

Repression of HIV-1 Reactivation Mediated By KRAB Fused CRISPR/dCas9 Proteins In Lymphoid And Myeloid Cell Models

Lendel Correia da Costa

Universidade Federal do Rio de Janeiro (UFRJ)

Larissa Maciel Bomfim

Universidade Federal do Rio de Janeiro (UFRJ)

Uilla Victoria Torres Dittz

Universidade Federal do Rio de Janeiro (UFRJ)

Camila de Almeida Velozo

Universidade Federal do Rio de Janeiro (UFRJ)

Rodrigo Delvecchio da Cunha

Universidade Federal do Rio de Janeiro (UFRJ)

Amilcar Tanuri (✉ atanuri1@gmail.com)

Universidade Federal do Rio de Janeiro (UFRJ)

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Abstract

Background: Despite antiretroviral treatment efficacy, it does not lead to the complete eradication of HIV infection. In addition, HIV-1 latency reactivation is a major challenge towards cure efforts. Two strategies to cure HIV-1 infection, the “shock and kill” is based on the eradication of the HIV-1 from the patient, and the “block and lock”. The “Block and Lock” methodology aims to control HIV-1 latency reactivation, promoting a functional cure. The KRAB fused CRISPR/dCas9 (pdCas9KRAB) system was previously produced to control cell transcription. Based on this construct we developed a CRISPR RNAs (sgRNAs), to guide the pdCas9KRAB up to five different sites in HIV-1 provirus sites to block HIV-1 latency reactivation. This process was mediated by phorbol esters and HDAC inhibitors.

Results: We found five sites in the HIV-1 provirus genome (LTR1-LTR5) that minimize CRISPR off-targets and transduced them in the lymphoid and myeloid HIV-1 latency models. One of the five sgRNAs (LTR5) which binds specifically in the HIV-1 LTR NFκB binding site was able to promote a robust repression of reactivation pattern in a HIV-1 latency lymphoid model stimulated with Phorbol 12-Myristate 13-Acetate (PMA) and Ingenol B (IngB), both potent protein kinase C (PKC) stimulators. Reactivation with HDAC inhibitors, such as SAHA and Panobinostat, showed the same strong inhibition of reactivation. Additionally, we observed a reduction of 100 times in HIV-1 RNA molecules, when reactivated IngB in myeloid HIV-1 latently infected U1 cells.

Conclusion: Taken together, our results show that the KRAB fused CRISPR/dCas9 system can robustly prevent the HIV-1 latency reactivation process, mediated by PMA or IngB and SAHA or Panobinostat, both in myeloid and lymphoid HIV-1 latency. In addition, we demonstrated that KRAB repressor protein is crucial to reactivation resistance phenotype, and we also have shown some useful hotspots sequences in HIV-1 LTR to design sgRNAs.

Background

By the end of 2020, around 38 million people were living with HIV throughout the world [1]. Although antiretroviral therapy (ART) increases the survival of people living with HIV, virologic failure, viral resistance and adverse effects still hamper treatment adherence. Moreover, lifelong therapy is required, and it does not lead to a complete eradication of HIV infection [2,3].

HIV-latency and viral reservoirs are the major challenges towards HIV cure. HIV reservoirs are established when active CD4+ T cells are infected and turn into a resting state as a memory CD4+ T cell, harboring a capable but non-replicative HIV-1 provirus [4,5]. Memory CD4+ T cells are highly stable, have long half-lives, lasting almost 44 months on average and are continually replenished by clonal expansion and homeostatic proliferation [6].

Viral latency can be established before or after the provirus integration [7] when a stable and replicative HIV-1 provirus is blocked in transcriptional or/and translational levels [8]. Blocking HIV-1 transcription is the most frequent mechanism to induce viral latency. Tat protein interacts with the trans-activation

response element (TAR) in HIV-1 mRNA, increasing RNA polymerase II (RNAPol II) processivity and transcription elongation. Therefore, low concentrations of Tat induce HIV-1 latency [9].

The modulation of host transcription factor (TF) required for HIV-1 gene expression is another mechanism that leads to HIV-1 latency. Nuclear factor-*kappa* B (NF- κ B) and nuclear factor of activated T cell (NFAT) are transcription factors involved in T cell activation and HIV-1 reactivation [10,11]. These two host proteins are inhibited in the cytoplasm of resting T cell, NF- κ B by inhibitors of NF- κ B (I κ B) and NFAT by phosphorylation [12,13]. HIV-1 transcription can also be negatively modulated by repressive TF, such as yin yang 1 (YY1), late SV40 factor (LSF and C-promoter binding factor (CBF) [14,15], that recruits histone deacetylases (HDACs) to HIV-1 long terminal repeat (LTR) promoter thus limiting RNAPol II access [16]. Chromatin structure and epigenetic markers can also modulate HIV-1 transcription and latency entry by the addition or withdrawal of post-translational modifications of histones [17]. Such modifications might impact the interaction of nucleosomes with the DNA sequence, modulating HIV-1 LTR more or less accessible to TF and cellular transcription machinery.

Histones can be modified by histone acetyl transferases (HAT) that are associated with transcription activation. Depending on which histone has been modified and which amount of methyl groups has been transferred, the histone methyltransferase (HMT) is implicated in transcription repression or activation [17]. While trimethylation of H3K27 and H3K9 promotes transcription repression, H3K36 is well documented to induce transcription activation. Other molecular processes may impact HIV-1 transcription and possibly induce its latency, such as distal transcription, multiple splicing, euchromatin and heterochromatin modulation. The location in which HIV-1 provirus has been integrated into the cellular genome is also relevant [18].

Although HIV-1 latency is the main barrier to find a cure for HIV infection, there are two major strategies to overcome this issue. "Shock and kill" eradicates latent reservoirs by the reactivation of dormant virus upon the administration of latency reversing agents (LRA). ART administration prevents *de novo* infections and purges cells harboring the reactivated virus via active viral replication or indirectly via the host immune system. In contrast, the "block and lock" strategy is based on the impairment of some HIV-1 transcription factors to promote a permanent latent state, even in the absence of ART. Despite the success of both models in primary cells, clinical applications must be further evaluated [19–21].

