFA protein detection was analyzed by 4%–12% SDS-PAGE (National Diagnostics), loaded with the indicated amount of total cellular protein extract. Samples were transferred onto a 0.2-μm Amersham Protran nitrocellulose membrane (GE Healthcare). ZFAs were detected by a horseradish peroxidase (HRP)-conjugated anti-HA monoclonal antibody (clone 3F10; Roche, Basel, Switzerland). Vinculin and β-tubulin loading controls were detected using, respectively, a mouse anti-Vinculin monoclonal antibody (clone 7F9; Santa Cruz Biotechnology, Dallas, TX, USA) and a mouse anti-β-tubulin monoclonal antibody (clone AA2; Sigma, St. Louis, MO, USA) and HRP-conjugated goat anti-mouse immunoglobulin G (IgG; Bio-Rad, Hercules, CA, USA). HIV capsid precursor p55 was detected using a mouse anti-p24 (clone 183-H12-5C; NIH AIDS Reagents) and HRP-conjugated goat anti-mouse IgG (Bio-Rad). Membranes were washed with TBS-0.2% Tween 20, and proteins were detected following incubation with Immobilon Western Chemiluminescent HRP substrate (Merck Millipore) and then revealed in a chemiluminescence film Amersham Hyperfilm Enhanced Chemiluminescence (ECL) (GE Healthcare).

Quantitative PCR (qPCR)

To determine HIV expression from J-Lat 10.6 and ACH-2 cell lines, total cellular RNA was extracted using the Trizol reagent (Thermo Fisher Scientific) and eluted in diethyl pyrocarbonate (DPEC)-treated water. RNA samples were treated with DNase I (Thermo Fisher Scientific), and cDNA was synthesized using the NZY reverse transcriptase (NZYTech) and random hexamer primer mix (NZYTech). qPCR was performed using the SensiFAST SYBR Hi-ROX Kit (Bioline, London, UK), according to the manufacturer's instructions. HIV viral gene transcription was detected using the primers 5′ HIV-Fwd and 3′ HIV-Rev. β-actin housekeeping gene transcription was detected using the primers 5′ β-actin-Fwd and 3′ β-actin-Rev. Primer sequences are provided in Table S3. HIV relative gene expression was determined by using the Delta-Delta Ct method and normalized to cells treated with ZF storage buffer alone (Mock).

Statistical Analysis

Statistical analyses for all experiments were performed from three independent experimental replicates (n = 3)—unless otherwise indicated—using a one-way ANOVA, followed by Bonferroni post hoc test, corrected for multiple comparisons (Prism Software 5.0; GraphPad Software). Statistically significant differences were expressed as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.05.016.

AUTHOR CONTRIBUTIONS

P.R.L.P., C.F.B. III, M.S.-M., and J.G. conceived and designed this study. P.R.L.P. and C.C.-S. performed the experiments and analyzed the data. P.R.L.P. drafted the manuscript. P.R.L.P., C.C.-S., M.S.-M., and J.G. discussed and reviewed the draft and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGMENTS

We thank Dr. Jia Liu for technical guidance on zinc-finger protein production. We thank Dr. Joel M. Gottesfeld for his generous mentorship. We thank Dr. Jacqueline van der Spuy for providing her laboratory facilities to complete this research. The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, and NIH: pNL4-3 from Dr. Malcolm Martin, Jurkat clone E6-1 from Dr. Arthur Wess, J-Lat 10.6 clone from Dr. Eric Verdin, and ACH-2 clone from Dr. Thomas Folks. This work was supported by grants from Alto Comissariado para a Saúde and Fundação para a Ciência e Tecnologia-Ministério da Educação e Ciência (FCT-MEC) VIH/SAU/0013/2011, VIH/SAU/0020/2011, and HIVERA/0002/2013; the Skaggs Institute for Chemical Biology; and the National Institutes of Health DP1CA174426. M.S.-M. was a recipient of a Ciencia 2008 contract from FCT-MEC. P.R.L.P. was a recipient of a PhD fellowship from FCT-MEC (SFRH/BD/81941/2011).

REFERENCES

1. Richman, D.D., Margolis, D.M., Delaney, M., Greene, W.C., Hazuda, D., and Pomerantz, R.J. (2009). The challenge of finding a cure for HIV infection. Science *323*, 1304–1307.

