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Original Article

A conditional RNA Pol II mono-promoter drives HIV-inducible, CRISPR-mediated cyclin T1 suppression and HIV inhibition

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Gene editing using clustered regularly interspaced short palindromic repeats (CRISPR) targeted to HIV proviral DNA has shown excision of HIV from infected cells. However, CRISPR-based HIV excision is vulnerable to viral escape. Targeting cellular co-factors provides an attractive yet risky alternative to render viral escape irrelevant. Cyclin T1 is a critical modulator of HIV transcription and mediates recruitment of positive transcription elongation factor-b (P-TEFb) kinase for transcriptional elongation. Hence, a CRISPR-mediated cyclin T1 inactivation will silence HIV transcription, locking it in an inactive form in the cell and thereby serving as an effective antiviral and possibly effecting a functional cure. However, cellular genes play important roles, and their uncontrolled inhibition can promote undesirable effects. Here, we demonstrate a conditional inducible RNA polymerase II (RNA Pol II) mono-promoter-based co-expression of a CRISPR system targeting cyclin T1 from a single transcription unit. Co-expression of guide RNA (gRNA) and CRISPR-associated protein (Cas9) is observed only in HIV-infected cells and leads to sustained HIV suppression in stringent chronically infected cell lines as well as in T cell lines. We further show that incorporation of *cis*-acting ribozymes immediately upstream of the gRNA further enhances HIV silencing.

INTRODUCTION

Clustered regularly interspacedshort palindromic repeats (CRISPR)-CRISPR-associated protein (Cas) systems have been extensively used for loss-of-function research as well as therapeutic disease to modulate disease outcomes. CRISPR has been employed for gene editing, epigenetic modulation, or transcriptional control (using modified Cas9 activators). CRISPR depends on complementarity between the guide RNA (gRNA) sequence and the DNA sequence to hybridize and direct Cas9-mediated cleavage of double-stranded DNA. Even though hybridization of the ∼20 bp region between the gRNA and the target DNA is necessary for CRISPR-mediated cleavage, unregulated CRISPR expression can lead to off-target effects,[1–4] resulting in genetic mutations that can cause loss of gene function, leading to carcinogenesis or toxicity. Moreover, all expression systems to date produce gRNAs from RNA polymerase III (RNA Pol III)-based promoters, resulting in uncontrolled overexpression of gRNAs that can also mediate antisense effects on cellular RNAs due to partial complementarity further contributing to off-target effects. While constitutive expression is acceptable for gene editing in functional genomic studies, therapeutic applications of CRISPR require temporal, conditional, or cell-type-specific CRISPR expression. Several drug-inducible systems have been reported that regulate transcriptional and post-transcriptional Cas9 expression (for review, see Zhang et al.[5]). Some studies have attempted inducible Tet-ON and Tet-OFF RNA Pol II promoters for temporal control of Cas9 expression.[6] Yet, others have used Cre-based transcriptional regulation.[7] These systems always involve RNA Pol II-mediated inducible Cas9 expression while retaining RNA Pol III-mediated constitutive gRNA expression, thereby requiring separate transcriptional units. While a drug-inducible system provides temporal control in a research setting, it is not suitable in a therapeutic setting, as every cell harboring the CRISPR system would express Cas9 (and the constitutively expressed gRNA) upon administration of the drug. Conditional or cell-type-specific expression has distinct advantages in that the expression system can be triggered only in specific cells or tissues under certain conditions, thereby allowing greater flexibility in targeting genes that can otherwise have important roles in cellular homeostasis. In this article, we demonstrate an RNA Pol II mono-promoter-based conditional CRISPR expression system that co-expresses a gRNA and Cas9 from a single expression cassette to effect negative feedback silencing of HIV.

The advent of combined antiretroviral therapy (cART) has led to a dramatic decline in morbidity and mortality from HIV/AIDS.

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However, cART is unable to eradicate HIV due to established HIV reservoirs. HIV replication persists even in presence of suppressive cART.[8,9] Moreover, a recent paradigm-shifting single genome study shows that the "silent majority" of the HIV reservoir harbors transcriptionally active HIV that continuously adapts to suppressive factors, allowing the reservoir to survive, proliferate, and persist even with suppressive cART.[10,11] These transcriptionally active reservoirs can express viral proteins like Tat and gp120 even with suppressive cART, leading to pleotropic effects. This is one of the primary causes of non-AIDS comorbidities of HIV. A number of cell types can serve as HIV reservoirs in diverse tissues namely resting CD4+ T cells, macrophages, astrocytes, and microglia.[12] While CRISPR-mediated HIV excision has shown promise, its application is stymied by some of the same limitations with previous gene therapy approaches targeting the viral RNA/genome, namely mutations within the hybridization and cleavage sites.[13] Targeting cellular co-factors critical to HIV replication remains the most attractive (to prevent HIV escape) yet risky strategy (due to cellular roles of these genes) to cure HIV or effect long-term suppression.

Positive transcription elongation factor-b (P-TEFb), a heterodimer of CDK9 and cyclin T1, serves as a master regulator of HIV transcription. HIV Tat recruits P-TEFb, which phosphorylates negative elongation factor (NELF) and the C-terminal domain of RNA Pol II to overcome promoter proximal pausing and make the polymerase elongation competent.[14] Viral reactivation by Tat-dependent and Tat-independent means requires cyclin T1-mediated CDK9 recruitment.[15–19] Hence, P-TEFb kinase-mediated phosphorylation of RNA Pol II serves as a master switch to turn on HIV replication, and inhibition of P-TEFb blocks HIV replication.[20–22] In our earlier reports, we have shown that an HIV long-terminal repeat (LTR)-minimal hsp70 fusion promoter can co-express a small RNA (small hairpin RNA [shRNA]) and a protein (RevM10).[23] In this study, we report that our HIV LTR-hsp70 fusion promoter directs HIV-inducible co-expression of gRNA and Cas9 from a single cassette. As proof of concept, we show that a CRISPR system targeting the HIV cellular co-factor cyclin T1 is expressed (and mediates Cyclin T1 inactivation) only in HIV-infected cells. This results in sustained suppression of HIV without affecting cell viability. We further demonstrate that inserting *cis*-acting ribozymes to remove the 5-methyl guanosine cap on gRNAs further improves efficacy and duration of HIV silencing. We posit that the gRNA and Cas9 expression would be self-limiting, as the CRISPR expression system also depends on Tat-cyclin T1 interaction. Once cyclin T1 is eliminated, transcription from both the proviral DNA as well as the fusion promoter would cease.

RESULTS

HIV Tat interacts with cyclin T1 to recruit CDK9 to the paused RNA Pol II, and this interaction is considered both necessary and sufficient for HIV transcription.[24–27] CRISPR efficiency depends on several factors including nucleotides near the protospacer-adjacent motif (PAM) site and the epigenetic assembly at or near the target site.[28,29] Moreover, the G-C percentage and secondary structures of the gRNA itself can play an important role in determining efficiency of CRISPR-mediated genome editing.[28]

To screen an effective editing site, we tested three different gRNA sequences designed by Genescript, gRNA-1, gRNA-2, and gRNA-3, which were purchased as lentiviral vector clones (pLentiCRISPR-gRNA-1, pLentiCRISPR-gRNA-2, or pLentiCRISPR-gRNA-3). A U6 promoter drives RNA Pol III-mediated gRNA expression with an RNA Pol II EFS promoter drives Cas9 expression. The three cyclin T1 gRNAs were tested individually in transient transfection assays in HeLa cells for their ability to inactivate cyclin T1 (lentiviral vector pHIV-7-GFP,[23,30] as control). 8 days post-transfection, total protein was analyzed for cyclin T1 by western blot analyses. pLentiCRISPR-gRNA-2 showed the best cyclin T1 inactivation (Figure 1A). Next, we determined if the extent of cyclin T1 inactivation correlates to HIV inhibition. For these experiments, we used more stringent post-infection models. Chronically HIV-infected HeLa-CD4 cells were transfected with either pHIV-7-GFP (control) or individually with pLentiCRISPR-gRNA-1, pLentiCRISPR-gRNA-2, or pLenti-CRISPR-gRNA-3. pHIV-7-GFP also served as an index of transfection efficiency. Experiments were allowed to proceed for 8 days to allow cyclin T1 inactivation to have a measurable effect on HIV p24 levels. Culture supernatants were analyzed on days 6 and 8 post-transfection by enzyme-linked immunosorbent assays (ELISAs) for HIV p24, a reliable indicator of HIV infection. Experiments were terminated on day 8, and cell viability and the number of live cells were determined. All three gRNAs suppressed HIV, with pLentiCRISPR-gRNA-2 showing the best suppression (Figure 1B). A ~96%–98% suppression of HIV is observed on days 6 and 8 post-transfection for p-LentiCRISPR-gRNA-2.

