

Specific and Stable Suppression of HIV Provirus Expression In Vitro by Chimeric Zinc Finger DNA Methyltransferase 1

Junxiao Deng,^{1,3} Xiyang Qu,^{1,3} Panpan Lu,¹ Xinyi Yang,¹ Yuqi Zhu,¹ Haiyan Ji,¹ Yanan Wang,¹ Zhengtao Jiang,¹ Xian Li,¹ Yangcheng Zhong,¹ He Yang,¹ Hanyu Pan,¹ Won-Bin Young,² and Huanzhang Zhu¹

¹State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, Shanghai 200438, China; ²Department of Radiology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15219, USA

HIV-1 inserts its proviral DNA into the infected host cells, by which HIV proviral DNA can then be duplicated along with each cell division. Thus, provirus cannot be eradicated completely by current antiretroviral therapy. We have developed an innovative strategy to silence the HIV provirus by targeted DNA methylation on the HIV promoter region. We genetically engineered a chimeric DNA methyltransferase 1 composed of designed zinc-finger proteins to become ZF2 DNMT1. After transient transfection of the molecular clone encoding this chimeric protein into HIV-1 infected or latently infected cells, efficient suppression of HIV-1 expression by the methylation of CpG islands in 5'-LTR was observed and quantified. The effective suppression of HIV in latently infected cells by ZF2-DNMT1 is stable and can last through about 40 cell passages. Cytotoxic caused by ZF2-DNMT1 was only observed during cellular proliferation. Taken together, our results demonstrate the potential of this novel approach for anti-HIV-1 therapy.

INTRODUCTION

With the development of highly active antiretroviral therapy (HAART), HIV-1 infection has been transformed from a deadly illness into a chronic disease. Although it is effective in suppressing viral replication to reduce viral load in HIV patients, it does not clear the proviral DNA integrated in the host genome. Thus, these latently infected cells can be reactivated to produce HIV for viremia rebound once ART is withdrawn.¹⁻⁴ Moreover, because of the side effect of long-term therapy, as well as the emergence of drug resistance, a new therapeutic strategy to eradicate HIV is needed in addition to HAART.^{5,6}

Human endogenous retroviruses (HERVs) and other retroviral elements consist of a total 8% of human genome, which are believed to be the result from the integrated by ancient retroviruses.⁷ HERVs share similar genetic structure with HIV-1. Most of the HERVs are defective and silenced, but certain members of HERVs still remain transcriptionally active. For example, HERV-K retains all open reading frames, suggesting the potential infectivity after reactivation under certain conditions. In fact, HERVs have been demonstrated

transcriptionally active in germ cells. After embryonic development, they are strictly repressed and become permanently silenced.^{8,9} Several studies have demonstrated that DNA methylation plays a crucial role in such timing and long-lasting control in silencing HERVs.¹⁰⁻¹⁴

DNA methylation is the first and the most studied epigenetic mechanism in establishing genomic imprinting, silencing transposon and retroviral elements, and regulating differential gene expression during embryogenesis and development. The classical DNA methylation maintenance model consists of the de novo DNA methylation initiated by the DNMT3 and maintained by DNMT1 throughout each cell division. However, substantial experimental evidence from the last decade indicates that DNMT1 has an important role in de novo DNA methylation as well.¹⁵⁻¹⁹ DNA methylation of CpG islands in the promoter region of a gene plays a significant role in regulating mammalian gene expression. DNA methylation of HIV-1 5'-LTR has been attributed to HIV-1 transcription silencing in vitro and in vivo studies.²⁰⁻²⁷ In addition, DNA methylation patterns are inheritable to the progeny and can perform long-term stable gene suppression by methylating the targeted genes.²⁸⁻³³ These facts prompted us to develop this innovative strategy of using DNA methylation to silence the invading HIV-1 proviral DNA by adding DNA methylation to the HIV-1 5'-LTR, the way similar to silencing HERVs for a peaceful coexistence between HIV-1 provirus and human host.³⁴

Martinez-Colom et al. have achieved inhibiting HIV-1 expression using chimeric protein that linked the N-terminal domain of the HIV integrase with the C-terminal domain of DNMT3b.³⁵ However, the N-terminal domain of the HIV integrase enzyme is not conservative and thus may cause great concerns on the safety of this approach for

Received 17 July 2016; accepted 9 January 2017;
<http://dx.doi.org/10.1016/j.omtn.2017.01.002>.

³These authors contributed equally to this work.

Correspondence: Huanzhang Zhu, State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, Shanghai 200438, China.

E-mail: hzzhu@fudan.edu.cn

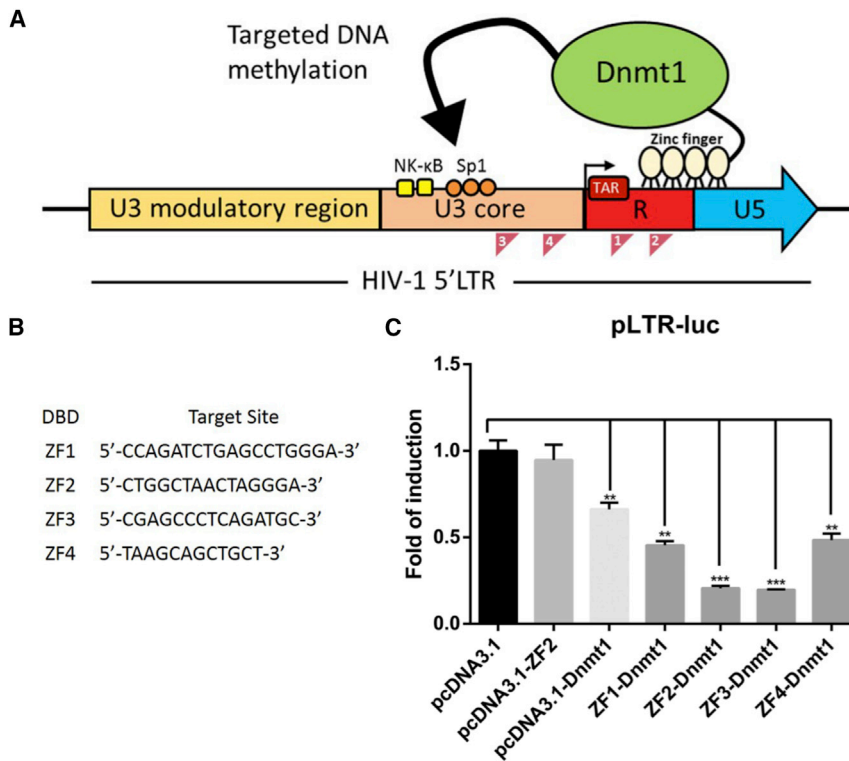


Figure 1. Design and Screen of ZF-DNMT1 Fusion Protein by Dual-Luciferase Assay

(A) Schematic representation of ZF-DNMT1-mediated DNA methylation of HIV-1 5'-LTR. The red triangles indicate the location of the target site of ZF-DNMT1. The black arrow denotes the transcription start site (TSS). The yellow squares and orange circles indicate NF- κ B and Sp-1 binding sites, respectively. TAR: transactivation responsive element. (B) Binding sites of ZF-DNMT1 to the HIV-1 5'-LTR. (C) Screen of ZF-DNMT1-mediated HIV-1 LTR suppression by dual-luciferase assay. The HEK293T cells were cotransfected with pcDNA3.1, pcDNA3.1-ZF2, pcDNA3.1-DNMT1, or ZF-DNMT1 and the LTR-luc reporter vector at the indicated time. The relative luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) 72 hr post transfection. The data represent the mean \pm SD of three independent experiments. ** $p < 0.01$ and *** $p < 0.001$; Student's *t* test.

clinical applications. In our previous studies, we have engineered four zinc-finger proteins (ZFP) targeted on the conserved region located at HIV LTR.^{36,37} The binding affinity and specificity have been previously defined. In this study, we fused ZFP, which was designed to target HIV-1 5'-LTR, to DNMT1 in order to methylate the CpG islands in 5'-LTR, to reach a long term silencing of HIV-1 provirus. We tested our ZF2-DNMT1 fusion protein on HIV-1 infected or latently infected cell culture models. The results indicate that this chimeric ZF2-DNMT1 can induce table suppression of HIV-1 provirus by adding methyl group to the targeted CpG islands in 5'-LTR. Our approach provides a new approach for suppressing gene expression of HIV-1 provirus in the hope to replace the daily HAART.