2. Ruelas, D.S., and Greene, W.C. (2013). An integrated overview of HIV-1 latency. Cell *155*, 519–529.

3. Spivak, A.M., and Planelles, V. (2016). HIV-1 Eradication: Early Trials (and Tribulations). Trends Mol. Med. *22*, 10–27.

4. Korin, Y.D., Brooks, D.G., Brown, S., Korotzer, A., and Zack, J.A. (2002). Effects of prostratin on T-cell activation and human immunodeficiency virus latency. J. Virol. *76*, 8118–8123.

5. Mehla, R., Bivalkar-Mehla, S., Zhang, R., Handy, I., Albrecht, H., Giri, S., Nagarkatti, P., Nagarkatti, M., and Chauhan, A. (2010). Bryostatin modulates latent HIV-1 infection via PKC and AMPK signaling but inhibits acute infection in a receptor independent manner. PLoS One *5*, e11160.

6. Lehrman, G., Hogue, I.B., Palmer, S., Jennings, C., Spina, C.A., Wiegand, A., Landay, A.L., Coombs, R.W., Richman, D.D., Mellors, J.W., et al. (2005). Depletion of latent HIV-1 infection in vivo: a proof-of-concept study. Lancet *366*, 549–555.

7. Contreras, X., Schweneker, M., Chen, C.-S., McCune, J.M., Deeks, S.G., Martin, J., and Peterlin, B.M. (2009). Suberoylanilide hydroxamic acid reactivates HIV from latently infected cells. J. Biol. Chem. *284*, 6782–6789.

8. Gaj, T., Gersbach, C.A., and Barbas, C.F., 3rd (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. *31*, 397–405.

9. Gersbach, C.A., Gaj, T., and Barbas, C.F., 3rd (2014). Synthetic zinc finger proteins: the advent of targeted gene regulation and genome modification technologies. Acc. Chem. Res. *47*, 2309–2318.

10. Doyle, E.L., Stoddard, B.L., Voytas, D.F., and Bogdanove, A.J. (2013). TAL effectors: highly adaptable phytobacterial virulence factors and readily engineered DNA-targeting proteins. Trends Cell Biol. *23*, 390–398.

11. Hsu, P.D., Lander, E.S., and Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. Cell *157*, 1262–1278.

12. Klug, A. (2010). The discovery of zinc fingers and their development for practical applications in gene regulation and genome manipulation. Q. Rev. Biophys. *43*, 1–21.

13. Isalan, M., Choo, Y., and Klug, A. (1997). Synergy between adjacent zinc fingers in sequence-specific DNA recognition. Proc. Natl. Acad. Sci. USA *94*, 5617–5621.

14. Thakore, P.I., Black, J.B., Hilton, I.B., and Gersbach, C.A. (2016). Editing the epigenome: technologies for programmable transcription and epigenetic modulation. Nat. Methods *13*, 127–137.

15. Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S., and Gregory, P.D. (2010). Genome editing with engineered zinc finger nucleases. Nat. Rev. Genet. *11*, 636–646.

16. Gaj, T., Sirk, S.J., and Barbas, C.F., 3rd (2014). Expanding the scope of site-specific recombinases for genetic and metabolic engineering. Biotechnol. Bioeng. *111*, 1–15.

17. 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.

18. Ebina, H., Misawa, N., Kanemura, Y., and Koyanagi, Y. (2013). Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. Sci. Rep. *3*, 2510.

19. Perez, E.E., Wang, J., Miller, J.C., Jouvenot, Y., Kim, K.A., Liu, O., Wang, N., Lee, G., Bartsevich, V.V., Lee, Y.L., et al. (2008). Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. Nat. Biotechnol. *26*, 808–816.

20. Holt, N., Wang, J., Kim, K., Friedman, G., Wang, X., Taupin, V., Crooks, G.M., Kohn, D.B., Gregory, P.D., Holmes, M.C., and Cannon, P.M. (2010). Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted to CCR5 control HIV-1 in vivo. Nat. Biotechnol. *28*, 839–847.

21. Tebas, P., Stein, D., Tang, W.W., Frank, I., Wang, S.Q., Lee, G., Spratt, S.K., Surosky, R.T., Giedlin, M.A., Nichol, G., et al. (2014). Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. N. Engl. J. Med. *370*, 901–910.

22. Isalan, M., Klug, A., and Choo, Y. (2001). A rapid, generally applicable method to engineer zinc fingers illustrated by targeting the HIV-1 promoter. Nat. Biotechnol. *19*, 656–660.