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are short sequences of 20 bases (b) small guide RNA (sgRNA) responsible for antiviral defense of bacteria. CRISPR-associated protein 9 (Cas9) uses CRISPR sequences to recognize and cleave specific strands of DNA [22,23]. CRISPR-Cas9 technology was first used as an engineered molecular scissor for gene disruption to promote protein knockout [24]. This molecular tool is improved to work not only with knockout but also with transcription activation and repression, by utilizing a deactivated Cas9 (dCas9) fused with a repressor or an activator protein [25].

In the context of CRISPR/dCas9 transcription repressive tools, the most frequent repressor is the kruppel associated box (KRAB) domain, which naturally occurs in association with zinc finger proteins (ZFP),

functioning as a transcription repression factor. KRAB plays a crucial role inducing heterochromatin state of the proximal DNA molecule by recruiting KRAB associated protein 1 (KAP1). This protein interacts with other repressive complexes such as heterochromatin protein 1 (HP1) and histone methyltransferases (HMT), which promotes the amplification of trimethylated H3K9 marker and chromatin remodeling. Altogether, these tools based on DNA specific recognition and repressive epigenetic markers induction could act as a deep latency strategy [26,27]. Hence, the present study aims to provide a proof of concept of a possible use of CRISPR/dCas9 DNA recognition system fused with KRAB domain to maintain a repressive state in a latent-infected lymphoid and myeloid cell, independently of the reactivation process.

Methods

Materials and reagents

The cell line HEK293T used in the present study was obtained from the cell bank ATCC, from the English American type of culture collection, and maintained in DMEM medium (Dulbecco's Modified Eagle Medium - 11995073 - ThermoFisher - USA) supplemented with 10% of fetal bovine serum (A31608, ThermoFisher, USA).

The lymphocyte lineage cells used in this study was J.Lat 10.6 obtained from the Aids reagent program of the National Health Institute of the United States of America (NIH, USA). The myeloid strain was the U1 cell, which comes from U937 myeloid cells chronically infected with an HIV-1 clone. The lymphocyte and myeloid lines described above were kindly provided by Dr. Lucio Gama (Johns Hopkins Medical School, MD, US). They were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. All cell lines were cultured without antibiotics and kept in a conventional cell cultural incubator at 37 °C and 5% CO₂. The cultured cells were tested for mycoplasma contamination using MycoAlert® Mycoplasma detection kit (LT07-318, Lonza, USA).

Ingenol B (IngB) (Kyolab - BR) and phorbol 12-myristate 13-acetate (PMA) (P8139, Merck, USA) are two phorbol esters that were used in the present work to induce the activation of HIV-1 promoter in latent cells. PMA is used at a concentration of 1 µg/µl and Ingenol B at 1 µM. Suberoylanilide hydroxamic acid (SAHA - SML0061) and Panobinosat (Pano) (SML3060 - Merck, USA) were used at concentration of 5 µM and 0,15 µM, respectively [28].

The CRISPR vector, pLV hU6-sgRNA hUBC-dCas9KRAB-T2a-Puro named here as pdCas9Krab (pLV_hU6-sgRNA_hUBC-dSaCas9-KRAB-T2A-PuroR) was kindly provided by Dr. Charles Gersbach (Addgene plasmid # 162334; <http://n2t.net/addgene:162334>; RRID: Addgene_162334). In addition, pVSV-G and psPAXv2 plasmids were also acquired through Addgene platform.

Selection of the best sgRNAs for the HIV-1 proviral genome and for AAVS1 control gene

To identify and design sgRNAs that would be complementary to the HIV-1 genome, the HIV-1 subtype B sequence, clone HXB2, accession number AF033819 was previously acquired from the Los Alamos

database was submitted in the CRISPR Pick tool which is made available in the Broad Institute genetic disorder platform (GPP). This platform designs the possible sgRNAs, align against the human genome sequence, GRCh38 (NCBI RefSeq v.10920210514), for minimize the off target and rank them. We also performed the same process described above to design a control sgRNA for AAVS1 DNA sequence.

Assembly of sgRNAs in the pdCas9Krab expression vector

The pdCas9Krab vector expresses the fused dCas9 and Krab proteins, both being regulated by the UbC promoter. The sgRNA is regulated by the U6 promoter that occurs in the same vector. To insert the nucleotide sequence for the sgRNA of choice, a digestion of 1.5 µg of the vector pdCas9KRAB with 10 UI/µL of BsmBI (R0739S, New England Biolabs, USA) was performed following the manufacturer's procedure. The sgRNA strings was synthesized as independent oligos by (Integrated DNA technologies, USA). After the digestion, lyophilized sgRNAs oligos (additional file 1) were resuspended for 100 µM in H₂O DEPC. The phosphorylation step was proceeded with the enzyme T4 PNK (M0201S, New England Biolabs, USA), accordingly manufacturer's protocols. Then, the reaction was submitted to 95 °C for 5 min and 5 °C for 1 min to join the two DNA strands.

The ligation of the sgRNAs sequences in the digested expression vector was performed T4 DNA Ligase enzyme (M0202S, New England Biolabs, USA), following manufacturer's procedure.

The ligation reaction was transformed in chemically competent bacteria JM109, previously prepared using Mix & Go Competent Cells - JM109 strain kit (T3003, Zymo Research, USA). Bacterial transformations were performed according to the manufacturer's recommendations. These were plated in LB medium supplemented with 50 µg/ml ampicillin and maintained at 37°C for 24 h.

Confirmation of positive colonies was performed by colony PCR using the U6 forward primer and the one of sgRNA strand as a reverse primer (LTR (1-5) Reverse). The PCR cycling consisted of 10 min at 95 °C, 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, ending with 10 min at 72 °C. The PCR product was evaluated by electrophoresis on a 2% agarose gel and fragments of approximately 280 bp were observed (data not shown).