HIV suppression was observed 8 days following transfection, by which time the transfected plasmid would have been eliminated from the cultures, suggesting that cyclin T1 inactivation can lead to HIV silencing even after CRISPR expression has ceased. While P-TEFb is a heterodimer of CDK9 and cyclin T1, targeting cyclin T1 has distinct advantages in that CDK9 can partner with other cyclins, namely cyclin T2A, cyclin T2B, and cyclin K, and provide redundancy for cellular transcription by P-TEFb.[17,31,32] However, cyclin T1 is critical for Tat-mediated recruitment of P-TEFb. Other reports have also shown that cyclin T1 silencing does not adversely affect cell viability.[20,33] Our data agree with these observations in that cyclin T1 suppression did not affect the percentage of cell viability even after 8 days (Figure 1C). However, there was a noticeable difference in the number of viable cells in all pLentiCRISPR-gRNA transfections (Figure 1C).

To mitigate any deleterious effects of uncontrolled CRISPR expression on cellular homeostasis, we designed a self-limiting RNA Pol II-based, HIV-inducible CRISPR expression system for CyclinT1 inactivation only in HIV infected cells. We had previously reported that an HIV LTR-minimal Drosophila hsp70 fusion promoter drives HIV-inducible expression of small RNA molecules like small hairpin RNAs (shRNAs).[30] The shRNA expression system utilizes a minimal polyadenylation (mpolyA) signal sequence reported by Xia et al. to

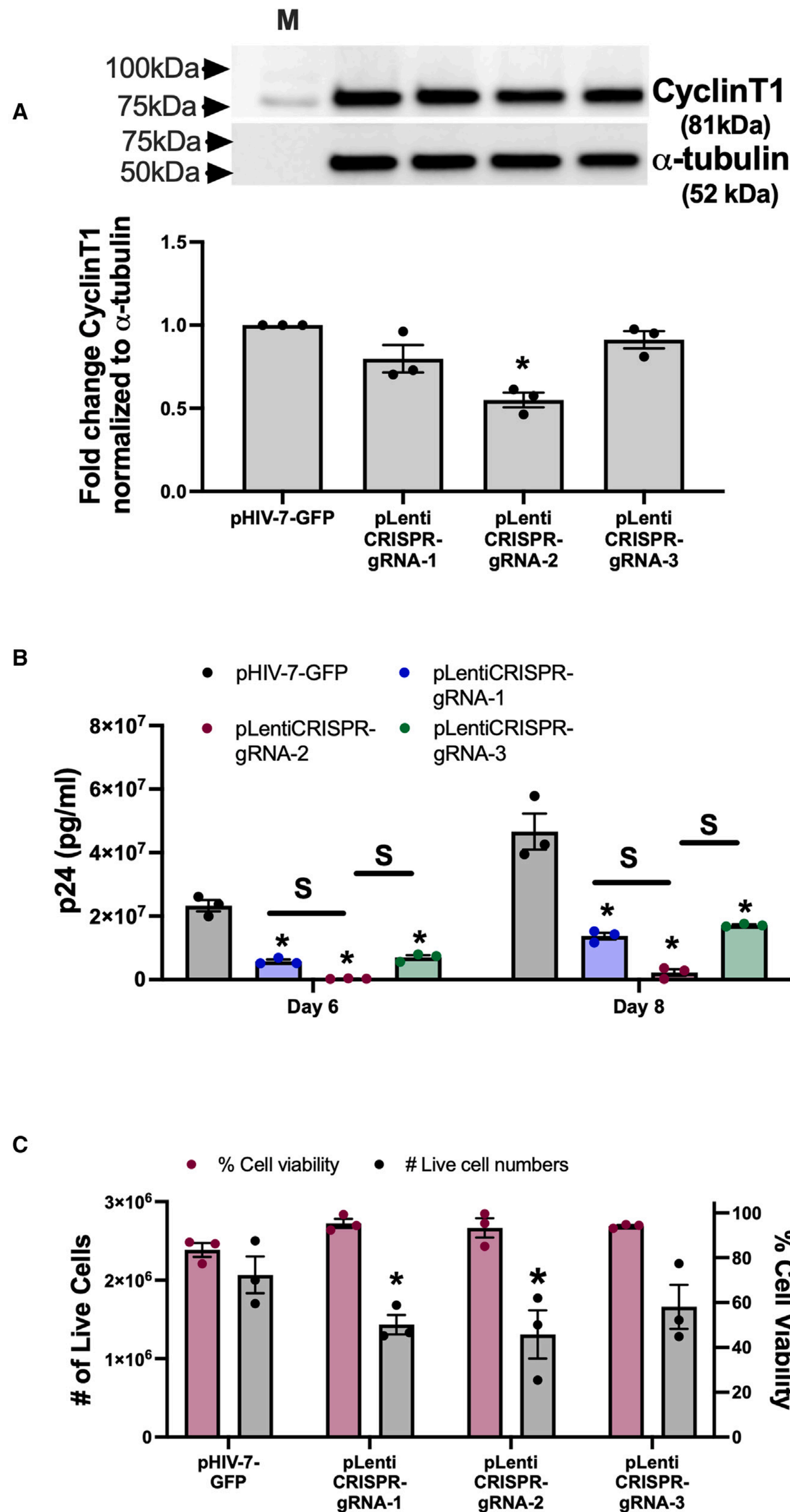


Figure 1. Screening effective gRNA target sites for cyclin T1 editing: HeLa cells were transfected with pLentiCRISPR-gRNA-1, pLentiCRISPR-gRNA-2, or pLentiCRISPR-gRNA-3 using lipofectamine 2000 as described in methods

Our previously reported lentiviral vector pHIV-7-GFP was transfected as control.[23,30] 8 days post-transfection, total protein was isolated and analyzed for cyclin T1 protein levels using western blot analyses. pLentiCRISPR-gRNA-2 demonstrates maximal cyclin T1 suppression (A). HIV-infected HeLa-CD4 cells were transfected with pLentiCRISPR-gRNA-1, pLentiCRISPR-gRNA-2, or pLentiCRISPR-gRNA-3. At designated time points, culture supernatants were collected and analyzed for HIV p24. All three gRNAs demonstrate HIV suppression, with pLentiCRISPR-gRNA-2 demonstrating maximal suppression at both time points (B). Cells were trypsinized, followed by trypsin neutralization buffer. Cells were washed to remove trypsin and resuspended in DMEM 10% FBS. The otal number of live cells and percentage of viability were determined by trypan blue staining as described in materials and methods. Cyclin T1 knockdown does not affect the percentage of cell viability. However, we observed a statistically significant decline in the number of live cells with all pLentiCRISPR-gRNAs, including our most efficient pLentiCRISPR-gRNA-2 (C). n = mean ± SEM from 3 independent experiments. *, significant from control; S, significant from each other ($p < 0.05$).

terminate transcription of the small RNA molecules.[34] We had further exploited the transcriptional readthrough of the weak mpolyA signal sequence for co-expressing an shRNA and GFP or the antiviral transdominant RevM10 protein, thereby effecting a co-expression of small RNA and a protein only in HIV-infected cells from a single transcriptional cassette.[23] We adapted this system for HIV-inducible expression of gRNA and Cas9 protein. We employed precise PCR-based cloning and ligation strategies to clone gRNA-2 immediately downstream of the minimal Drosophila hsp70 promoter such that transcription of gRNA begins from +1 of the minimal Drosophila hsp70 promoter. The fusion promoter-gRNA-mpolyA cassette was then inserted upstream of the Cas9 open reading frame (ORF) in the pLentiCRISPRV2, while deleting the U6 gRNA expression cassette and the EFS promoter, to generate a LTRhsp-CRISPR (LTRhsp-gRNA-mpolyA-Cas9pA) cassette (Figure 2A). The HIV LTR contains two nuclear factor κB (NF-κB) and three SP1 sites each within the LTR that may lead to Tat-independent and consequently leaky CRISPR expression. We tested NF-κB (LTRhsp(ΔNF-κB)-CRISPR) or SP1 site (LTR(ΔSP1)-CRISPR) deletion versions of our fusion promoter for HIV-inducible Cas9 expression. HIV-infected HeLa-CD4 cells were transfected with our LTRhsp-CRISPR (or the ΔNF-κB or ΔSP1 mutants). Uninfected Hela-CD4 cells were transfected identically for comparison. LTRhsp-CRISPR demonstrates Cas9 expression only in HIV-infected HeLa cells and not in uninfected HeLa cells (Figure 2B). Deleting the NF-κB sites completely abolishes Cas9 expression from the fusion promoter, suggesting that NF-κB may be essential for transcription from the fusion promoter even in the presence of HIV Tat. We found detectable Cas9 expression in uninfected HeLa-CD4 cells transfected with LTR(ΔSP1)-CRISPR, suggesting that deleting SP1 sites promotes leaky expression from the fusion promoter (Figure 2B). Hence, we restricted future experiments to the LTRhsp-CRISPR construct.