RESULTS

Construction and Screening of ZF-DNMT1 Fusion Protein

The HIV-1 5'-LTR represents an attractive and ideal target for chimeric ZF2-DNMT1 because it is highly conserved across most of the HIV-1 strains with two CpG islands flanking the viral transcription initiation site in 5'-LTR. We have previously designed and assembled four zinc-finger motifs specifically targeting the 5'-LTR.³⁷ To construct chimeric ZF2-DNMT1 targeting the 5'-LTR, zinc-finger motif with N-terminal 3 \times FLAG, nuclear localization signal (NLS), and C-terminal linker (LRGS) were amplified by PCR and cloned into *NheI/EcoRI*-digested pcDNA3.1-DNMT1. We obtained four different clones: ZF1-DNMT1, ZF2-DNMT1, ZF3-DNMT1, and ZF4-DNMT1. The basic structure and target sites of ZF-DNMT1 were shown in Figure 1.

To screen for the most effective one for suppressing 5'-LTR, we cotransfected HEK293T cells with the ZF-DNMT1 expression vectors and a reporter vector containing the luciferase gene driven by a HIV-1 LTR promoter (LTR-luc). The luciferase levels were measured 72 hr post transfection. An empty vector (pcDNA3.1) was used as a null negative control in the place of ZF-DNMT1. We

also used a DNMT1 expression vector (pcDNA3.1-DNMT1) and a zinc-finger motif expression vector (pcDNA3.1-ZF2) as additional negative control for comparison. The results showed that all four ZF-DNMT1 significantly suppressed the promoter function of LTR, which drove reporter gene expression. ZF2-DNMT1 and ZF3-DNMT1 showed 80% reduction of reporter gene expression relative to the null negative control. A DNMT1 expression vector showed only a modest reduction of reporter gene expression while zinc-finger motif alone had no effect on LTR promoter (Figure 1C). Based on these results, we chose ZF2-DNMT1 and ZF3-DNMT1 for further experiments.

ZF-DNMT1 Fusion Protein Mediates Suppression of the HIV-1 5'-LTR in TZM-bl Cells

The suppression of 5'-LTR promoter in the aforementioned transient transfection assay may be different from genomic expression of ZF2-DNMT1 because the reporter plasmid is not integrated into genome.³⁸ To address this issue, we performed an luciferase reporter assay in TZM-bl cells, a reporter cell line for HIV infection, in which a luciferase reporter expression cassette driven by HIV-1 5'-LTR was inserted in the parental HeLa cellular genome.³⁹ To express the luciferase without HIV infection in this TZM-bl, we gene transferred a plasmid containing Tat (the name of the plasmid) to induce the expression of luciferase for testing the suppression function of the two ZF-DNMT1s on Tat-activated 5'-LTR. Based on the reduction of luciferase expression, we only observed a significant suppression of the HIV-1 5'-LTR by ZF2-DNMT1, but not ZF3-DNMT1 (Figure 2A). ZF2-DNMT1 yielded a 56% reduction of reporter gene,

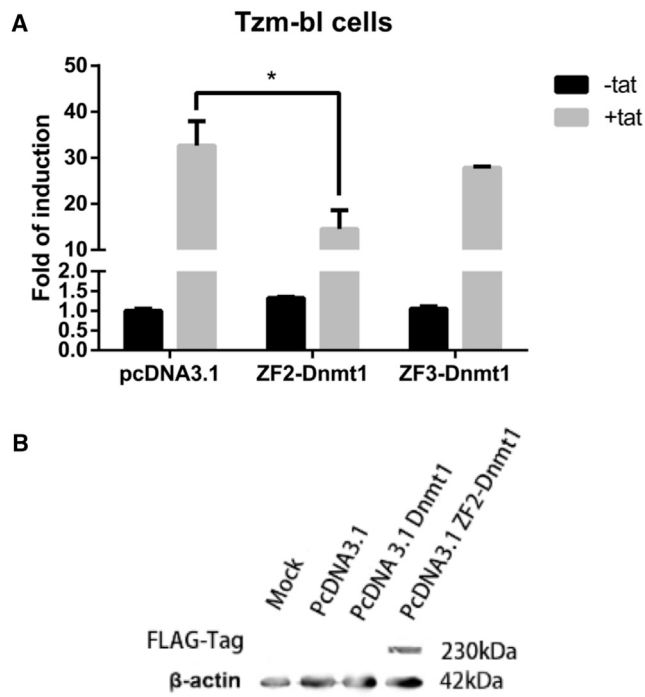


Figure 2. Protein Expression and Repression Activity of ZF-DNMT1 in HEK293T Cells and TZM-bl Cells, Respectively

(A) ZF-DNMT1 fusion protein mediates repression of the HIV-1 5'-LTR in TZM-bl cells. The TZM-bl cells were cotransfected with ZF2-DNMT1 or ZF3-DNMT1 at the indicated time. The cells were harvested and then subjected to luciferase activity assay at 72 hr post transfection. The data represent the mean \pm SD of three independent experiments. * $p < 0.05$; Student's *t* test. (B) Protein expression of ZF2-DNMT1. The HEK293T cells were transfected with ZF2-DNMT1 or an empty vector, pcDNA3.1. The cells were harvested 72 hr post-transfection and western blot was performed using anti-FLAG antibody. An anti- α -tubulin antibody was used to detect α -tubulin for quantitative measurement.

which was lower than that observed 80% reduction in the transient assay. Thus, we chose ZF2-DNMT1 for the rest of the study.

To monitor the protein expression of ZF2-DNMT1, western blot analysis was performed using anti-FLAG antibody. We detected the fusion protein in the sample that was transfected with ZF2-DNMT1, but not the ones transfected with pcDNA3.1 or pcDNA3.1-DNMT1 (Figure 2B).

ZF-DNMT1 Induces a Significant and Stable Silencing Effect on HIV-1 Expression

To validate the effects of ZF2-DNMT1 in HIV-1 infected cell models, YA cells, in which an integrated provirus carrying the GFP gene under the control of the HIV-1 LTR was previously introduced by a single round of HIV transduction. Under normal conditions, the majority of YA cells express GFP because the HIV LTR is active. To demonstrate the ability of ZF2-DNMT1 on silence active HIV LTR and the entire provirus including GFP, we nucleofected YA cells with the empty vector pcDNA3.1 or ZF2-DNMT1 expression vector. We

found that the percentage of GFP-positive cells measured by flow cytometry at 72 hr post-transfection was dramatically reduced to 52.8% by ZF2-DNMT1 transfection, while 77.3% by the empty vector group (Figure 3A). To confirm this result, total RNA from the transfected YA cells was extracted at 72 hr post-transfection for measuring HIV-1 mRNA using real-time PCR assay. The average amount of HIV-1 mRNA detected in YA cells transfected with ZF2-DNMT1 was 80% less than that measured in YA cells transfected with the empty vector (Figure 3B). To measure HIV-1 replication, we quantified HIV p24 protein in the supernatant using ELISA assays. The amount of HIV-1 p24 protein detected in YA cells transfected with ZF2-DNMT1 was 50% less than what was detected in YA cells transfected with the empty vector (Figure S1). In summary, expression of ZF-DNMT1 fusion protein in YA cells can significantly reduce HIV-1 expression in infected cells.