23. Reynolds, L., Ullman, C., Moore, M., Isalan, M., West, M.J., Clapham, P., Klug, A., and Choo, Y. (2003). Repression of the HIV-1 5′ LTR promoter and inhibition of HIV-1 replication by using engineered zinc-finger transcription factors. Proc. Natl. Acad. Sci. USA *100*, 1615–1620.

24. Segal, D.J., Gonçalves, J., Eberhardy, S., Swan, C.H., Torbett, B.E., Li, X., and Barbas, C.F., 3rd (2004). Attenuation of HIV-1 replication in primary human cells with a designed zinc finger transcription factor. J. Biol. Chem. *279*, 14509–14519.

25. Eberhardy, S.R., Goncalves, J., Coelho, S., Segal, D.J., Berkhout, B., and Barbas, C.F., 3rd (2006). Inhibition of human immunodeficiency virus type 1 replication with artificial transcription factors targeting the highly conserved primer-binding site. J. Virol. *80*, 2873–2883.

26. Sakkhachornphop, S., Barbas, C.F., 3rd, Keawvichit, R., Wongworapat, K., and Tayapiwatana, C. (2012). Zinc finger protein designed to target 2-long terminal repeat junctions interferes with human immunodeficiency virus integration. Hum. Gene Ther. *23*, 932–942.

27. Moonmuang, S., Saoin, S., Chupradit, K., Sakkhachornphop, S., Israsena, N., Rungsiwiwut, R., and Tayapiwatana, C. (2018). Modulated expression of the HIV-1 2LTR zinc finger efficiently interferes with the HIV integration process. Biosci. Rep. *38*, BSR20181109.

28. Perdigão, P., Gaj, T., Santa-Marta, M., Barbas, C.F., 3rd, and Goncalves, J. (2016). Reactivation of Latent HIV-1 Expression by Engineered TALE Transcription Factors. PLoS One *11*, e0150037.

29. Wang, P., Qu, X., Wang, X., Zhu, X., Zeng, H., Chen, H., and Zhu, H. (2014). Specific reactivation of latent HIV-1 with designer zinc-finger transcription factors targeting the HIV-1 5′-LTR promoter. Gene Ther. *21*, 490–495.

30. Wang, X., Wang, P., Fu, Z., Ji, H., Qu, X., Zeng, H., Zhu, X., Deng, J., Lu, P., Zha, S., et al. (2015). Designed transcription activator-like effector proteins efficiently induced the expression of latent HIV-1 in latently infected cells. AIDS Res. Hum. Retroviruses *31*, 98–106.

31. Geissler, R., Hauber, I., Funk, N., Richter, A., Behrens, M., Renner, I., Chemnitz, J., Hofmann-Sieber, H., Baum, H., van Lunzen, J., et al. (2015). Patient-adapted, specific activation of HIV-1 by customized TAL effectors (TALEs), a proof of principle study. Virology *486*, 248–254.

32. Zhang, Y., Yin, C., Zhang, T., Li, F., Yang, W., Kaminski, R., Fagan, P.R., Putatunda, R., Young, W.B., Khalili, K., and Hu, W. (2015). CRISPR/gRNA-directed synergistic activation mediator (SAM) induces specific, persistent and robust reactivation of the HIV-1 latent reservoirs. Sci. Rep. *5*, 16277.

33. Saayman, S.M., Lazar, D.C., Scott, T.A., Hart, J.R., Takahashi, M., Burnett, J.C., Planelles, V., Morris, K.V., and Weinberg, M.S. (2016). Potent and Targeted Activation of Latent HIV-1 Using the CRISPR/dCas9 Activator Complex. Mol. Ther. *24*, 488–498.

34. Limsirichai, P., Gaj, T., and Schaffer, D.V. (2016). CRISPR-mediated Activation of Latent HIV-1 Expression. Mol. Ther. *24*, 499–507.

35. Ji, H., Jiang, Z., Lu, P., Ma, L., Li, C., Pan, H., Fu, Z., Qu, X., Wang, P., Deng, J., et al. (2016). Specific Reactivation of Latent HIV-1 by dCas9-SunTag-VP64-mediated Guide RNA Targeting the HIV-1 Promoter. Mol. Ther. *24*, 508–521.