Next, we tried to determine if HIV-inducible gRNA and Cas9 expression from LTRhsp-CRISPR translates to conditional cyclin T1 inactivation only in HIV-infected cells. HIV-infected HeLa-CD4 cells (HeLa-CD4 cells as uninfected controls) were transfected with our LTRhsp-CRISPR. Separately, pLentiCRISPR-gRNA-2 was transfected for comparison. 8 days post-transfection, experiments were terminated, and total protein was analyzed for cyclin T1 suppression by western blot analysis. pLentiCRISPR-gRNA-2 suppressed cyclin T1 protein levels in both infected and uninfected HeLa-CD4 cells, while LTRhsp-CRISPR demonstrates cyclin T1 suppression only in HIV-infected HeLa-CD4 cells. No suppression of cyclin T1 is observed by LTRhsp-CRISPR in uninfected HeLa-CD4 cells (Figures 2C and 2D). To determine the extent of LTRhsp-CRISPR efficacy for HIV suppression, we transiently transfected HIV-infected HeLa-CD4 cells with LTRhsp-CRISPR (pHIV-7-GFP as control). LTRhsp-CRISPR showed ∼75% suppression of HIV p24 (Figure 2E). However, the suppression efficacy was lower than that observed with the constitutive pLentiCRISPR-gRNA-2 (Figure 1B).

One of the limitations of our expression system is a paradox where the gRNA must be retained in the nucleus while the Cas9 mRNA must be exported to the cytoplasm for translation. mRNA processing of RNA Pol II-based transcripts results in addition of the 5’ cap to all mRNAs, which facilitates their nuclear export and translation. Hence for gRNAs, which require nuclear retention, the 5′-methyl guanosine cap must be removed for nuclear retention. Conversely, Cas9-expressing mRNA needs to retain the cap for nuclear export. However, LTRhsp-CRISPR would generate 5′-cap transcripts for both gRNA and Cas9, leading to much of the gRNA being exported from the nucleus to the cytoplasm, thereby decreasing CRISPR efficacy. To overcome this limitation and to improve HIV suppression, we inserted a *cis*-cleaving ribozyme, previously reported by us,[35,36] immediately upstream of the gRNA (Figure 3A). The *cis*-cleaving ribozyme would remove the 5′ cap from a proportion of transcripts, thereby increasing nuclear retention of the gRNA. It is necessary to ensure that a proportion of transcripts, especially the Cas9 mRNA, still retain the 5′ cap for cytoplasmic export and Cas9 expression. We used modified hammerhead ribozymes called minizymes to attenuate the ribozyme efficacy (LTRhsp-MzCRISPR). Minizymes are variants of hammerhead ribozymes in which stem II has been replaced by a shorter linker sequence.[37] Like hammerhead ribozymes, they target the NUX site (X = A, U, or C). They demonstrate lower RNA cleavage efficiency compared with full-length hammerhead ribozymes. We also designed LTRhsp-CRISPR with a weaker version of the minizyme (LTRhsp-Mz(Wk)CRISPR) that uses a non-canonical cleavage site (X = G) to determine the optimal balance between cap retention and cap removal for maximal CRISPR efficiency. The lower activity of minizymes would mediate cap removal and nuclear retention of a proportion of the transcripts including the gRNA, while the uncleaved transcripts with an intact 5′ cap would be exported to the cytoplasm for Cas9 translation. Figure 3B shows a schematic of the *cis*-cleaving ribozymes and cleavage upstream of the gRNA to remove the 5′ cap. Next, we tested LTRhsp-MzCRISPR and LTRhsp-Mz(Wk)CRISPR for their ability to suppress HIV in HIV-infected Hela-CD4 cells. LTRhsp-CRISPR was transfected for comparison. Culture supernatants were collected every 72 h and analyzed for p24 analysis as an index of HIV infection. LTRhsp-MzCRISPR and LTRhsp-Mz(Wk)CRISPR show some improvement in HIV suppression compared with the original LTRhsp-CRISPR (Figure 3C). After 12 days, experiments were terminated, and cell viability and live cell numbers were determined. We did not observe any differences in cell viability or number of live cells with our HIV-inducible CRISPR constructs (Figure 3D). This is in line with other reports also demonstrating that cyclin T1 silencing does not adversely affect cell viability.[20,33] This demonstrates that embedding ribozymes in our RNA Pol II CRISPR cassette improves its efficacy.

To investigate the potential of this system in a more physiologically relevant post-infection model CEM T cell lines were infected with the X4-tropic HIV IIIB strain. The infection was allowed to proceed for 12 days. HIV p24 levels were monitored to follow the progress of infection. Following 12 days of infection, infected cultures were divided into different experimental sets and then electroporated with either the original LTRhsp-CRISPR or the minizyme-embedded construct

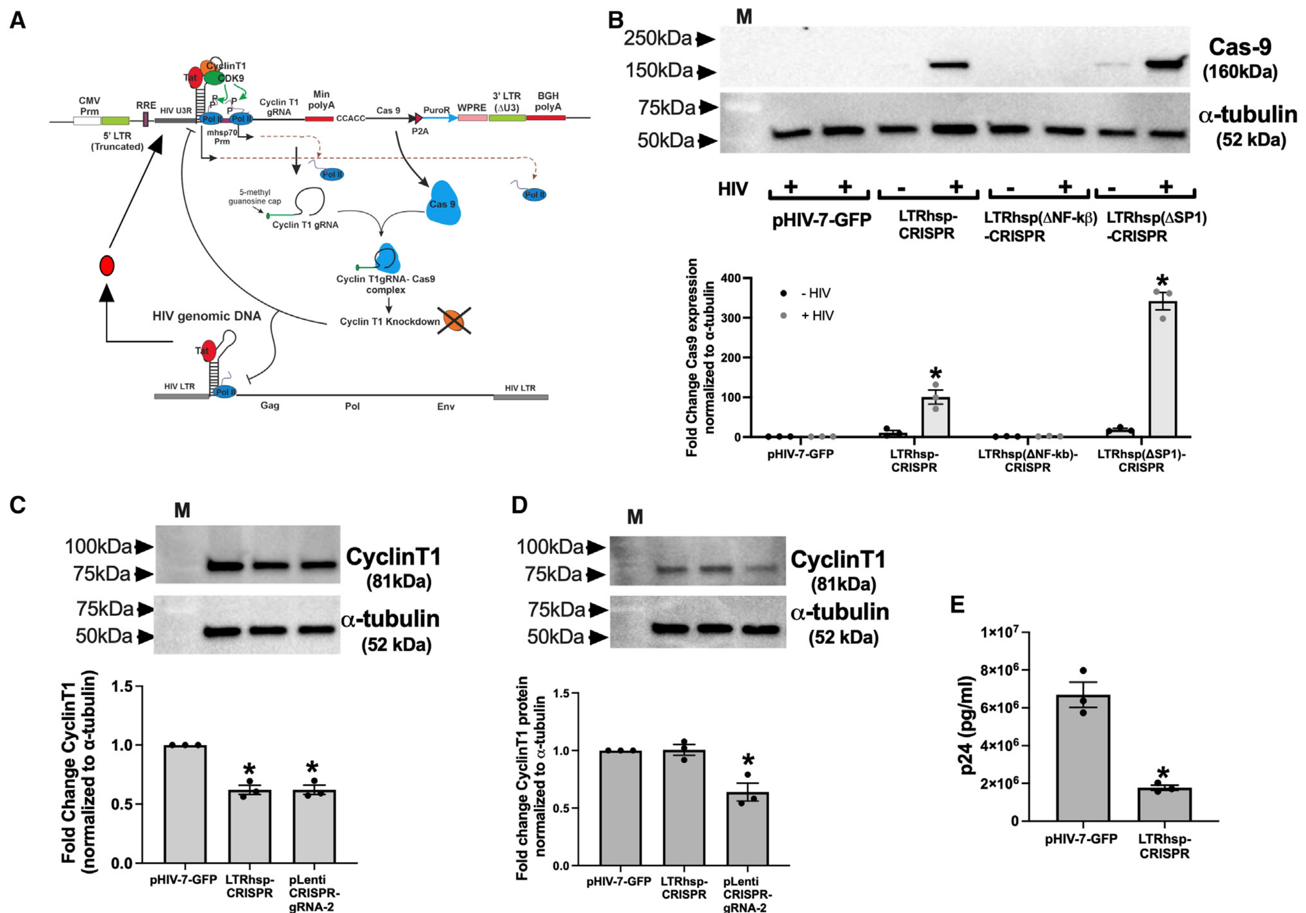


Figure 2. HIV LTR-hsp70 fusion promoter demonstrates HIV-inducible co-expression of cyclin T1-targeting gRNA and Cas9