To determine whether ZF-DNMT1 expression could result in substantial suppression of HIV-1, we subcultured the transfected YA cells and measured GFP-expressing cells 5 days, 10 days, and 15 days after transfection. The suppression of HIV-1 expression by ZF-DNMT1, which is represented by GFP-negative population, increased over the 15 days from about 34% to 50%, while it remained constantly at about 20% in the negative control (Figure 3C). This result indicates that suppression caused by ZF-DNMT1 fusion protein can persist in transfected cells for at least 15 days which is possibly long enough for 15 cell divisions if the cell doubling time is estimated for 24 hr per division.

ZF-DNMT1 Induces Significant and Stable Suppression of HIV-1 Reactivation in Latently Infected Cells

HIV-1 latency remains the major obstacle to HIV eradication. To determine if ZF-DNMT1 could induce suppression of provirus in latently infected cells, we transferred ZF2-DNMT1 into C11 cells, which contain a single copy of HIV provirus carrying GFP in an intron of RNPS1 gene. The GFP is also under the control of the HIV-1 LTR.³⁶ Under normal conditions, the expression of HIV provirus is silenced in C11 cells based on the GFP expression. Different latency reversing agents (LRAs) have been demonstrated to reactivate the HIV provirus in C11 cells to different degrees. We nucleofected C11 cells with the empty vector or ZF2-DNMT1 expression vector. At 72 hr post-transfection, three different LRAs, SAHA, Prostratin, or 5-Aza, were added to the culture, respectively, and the percentage of GFP-positive cells was measured using flow cytometry after 48 hr of incubation. The results showed that whether in mock, SAHA, Prostratin, or 5-Aza treatment, ZF2-DNMT1 dramatically decreased the percentage of GFP-positive cells compared to the negative group transfected with pcDNA3.1 vector (Figure 4A). We also extracted RNA from transfected C11 cells after the incubation with different LRAs. The average amount of HIV-1 mRNA was suppressed by 33-fold by ZF2-DNMT1 without any LRAs (mock), 13-fold in SAHA treatment, 81-fold in Prostratin treatment, and 12-fold in 5-Aza treatment, while compared to the same treatment group transfected with the pcDNA3.1 vector (Figure 4B). To more accurately measure HIV-1 replication, we quantified p24 protein in the supernatants using

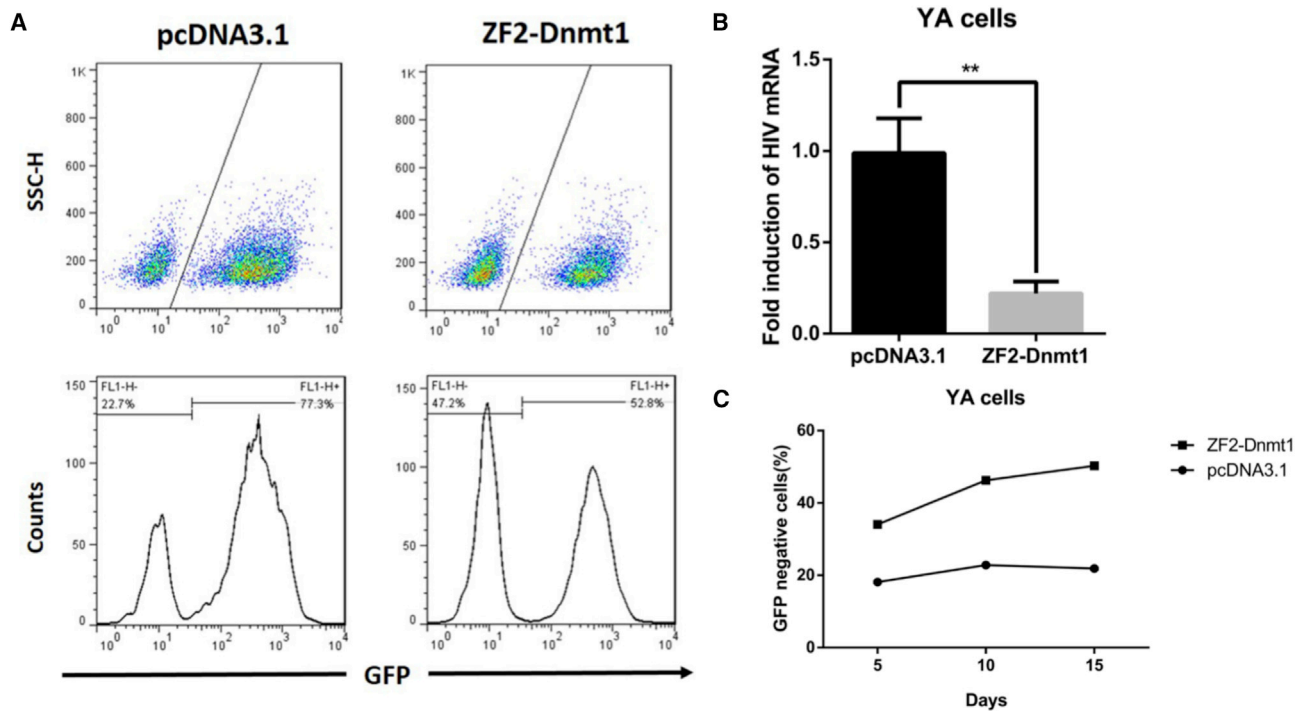


Figure 3. ZF-DNMT1 Can Suppress HIV-1 Provirus in Infected Cells Longitudinally

YA cells were nucleofected with 2 μ g of ZF2-DNMT1 plasmid or an empty vector. (A) At 72 hr post-transfection, the percentage of GFP-positive cells was measured by flow cytometry. The results are presented as fluorescence histograms. ** $p < 0.01$; Student's *t* test. (B) At 72 hr post-transfection, mRNA from the transfected YA cells was extracted and RT-qPCR was performed to detect the HIV-1 mRNA. The data represent the mean \pm SD of four independent experiments. (C) Time-dependent effects of ZF-DNMT1-mediated repression of HIV-1 provirus in YA cells. The GFP expression was monitored in gated live cells at 5 days, 10 days, and 15 days. The data were presented with the mean \pm SD of three independent experiments.

ELISA assays. We found that whether in SAHA, Prostratin, or 5-Aza treatment C11 cells, ZF-DNMT1 can decrease the expression of p24 protein in the supernatants, while compared to the same treatment group transfected with a pcDNA3.1 vector (Figure S2).

To determine how long ZF2-DNMT1 can suppress HIV-1 reactivation after LRA treatment, we subcultured transfected C11 cells and treated them with the most potent LRA, SAHA, to the culture. On days 7, 14, 20, 30, and 40 after transfection, cells were harvested 48 hr after being incubated with SAHA for measuring HIV reactivation by analyzing the GFP-positive population using flow cytometry. Regardless the incubation with SAHA, the proportion of GFP-positive cells in the ZF2-DNMT1 group was significantly lower than that in the pcDNA3.1 group at each indicated time point (Figure 4C). Our results indicate that the ZF-DNMT1 fusion protein can induce stable and persistent suppression on HIV-1 reactivation in latently infected cells for about 40 cell passages.