KRAB sequence excision

The sequence of the repressor protein KRAB was removed using 10 UI of NheI (Promega, USA) and after the resulting plasmid was relinked with 1 UI of T4 ligase (M1794, PROMEGA, USA), generating the vector pdCas9koKRAB. The excision was confirmed by sequencing the plasmid using the BigDye Terminator protocol (ThermoScientific, USA), according to the manufacturer's recommendation (data not shown).

Lentiviral vectors production

HEK293T cells were seeded at 8.0×10^5 cells/mL per well in 6-well plates, 24 h before transfection procedures. The LTR1-5sgRNA/dCas9KRAB expression vectors were incubated with a mix solution containing 9 µL of 2.5 M CaCl₂, 8 µg of total plasmid (3.53 µg of each expression vectors, 1.76 µg of

VSV-G and 2.7 µg of psPAX) and H₂O DEPC for a final volume of 90 µL, finally 90 µL of 2X Hepes Buffer Saline (HBS) was added for DNA precipitation. The mixture was incubated at room temperature for 15 min and dripped into the well, which had the culture medium previously replaced by a 1 mL of Opti-MEM (31985070, ThermoFisher, USA). The transfection solution was kept in contact with cells for 6 h and after this time it was replaced by 2 ml of complete medium. The transfected cells were kept for 48 h in an incubator at 37 °C, in an atmosphere with 5% CO₂. After two days, the supernatant from each well was collected and filtered in a 0.22 µm. The solution with the lentiviruses was frozen at -80 °C until the moment of transduction.

Transduction of lentiviral vectors in lymphoid and myeloid cells

The cells used for virus transduction containing sgRNA/dCas9KRAB construct were J.Lat 10.6 and U1. For this purpose, 2.5x10⁵ cells were plated in a 12-well plate in a final volume of 1 mL. Then, 8 µg/mL polybrene (Merck - H9868) and 500 µL of the lentivirus solution was added to the culture medium. The virus and cell solution were then submitted to centrifugation at 1000 g for 2 h at room temperature. The plates were then kept for 24 h in an oven at 37 °C, in an atmosphere with 5% CO₂, and 0.5 µg / mL of puromycin (Merck - USA - P8833) was added to select only the cells in which the construct was present. The optimum puromycin concentration was obtained by testing the drug sensitivity curve (data not shown). The transduced culture was kept in check until all sensitive cells had been removed and satisfactory cell growth was observed. This entire selection process took up to two weeks.

In addition to puromycin selection, transduction was confirmed by PCR. The PCR product was evaluated by electrophoresis on a 2% agarose gel and fragments of approximately 100 bp were observed (**supplemental figure 1**).

Measurement of green fluorescent protein (GFP) in J-Lat 10.6 cell line model

Wild-type (WT) and transduced (LTRs) J.Lat 10.6 cells were plated at a concentration of 10⁵ cells/mL, in 24-well plates and separated into three experimental groups: the untreated group, which did not receive the reactivating drug (negative control), the PMA group which received 1 µM of PMA and the IngB group which received 1 µM of ingenol B. The plates were kept for 24h at 37°C, in an atmosphere with 5% CO₂. After incubation, cells were centrifuged at 300 G for 4 min and resuspended in 100 µL of PBS. The analysis was performed using flow cytometry through BD Accuri C6 device (BD Biosciences, USA).

HIV-1 RNA detection in U1 cell line model

The U1 cell activation process was conducted as previously described by Abreu *et al* [29]. U1 WT cells and U1 LTRs were plated, in duplicate, at a concentration of 2.0x10⁵ per well. Two conditions per cell type were adopted: without treatment and with 1 µM IngB. Each group was kept for 48 h and then frozen at -80°C. HIV-1 viral load was estimated by HIV-1 RealTime Amplification Kit through m2000 RealTime System (Abbott, IL, USA).

Statistical analysis

Statistical calculations were performed with one-way or two-tailed unpaired Student T test using Graph Pad Prism version 9.0.0. P values ≤ 0.05 were considered statistically significant. Experiments were performed in three independent replicates with two replicates per samples. Statistical data analysis of untreated J.Lat 10.6 ko/KRAB cells experiments were performed in two independent replicates with two replicates per samples.

Results

CRISPR sgRNA binds specific to HIV-1 LTR in silico

In order to find the best sgRNAs that bind to HIV-1 provirus genome with maximum on target and minimum human genome off target ligation, we performed an *in silico* screening using the GPP (Genetic Perturbation Platform) sgRNA designer from Broad Institute. Through *in silico* analysis of the files generated by the platform, 98 possible sgRNAs were obtained (**additional file 2**). Firstly, we performed a screen to analyse only the sgRNA which bind in HIV-1 LTR or near locations. Among these, the five best ranked sgRNAs were chosen based on the major parameter established by the database: sgRNA's ability to do not bind within the human genome. More off-targets mean a lower score assigned by the program and a lower placement of this sgRNA. Therefore, the sgRNAs chosen for the subsequent assays were LTR1 to LTR5 (**table 1**).

LTR1 and LTR2 sgRNAs bind to the U3 region of the 3' LTR. LTR5 sgRNA binds to NF-kB transcription factor binding site in the 5'LTR U3R region. In turn, LTR4 binds to the U3 region of the 5'LTR, and LTRs 3 bind to the region encoding NEF viral protein (Fig. 1A).