(A) Schematic of LTRhsp-CRISPR cassette (not drawn to scale): in presence of HIV Tat, P-TEFb kinase is recruited to the HIV LTR-minimal Drosophila hsp70 fusion promoter, inducing transcription from both promoters. Most of the transcription from the minimal hsp70 promoter will terminate at the minimal polyA to express the cyclin T1-targeting gRNA-2, while transcriptional readthrough will encode the Cas9. The strong eukaryotic translation initiation signal CCACC ensures that the first ATG after this sequence is used for translation initiation. Given the critical importance of cyclin T1 for P-TEFb recruitment by HIV Tat, inactivation of cyclin T1 will irreversibly block all transcription from HIV locking it in a transcriptionally inactive state. Since the fusion promoter also requires Tat-cyclin T1 interaction for co-expression of cyclin T1 gRNA and Cas9, once cyclin T1 is knocked down, transcription from both the HIV proviral DNA as well as the fusion promoter will be completely inhibited, possibly affecting a functional cure and limiting any further expression from the fusion promoter. (B) Uninfected HeLa-CD4 cells or HIV-infected HeLa-CD4 cells were transfected with LTRhsp-CRISPR or the NF-κB or SP1 deletion mutants, (LTRhsp(ΔNF-κB)-CRISPR or LTRhsp(ΔSP1)-CRISPR respectively). 72 h post-transfection, total protein was isolated and analyzed for Cas9 expression by western blot analyses. HIV LTR-hsp70 fusion promoter demonstrates Cas9 expression only in HIV-infected HeLa-CD4 cells and not in uninfected HeLa-CD4 cells, demonstrating HIV-inducible expression. No expression of Cas9 is observed in LTRhsp(ΔNF-κB)-CRISPR-transfected uninfected or HIV-infected HeLa-CD4 cells, suggesting that NF-κB sites are important for transcription of Cas9 from the fusion promoter even in the presence of HIV Tat. Some expression is observed in uninfected HeLa-CD4 cells transfected with LTRhsp(ΔSP1)-CRISPR, suggesting that deleting the SP1 sites promotes leaky transcription from the fusion promoter. (C and D) Uninfected or HIV-infected HeLa-CD4 cells were transfected with LTRhsp-CRISPR or the constitutive pLentiCRISPR-gRNA-2. 6 days post-transfection, total protein was isolated and analyzed for cyclin T1 suppression by western blot analyses. Both LTRhsp-CRISPR and pLentiCRISPR-gRNA-2 demonstrate cyclin T1 suppression in HIV-infected HeLa-CD4 cells (C). However, only the constitutive pLentiCRISPR-gRNA-2 demonstrates cyclin T1 suppression in uninfected HeLa-CD4 cells. LTRhsp-CRISPR does not suppress cyclin T1 in uninfected HeLa-CD4 cells, demonstrating HIV-inducible cyclin T1 knockdown from our fusion promoter (D). (E) HIV-infected HeLa-CD4 cells were transfected with LTRhsp-CRISPR as described in the materials and methods. Cells were washed four times to remove any residual HIV p24 and resuspended in fresh DMEM with 10% FBS. On day 6 post-infection, culture supernatants were collected and analyzed for HIV p24 by ELISA. LTRhsp-CRISPR suppresses HIV p24 in our stringent model of HIV infection. n = mean ± SEM from 3 independent experiments. *, significant from control ($p < 0.05$).

LTRhsp-MzCRISPR or LTRhsp-Mz(Wk)CRISPR. Lentiviral vector pHIV-7-GFP was electroporated as control and also allowed us to follow electroporation efficiency. The infection was allowed to proceed for a further 12 days, and culture supernatants were collected every 3 days for p24 analyses. We observed HIV silencing by all three CRISPR constructs that persist up to 12 days post-electroporation (Figure 4A). However, maximal suppression was observed on day 6, followed by a progressive increase in viral output on days 9 and 12 with

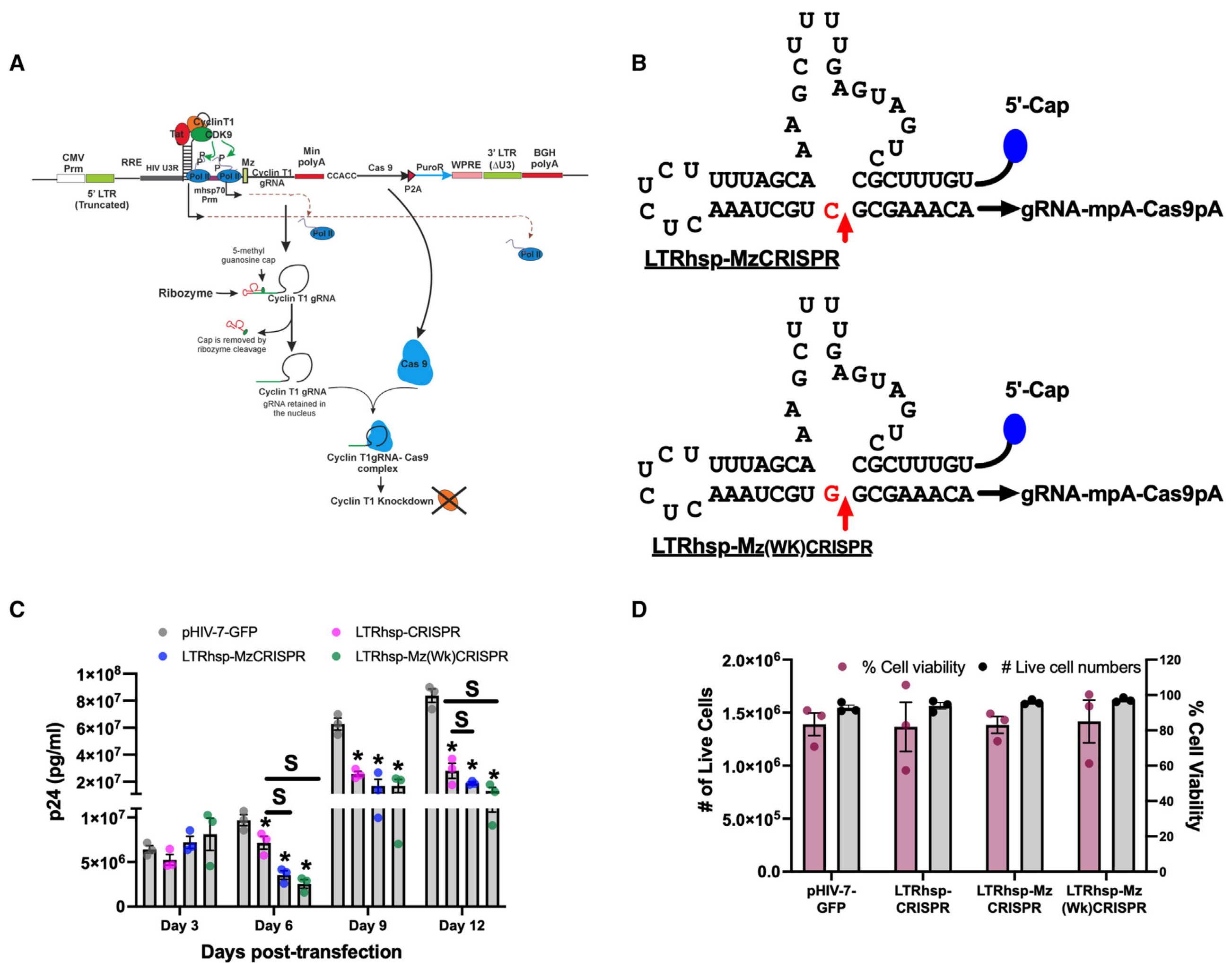
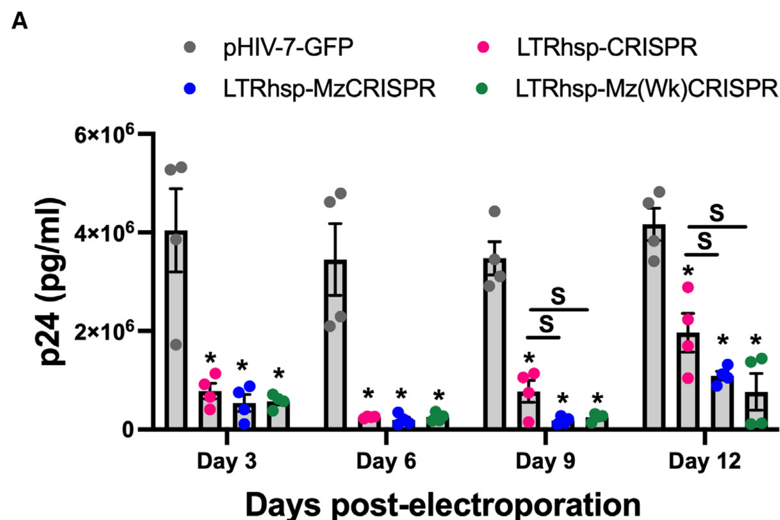


Figure 3. *Cis*-cleaving minizymes improve RNA Pol II CRISPR-based HIV suppression