ZF-DNMT1-Mediated DNA Methylation of CpG Islands in HIV-1 5'-LTR

To demonstrate that ZF2-DNMT1 can increase the DNA methylation status of targeted CpG islands in 5'-LTR, we extracted the genomic DNA from transfected YA and C11 cells 5 days after transfection. Af-

ter bisulfite conversion, the sequence containing the region of two CpG islands (there are a total of 30 CpG sites in 777 bp) was amplified by nest-PCR (Figure 5B). The methylation status was analyzed by sequencing of each individual clone. In YA cells or C11 cells, the cells transfected with empty vector showed a basal level of 1.4%–1.8% of CpG was methylated. However, in ZF2-DNMT1 transfected cells, the targeted CpG methylation was increased to 6.8% in YA cells and 4.8% in C11 cells (Figures 5C and 5D). Up to 22.2% or 21.4% of DNA methylation was achieved at certain clones of YA or C11 cells, respectively (data not shown). Some methylation hot sites (>30% of DNA methylation) were observed in YA (sites 19–20) or C11 (site 6) cells (Figure 5A).

Since the methylation experiment was conducted on the genomic DNA extracted from transiently transfected cells, the transfection efficiency, which no more than 60% at the most, might have affected the outcomes. Thus, the efficacy of ZF2-DNMT1 and DNA methylation status may have been underestimated.

ZF-DNMT1 Does Not Affect the Methylation in the Promoter of ECL1, which Located around HIV

To investigate the binding specificity of ZF-DNMT1 to the HIV-1 LTR, we analyzed methylation of CpG islands in the promoter of a

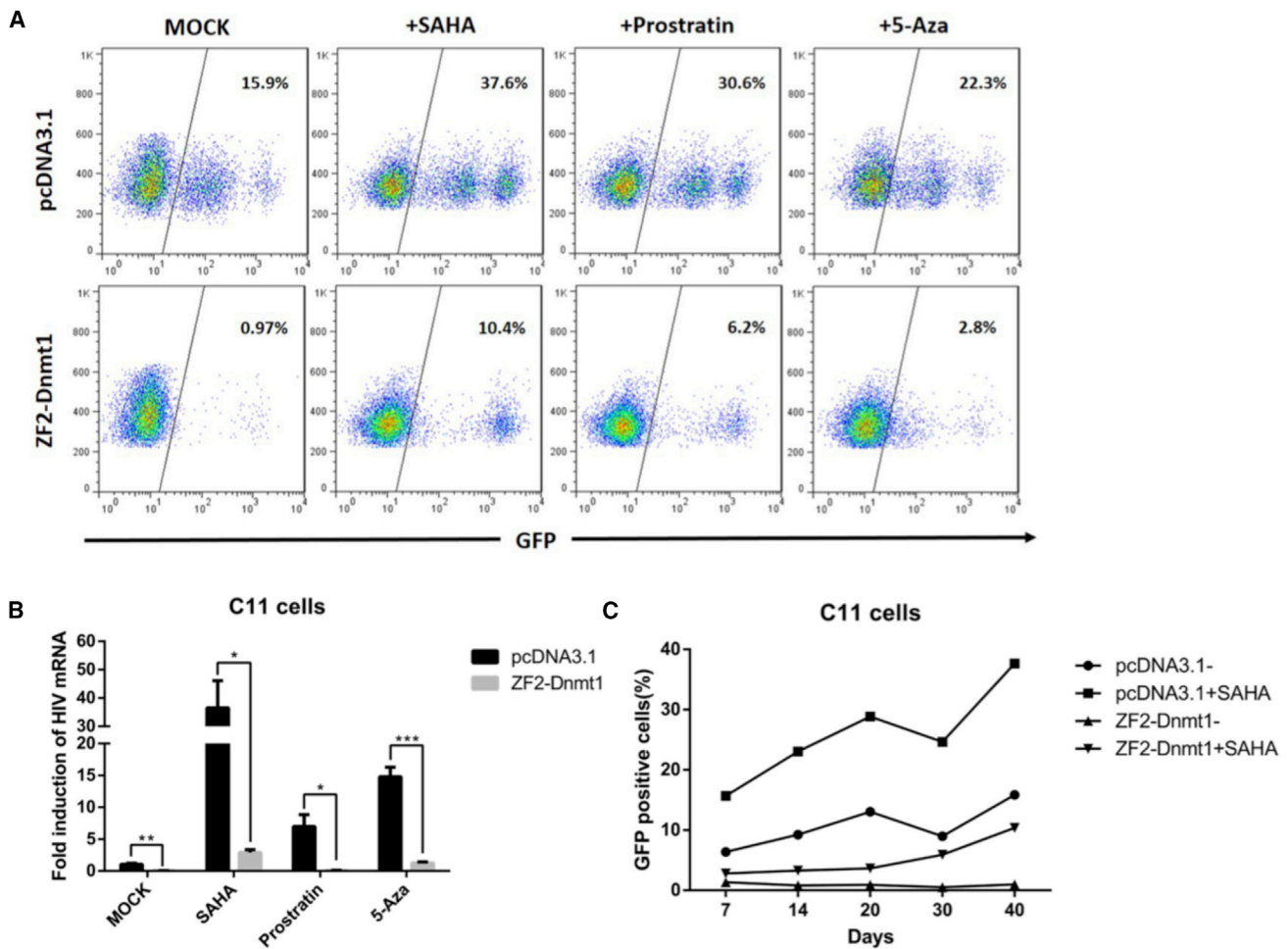


Figure 4. ZF-DNMT1 Mediates Stable Repression of HIV-1 Provirus in Latently Infected Cells

C11 cells were nucleofected with 2 μg of ZF2-DNMT1 plasmid or an empty vector. (A) At 72 hr post-transfection, the cell culture was treated with SAHA (500 nM), Prostratin (500 nM), or 5-Aza (500 nM). The percentage of GFP-positive population was measured by flow cytometry after 48 hr of incubation. The results are presented as fluorescence histograms. (B) After 48 hr of incubation with LRAs, RNA was extracted from the transfected C11 cells for RT-PCR assay to reveal the presence of HIV-1 RNA. The data represent the mean ± SD of four independent experiments. **p < 0.01; Student's t test. (C) Time-dependent effects of ZF-DNMT1-mediated repression of HIV-1 provirus in C11 cells. The cell culture was treated with SAHA at 7 days, 14 days, 20 days, 30 days, and 40 days post-transfection. After 48 hr of incubation, the GFP expression representing HIV LTR activity was monitored in gated live cells using flow cytometry. The data represent the mean ± SD of three independent experiments.

cellular gene, ECL1. The genomic DNA was extracted from the transfected C11 cells 5 days after transfection. After bisulfite conversion, the sequence containing the promoter region with total 20 CpG sites dispersing across 485 bp was amplified by nest-PCR for measuring DNA methylation using bisulfite sequencing (Figure S3). In C11 cells, the cells transfected empty vector showed a basal level of 2% DNA methylation, and in ZF2-DNMT1 transfected cells, the methylation was 3% in C11 cells (Figure S3) without any statistical significance between these two treatments.

ZF-DNMT1 Does Not Affect Cell Proliferation or Cell Cycle Progression

Safety is a critical concern of agents for HIV-1 treatment. To address this issue, we evaluated the safety in the Jurkat T cells transfected with

ZF-DNMT1. The CCK-8 assay was used to evaluate cell proliferation 72 hr after Jurkat T cells were transfected with pcDNA3.1(–) or with ZF-DNMT1. Our results indicated that ZF-DNMT1 did not affect cell proliferation (Figure 6A). The potential of ZF-DNMT1 to induce cell cycle progression was further investigated by staining these cells with propidium iodide 72 hr post-transfection and analyzing the DNA content using flow cytometry. Compared with the control group, the transfection of the ZF-DNMT1 did not affect cell cycle progression (Figure 6B).