HIV-1 latency can be maintained by specific CRISPR action in a lymphoid model

To understand whether the sgRNA inserted in the repression vector could limit HIV-1 replication, J.Lat 10.6 cells were transduced with the repressor constructs LTRs and subjected to PMA and IngB reactivation. A statistically significant reduction in GFP expression was observed in clones LTR1, LTR2, LTR4 and LTR5, in both reactivation treatments compared to treated WT and AAVS1 control. There is a significant reduction ($p < 0.0001$) in the percentage of positive GFP LTR5 cells with a decrease of 72.31% and 78.66% in the PMA and IngB treatments, respectively, when compared with the WT treated with reactivators (Fig. 1). LTR1, LTR2, LTR4 clones treated with LRA drugs also had a statistically significant reduction in GFP expression ($p < 0.05$) compared to the treated WT, of 28.70%, 16.87% and 38.27%, respectively, despite being less effective than LTR5 in inducing a repressive state. LTR3 clone showed no statistically significant difference in reducing GFP expression when compared to treated WT and AAVS1 control (Fig. 1). The cells which were transduced with the empty control vector and with the AAVS1 control had no repressive effect in the GFP positive cells amount when compared with the WT clone.

KRAB protein impact in the maintenance of HIV-1 latency

To understand whether the suppression of GFP expression in J.Lat 10.6 cells would be only by the presence of the CRISPR/KRAB construct or it would be possible that only the sgRNA and dCas9 expression could impact the HIV reactivation. Thus, we removed KRAB repressor sequence of the LTR2, LTR3, LTR4, LTR5 vectors, generating sgRNA knockout KRAB (LTR2-LTR5koKRAB) and treated them with PMA or IngB. For LTR2koKRAB, LTR3koKRAB we did not observe a significant reduction of GFP positive cells when comparing with WT and sgRNA negative control. In turn, both LTR4koKRAB and LTR5koKRAB have shown significant GFP reduction, 44.04% (PMA) / 53.6% (IngB) and 33.43% (PMA) / 31.95% (IngB), when treated with PMA or IngB, respectively (Fig. 2).

CRISPR sgRNA LTR5 repressor prevents HIV-1 reactivation by NFκB independent manner

After confirmation of which was the best clone cell for repressing HIV-1 reactivation, we investigated whether this repression was obtained only because we administered PKC stimulation drugs. To eliminate the bias created due to use of phorbol ester drugs, LTR5 J.Lat 10.6 cells were treated with SAHA and Panobinostat (Pano), both potent HDAC inhibitors. We found that the LTR5 J.Lat 10.6 cells when treated with SAHA and Pano

express an amount of GFP positive cells 35 and 24 times less, respectively when compared with WT cells (Fig. 3). These results show that the CRISPRdCas9-KRAB system can repress the latency reactivation through a NFκB independent mechanism.

HIV-1 latency can be maintained by specific CRISPR action in a myeloid cell model

To examine whether the reactivation repression visualized in the J.Lat cell model could be replicated in a myeloid lineage, we transduced U1 cells with the LTR repressors that worked best in J.Lat10.6 (LTR1, LTR4, LTR5), stimulated or not IngB and measured HIV-1 RNA reactivation by qRT PCR (Fig. 4). In a non-reactivated state, U1 WT cells express 400000 RNA copies/mL, while LTR1 and LTR4 repressor cells express respectively 159446 and 190005 RNA copies/mL, almost two times less HIV-1 RNA molecules when compared to U1 WT cells. Additionally, when we observed the LTR5 repressor cells, in non-reactivated state, the expression of HIV-1 RNA molecules was a mean of 157036 RNA copies/mL, more than 2 times less expression compared with U1 WT cells in all three replicates ($p < 0.09$). When we reactivated the U1 cells with IngB, we observed an expression reduction about 100 times in HIV-1 RNA molecules only for the U1 LTR5 cells, 1.5×10^6 RNA molecules/mL for IngB treatments compared with U1 Wt cells, 1.5×10^8 molecules/mL IngB treated. There was no significant alteration in the RNA molecules production of U1 LTR1 and LTR4 cells in comparison of U1 Wt cells in terms of HIV-1 RNA expression.

Discussion

Here, we describe a potential use of the repressive KRAB protein in association of CRISPR/dCas9 DNA recognition system. This system is addressed to HIV-1 proviral genome, almost all in the LTR sequence, in order to maintain a latent state despite LRA induction as a strategy of deep latency induction in lymphocytes and monocytes lineages. The HIV-1 latency reactivation of reservoirs is the major issue

against a cure for HIV-1 infection. HIV-1 latency maintenance resembles endogenous retrovirus (HERVs) controlled by cells through repressive epigenetics mechanisms, such as HDACs, DNMTs and HMTs, which recruit chromatin remodelling systems inducing heterochromatin formation [30]. After such alterations, the chromatin conformation imposes a structural impermeant to the access of transcription factors in HERVs and HIV-1 promoter, which implicates in transcription disruption. The molecular interplay between HERVs and HIV-1 has been demonstrated by the reactivation of some types of HERVs by HIV-1 Tat protein [31]. In contrast, somatic cells can efficiently control viral reactivation, unlike HIV-1, which under certain T cell stimulation overcomes these repressive epigenetic mechanisms and can be successfully replicated [32,33]. J.Lat is the most used lymphocyte lineage to test LRA response since it carries a latent integrated HIV-1 with a GFP reporter instead of the Env gene. Different types of J.Lat cells show different reactivation patterns. J.Lat 10.6 is one of the most responsive clone, with 80-90% GFP+ cells after phorbol ester (PMA and IngB) stimulation on average [28,29,34] [25–27]. These LRAs act through the PKC pathway, stimulating nuclear internalization of the NFκB transcription factor and simultaneously HIV-1 LTR activation. Saayman *et al.* have shown that CRISPR-sgRNA dCas9, when associated with VP64 or with a synergistic activation mediator (SAM) is capable of stimulating HIV-1 latency reactivation in J.Lat clones cells. J.Lat 10.6 showed 40-fold activation when compared with the control sgRNA cell [35]. Nevertheless, rather the reactivation induction, the present study shows a distinct approach, to prevent HIV-1 RNA reactivation even in the presence of an optimal concentration of LRAs. One of our six designed CRISPR constructs for the HIV-1 subtype B genome could inhibit latency reactivation process 160 times. The best sgRNA interacts exactly in the LTR enhancer region, in the NFκB ligation point thus it could explain why the repression rate was so robust when J.Lat 10.6 LTR5 cells were treated with PMA and IngB, both NFκB stimulation drugs. This finding is consistent with the results obtained by Saayman *et al.*, which showed that the most responsive sgRNA was designed to the NFκB ligation LTR's sequence [35].