(A) Schematic of LTRhsp-CRISPR minizyme-embedded variants. In the original clone (Figure 2A), co-expression of cyclin T1 gRNA and Cas9 results in cyclin T1 gRNA with a 5-methyl guanosine cap, which can export the gRNA from the nucleus to the cytoplasm. This can lead to suboptimal CRISPR-mediated knockdown of cyclin T1. Embedding a *cis*-acting minizyme just downstream of the transcriptional start such that it would cleave the 5′ cap will result in cap removal and retention of the gRNA in the nucleus. The weak ribozyme will ensure that part of the RNA is cleaved to remove the cap while some of the RNA retains the cap and is exported to the cytoplasm to express the Cas9 protein. (B) A schematic representation of the *cis*-cleaving minizyme-embedded variants and cleavage site upstream of the gRNA to remove the 5′ cap and improve RNA Pol II CRISPR efficacy. Hammerhead ribozymes can cleave any RNA as long as the ribozyme arms can hybridize with the target RNA, and the target contains an NUX triplet where N = A, G, C, or U and X = A, U, or C for optimal cleavage. LTRhsp-MzCRISPR recognizes a canonical GUC cleavage site, while LTRhsp-Mz(Wk)CRISPR recognizes a weaker non-canonical GUG cleavage site. (C and D) HIV-infected HeLa-CD4 cells were transfected with LTRhsp-MzCRISPR or LTRhsp-Mz(Wk)CRISPR. Transfection with the lentiviral backbone pHIV-7-GFP was used as control and to monitor transfection. Transfection with LTRhsp-CRISPR was used for comparison. To mimic a more physiological setting, only the transfection medium was replaced with fresh DMEM with 10% FBS. At designated time points, culture supernatants were collected and analyzed for HIV p24. Both ribozyme *cis*-cleaving minizyme-embedded variants demonstrate slightly improved HIV suppression compared with LTRhsp-CRISPR (C). Following 12 days of transfection, experiments were terminated, and cell viability was determined. LTRhsp-CRISPR or the minizyme-embedded LTRhsp-MzCRISPR or LTRhsp-Mz(Wk)CRISPR did not affect cell viability and demonstrated similar live cell counts. n = mean ± SEM from 3 independent experiments. *, significant from control; S, significant from each other ($p < 0.05$).

only 53% HIV silencing observed on day 12. Both minizyme-embedded constructs demonstrated slightly better efficacy of HIV silencing compared with the original LTRhsp-CRISPR. Differences in HIV suppression between the minizyme constructs were not statistically significant. Cell viability and cell numbers were determined on day 12 using trypan blue staining. Figure 4B shows that HIV-inducible CRISPR constructs did not demonstrate any decrease in cell viability or the number of live cells. One of the limitations of any DNA delivery in *in vitro* experiments is that the best reagents do not allow 100% transfection of cells. This would allow *de novo* infection of



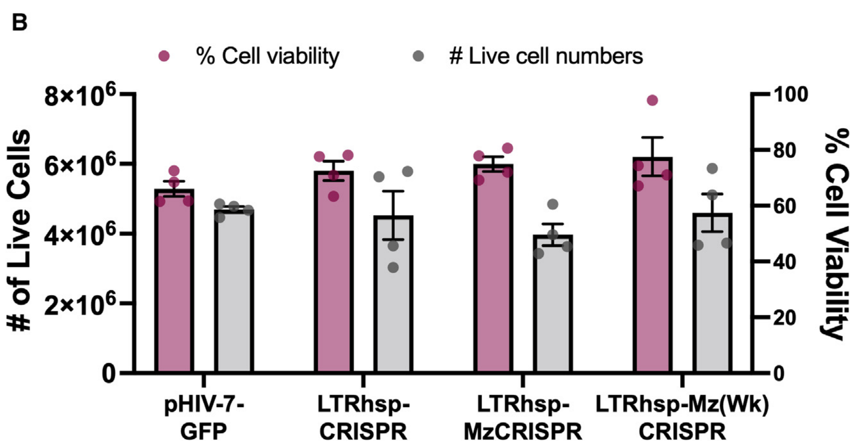


Figure 4. HIV-inducible CRISPR systems demonstrate sustained HIV suppression in T cell lines

CEM T cells were infected with HIV IIIB strain as described in the materials and methods. A million cells each from this infection were used for electroporation with LTRhsp-MzCRISPR or LTRhsp-Mz(Wk)CRISPR using the Neon electroporation kit as described in the materials and methods. Electroporation with the lentiviral vector pHIV-7-GFP was used as control and to monitor transfection efficiency. All three HIV-inducible CRISPR systems demonstrated HIV suppression that persisted up to 12 days post-electroporation. The original LTRhsp-CRISPR demonstrated maximum suppression by day 6, followed by a progressive increase in viral output on days 9 and 12 with only a 53% HIV suppression observed on day 12. Both minizyme-embedded constructs demonstrate slightly better efficacy compared with the original LTRhsp-CRISPR (A). On day 12, experiments were terminated, and cell viability was determined by trypan blue staining as described in the materials and methods. HIV-inducible CRISPR systems did not affect the percentage of cell viability or the number of live cells (B). n = mean ± SEM from 4 independent experiments. *, significant from control; S, significant from each other ($p < 0.05$).

untransfected/unelectroporated cells in a post-infection model and a progressive rebound in viral titers over time. To circumvent this problem, we attempted electroporation in combination with an initial 3 day treatment with the anti-HIV retroviral tenofovir. Tenofovir inhibits reverse transcription (and hence *de novo* infection) but will not affect HIV output from cells that are already infected. Following electroporation in post-infection CEM cells, Tenofovir was added to the culture media. On day 3, culture medium was replaced with fresh medium without tenofovir, and CEM cells were propagated in the absence of tenofovir for the remainder of the experiment. Figure 5A shows that a single electroporation of our HIV-inducible CRISPR systems when used in combination with an initial tenofovir treatment demonstrates 85%–95% suppression of HIV up to day 21 compared with pHIV-7-GFP alone. pHIV-7-GFP along with tenofovir treated shows a viral rebound by day 6.. Using this combinatorial approach, we did not detect any difference in HIV suppression between our constructs with and without ribozyme inserts. Figure 5B shows that CEM cells electroporated with HIV-inducible CRISPR constructs showed comparable cell viability and number of live cells to tenofovir alone- and lentiviral vector alone-transduced cells.

While electroporation or naked DNA-based delivery would result in elimination of the CRISPR plasmid from the cells in a few days, in a physiological setting, our CRISPR constructs may need to be delivered using viral vectors. All CRISPR constructs including pHIV-7-GFP and the constitutive pLentiCRISPR-gRNA-2 were packaged as lentiviral vectors using the approach described by us.[23,30] Post-infection model of CEM cells was generated as described above. Cells were transduced with pHIV-7-GFP or the CRISPR constructs at 100 MOI. After 24 h, medium was changed to fresh medium containing tenofovir. 3 days post-transduction, the medium was replaced with fresh medium without tenofovir, and CEM cells were propagated in the absence of tenofovir for the remainder of the experiment. Culture supernatants were collected at designated time points. As seen in Figure 6A, lentiviral-based delivery enhances the HIV silencing by our HIV-inducible CRISPR system compared with electroporation- or transfection-based delivery. LTRhsp-CRISPR and LTRhsp-Mz(Wk)CRISPR demonstrate comparable silencing (~90% HIV suppression) up to day 24. LTRhsp-MzCRISPR-transduced cells demonstrated the best suppression, with HIV p24 becoming undetectable by day 12 and remaining undetectable until day 21. The constitutive pLentiCRISPR-gRNA-2 demonstrated undetectable HIV on days 21 and 24. However, this was most likely due to extensive cell death in this group (Figure 6B). LTRhsp-CRISPR and LTRhsp-MzCRISPR improved cell viability

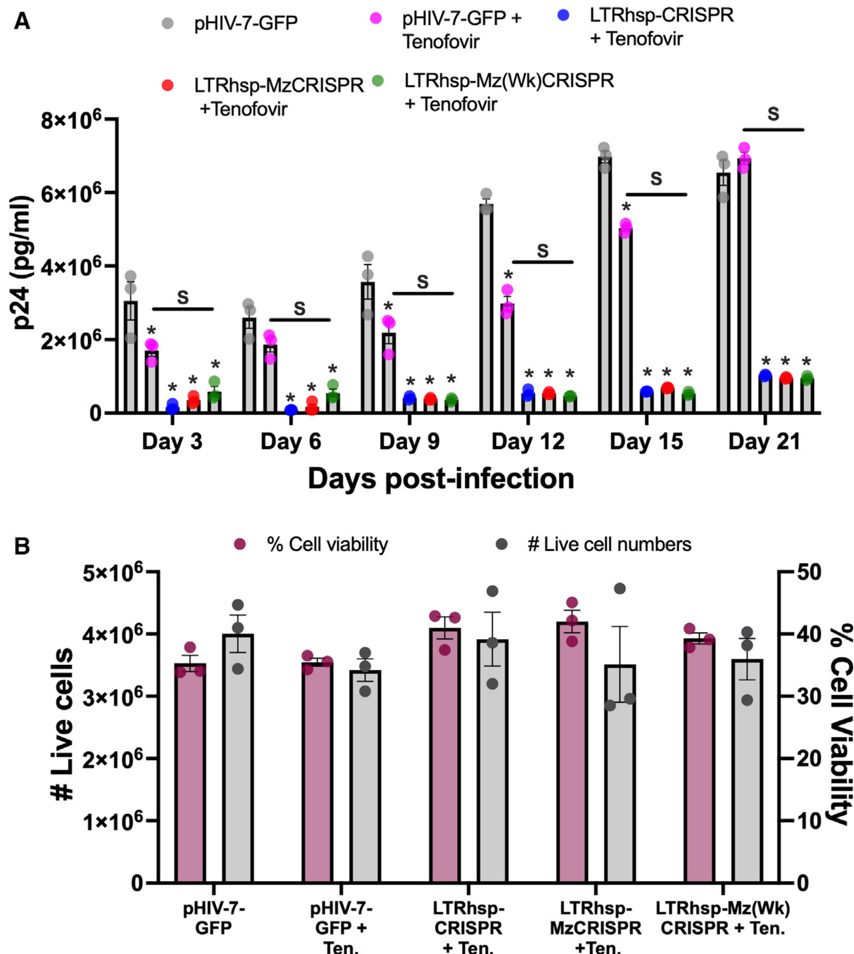


Figure 5. HIV-inducible CRISPR systems demonstrate prolonged and sustained HIV suppression in T cell lines when used in combination with initial antiretroviral treatment