DISCUSSION

HAART is the most successful achievement against HIV infection thus far, however, HAART cannot eliminate the HIV-1 reservoirs and viremia rebound occurs as soon as the treatment is interrupted.

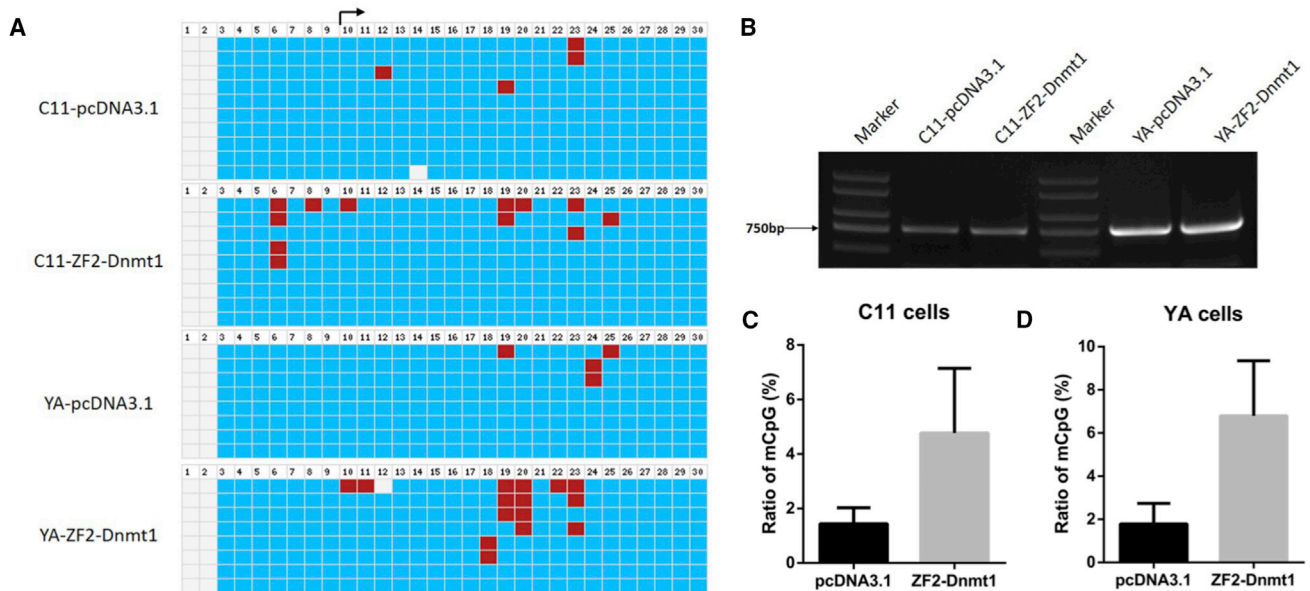


Figure 5. ZF-DNMT1 Induces Targeted DNA Methylation in HIV-1 5'-LTR

(A) The genomic DNA extracted from the transfected YA and C11 cells 5 days after transfection was bisulfite sequenced to reveal the DNA methylation status of two CpG islands in the HIV-1 5'-LTR. The horizontal rows indicate the CpG sites in the amplicon analyzed, and the vertical rows represent individual clones, which were sequenced. The blue and red colors represent unmethylated CpG and methylated CpG, respectively. The one colored in white represents that the methylation state is undetermined due to sequencing ambiguity. The transcription start site is indicated with a black arrow. (B) The amplicons containing two CpG islands were amplified by nest-PCR. (C) The level of DNA methylation of HIV LTR in C11 cells transfected with ZF2-DNMT1 or an empty vector in C11 cells or in YA cells (D). The data represent the mean \pm SD of all individual clones.

Furthermore, long-term use of HAART may lead to various side effects, because of their cytotoxicity. The most recognized approach thus far for eliminating latent HIV-1 reservoirs is “shock and kill”, an innovative approach of reactivating these reservoirs by inducing the transcription of the quiescent provirus followed by HAART to eliminate the reactivated cells harboring HIV provirus via viral cytopathic effect or host immune response. The HAART in this shock and kill approach is basically for preventing the spread of the nascent HIV from infecting neighboring cells. Although some drugs based on this strategy have already been tested in human clinical trials, the outcomes are not as promising as previously expected. The adverse effects and limitations were also observed, such as cytotoxicity, mutagenicity, or a lack of target specificity, still exist.^{40–42} Therefore, new anti-viral approaches would need to be explored.

In this study, we constructed a new chimeric DNA methyltransferases 1 (DNMT1) with ZFP targeting HIV-1 5'-LTR. We hypothesized that the binding specificity of ZFP to HIV LTR will limit the ligated DNMT1 to methylate the HIV LTR, but not other regions. Several researches have reported that ZFP could mediate the targeted binding of HIV-1 LTR.^{36–38,43} We used a ZFP which was designed to target HIV LTR and has been well characterized in our studies^{36,37} as the DNA binding domain to direct DNMT1 to land on HIV LTR. A conventional paradigm of the mammalian DNA methylation model is that DNMT3 initiates de novo methylation, while DNMT1 is responsible for maintaining the heritage of the DNA methylation pattern af-

ter DNA duplication during cell proliferation. Thus, previous studies were focused on using DNMT3 as catalytic domain to initiate DNA methylation.^{44–48} However, most recent studies demonstrated that DNMT1 can also initiate de novo DNA methylation without the presence of DNMT3.^{15–19} Therefore, we used DNMT1 as the catalytic domain for DNA methylation in this chimeric ZNF-DNMT1 to increase the methylation status on HIV LTR to suppress HIV-1 expression.

Our results indicate that ZF2-DNMT1 could repress HIV-1 expression by increasing the DNA methylation of CpG islands in 5'-LTR in both infected cells and latently infected cells. The similar results were also found by Martinez-Colom et al.³⁵ These data are in agreement with the other studies which correlated the DNA methylation status in 5'-LTR to transcriptional silencing.^{23–25,49} Furthermore, we found that the repression of HIV-1 expression caused by ZF2-DNMT1 was stable and could be maintained through several cell divisions, which was consistent with the results published by Blancafort et al.^{45,47} We noticed that the increase of DNA methylation in the 5'-LTR by ZF2-DNMT1 was modest. Some recent works reported higher efficiency of targeted DNA methylation on the promoter of endogenous genes. This was probably attributed to the modest transfection efficiency and inferior ability of de novo methylation of DNMT1. In addition, the methylation status of certain methylated CpG sites, rather than the density of CpG methylation, plays a more important role in controlling the promoter activities of HIV-1.⁵⁰

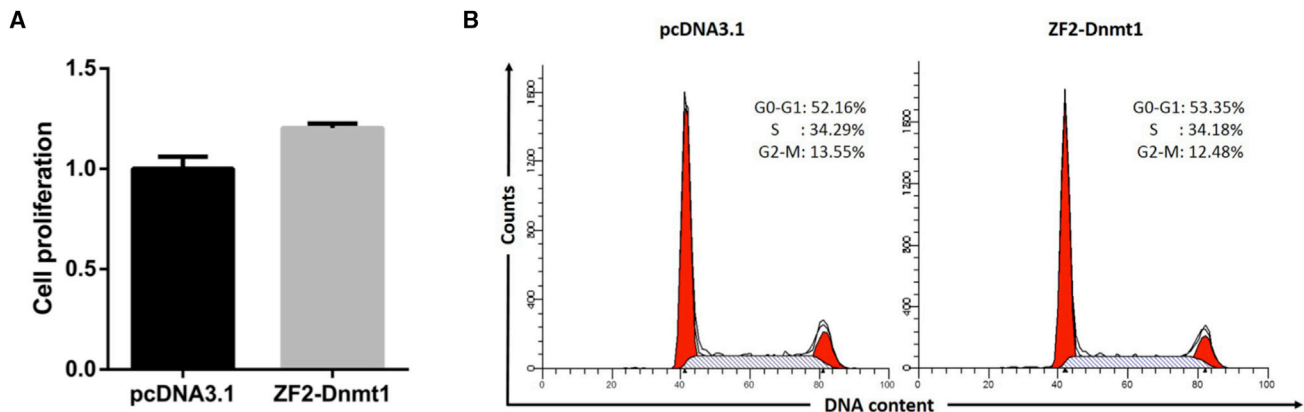


Figure 6. ZF-DNMT1 Has No Effect on Cell Proliferation or Cell Cycle Progression

Jurkat T cells were nucleofected with ZF2-DNMT1 plasmid or an empty vector as a negative control. (A) At 72 hr post-transfection, cell viability was measured using a CCK-8 kit (Dojindo). The data represent the mean \pm SD of three independent experiments. (B) Cell cycle progression was analyzed by propidium iodide staining and flow cytometry. A representative experiment of three independent assays is shown.