The bias produced for using a NFκB agonist to analyse the reactivation capability of latent-integrated HIV-1 in cells with the LTR5 construct was mitigated by using HDACi, which promotes the HIV-1 reactivation by a NFκB independent mechanism [36,37]. When we used SAHA and Panobinostat, both HDACi, in LTR5 JLat cells, we observed respectively 1% and 3% of GFP+ cells, showing a robust repression.

Despite being controversial whether myeloid cells act as latency reservoirs, some research showed that myeloid cells could promote viral latency in Gut Associated Lymphoid Tissue (GALT), lung, adipose tissue, and central nervous system (CNS) Myeloid cells as a latency sanctuary were also demonstrated in SIVmac infections *in vivo* [38]. Given the importance of myeloid cells in HIV-1 latency, our work shows that LTR5 sgRNA/dCas9 KRAB can attenuate phorbol-ester HIV-1 reactivation. Although we did not see the same range of repression as in J.Lat 10.6 cells, we realize that the same LTR5 sgRNA can function similarly in both lineages. The difference between the repressive state in J.Lat and in U1 cells could be explained by the distinct molecular mechanism of latency establishment [39,40]. In terms of U1 untreated cells we were unable to observe a statistically significant reduction ($p < 0.09$) of HIV-1 RNA molecules in U1 LTR5 clones due to one of three replicates showed an outlier result. Probably, if we have performed

more replicates, we may have observed a decrease of HIV-1 RNA molecules in U1 LTR5 clone supernatant.

The impact of KRAB protein in the HIV-1 latency establishment was demonstrated by several studies. Genome-intact provirus reservoirs of 64 HIV-1 elite controllers were often integrated in centromeric satellite DNA and in genes encoding for KRAB-ZNF proteins. Jiang and colleagues observed HIV-1 provirus from elite controllers being harboured in a repressive location and showing the robust repressive histone markers H3K9me3 and H3K4me1, which induce heterochromatin formation and impact provirus reactivation [41]. Interestingly, both repressive markers found in elite controllers are also induced by KRAB proteins that are encoded in our repressive vector. Moreover, KRAB-containing zinc finger protein ZNF304 was found as a naturally robust HIV-1 latency inducer in a genome-wide CRISPR knockout screening of HIV-1 infected Jurkat cell line [42]. Another KRAB-containing zinc finger, ZNF10, was demonstrated to repress LTR activity through interaction with NF- κ B and SP1 binding sequence [43]. According to our hypothesis, KAP1, a protein recruited by KRAB, was reported to be responsible by the impairment of HIV-1 gene transcription, especially by inhibiting Tat's P-TEFb induction in both myeloid and lymphoid cells [44,45]. In myeloid cells, KAP1, in association with CTIP2, was observed to stimulate Tat degradation by SUMOylation throughout the proteasome pathway [44,46]. Recent findings, in addition to corroborate our results of CRISPR/dCas9 fused KRAB protein, improved the knowledge of the impact of KRAB protein in retrovirus transcription such as prototype foamy virus (PFV) showing that this protein negatively regulates PFV transactivator protein (Tas) and leading to its degradation through ubiquitination [47]. Our KRAB fused CRISPR constructs, in agreement with previous studies, show a robust HIV-1 transcription impairment, especially when targeted to the NF κ B region. Furthermore, when KRAB protein was removed from the construct, the impact of HIV-1 reactivation was more prominent for all the LTR recognition CRISPR clones, although in J.Lat 10.6 LTR5 ko/KRAB clone we observed a significant decrease in HIV-1 reactivation compared with Wt clone. This effect is in accordance with previous works that demonstrated the allosteric inhibition of transcription promoted by dCas9 [48,49].

Our results are also consistent with those observed by Olson *et al.* which suggest a relevant inhibition of KRAB-fused CRISPR/dCas9 system in J.Lat 6.3 model, when reactivated with PMA plus ionomycin. Moreover, this study showed the promotion of H3K9me3 modification mediated by their constructs in HEK293T cells [50]. Our work went a step further from Olson *et al.*, showing the KRAB-fused CRISPR/dCas9 mediated repression in a robust activation responsive J.Lat model, J.Lat 10.6, and in an infective competent HIV-1 myeloid latency model, U1.

The present study does not access all epigenetics patterns stimulated by the ligation of KRAB fused CRISPR/dCas9 systems on HIV-1 LTR. However, we hypothesize that KRAB-KAP1 interaction plays a critical role in the reactivation repression since removing the KRAB protein from the system does not show the same repressive state upon LRA stimulus.

Further experiments such as a chromatin immunoprecipitation assay associated with a next generation sequencing could be done to access the LTR5 sgRNA ligation specificity and off-target possibility and

whether this repressor could impact any cellular gene expression. An *ex vivo* approach using CD4⁺T cells and macrophage cells from HIV-1 infected patients could be done to test with the repression we observed in cell culture could be replicated *in vivo*.

Conclusion

In summary, we demonstrate the robust impact of our KRAB-fused CRISPRdCas9 system in HIV-1 latency maintenance in both lymphoid and myeloid HIV-1 latency models. Additionally, we demonstrate that this system can be resistant to both types of HIV-1 LRA such as PKC agonists and HDAC inhibitors. Altogether, our results contribute to further studies investigating the development of a useful block and lock strategy and to make a step forward on the direction of a cure for HIV-1 infection.

List Of Abbreviations

KRAB – Krüppel associated box.

KAP1 – KRAB associated protein 1.

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats.

PMA - Phorbol 12-Myristate 13-Acetate.