CEM T cells were infected with HIV IIIB strain as described in the materials and methods. Cells were electroporated with LTRhsp-CRISPR, LTRhsp-MzCRISPR, or LTRhsp-Mz(Wk) CRISPR using the Neon electroporation kit as described in the materials and methods. After 24 h, medium was changed to fresh medium containing tenofovir (5 μM). Electroporation with the lentiviral vector pHIV-7-GFP was used as control and to monitor transfection efficiency. One set of infected cells was electroporated with pHIV-7-GFP and treated with tenofovir alone to compare HIV suppression by tenofovir. Medium was changed following 3 days, fresh medium without tenofovir was added, and the experiment was allowed to proceed for 21 days. At designated time points, HIV p24 was quantitated by ELISA as an index of viral replication. All three HIV-inducible CRISPR systems demonstrated HIV suppression (85%–90%) that persisted up to 21 days following a single electroporation of the inducible CRISPR plasmids. No differences were observed in suppression by minizyme constructs and the original LTRhsp-CRISPR (A). On day 21, experiments were terminated, and cell viability was determined by trypan blue staining as described in the materials and methods. HIV-inducible CRISPR systems did not affect the percentage of cell viability or the number of live cells (B). n = mean ± SEM from 3 independent experiments. *, significant from control; S, significant from each other ($p < 0.05$).

over controls. However, they did show a small, but statistically significant, decrease in the number of live cells.

DISCUSSION

In this article, we provide proof of concept demonstrating an RNA Pol II mono-promoter-single transcription unit-based conditional CRISPR expression system using our previously reported HIV LTR-minimal Drosophila hsp70 fusion promoter.[23] Using this expression system, we demonstrate conditional CRISPR expression that inhibits cyclin T1 and mediates HIV silencing. Given the propensity of HIV to mutate and escape gene therapy approaches directed against the viral genome, knocking down cellular co-factors remains an attractive approach if the knockdown is restricted only to HIV-infected cells. P-TEFb kinase-mediated phosphorylation of RNA Pol II serves as a master switch to turn on HIV replication.[20–22] P-TEFb is a heterodimer of CDK9 and one of the regulatory cyclins. Cyclin T1 is one of four regulatory cyclins (cyclin T1, -T2a, -T2b, or -K) that bind to and activate CDK9.[17,31,32] However, HIV-mediated recruitment and activation of CDK9 specifically requires cyclin T1, and knocking down either component has been shown to inhibit HIV transcription.[20,21] While CDK9 inhibitor Flavopiridol has already shown clinical efficacy in non-small cell lung cancer without any adverse effects,[38] we chose to target the cyclin T1 partner instead of CDK9 as CDK9 can partner with other cyclins, thereby providing redundancy for cellular transcription by P-TEFb.[17,31,32] This would mitigate any toxicities associated with cyclin T1 inactivation. Cyclin T1 is critical for HIV transcription by both Tat-dependent and Tat-independent mechanisms.[16–19,24] This criticality for HIV transcription and redundancy for cellular transcription makes cyclin T1 an attractive target to silence HIV proviral DNA.

We first selected the best cyclin T1-targeting gRNA in chronically HIV-infected HeLa-CD4 cells. These cells can be considered ideal for pilot testing of therapeutics as most, if not all, cells harbor the provirus and express microgram quantities of HIV p24 output. In line with other reports,[20,33] we did not observe any change in the percentage of cell viability, possibly due to redundancy of CDK9 for other cyclin partners.[17,31,32] However, we did observe a statistically significant decrease in the number of live cells with constitutive expression of our most effective CRISPR cassette. Surprisinglywhile the western blot provided ∼50% knockdown, this translated to over 90% suppression of HIV. This confounding observation can be partly explained due to normalization. We observed that expression of normalizing control (α-tubulin) also dovetails to some extent with that of cyclin T1.

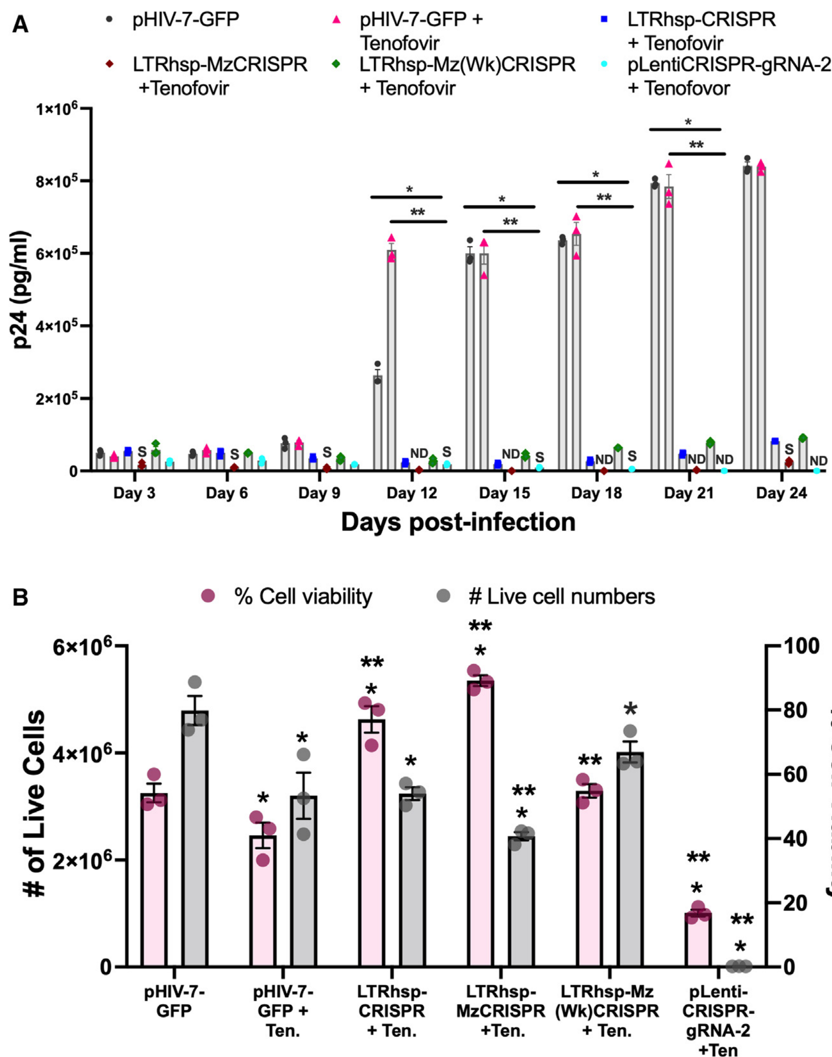


Figure 6. HIV-inducible CRISPR systems were packaged, as lentiviral vectors demonstrate prolonged and sustained HIV suppression in T cell lines when used in combination with initial antiretroviral treatment

CEM T cells were infected with HIV IIIB strain as described in methods. All CRISPR constructs including pHIV-7-GFP and the constitutive pLentiCRISPR-gRNA-2 were packaged as lentiviral vectors. (A) Cells were transduced with HIV-7-GFP or the CRISPR constructs at 100 MOI. After 24 h, medium was changed to fresh medium containing tenofovir (5 μM). 3 days post-transduction, the medium was replaced with fresh medium without tenofovir, and CEM cells were propagated in the absence of tenofovir for the remainder of the experiment. Culture supernatants were collected at the designated time points. Lentiviral vector-based delivery significantly enhances the suppressive effects of our HIV-inducible CRISPR system compared with electroporation- or transfection-based delivery. LTRhsp-CRISPR and LTRhsp-Mz(Wk)CRISPR demonstrated comparable suppression (~90% HIV suppression) up to day 24. LTRhsp-MzCRISPR-transduced cells demonstrated the best suppression, with viral titers becoming undetectable by day 12 and remaining undetectable until day 21. The constitutively expressed LentiCRISPR-gRNA-2 demonstrated undetectable HIV on days 21 and 24. However, this was most likely due to extensive cell death in this group (B). LTRhsp-CRISPR and LTRhsp-MzCRISPR improved cell viability over controls. However, they demonstrated some decrease in the number of live cells. n = mean ± SEM from 3 independent experiments. *, significant from control; **, significant from pHIV-7-GFP + tenofovir; S, significant from all other CRISPR constructs; ND, not detectable ($p < 0.05$).