Based on the fact that the majority of HERVs stay silent in the human genome throughout the evolution, we believe it is possible that HIV can be tightly suppressed or even be permanently silenced in the human genome. The data showed that ZF2-DNMT1 could efficiently and stably suppress HIV-1 expression by targeted DNA methylation without affecting the cell proliferation. In order to utilize the full potential of this toolset, a number of questions and challenges are needed to be addressed, such as what the most effective method is for delivering this technology and what the specificity is and if there is any toxicity or adverse effect associated with it while being used in vivo. Taken together, the DNA methylation-based silencing strategy is of great potential for gene therapy on HIV infection.

MATERIALS AND METHODS

Cell Culture

YA cells and C11 cells, which are HIV-1-infected or latently infected Jurkat T cells, respectively, were first created in our laboratory and are now used widely in the research community.^{36,37,51–54} The C11 and YA cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 U mL⁻¹ penicillin, and 100 μ g mL⁻¹ streptomycin (Invitrogen) at 37°C under 5% CO₂. HEK293 cells stably expressing the SV40 large T antigen (HEK293T) were purchased from the American Type Culture Collection (Manassas, VA). TZM-bl cells obtained from the NIH AIDS Research and Reference Reagent Program were previously generated by introducing separate integrated copies of the luciferase and β -galactosidase genes driven by HIV-1 LTR.³⁹ HEK293T and TZM-bl cells were maintained in DMEM (Gibco) with 10% fetal bovine serum, 100 U mL⁻¹ penicillin and 100 μ g mL⁻¹ streptomycin at 37°C under 5% CO₂.

Vector Constructs

There were four ZFP expression plasmids targeting the HIV-1 LTR that were designed and assembled by Sigma-Aldrich. Plasmids encoding DNMT1 (pcDNA3.1-DNMT1) were a gift from Edward Seto.⁵⁵

There were four ZFPs with 3FLAG, NLS, linker (LRGS) that were subcloned from ZFP expression plasmids into NheI/EcoRI-digested pcDNA3.1-DNMT1, respectively. DNMT1 was cloned into the C-terminal of the zinc-finger motifs in frame to become ZF-DNMT1 in these new constructs. The target sites for these constructs in the HIV-1 LTR are shown in Figure 1. The pcDNA3.1-ZF2 was generated by deleting DNMT1 fragment from the ZF2-DNMT1. The pLTR-luc reporter vector was constructed previously.³⁶ pRL-SV40 plasmid was purchased from Promega.

Luciferase Reporter Assay

To examine the effects of ZF-DNMT1 on the HIV-1 LTR, each ZF-DNMT1 construct, pcDNA3.1 (mock), pcDNA3.1-ZF2, or pcDNA3.1-DNMT1 (700 ng) was cotransfected into HEK293T cells with pLTR-luc (100 ng) and internal control pRL-SV40 (40 ng) using ViaFect reagent (Promega) following the manufacturer's instructions. The cells were harvested 72 hr post-transfection, and the lysate was analyzed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). There were three independent transfection experiments that were performed, and each of them was performed in triplicate.

Since the genome of the TZM-bl cells was integrated with a luciferase gene driven by the HIV-1 5'-LTR promoter, we only needed to cotransfect pcDNA3.1 (mock) or ZF-DNMT1 construct (700 ng) with pCMV-Tat (200 ng) into TZM-bl cells using ViaFect reagent (Promega). Cells were harvested at 72 hr post-transfection, and the lysate was analyzed for luciferase activity. There were three independent transfection experiments that were performed, and each assay was performed in triplicate.

Western Blot

HEK293 T cells were plated in a 60-mm dish and then transfected with 8 μ g of pcDNA3.1, pcDNA3.1-DNMT1, or ZF-DNMT1 plasmid, respectively, using ViaFect reagent (Promega). After 3 days

of transfection, cells were harvested, lysed, and subjected to western blotting. The membrane was detected by primary antibody against FLAG for detecting ZF-DNMT1 or α -tubulin as reference. Immun-Star WesternC chemiluminescence Kit (Bio-Rad) was used to detect aforementioned proteins, and the signal of chemiluminescence was captured using a ChemiDoc XRS+ System and analyzed using Image-Lab software (Bio-Rad).

Nucleofection, Flow Cytometry Assays, and ELISA Assays

To analyze the HIV-1 expression regulated by ZF-DNMT1 in YA cells or C11 cells, the cells were nucleofected with ZF-DNMT1 or pcDNA3.1 empty vector using the Amaxa Cell Line Nucleofector Kit V (Lonza). The expression of GFP was used as a marker for HIV-1 expression and was observed under fluorescence microscopy. To determine the level of HIV-1 LTR promoter activity based on GFP expression, at each indicated time point after transfection, the cells were washed and resuspended in phosphate-buffered saline, and the percentage of GFP-positive cells was measured using FACScan flow cytometer (Becton Dickinson). To determine the effects of ZF-DNMT1 on inhibiting HIV reactivation by different LRAs, C11 cells were harvested 48 hr after incubation with LRAs. The FACS data were analyzed using CellQuest software (Macintosh). The expression of viral protein p24 was quantified using the HIV-1 p24 Antigen ELISA Kit (ZeptoMetrix) at each indicated time point.

RT-PCR Assay

To detect the changes in the level of HIV-1 RNA, RT-PCR assay was performed as described previously.⁵⁶ Total RNA was extracted using RNeasy Mini Kit (QIAGEN). cDNA synthesis was performed using the GoScript Reverse Transcription System containing an oligo(dT)₁₅ primer (Promega). Quantitative real-time PCR was performed in triplicate using the QuantiFast SYBR Green PCR Kit (QIAGEN) on a Roche LightCycler 480 II machine. Primers specific for the poly(A) tail of HIV-1 mRNA were designed as described: forward, 5'-CAGAT GCTGCATATAAGCAGCTG-3' (9501-9523) and reverse, 5'TTTT TTTTTTTTTTTTTTTTTTTTGAAGCAC-3' (9629-poly A). The GPDH gene was used for normalization.

Cytosine Methylation Analysis

The methylation status of HIV LTR in the transfected YA or C11 cells was analyzed using EZ DNA Methylation-Gold Kit (Zymo Research). Modified DNA was amplified by PCR application using two pairs of primers as described.⁵⁷ PCR was performed by 40 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 60 s.