HDAC – Histone deacetylase

LRA – Latency Reactivation Agents.

DNMT – DNA methyl-transferase.

HMT – Histone methyl-transferase.

LTR – Long Terminal Repeat

h – Hours

min – Minutes

s - seconds

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests

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Authors' information

Affiliations

Department of Genetics, Biology Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.

Dr. Amilcar Tanuri is a professor, Lendel Correia da Costa is a technician and Phd student, Larissa Maciel Bomfim and Camila de Almeida Velozo are Phd students, Uilla Victoria Torres Ditz is undergraduate student & Rodrigo Delvecchio da Cunha is a assistant researcher.

Authors' contributions

LCC, RDC and AT conceived this work. LCC, RDC, UVTD, CAV performed experiments. LCC and LBM analysed experiments data and performed the figures. LCC prepare the original manuscript. All authors revised and edited the paper. All authors read and approved the final manuscript.

Web Links:

CRISPR Pick web site: <https://portals.broadinstitute.org/gppx/crispick/public>

Addgene CRISPR vectors: <http://www.addgene.org/crispr/>

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Table

Table 1: Constructs sequences and ligation sites.

Vector name	sgRNA sequence	HIV-1 provirus ligation site*	HIV-1 genome position
pLTR1	5'CCACGTGATGAAATGCTAGG3'	3'LTR U3	9360-9379
pLTR2	5'CCGCCTAGCATTTTCATCACG3'	3'LTR U3	9357-9376
pLTR3	5'TGCCTGGCTAGAAGCACAAAG3'	NEF	8961-8980
pLTR4	5'CTGTGGATCTACCACACACA3'	5'LTR U3	45-64
pLTR5	5'CTACAAGGGACTTTCCGCTG3'	5'LTR U3R	350-359

*sequence from subtype B group M HIV-1.

pLTR – expression vector containing sgRNA, the dCas9 and KRAB protein.

Figures

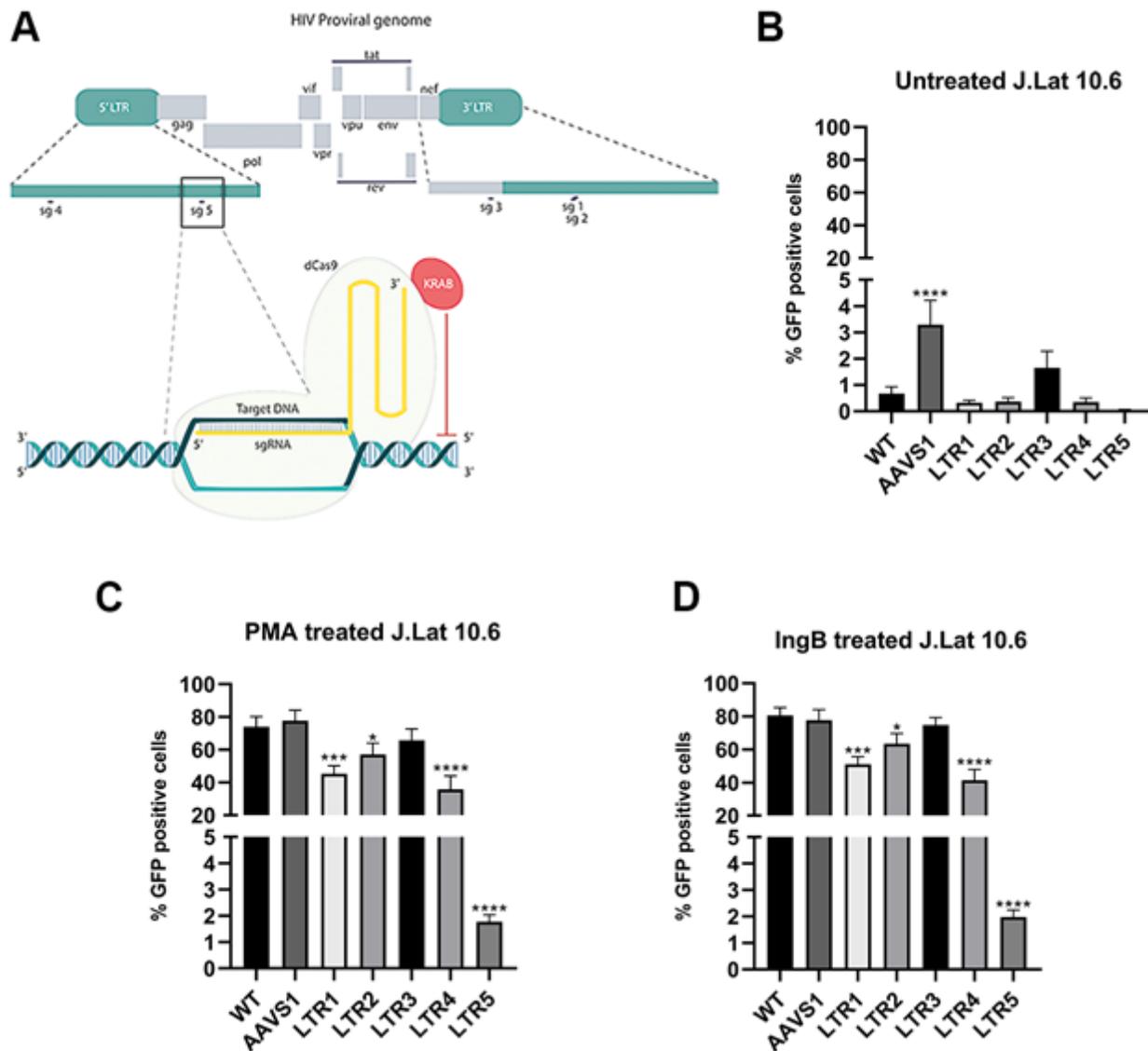


Figure 1

CRISPR sgRNAs repress GFP⁺ expression in J.Lat 10.6 cells. **A.** A schematic representation of HIV-1 provirus genome (HXB2) pointing five predicted sgRNA ligation sites. **B.** An illustrative scheme of the used CRISPR system: CRISPR sgRNA, dCas9 and KRAB protein B GFP background expression of each previous sgRNA (LTR1-LTR5) transduced untreated control J.Lat 10.6 cells. **C.** GFP expression of each previous sgRNA (LTR1-LTR5) transduced J.Lat 10.6 cells treated with 1 μ M PMA. **D.** GFP expression of each previous sgRNA (LTR1-LTR5) transduced J.Lat 10.6 cells treated with 1 μ M IngB. The statistical analysis was estimated by one-way ANOVA comparing mean of Wt or AAVS1 with LTR1 to LTR5 (* p <0.03; ** p <0.0053; *** p <0.0003; **** p <0.0001). The mean values of two duplicates of three independent experiments are shown.