36. Bialek, J.K., Dunay, G.A., Voges, M., Schäfer, C., Spohn, M., Stucka, R., Hauber, J., and Lange, U.C. (2016). Targeted HIV-1 Latency Reversal Using CRISPR/Cas9-Derived Transcriptional Activator Systems. PLoS One *11*, e0158294.

37. Gaj, T., Liu, J., Anderson, K.E., Sirk, S.J., and Barbas, C.F. (2014). Protein delivery using Cys2-His2 zinc-finger domains. ACS Chem. Biol. *9*, 1662–1667.

38. Gaj, T., Guo, J., Kato, Y., Sirk, S.J., and Barbas, C.F., 3rd (2012). Targeted gene knockout by direct delivery of zinc-finger nuclease proteins. Nat. Methods *9*, 805–807.

39. Liu, J., Gaj, T., Wallen, M.C., and Barbas, C.F., 3rd (2015). Improved cell-penetrating zinc-finger nuclease proteins for precision genome engineering. Mol. Ther. Nucleic Acids *4*, e232.

40. Hossain, M.A., Shen, Y., Knudson, I., Thakur, S., Stees, J.R., Qiu, Y., Pace, B.S., Peterson, K.R., and Bungert, J. (2016). Activation of Fetal γ-globin Gene Expression via Direct Protein Delivery of Synthetic Zinc-finger DNA-Binding Domains. Mol. Ther. Nucleic Acids *5*, e378.

41. Mandell, J.G., and Barbas, C.F., 3rd (2006). Zinc Finger Tools: custom DNA-binding domains for transcription factors and nucleases. Nucleic Acids Res. *34*, W516–W523.

42. Dreier, B., Segal, D.J., and Barbas, C.F., 3rd (2000). Insights into the molecular recognition of the 5′-GNN-3′ family of DNA sequences by zinc finger domains. J. Mol. Biol. *303*, 489–502.

43. Dreier, B., Beerli, R.R., Segal, D.J., Flippin, J.D., and Barbas, C.F., 3rd (2001). Development of zinc finger domains for recognition of the 5′-ANN-3′ family of DNA sequences and their use in the construction of artificial transcription factors. J. Biol. Chem. *276*, 29466–29478.

44. Carroll, D., Morton, J.J., Beumer, K.J., and Segal, D.J. (2006). Design, construction and in vitro testing of zinc finger nucleases. Nat. Protoc. *1*, 1329–1341.

45. Dreier, B., Fuller, R.P., Segal, D.J., Lund, C.V., Blancafort, P., Huber, A., Koksch, B., and Barbas, C.F., 3rd (2005). Development of zinc finger domains for recognition of the 5′-CNN-3′ family DNA sequences and their use in the construction of artificial transcription factors. J. Biol. Chem. *280*, 35588–35597.

46. Shimizu, Y., Şöllü, C., Meckler, J.F., Adriaenssens, A., Zykovich, A., Cathomen, T., and Segal, D.J. (2011). Adding fingers to an engineered zinc finger nuclease can reduce activity. Biochemistry *50*, 5033–5041.

47. Liu, Q., Segal, D.J., Ghiara, J.B., and Barbas, C.F., 3rd (1997). Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. Proc. Natl. Acad. Sci. USA *94*, 5525–5530.

48. Beerli, R.R., Segal, D.J., Dreier, B., and Barbas, C.F., 3rd (1998). Toward controlling gene expression at will: specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. Proc. Natl. Acad. Sci. USA *95*, 14628–14633.

49. Hirai, H., Tani, T., and Kikyo, N. (2010). Structure and functions of powerful transactivators: VP16, MyoD and FoxA. Int. J. Dev. Biol. *54*, 1589–1596.

50. Liu, P.Q., Rebar, E.J., Zhang, L., Liu, Q., Jamieson, A.C., Liang, Y., Qi, H., Li, P.X., Chen, B., Mendel, M.C., et al. (2001). Regulation of an endogenous locus using a panel of designed zinc finger proteins targeted to accessible chromatin regions. Activation of vascular endothelial growth factor A. J. Biol. Chem. *276*, 11323–11334.

51. Gräslund, T., Li, X., Magnenat, L., Popkov, M., and Barbas, C.F., 3rd (2005). Exploring strategies for the design of artificial transcription factors: targeting sites proximal to known regulatory regions for the induction of gamma-globin expression and the treatment of sickle cell disease. J. Biol. Chem. *280*, 3707–3714.

