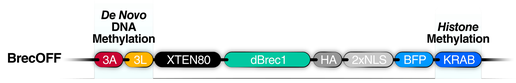
DEVELOPMENT AND DELIVERY OF NEXT-GENERATION EPIGENOME EDITORS FOR LONG-TERM HIV SILENCING

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Introduction

HIV infection establishes viral latency in CD4+ T-cells and microglia. While combination antiretroviral therapy (cART) suppresses viral replication, cARTs do not eliminate integrated viral reservoir. Importantly, recent studies have shown that up to 81% of this viral reservoir remains transcriptionally active, causing chronic immune activation and rapid viral rebound after treatment interruption. Therefore, novel strategies to target this critical loophole in current cART treatment are urgently needed. Here, we develop a gene therapy approach to permanently silence the active HIV reservoir using a novel fusion of two technologies: CRISPRoff, which enables heritable gene silencing through DNA and histone methylation both *in vitro* and *in vivo*, and Brec1, a small recombinase specifically evolved to specifically target a highly conserved region of the HIV Long-tandem repeat (LTR). By combining the Brec1's precise targeting with the CRISPRoff's silencing capabilities, we created **"BrecOFF"** - an epigenome editor that effectively silences HIV-1. This novel approach could lead to a functional HIV cure by turning HIV into a permanently silenced endogenous retrovirus.

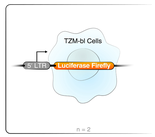


Methods

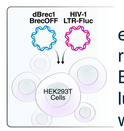
To characterize the silencing potential of BrecOFF, we study:

1. Binding and repression of integrated HIV-1 LTR:

For LTR binding, we ChIP on cells expressing BrecOFF or negative control (dBrec1). Protein-DNA complexes were pulled-down with a HA-tag specific ab and quantified interaction by qPCR. For transcription silencing, we sorted expressing cells and measured basal transcription at different points.

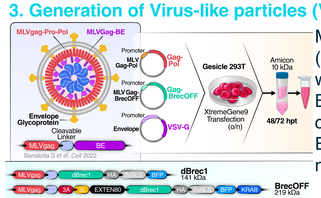


2. Episomal LTR-driven transcriptional silencing



HEK293T cells were co-transfected with a plasmid encoding for a LTR-driven Firefly luciferase (FLuc) reporter and a plasmid encoding for dBrec1 or BrecOFF. 48h hours after, cells were lysed and luciferase activity measured. Transcription silencing was determined as the reduction of FLuc to control.

3. Generation of Virus-like particles (VLPs) packaging BrecOFF

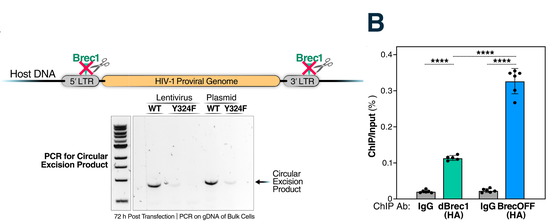


Moloney Leukemia Virus (MLV)-based VLP system was engineered to package BrecOFF inside the VLP. We confirmed packaging by ELISA, WB, and flow nanoanalyzer.

Results

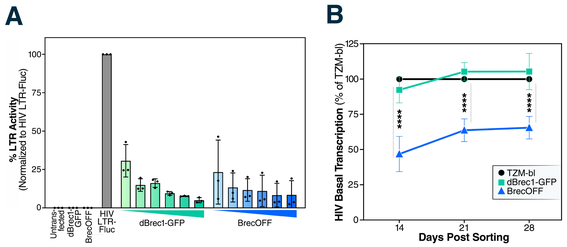
1. Catalytically dead Brec1 (dBrec1) and BrecOFF binds to HIV LTR

(**A**) **Generation of a catalytically dead mutant Brec1.** Based on Cre studies, we introduced a Y324F mutation on the catalytic site of Brec1 to generate dead Brec1 (dBrec1). WT Brec1 or dBrec1 were expressed on HeLa-smurf harboring a HIV-1 proviral genome by either lentivirus transduction or by plasmid expression. To evaluate HIV-1 proviral cleavage by Brec1, a PCR to detect excised circular by-products was performed. Two different cell clone transduced wit dBrec1 were assessed for cleavage activity. (**B**) **dBrec1 and BrecOFF binds the HIV-1 LTR**. TZM-bl cells were transfected with dBrec1-GFP or BrecOFF. 24 hours post transfection, cells were fixed with PFA and quenched with glycine. ChIP-qPCR assay was performed using HA tag as bait and HIV LTR as probe. Graph shows 2 independent transfections with individual values represent independent qPCR Ct values normalized to % of DNA input from a total of 3 experiments per cell line.