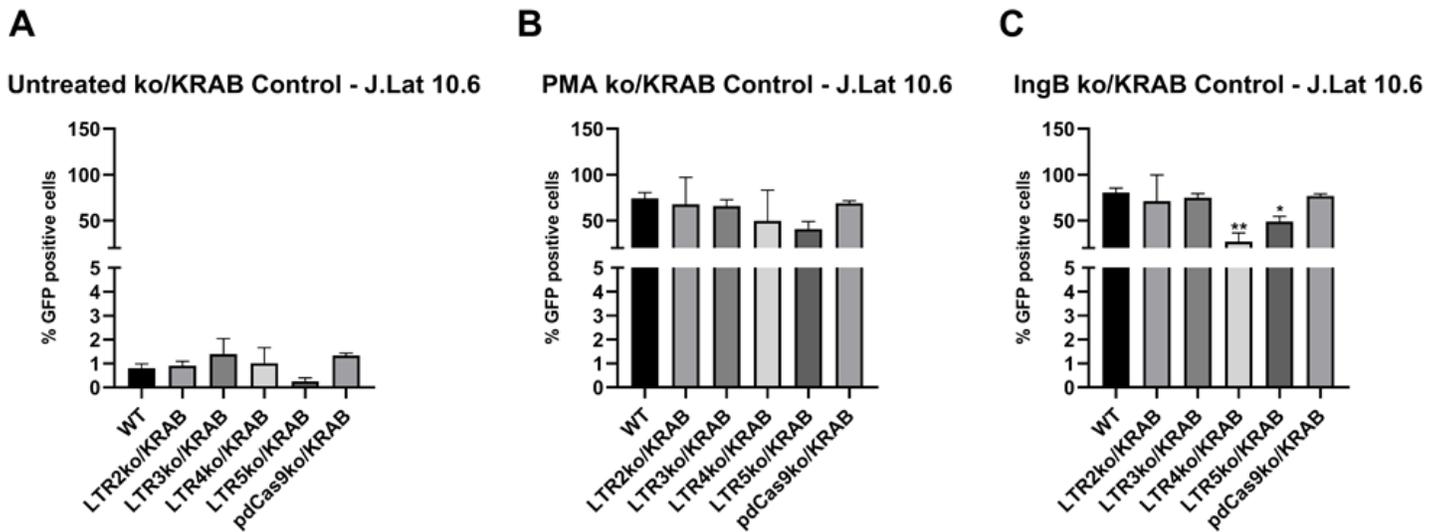


Figure 2

KRAB depletion lost CRISPR/dCas9 system repression. **A.** GFP expression of each sgRNA (LTR2-LTR5) transduced J.Lat 10.6 cells treated with 1 μ M PMA. pdCas9ko/KRAB is the sgRNA and dCas9 expression vector without the KRAB protein sequence and was used as a negative control **B.** GFP expression of each previous sgRNA (LTR2-LTR5) transduced J.Lat 10.6 cells treated with 1 μ M PMA. **C.** GFP background expression of each previous sgRNA (LTR2-LTR5) transduced treated with 1 μ M of IngB. The statistical analysis was estimated by one-way ANOVA comparing mean of Wt or AAVS1 with LTR2 to LTR5 (* $p < 0.0384$; ** $p < 0.0011$). The mean values of two duplicates of three independent experiments are shown.