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

53. Mak, J., and Kleiman, L. (1997). Primer tRNAs for reverse transcription. J. Virol. *71*, 8087–8095.

54. Frecha, C., Lévy, C., Costa, C., Nègre, D., Amirache, F., Buckland, R., Russell, S.J., Cosset, F.L., and Verhoeyen, E. (2011). Measles virus glycoprotein-pseudotyped lentiviral vector-mediated gene transfer into quiescent lymphocytes requires binding to both SLAM and CD46 entry receptors. J. Virol. *85*, 5975–5985.

55. Tritel, M., and Resh, M.D. (2000). Kinetic analysis of human immunodeficiency virus type 1 assembly reveals the presence of sequential intermediates. J. Virol. *74*, 5845–5855.

56. Clouse, K.A., Powell, D., Washington, I., Poli, G., Strebel, K., Farrar, W., Barstad, P., Kovacs, J., Fauci, A.S., and Folks, T.M. (1989). Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. J. Immunol. *142*, 431–438.

57. Folks, T.M., Justement, J., Kinter, A., Dinarello, C.A., and Fauci, A.S. (1987). Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. Science *238*, 800–802.

58. Saayman, S., Ali, S.A., Morris, K.V., and Weinberg, M.S. (2015). The therapeutic application of CRISPR/Cas9 technologies for HIV. Expert Opin. Biol. Ther. *15*, 819–830.

59. Sarkar, I., Hauber, I., Hauber, J., and Buchholz, F. (2007). HIV-1 proviral DNA excision using an evolved recombinase. Science *316*, 1912–1915.

60. Wang, G., Zhao, N., Berkhout, B., and Das, A.T. (2016). CRISPR-Cas9 Can Inhibit HIV-1 Replication but NHEJ Repair Facilitates Virus Escape. Mol. Ther. *24*, 522–526.

61. Yoder, K.E., and Bundschuh, R. (2016). Host Double Strand Break Repair Generates HIV-1 Strains Resistant to CRISPR/Cas9. Sci. Rep. *6*, 29530.

62. Polstein, L.R., Perez-Pinera, P., Kocak, D.D., Vockley, C.M., Bledsoe, P., Song, L., Safi, A., Crawford, G.E., Reddy, T.E., and Gersbach, C.A. (2015). Genome-wide specificity of DNA binding, gene regulation, and chromatin remodeling by TALE- and CRISPR/Cas9-based transcriptional activators. Genome Res. *25*, 1158–1169.

63. Perez-Pinera, P., Kocak, D.D., Vockley, C.M., Adler, A.F., Kabadi, A.M., Polstein, L.R., Thakore, P.I., Glass, K.A., Ousterout, D.G., Leong, K.W., et al. (2013). RNA-guided gene activation by CRISPR-Cas9-based transcription factors. Nat. Methods *10*, 973–976.

64. Moore, M., Klug, A., and Choo, Y. (2001). Improved DNA binding specificity from polyzinc finger peptides by using strings of two-finger units. Proc. Natl. Acad. Sci. USA *98*, 1437–1441.

65. Agustín-Pavón, C., Mielcarek, M., Garriga-Canut, M., and Isalan, M. (2016). Deimmunization for gene therapy: host matching of synthetic zinc finger constructs enables long-term mutant Huntingtin repression in mice. Mol. Neurodegener. *11*, 64.

66. De Baar, M.P., De Ronde, A., Berkhout, B., Cornelissen, M., Van Der Horn, K.H., Van Der Schoot, A.M., De Wolf, F., Lukashov, V.V., and Goudsmit, J. (2000). Subtype-specific sequence variation of the HIV type 1 long terminal repeat and primer-binding site. AIDS Res. Hum. Retroviruses *16*, 499–504.

67. Chen, X., and Gonçalves, M.A.F.V. (2016). Engineered Viruses as Genome Editing Devices. Mol. Ther. *24*, 447–457.

68. Liu, J., and Shui, S.L. (2016). Delivery methods for site-specific nucleases: Achieving the full potential of therapeutic gene editing. J. Control. Release *244* (Pt A), 83–97.

69. Karn, J. (2011). The molecular biology of HIV latency: breaking and restoring the Tat-dependent transcriptional circuit. Curr. Opin. HIV AIDS *6*, 4–11.