PCR products were purified and cloned into pMD18-T Vector (Takara) for bacteria transformation. Plasmid DNA from each individual clone was sequenced to reveal the methylation status of CpG islands. A sequence was excluded if it shows less than 95% conversion rate. Data were analyzed using the BISMA software.⁵⁸

Cell Proliferation and Cell Cycle Analysis

The Cell Counting Kit-8 (CCK-8) (Dojindo) was used to measure cell proliferation after the ZF-DNMT1 treatment. Briefly, Jurkat T cells

were seeded at the density of 4×10^5 cell per well in 96-well plates and transfected with pcDNA3.1 (mock) or ZF-DNMT1 construct, respectively. At 72 hr post-transfection, 10 μ L of CCK-8 solution was added to each well. After 4 hr of incubation at 37°C, the absorbance at 450 nm was measured using a microplate reader. Each experiment was performed independently in triplicate.

To generate the cell cycle profiles, 1×10^6 Jurkat T cells were transfected and then fixed with 500 μ L 70% ethanol at -20°C for 2 hr. Subsequently, the cells were washed twice with cold $1 \times$ PBS and then stained with propidium iodide (50 μ g/mL propidium iodide and 100 μ g/mL RNase A in $1 \times$ PBS) at 37°C for 30 min. The cell cycle analysis was performed using a FACScan flow cytometer. All experiments were performed independently in triplicate.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at <http://dx.doi.org/10.1016/j.omtn.2017.01.002>.

AUTHOR CONTRIBUTIONS

H.Z. conceived and designed the experiments. J.D. and X.Q. carried out most experiments. P.L., X.Y., Y.Z., H.J., Y.W., Z.J., X.L., Y.Z., H.Y., and H.P. participated in some of the experiments. W-B.Y. and H.Z. directed and supervised the experiments and interpretation of data. The manuscript was prepared by J.D., W-B.Y., and H.Z.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the Natural Science Foundation of China (no. 31501032) and the National High-Tech Research and Development Program of China (2015AA020307).

REFERENCES

- Cohen, J. (2012). Infectious disease. HIV prevention and cure insights come from failure and success. *Science* 335, 1291.
- Cohen, J. (2011). Understanding HIV latency to undo it. *Science* 332, 786.
- Ruelas, D.S., and Greene, W.C. (2013). An integrated overview of HIV-1 latency. *Cell* 155, 519–529.
- Deeks, S.G. (2012). HIV: Shock and kill. *Nature* 487, 439–440.
- Carr, A. (2003). Toxicity of antiretroviral therapy and implications for drug development. *Nat. Rev. Drug Discov.* 2, 624–634.
- Zaccarelli, M., Tozzi, V., Lorenzini, P., Trotta, M.P., Forbici, F., Visco-Comandini, U., Gori, C., Narciso, P., Perno, C.F., and Antinori, A.; Collaborative Group for Clinical Use of HIV Genotype Resistance Test (GRT) at National Institute for Infectious Diseases Lazzaro Spallanzani (2005). Multiple drug class-wide resistance associated with poorer survival after treatment failure in a cohort of HIV-infected patients. *AIDS* 19, 1081–1089.
- van der Kuyl, A.C. (2012). HIV infection and HERV expression: a review. *Retrovirology* 9, 6.
- Rowe, H.M., Jakobsson, J., Mesnard, D., Rougemont, J., Reynard, S., Aktas, T., Maillard, P.V., Layard-Liesching, H., Verp, S., Marquis, J., et al. (2010). KAP1 controls endogenous retroviruses in embryonic stem cells. *Nature* 463, 237–240.