Upon normalisation, this manifests as a decrease in observed suppression. We also observed this decrease with another normalizing control (β-actin; data not shown). It is also possible that cyclin T1 suppression leads to decreased infectivity of the released virus by altering cellular factors that facilitate the next round of viral infection. Indeed, a genome-wide screen for positive and restrictive factors regulating HIV-1 replication concludes that potentially 2,410 protein-coding genes (9.5% all human genes) may be involved in the replication of HIV.[39] The experiments required to identify this factor(s) are beyond the scope of this article.

In our earlier report, we had demonstrated HIV-inducible co-expression of a small RNA (shRNA) and a protein (RevM10).[23] We adapted our fusion promoter small RNA-protein co-expression system[23] for co-expression of gRNA and Cas9. We show that Cas9 expression is restricted to HIV-infected HeLa-CD4 cells. In our previous reports with the fusion promoter co-expressing anti-HIV shRNA and RevM10, we had observed a very faint band of RevM10 mRNA in the absence of HIV in our northern blot analyses.[23] It is possible that overexpression of our blots may show a similar faint band in uninfected HeLa-CD4 cells. However, this does not translate to cyclin T1 inactivation in these cells, suggesting that low-level leakiness, if any, does not mediate functional effects. A characteristic feature of RNA Pol II transcription is the addition of a 5′ cap to all transcripts that is essential for mRNA nuclear export. This can diminish CRISPR efficacy by limiting gRNA availability within the nucleus. To circumvent this limitation, we incorporated weak (and weaker) *cis*-cleaving ribozymes to excise the 5′ cap from the gRNA from a proportion of transcripts to increase nuclear retention of gRNA.

All CRISPR constructs silenced HIV in transient transfection or electroporation assays. The minizyme-incorporated CRISPR constructs demonstrated a small but statistically significant improvement in the extent of HIV silencing, following a single transfection in our post-infection models with HIV-infected HeLa-CD4 cells and CEM T-cells. However, this was offset by a viral rebound by day 9 in

HIV-infected HeLa-CD4 cells and by day 12 in CEM T cells. We posited that this could be due to virus release and *de novo* infection from untransfected or unelectroporated cells. To inhibit *de novo* infection during treatment and to prolong HIV silencing, we included an initial 3 day treatment with the reverse transcriptase inhibitor tenofovir in combination with our CRISPR constructs. We observed a sustained 85%–90% suppression in p24 output for 21 days following a single electroporation with our CRISPR cassettes with no effects on cell viability. Since plasmid DNA is eliminated by cells within 6 days, a sustained suppression up to 21 days suggests that cyclin T1 inactivation prevents further HIV transcription even after the CRISPR constructs are eliminated from cells. When the CRISPR constructs were delivered by lentiviral vector-based transduction, the differences in suppression by LTRhsp-MZCRISPR versus other HIV-inducible CRISPR constructs became more evident. We believe this is due to differences in copy numbers of CRISPR constructs delivered by transfection/electroporation versus lentiviral transduction. Transfection/electroporation has been shown to deliver anywhere between 75 and 50,000 copies per cell.[40] On the other hand, transduction is known to deliver anywhere between 2 and 10 copies. Hence, with transduction, the available gRNA within the nucleus becomes a limiting step for CRISPR efficacy. LTRhsp-MzCRISPR demonstrated the best suppression with undetectable HIV p24 by day 12 up to day 21. LTRhsp-MzCRISPR and LTRhsp-CRISPR also improved the percentage of cell viability over controls (vector alone/vector alone + tenofovir). However, we also observed a small but statistically significant decrease in the number of live cells with LTRhsp-MzCRISPR when compared with vector alone-/vector alone + tenofovir-treated cells. This paradox can be explained by differences in the transcriptional kinetics of CDK9/cyclin T1 and CDK9 with other cyclin partners. CDK9/cyclin T1 complexes have slightly higher activity compared with CDK9/cyclin T2a and CDK9/cyclin T2b complexes.[31] We anticipate this difference to be nominal and do not believe this will affect cellular homeostasis. On the contrary, the constitutively expressed LentiCRISPR-gRNA-2-transduced CEM cells demonstrated extensive cell death. It is possible that this effect is due to unregulated expression of Cas9 rather than a side effect of cyclin T1 inactivation. This is because transient delivery approaches using electroporation, where the plasmid DNA is expected to be eliminated from cells by day 5, did not show similar cellular toxicity in cells electroporated with the constitutively expressed pLentiCRISPR-gRNA-2. At least one report has shown that constitutive Cas9 expression, even when delivered by lentiviral vector, is toxic to hematopoietic stem cells.[41] This shows that unregulated long-term CRISPR expression can lead to toxicity and cell death.

We believe that further optimization for *in vivo* delivery with respect to a combinatorial approach using appropriate viral vectors, concurrent antiretroviral therapy, and/or latency reactivating agents can silence HIV in reservoirs, possibly effecting a functional cure. At the most, we anticipate that silencing HIV transcription will decrease expression of viral proteins like Tat, nef, and HIV gp120 from these reservoirs, thereby mitigating the development of non-AIDS comorbidities. To our knowledge, there is only one other report of an HIV-inducible CRISPR system in which Kaminski et al. expressed Cas9 from a truncated HIV LTR.[42] In this system, only Cas9 expression is HIV inducible, while the multiplexed gRNAs targeting HIV LTR are expressed from a constitutive RNA Pol III promoter. We do not know the long-term effects of constitutive gRNA expression in the cells given that these RNAs can also serve in an antisense role or for transcriptional gene silencing due to partial hybridization with other cellular RNAs or promoter regions of genes. Inducible gRNAs could provide a level of safety that may be required for long-term treatment of HIV-1. Our RNA Pol II expression system can also be adapted to target HIV with multiple gRNAs by inserting *cis*-acting ribozymes between two contiguous gRNAs targeting HIV proviral DNA itself at two distinct sites to prevent viral escape.

MATERIALS AND METHODS

Cyclin T1 CRISPR gRNAs

Three gRNA sequences targeting cyclin T1 were obtained from Genscript (https://www.genscript.com/gRNA-detail/904/CCNT1-CRISPR-guide-RNA.html). The CCNT1 (Cyclin T-1) CRISPR gRNA sequences from Genscript were designed to efficiently target the CCNT1 gene with minimal risk of off-target Cas9 binding elsewhere in the genome using the approach described by Sanjana et al.[43] CCNT1 gRNA sequences, 5′-AATAGCCCATCCCGTCGTTT-3′ (pLentiCRISPR-gRNA-1), 5′-TCCACGCCAAAACGACGGGA-3′ (pLentiCRISPR-gRNA-2), and 5′CCTACCTCACTTCTAGTATC-3′ (pLentiCRISPR-gRNA-3), were obtained pre-cloned in the pLentiCRISPRv2 plasmid (Genscript Biotechnology), in which the gRNAs were expressed from the U6 promoter and Cas9 was expressed from the EFS promoter, with the lentiviral LTR polyA signal sequence serving as transcriptional termination for Cas9. All work was performed with required Institutional Biosafety approvals from the Florida International University IBC committee.

HIV-inducible RNA Pol II expression constructs

Construction and characteristics of the LTR-minimal hsp70 promoter have been previously reported.[23,30] Briefly, the ecdysone and glucocorticoid response elements upstream of the minimal Drosophila hsp70 promoter component were removed from the pIND vector (Invitrogen) and replaced with the HIV-1 LTR up to and including the transactivation response element. The HIV LTR-mhsp70 fusion promoter was PCR amplified using an HIV LTR 5-primer (HU-1: 5′-CCGGTACCTGGAAGGGCTAATTTGGTCC-3′) and hsp70 promoter 3′ primer (HU-2: 5′-GAGGCGCTTCGTCTACGGA-3′) from the LTRhsp-shRNA plasmid reported by us earlier.[30] The gRNA-2 (comprising the guide and scaffold region) sequence was PCR amplified using flanking primers (HU-3: 5′-GAAACACCGTCCACGCCAAAACG-3′ and HU-4: 5′-CACCGACTCGGTGCCACTTTTTCA-3′) based on a sequence provided by Genscript. The PCR products were kinased and blunt-end ligated to each other, and the ligated product was PCR amplified using HU-1 and HU-4 to obtain the LTRhsp-gRNA fragment. The mpolyA signal sequence was likewise PCR amplified from the LTRhsp-shRNA plasmid using primers flanking mpolyA, HU-5: (5′-CTAGAACTAGTAATAAAGG-3′), and HU-6 with an XbaI site: (5′-TCTAGATCTAGACGCGGCCGCACAC-3′). The PCR product was kinased and ligated to the LTRhsp-gRNA fragment, and the ligated product was reamplified using HU-1 and HU-6. The

resulting PCR product with KpnI and XbaI terminal sites was digested with KpnI and XbaI and ligated in a similarly digested pLentiCRISPRV2-gRNA-2. This substitutes the U6 gRNA expression cassette and the EFS promoter upstream of Cas9 with the LTRhsp-gRNA-mpolyA to obtain LTRhsp-CRISPR. A strong eukaryotic translation initiation site CCACC serves as a Kozak sequence immediately upstream of Cas9 in the vector and ensures appropriate Cas9 translation initiation. LTRhsp(ΔNF-κB)-CRISPR and SP1 sites LTRhsp(ΔSP1)-CRISPR were generated by PCR-based deletion of the NF-κB or SP1 sites in the HIV LTR using LTRhsp-CRISPR as template. Ribozyme insertions were done by sequential PCRs and ligations mentioned below.