70. Yin, H., Kauffman, K.J., and Anderson, D.G. (2017). Delivery technologies for genome editing. Nat. Rev. Drug Discov. *16*, 387–399.

71. Cillo, A.R., Sobolewski, M.D., Bosch, R.J., Fyne, E., Piatak, M., Jr., Coffin, J.M., and Mellors, J.W. (2014). Quantification of HIV-1 latency reversal in resting CD4+ T cells from patients on suppressive antiretroviral therapy. Proc. Natl. Acad. Sci. USA *111*, 7078–7083.

72. Bonczkowski, P., De Scheerder, M.-A., De Spiegelaere, W., and Vandekerckhove, L. (2016). Minimal Requirements for Primary HIV Latency Models Based on a Systematic Review. AIDS Rev. *18*, 171–183.

73. Duyne, R.V., Narayanan, A., K-Hall, K., Saifuddin, M., Shultz, L., and Kashanchi, F. (2011). Humanized mouse models of HIV-1 latency. Curr. HIV Res. *9*, 595–605.

74. Chavez, A., Scheiman, J., Vora, S., Pruitt, B.W., Tuttle, M., P R Iyer, E., Lin, S., Kiani, S., Guzman, C.D., Wiegand, D.J., et al. (2015). Highly efficient Cas9-mediated transcriptional programming. Nat. Methods *12*, 326–328.

75. Tachikawa, K., Schröder, O., Frey, G., Briggs, S.P., and Sera, T. (2004). Regulation of the endogenous VEGF-A gene by exogenous designed regulatory proteins. Proc. Natl. Acad. Sci. USA *101*, 15225–15230.

76. Bailus, B.J., Pyles, B., McAlister, M.M., O'Geen, H., Lockwood, S.H., Adams, A.N., Nguyen, J.T., Yu, A., Berman, R.F., and Segal, D.J. (2016). Protein Delivery of an Artificial Transcription Factor Restores Widespread Ube3a Expression in an Angelman Syndrome Mouse Brain. Mol. Ther. *24*, 548–555.

77. Marban, C., Forouzanfar, F., Ait-Ammar, A., Fahmi, F., El Mekdad, H., Daouad, F., Rohr, O., and Schwartz, C. (2016). Targeting the Brain Reservoirs: Toward an HIV Cure. Front. Immunol. *7*, 397.

78. Cunha-Santos, C., Perdigao, P.R.L., Martin, F., Oliveira, J.G., Cardoso, M., Manuel, A., Taveira, N., and Goncalves, J. (2019). Inhibition of HIV replication through siRNA carried by CXCR4-targeted chimeric nanobody. Cell. Mol. Life Sci. Published online October 22, 2019.

79. Zhou, J., Lazar, D., Li, H., Xia, X., Satheesan, S., Charlins, P., O'Mealy, D., Akkina, R., Saayman, S., Weinberg, M.S., et al. (2018). Receptor-targeted aptamer-siRNA conjugate-directed transcriptional regulation of HIV-1. Theranostics *8*, 1575–1590.

80. Chen, Z., Jaafar, L., Agyekum, D.G., Xiao, H., Wade, M.F., Kumaran, R.I., Spector, D.L., Bao, G., Porteus, M.H., Dynan, W.S., and Meiler, S.E. (2013). Receptor-mediated delivery of engineered nucleases for genome modification. Nucleic Acids Res. *41*, e182.

81. Descours, B., Petitjean, G., López-Zaragoza, J.-L., Bruel, T., Raffel, R., Psomas, C., Reynes, J., Lacabaratz, C., Levy, Y., Schwartz, O., et al. (2017). CD32a is a marker of a CD4 T-cell HIV reservoir harbouring replication-competent proviruses. Nature *543*, 564–567.

82. Gonzalez, B., Schwimmer, L.J., Fuller, R.P., Ye, Y., Asawapornmongkol, L., and Barbas, C.F., 3rd (2010). Modular system for the construction of zinc-finger libraries and proteins. Nat. Protoc. *5*, 791–810.

83. Cunha-Santos, C., Figueira, T.N., Borrego, P., Oliveira, S.S., Rocha, C., Couto, A., Cantante, C., Santos-Costa, Q., Azevedo-Pereira, J.M., Fontes, C.M., et al. (2016). Development of synthetic light-chain antibodies as novel and potent HIV fusion inhibitors. AIDS *30*, 1691–1701.