9. Gimenez, J., Montgiraud, C., Oriol, G., Pichon, J.P., Ruel, K., Tsatsaris, V., Gerbaud, P., Frendo, J.L., Evain-Brion, D., and Mallet, F. (2009). Comparative methylation of ERVWE1/syncytin-1 and other human endogenous retrovirus LTRs in placenta tissues. *DNA Res.* 16, 195–211.
10. Lavie, L., Kitova, M., Maldener, E., Meese, E., and Mayer, J. (2005). CpG methylation directly regulates transcriptional activity of the human endogenous retrovirus family HERV-K(HML-2). *J. Virol.* 79, 876–883.
11. Schulz, W.A., Steinhoff, C., and Florl, A.R. (2006). Methylation of endogenous human retroelements in health and disease. *Curr. Top. Microbiol. Immunol.* 310, 211–250.
12. Yoder, J.A., Walsh, C.P., and Bestor, T.H. (1997). Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet.* 13, 335–340.
13. Matousková, M., Vesely, P., Daniel, P., Mattiuzzo, G., Hector, R.D., Scobie, L., Takeuchi, Y., and Hejnar, J. (2013). Role of DNA methylation in expression and transmission of porcine endogenous retroviruses. *J. Virol.* 87, 12110–12120.
14. Chiu, S., Hsu, K., Greenhalgh, D.G., and Cho, K. (2010). Post-injury stress signals alter epigenetic profile of cytosine methylation in the proviral genome of endogenous retroviruses. *Exp. Mol. Pathol.* 89, 291–300.
15. Goyal, R., Reinhardt, R., and Jeltsch, A. (2006). Accuracy of DNA methylation pattern preservation by the Dnmt1 methyltransferase. *Nucleic Acids Res.* 34, 1182–1188.
16. Binizkiewicz, D., Gribnau, J., Ramsahoye, B., Gaudet, F., Eggan, K., Humpherys, D., Mastrangelo, M.A., Jun, Z., Walter, J., and Jaenisch, R. (2002). Dnmt1 overexpression causes genomic hypermethylation, loss of imprinting, and embryonic lethality. *Mol. Cell. Biol.* 22, 2124–2135.
17. Arand, J., Spieler, D., Karius, T., Branco, M.R., Meilinger, D., Meissner, A., Jenuwein, T., Xu, G., Leonhardt, H., Wolf, V., and Walter, J. (2012). In vivo control of CpG and non-CpG DNA methylation by DNA methyltransferases. *PLoS Genet.* 8, e1002750.
18. Jones, P.A. (2012). Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat. Rev. Genet.* 13, 484–492.
19. Jeltsch, A., and Jurkowska, R.Z. (2014). New concepts in DNA methylation. *Trends Biochem. Sci.* 39, 310–318.
20. Bednarik, D.P., Cook, J.A., and Pitha, P.M. (1990). Inactivation of the HIV LTR by DNA CpG methylation: evidence for a role in latency. *EMBO J.* 9, 1157–1164.
21. Schulze-Forster, K., Götz, F., Wagner, H., Kröger, H., and Simon, D. (1990). Transcription of HIV1 is inhibited by DNA methylation. *Biochem. Biophys. Res. Commun.* 168, 141–147.
22. Gutekunst, K.A., Kashanchi, F., Brady, J.N., and Bednarik, D.P. (1993). Transcription of the HIV-1 LTR is regulated by the density of DNA CpG methylation. *J. Acquir. Immune Defic. Syndr.* 6, 541–549.
23. Blazkova, J., Trejbalova, K., Gondois-Rey, F., Halfon, P., Philibert, P., Guiguen, A., Verdin, E., Olive, D., Van Lint, C., Hejnar, J., and Hirsch, I. (2009). CpG methylation controls reactivation of HIV from latency. *PLoS Pathog.* 5, e1000554.
24. Li, F., Li, L., Zhong, Y., Xie, Q., Huang, J., Kang, X., Wang, D., Xu, L., and Huang, T. (2013). Relationship between LTR methylation and gag expression of HIV-1 in human spermatozoa and sperm-derived embryos. *PLoS ONE* 8, e54801.
25. Kauder, S.E., Bosque, A., Lindqvist, A., Planelles, V., and Verdin, E. (2009). Epigenetic regulation of HIV-1 latency by cytosine methylation. *PLoS Pathog.* 5, e1000495.
26. Baubec, T., Colombo, D.F., Wirbelauer, C., Schmidt, J., Burger, L., Krebs, A.R., Akalin, A., and Schübeler, D. (2015). Genomic profiling of DNA methyltransferases reveals a role for DNMT3B in genic methylation. *Nature* 520, 243–247.
27. Schübeler, D. (2015). Function and information content of DNA methylation. *Nature* 517, 321–326.
28. Jones, P.A., and Liang, G. (2009). Rethinking how DNA methylation patterns are maintained. *Nat. Rev. Genet.* 10, 805–811.
29. Reik, W. (2007). Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature* 447, 425–432.
30. Franklin, T.B., Russig, H., Weiss, I.C., Gräff, J., Linder, N., Michalon, A., Vizi, S., and Mansuy, I.M. (2010). Epigenetic transmission of the impact of early stress across generations. *Biol. Psychiatry* 68, 408–415.
31. Li, Y., and O'Neill, C. (2012). Persistence of cytosine methylation of DNA following fertilisation in the mouse. *PLoS ONE* 7, e30687.
32. Jiang, L., Zhang, J., Wang, J.J., Wang, L., Zhang, L., Li, G., Yang, X., Ma, X., Sun, X., Cai, J., et al. (2013). Sperm, but not oocyte, DNA methylome is inherited by zebrafish early embryos. *Cell* 153, 773–784.
33. Kelsey, G., and Feil, R. (2013). New insights into establishment and maintenance of DNA methylation imprints in mammals. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 368, 20110336.
34. Fior, J. (2013). Is a pacific coexistence between virus and host the unexploited path that may lead to an HIV functional cure? *Viruses* 5, 753–757.
35. Martinez-Colom, A., Lasarte, S., Fernández-Pineda, A., Rellosa, M., and Muñoz-Fernández, M.A. (2013). A new chimeric protein represses HIV-1 LTR-mediated expression by DNA methylase. *Antiviral Res.* 98, 394–400.
36. 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.
37. 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.
38. 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.
39. Platt, E.J., Bilska, M., Kozak, S.L., Kabat, D., and Montefiori, D.C. (2009). Evidence that ecotropic murine leukemia virus contamination in TZM-bl cells does not affect the outcome of neutralizing antibody assays with human immunodeficiency virus type 1. *J. Virol.* 83, 8289–8292.
40. Rasmussen, T.A., Schmeltz Sogaard, O., Brinkmann, C., Wightman, F., Lewin, S.R., Melchjorsen, J., Dinarello, C., Østergaard, L., and Tolstrup, M. (2013). Comparison of HDAC inhibitors in clinical development: effect on HIV production in latently infected cells and T-cell activation. *Hum. Vaccin. Immunother.* 9, 993–1001.
41. Elliott, J.H., Wightman, F., Solomon, A., Ghneim, K., Ahlers, J., Cameron, M.J., Smith, M.Z., Spelman, T., McMahon, J., Velayudham, P., et al. (2014). Activation of HIV transcription with short-course vorinostat in HIV-infected patients on suppressive antiretroviral therapy. *PLoS Pathog.* 10, e1004473.
42. Olesen, R., Vigano, S., Rasmussen, T.A., Sogaard, O.S., Ouyang, Z., Buzon, M., Bashirova, A., Carrington, M., Palmer, S., Brinkmann, C.R., et al. (2015). Innate immune activity correlates with CD4 T cell-associated HIV-1 DNA decline during latency-reversing treatment with panobinostat. *J. Virol.* 89, 10176–10189.
43. 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.
44. Li, F., Papworth, M., Minczuk, M., Rohde, C., Zhang, Y., Ragozin, S., and Jeltsch, A. (2007). Chimeric DNA methyltransferases target DNA methylation to specific DNA sequences and repress expression of target genes. *Nucleic Acids Res.* 35, 100–112.
45. Rivenbark, A.G., Stolzenburg, S., Beltran, A.S., Yuan, X., Rots, M.G., Strahl, B.D., and Blancafort, P. (2012). Epigenetic reprogramming of cancer cells via targeted DNA methylation. *Epigenetics* 7, 350–360.
46. Nunna, S., Reinhardt, R., Ragozin, S., and Jeltsch, A. (2014). Targeted methylation of the epithelial cell adhesion molecule (EPCAM) promoter to silence its expression in ovarian cancer cells. *PLoS ONE* 9, e87703.
47. Stolzenburg, S., Beltran, A.S., Swift-Scanlan, T., Rivenbark, A.G., Rashwan, R., and Blancafort, P. (2015). Stable oncogenic silencing in vivo by programmable and targeted de novo DNA methylation in breast cancer. *Oncogene* 34, 5427–5435.
48. Vojta, A., Dobričić, P., Tadić, V., Bočkor, L., Korać, P., Julg, B., Klasić, M., and Zoldoš, V. (2016). Repurposing the CRISPR-Cas9 system for targeted DNA methylation. *Nucleic Acids Res.* 44, 5615–5628.
49. Palacios, J.A., Pérez-Piñar, T., Toro, C., Sanz-Minguela, B., Moreno, V., Valencia, E., Gómez-Hernando, C., and Rodés, B. (2012). Long-term nonprogressor and elite controller patients who control viremia have a higher percentage of methylation in their HIV-1 proviral promoters than aviremic patients receiving highly active antiretroviral therapy. *J. Virol.* 86, 13081–13084.

50. Ishida, T., Hamano, A., Koiwa, T., and Watanabe, T. (2006). 5' long terminal repeat (LTR)-selective methylation of latently infected HIV-1 provirus that is demethylated by reactivation signals. *Retrovirology* 3, 69.
51. Lu, P., Qu, X., Shen, Y., Jiang, Z., Wang, P., Zeng, H., Ji, H., Deng, J., Yang, X., Li, X., et al. (2016). The BET inhibitor OTX015 reactivates latent HIV-1 through P-TEFb. *Sci. Rep.* 6, 24100.
52. 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.
53. Wang, P., Qu, X., Zhou, X., Shen, Y., Ji, H., Fu, Z., Deng, J., Lu, P., Yu, W., Lu, H., and Zhu, H. (2015). Two cellular microRNAs, miR-196b and miR-1290, contribute to HIV-1 latency. *Virology* 486, 228–238.
54. Wang, P., Qu, X., Wang, X., Liu, L., Zhu, X., Zeng, H., and Zhu, H. (2013). As2O3 synergistically reactivate latent HIV-1 by induction of NF-kappaB. *Antiviral Res.* 100, 688–697.
55. Peng, L., Yuan, Z., Ling, H., Fukasawa, K., Robertson, K., Olashaw, N., Koomen, J., Chen, J., Lane, W.S., and Seto, E. (2011). SIRT1 deacetylates the DNA methyltransferase 1 (DNMT1) protein and alters its activities. *Mol. Cell. Biol.* 31, 4720–4734.
56. Shan, L., Rabi, S.A., Laird, G.M., Eisele, E.E., Zhang, H., Margolick, J.B., and Siliciano, R.F. (2013). A novel PCR assay for quantification of HIV-1 RNA. *J. Virol.* 87, 6521–6525.
57. Pion, M., Jordan, A., Biancotto, A., Dequiedt, F., Gondois-Rey, F., Rondeau, S., Vigne, R., Hejnar, J., Verdin, E., and Hirsch, I. (2003). Transcriptional suppression of in vitro-integrated human immunodeficiency virus type 1 does not correlate with proviral DNA methylation. *J. Virol.* 77, 4025–4032.
58. Rohde, C., Zhang, Y., Reinhardt, R., and Jeltsch, A. (2010). BISMA—fast and accurate bisulfite sequencing data analysis of individual clones from unique and repetitive sequences. *BMC Bioinformatics* 11, 230.