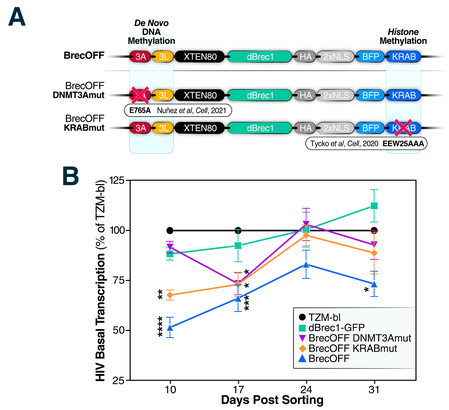
2. Both dBrec1 and BrecOFF Blocks Episomal HIV LTR Transcription by Promoter Occupancy but only BrecOFF Durably Silences Basal HIV-1 LTR Transcription

A) dBrec1 and BrecOFF block episomal HIV LTR transcription by promoter occupancy. 293T cells were co-transfected with a Firefly luciferase gene under the HIV-1 LTR promoter together with either dBrec1 or BrecOFF. 48 hours post-transfection, cells were lysed and luciferase activity measured. Relative light units (RLUs) were normalized to HIV LTR-FLuc plasmid alone to determine the % LTR activity. **B) BrecOFF transient expression durably silences basal HIV LTR transcription.** TZM-bl cells contain an integrated copy of an HIV defective provirus with a FLuc gene under the LTR promoter. TZM-bl cells were transfected with dBrec1-GFP or BrecOFF and sorted after 24 h for positive cells. Basal HIV transcription was measured at the indicated time points by luciferase activity measurements. Repression was determined as the % of basal transcription normalized to untransfected TZM-bl.



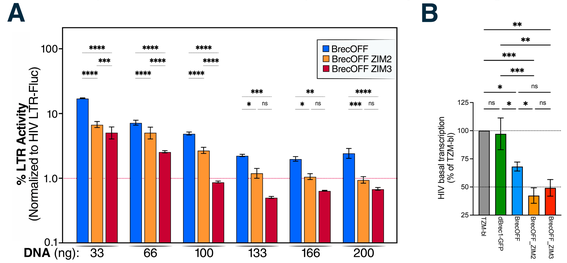
3. BrecOFF Repression Relies on DNA Methylation, but Needs Histone Methylation for Durable Effect

A) BrecOFF and generated BrecOFF mutants. BrecOFF DNMT3Amut and KRABmut were previously described in the literature and cannot deposit DNA or histone methylation marks, respectively. **B) BrecOFF long-term repression requires DNA and histone methylation.** TZM-bl cells were transfected with dBrec1-GFP, BrecOFF, or BrecOFF mutants and sorted after 24 h for positive cells. Basal HIV transcription was measured at the indicated time points by luciferase activity measurements. Repression was determined as the % of basal transcription normalized to untransfected TZM-bl. C-D experiments were performed three times in triplicates. Error bars represent SD. Statistical analysis for (C) was performed using a 2-way ANOVA. **** p-value <0.0001.



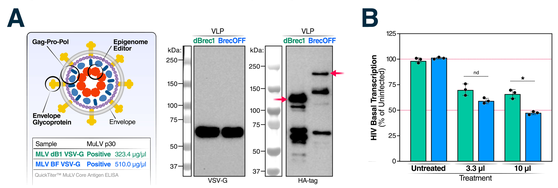
4. ZIM2 and ZIM3 are potent repressors of HIV-1 LTR-driven transcription

A) BrecOFF variants with a ZIM2 or a ZIM3 KRAB domain are more potent than WT BrecOFF to block episomal and integrated HIV LTR transcription. 293T cells were co-transfected with a Firefly luciferase gene under the HIV-1 LTR promoter together with either WT BrecOFF, BrecOFF_ZIM2 or BrecOFF_ZIM3. 48 hours post-transfection, cells were lysed and luciferase activity measured. Relative light units (RLUs) were normalized to HIV LTR-FLuc plasmid alone to determine the % LTR activity. (**B**). TZM-bl cells were transfected with dBrec1-GFP, BrecOFF , or BrecOFF_ZIM2/ZIM3 and sorted after 24 h for positive cells. Basal HIV transcription was 10 days post sorting.



5. dBrec1 and BrecOFF are packaged into VLPs and inhibits HIV-1 LTR transcription

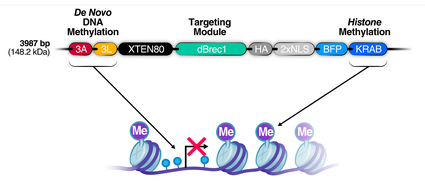
A) BrecOFF packages inside MLV VLPs. Upper left panel represents the components of MLV BrecOFF VLPs. Lower left panels show ELISA values of MLV Gag from purified VLPs. Right panels show western blots of purified VLPs probed for envelope glycoprotein (VSV-G) and epigenome editor (HA tag). Uncleaved Gag-Epigenome editor fusion proteins are marked with red arrows. **B) VLPs carrying dBrec1 (green bars) and BrecOFF (blue bars) silences HIV transcription in a dose-dependent manner.** Different volumes of purified VLPs were used to treat TZM-bl for 48 h without removal of the inoculum. 48 hour after inoculation, cells were then lysed and luciferase measured to quantify HIV basal transcription.



Summary & Outlook

In this study, we have engineered **BrecOFF**, a novel *HIV-specific* epigenome editor. We have shown that BrecOFF:

1) Strongly **binds** HIV LTR and silences by **promoter occupancy**
2) Durably repress HIV basal transcription by **epigenetic modifications**
3) Depends on **both** DNA and histone methylation for repression
4) Can be improved by **module optimization** (ZIM2 and ZIM3 KRAB domains)
5) Is **packaged in VLPs** that repress HIV transcription



Next:

1) BrecOFF DNA/histone **methylation studies**
2) Optimization and evaluation of BrecOFF-VLPs in **T cells**
3) Assessment of BrecOFF in **active infection** (HIV GKO)
4) Addition of novel **epigenome modules** (KDM5A, EZH2, G9a, LSD1, etc.)

Acknowledgments

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