PCR for MzgRNA-mpolyA

Step 1: PCR with 5′ primer HU-23: (5′-TTCGAAACGATTTTCTCTCAAATCGTCGCGAAACACCGTCCACGCCAAAACG-3′) and HU-6 creates a fragment with part of the ribozyme with gRNA and mpolyA.

Step 2: PCR with 5′ primer HU-24 having an EcoR1 site (5′-CCGAATTCTGTTTCGCCTGATGAGTTTTCGAAACGATTTTCTCTCAAATCG-3′) and HU-6 generates MzgRNA-mpolyA ribozyme with the minimal polyA with a 5′-EcoR1 site.

PCR for LTRhsp70 fusion promoter with a 3′-EcoR1 site

LTRhsp fusion promoter is amplified using HU-1 above and HU-7 (HU-2 with 3′-EcoR1 site).

Both PCR products are digested with EcoR1 and ligated. Following ligation, the entire LTRhsp-MzCRISPR is amplified using primers HU-1 and HU-6.

The LTRhsp-MzCRISPR is then digested with KpnI and XbaI and then ligated in similarly digested LTRhsp-CRISPR. This substitutes LTRhsp-gRNA-mpolyA with LTRhsp-MzgRNA-mpolyA in pLenti CRISPR.

For LTRhsp-Mz(Wk)CRISPR

In pLentiCRISPR, identical steps are used except that HU-23 primer is substituted with HU-21 primer containing the weak GUG site (5′-TTCGAAACGATTTTCTCTCAAATCGTGGCGAAACACCGTCCACGCCAAAACG-3′). HU-24 is a common primer that amplifies both the partial forms of the weak and the strong ribozyme to generate the full forms. Note that the weak and strong ribozymes differ by only one nucleotide. All PCR amplifications were performed using the high-fidelity Vent polymerase (NEB #M0254S).

Cell culture experiments

HeLa-CD4 and HIV-infected HeLa-CD4 cells were obtained from NIH AIDS Reagent Program (cat #153 and cat #1301, respectively) and maintained in DMEM with 10% fetal bovine serum (FBS). The human T cell line CEM was maintained in RPMI medium 1640 (GibcoBRL) supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% (vol/vol) FBS. For all plasmid transfections in HeLa cells (infected/uninfected), cells were grown to 60% confluence in a 6-well plate, and 1 μg plasmid DNA was complexed with lipofectamine 2000 in OPTIMEM according to manufacturers' protocol. Plasmid transfections in CEM cells were done by electroporation using the Neon electroporation system and kit (Thermo Fisher Scientific, cat #MPK1025) using a protocol standardized for CEM cells by the manufacturer (Thermo Fisher Scientific). At designated time points, culture supernatants were collected for HIV p24 analysis as an index of HIV infection. The lentiviral backbone plasmid pHIV-7-GFP reported by us[23,30] was transfected as control for all transfection experiments.

HIV-1 antiviral assay

Culture supernatants were collected on designated days, and HIV p24 viral antigen was measured from cultured supernatants using the p24 ELISA kit (ZeptoMetrix, cat #0801200) according to the manufacturer's protocol.

Cell viability assay

Trypan blue staining was used to determine viability and live cell counts for HeLa cells (infected/uninfected) as well as CEM T cells. For HeLa cells, the cells were trypsinized with TE and TNS, cells were resuspended in growth medium, and 10 μL suspension was mixed with an equal volume of trypan blue and loaded onto counting slides (Bio-Rad, cat #1450011). Cells were counted within 10 s of trypan blue staining by the TC20 Automated cell counter (Bio-Rad). For CEM T cells, 10 μL culture suspension was mixed with an equal volume of trypan blue and loaded onto counting slides, and cells counts were determined using the TC20 Automated cell counter within 10 s of trypan blue staining.

Infection of CEM cells

To infection 3×10^6 CEM cells were infected with 100 ng p24 equivalent of X4-tropic viral strain HIV IIIB and 2 mg/mL polybrene. After 24 h, cells were centrifuged, and medium was replaced with 5 mL complete growth medium and allowed to propagate in a T-25 culture flask for 12 days. Every 72 h culture supernatant was collected and analyzed for HIV p24 to monitor infection. Cells were then divided into aliquots of 10^6 infected cells in RPMI with 10% FBS media devoid of antibiotics before electroporation or transduction with each CRISPR construct (lentiviral vector as control). Electroporation was performed using the Neon transfection system (Thermo Fisher Scientific) using a protocol standardized by the manufacturer for CEM cells (voltage: 1,230 V; width: 45 ms; pulses: 1). Following electroporation, cells were resuspended in RPMI with 10% FBS in a 24-well plate. After O/N incubation, the medium was replaced with complete medium including antibiotics. For experiments involving tenofovir, tenofovir (5 μM), based on Musumeci et al.,[44] was added immediately following infection and retained for 3 days in the culture media.

Packaging of CRISPR lentivirus

293T cells were used for the lentiviral vector packaging and cultured in a 100 mm culture dish up to 80% confluency. Cells were co-transfected with 9 μg lentiviral vector plasmid pHIV-7-GFP or lentiviral vector plasmids with an appropriate insert such as pLTRhsp-CRISPR,

pLTRhsp-MzCRISPR, pLTRhsp-Mz(Wk)CRISPR, pLentiCRISPR-gRNA-2, pCHGP-2 (7.5 μg), pCMV-VSV-G (4.5 μg), and pCMV-Rev (3.5 μg) using the calcium phosphate precipitation kit (Thermo Fisher Scientific, cat #440052; manufacturer's instructions) as described by us before.[23,30] 6 h after transfection, the culture medium was replaced, and supernatants were collected at 24 and 48 h. The collected supernatants were pooled together and passed through a 0.45 μm filter. Virus preparation was concentrated by mixing with filtered 40% PEG solution followed by chilling at 4°C incubation for 24 h. Virus was pelleted by centrifugation at 1,650 g/20 min. Concentrated virus preparation was aliquoted and stored at −80°C until further use.

Determination of lentiviral vector titers

p24 levels in 10 μL virus preparation for all lentivirus preparations was determined including HIV-7-GFP. Separately, 10 μL HIV-7-GFP lentivirus was used to transduce HeLa cells, and infectious vector titers were determined by flow cytometry. The p24 levels corresponding to the HIV-7-GFP titer in 10 μL was then used to extrapolate and calculate virus titers in other lentiviral preparations. For transduction, infected CEM T cells were transduced with HIV-7-GFP or the CRISPR constructs at 100 MOI. After 24 h, medium was replaced with fresh medium containing tenofovir. On day 3, culture supernatants were collected, and fresh medium without tenofovir was added. Experiments were allowed to proceed in the absence of tenofovir, and culture supernatants were collected at the designated time points.

Western blot

Cells were lysed with RIPA (radioimmunoprecipitation assay) buffer (Thermo Fisher Scientific, cat #89901) with protease inhibitor cocktail (ThermoFisher Scientific, cat #78429). The protein concentration was determined by the method of BCA protein assay kit (Pierce, Thermo Fisher Scientific, cat #23225) in accordance with the manufacturer's instructions. Equal amounts of total protein were loaded onto 4%–20% precast polyacrylamide gel (Bio-Rad, cat #4568094) and run at 100 V. After protein was separated, it was transferred onto a polyvinylidene difluoride (PVDF) membrane. Afterward, the transfer blot was blocked by 10% blocking solution for 1 h. Then, the blot was incubated overnight in primary antibodies CRISPR-Cas9 (1:1,000; Thermo Fisher Scientific, cat #MA1-202), cyclin T1 (1:1,000; Cell Signaling, cat #81464), and α-tubulin (1:1,000; Cell Signaling, cat #2125), with 5% blocking solution. After incubation, the blot was washed with TBS-T and incubated for 1 h with horseradish-peroxidase-conjugated anti-rabbit/anti-mouse secondary antibody, which was diluted 1:2,500 with 1% blocking solution. The blotted protein bands were detected in Chemidoc (Bio-Rad) using supersignal west femto maximum sensitivity substrate (Thermo Fisher Scientific, cat #34095) following the kit manufacturer's recommendations. The blotted protein was quantified using the Quantity One software system (Bio-Rad), and values are normalized to α-tubulin.

Statistical analysis

Unless otherwise mentioned, data were expressed as mean ± SEM from at least 3 different experiments. The data were subjected to statistical analysis using unpaired t tests or ANOVA followed by Tukey Kramer honestly significant difference test for multiple comparisons as appropriate. The significance was considered at the level of $p < 0.05$.

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AUTHOR CONTRIBUTIONS

S.C. prepared the manuscript and performed the experiments. M.-J.S., K.P., M.S.R., J.A., and H.J.U. performed experiments. J.R. helped with experimental planning. H.J.U. designed and cloned the fusion promoter and the minizyme variants, performed experiments, and handled overall planning and design of the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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