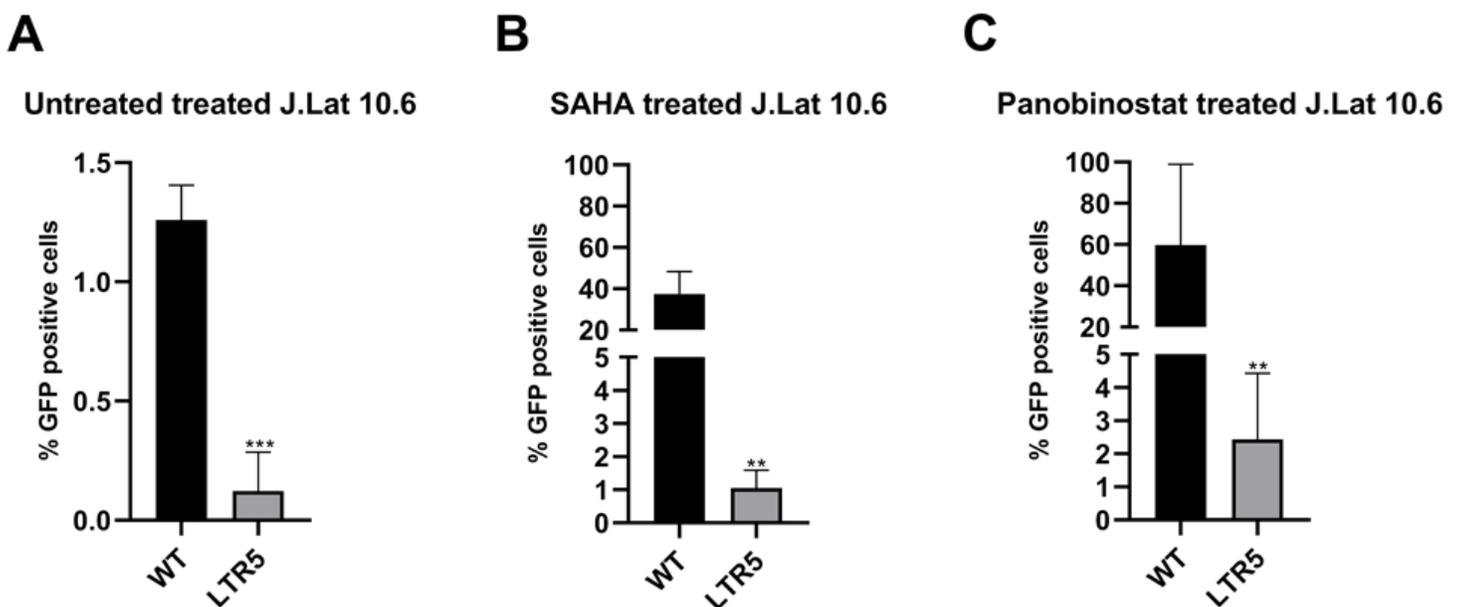


Figure 3

CRISPR LTR5 J.Lat 10.6 cells still promotes HIV-1 latency repression even stimulated with HDAC inhibitors. **A.** GFP expression of each sgRNA LTR5 transduced J.Lat 10.6 cells untreated. **B.** GFP expression of each sgRNA LTR5 transduced J.Lat 10.6 cells treated with 5 μ M SAHA. **C.** GFP expression of each sgRNA LTR5 transduced J.Lat 10.6 cells treated with 0.15 μ M Panobinostat. The statistical analysis was estimated by Student-T test comparing mean LTR5 with Wt (** $p < 0.05$; *** $p < 0.0008$). The mean values of two duplicates of three independent experiments are shown.

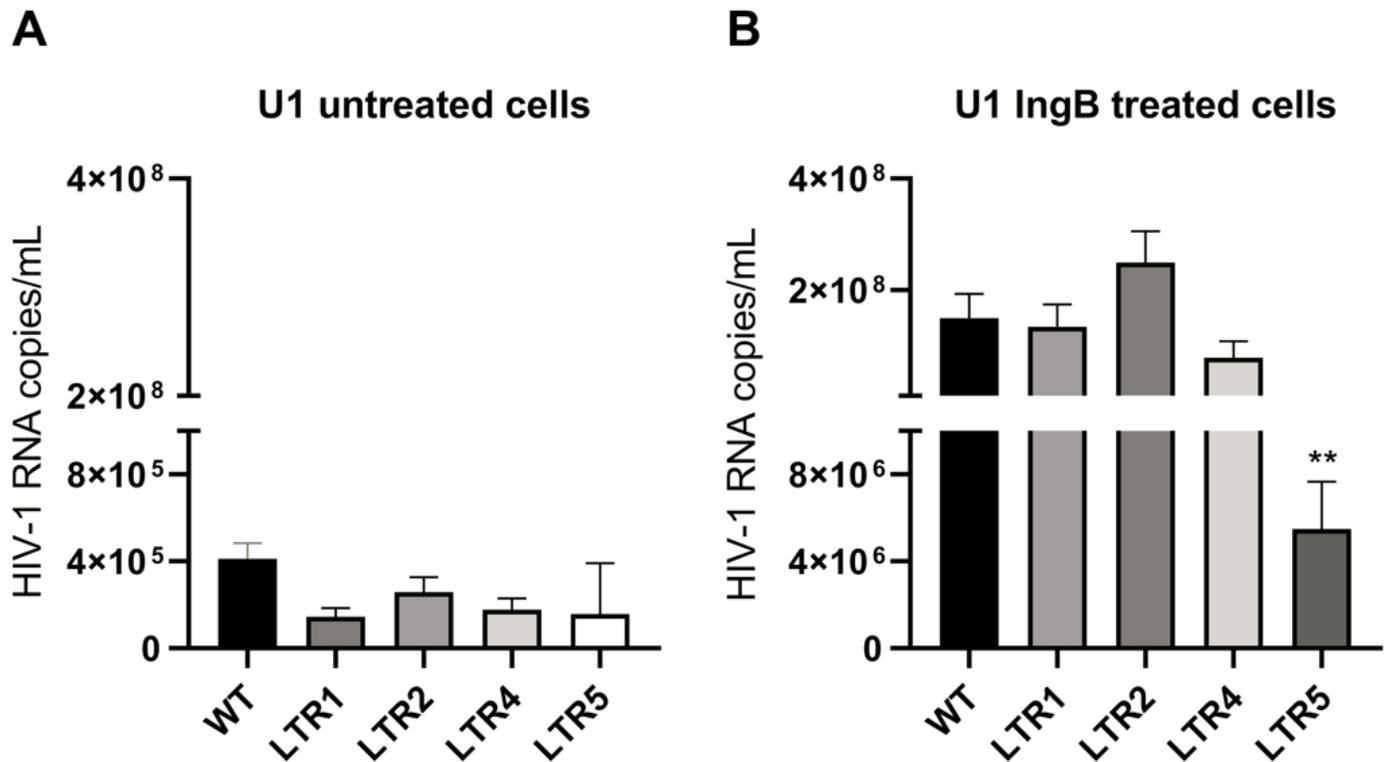


Figure 4

CRISPR sgRNAs repress HIV-1 RNA molecule expression in U1 cells. **A** Basal expression of HIV-1 RNA molecules in untreated U1 (Wt, LTR1, LTR4, LTR5) cells. **B.** HIV-1 RNA molecules of U1 (Wt, LTR1, LTR4, LTR5) cells reactivated with 1 μ M of IngB. The statistical analysis was performed by one-way ANOVA comparing mean of Wt with LTR1 to LTR5 (* $p < 0.05$; ** $p < 0.003$ ***, $p < 0.0003$). The mean values of two duplicates of three independent experiments are shown.

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