The HIV-1 provirus excised by a single CRISPR/Cas9 RNA guide persists in the host cell and may be reactivated

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24 **Abstract**

25 Gene editing may be used to cut out the human immunodeficiency virus type-1 (HIV-1) provirus
 26 from the host cell genome and eradicate infection. Here, using cells acutely or latently infected by
 27 HIV and treated with long terminal repeat-targeting CRISPR/Cas9, we show that the excised HIV
 28 provirus persists for a few weeks and, by means of HIV Integrase, rearranges in circular molecules.
 29 Circularization and integration restore proviral transcriptional activity that is enhanced in the
 30 presence of exogenous Tat and Rev or tumor necrosis factor- α , respectively, in acutely or latently
 31 infected cells. Although confirming that gene editing is a powerful tool to eradicate HIV infection,
 32 this work highlights that, to achieve this goal, the provirus has to be cleaved in several pieces and
 33 the infected cells treated with antiviral therapy before and after editing.

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36 **Keywords:** CRISPR/Cas9, Gene therapy, Endonucleases, Gene editing, HIV, Latent reservoir,
 37 Integrase, Tat, Rev, J-Lat

38

Introduction

The highly active anti-retroviral therapy (HAART) efficiently abates human immunodeficiency virus type-1 (HIV-1) replication and has transformed a deadly infection into a chronic illness. Unfortunately, HAART does not provide a cure. By stalling viral replication, HAART halts HIV spread to other cells but allows HIV to persist and reactivate at any time. Clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9), a technique that is changing paradigms and expectancies to cure genetic maladies¹, holds promises to provide a cure for HIV too². CRISPR/Cas9 cuts out the integrated HIV-1 genome (provirus) from the host cell genome³ and has proved effective to eliminate, within certain limits, infection *in vitro* and *in vivo*⁴.

The HIV long terminal repeats (LTRs) are highly conserved among HIV strains and comprise sequence domains recognized by cellular and viral proteins driving viral replication. Initial studies to eliminate HIV infection were performed using a single CRISPR/Cas9 RNA guide recognizing a sequence domain present in both LTRs of nearly all strains³. This approach, while effective at curing some infected cells, has been recently demonstrated to facilitate virus escape^{5,6,7} through the ensuing non-homologous end joining (NHEJ) repair mechanism⁸. It has also been shown that CRISPR/Cas9 alone is not always sufficient to eliminate HIV infection⁴. At the moment, however, CRISPR/Cas9 is the most effective gene editing method to cure HIV-infected cells, it has proved safe for human cells and animal models and has, therefore, good prerequisites for clinical use^{9,10,11}.

The rationale of the present study is based on previous observations showing that most linear or circularized reverse transcribed HIV RNA genome (cDNA) produced during viral replication does not integrate^{12,13}. Such unintegrated viral molecules are thought to either be destroyed, or aid productive infection through expression of several genes¹⁴, or have a second chance of integrating through complementation or rearrangement events¹⁵. The aim of this study is to investigate the fate of the provirus once excised from the host cell genome. To this purpose, and taking into account current difficulties to deliver multiple RNA guides per cell *in vivo*¹⁶, we used a single CRISPR/Cas9 RNA guide targeting both LTRs. Experiments were conducted in human embryonic kidney 293

65 (293T) cells bearing integrated, labeled HIV molecular clones and then extended to human T-
66 lymphoid cells actively replicating HIV-1, as occurs during acute infection, or latently infected J-lat
67 cells, as observed in the asymptomatic phase. The results show that the excised provirus persists in
68 the nucleus for a prolonged period of time; depending on the number of copies per cell, it closes as
69 single or rearranged inter-molecular circular elements that can form complete LTRs again with the
70 aid of HIV Integrase (IN), thereby reducing the efficiency of HIV eradication by gene editing. We
71 also show that pretreatment with Raltegravir (RAL) and Efavirenz (EFV) prevents such events.
72 Circular forms generated by inter-molecular joining exhibit functional LTRs that drive viral
73 transcriptional activity and respond to exogenous Tat and Rev, as occurs naturally during
74 superinfection.

75

Results

CRISPR/Cas9 treatment efficiently excises the HIV-1 provirus and triggers non-homologous

end-joining mechanisms to repair cellular DNA breaks. Human 293T cells were transduced with NL4-3/Luc or NL4-3/GFP, two HIV-1 NL4-3 reporter lentiviral particles obtained by pseudotyping with glycoprotein of the vesicular stomatitis virus (VSV-G). NL4-3/Luc is derived from pNL4-3 Luc.R-E- and encodes luciferase (Luc)¹⁷. NL4-3/GFP is derived from pNL4-3 ΔEnv EGFP and expresses the green fluorescent protein (GFP) as Env-GFP fusion protein that is retained in the endoplasmic reticulum¹⁷. The NL4-3-based constructs express their genes and GFP or Luc reporter genes under the control of 5'-LTR, but they are defective of *env* and, therefore, do not produce infectious particles unless *env* is provided *in trans*. The transduced cells were maintained in culture for two weeks to obtain stably integrated lines. Cells were then transfected with a plasmid expressing CRISPR/Cas9 and Puromycin resistance gene, and HIV-1 specific (T5) or scramble (SC) guide RNAs (gRNAs). The T5 gRNA targeted a highly conserved region between TATA box and NF-κB binding site in LTRs (Supplementary Information). CRISPR/Cas9-transfected cells were selected using high dose Puromycin, which eliminated non-transfectants within three days of treatment (data not shown) and were monitored for reduction of Luc activity. As shown in Fig. 1a, in cells treated with CRISPR/Cas9 and T5 gRNA, Luc activity dropped abruptly to less than 25% between day 2 and 3 after transfection and declined to nearly undetectable levels thereafter.

To rule out that Luc reduction was caused by cell death or arrest of cell growth as a consequence of transfection, we performed a cell viability assay (WST-8) 48 h post-transfection. As shown in Fig. 1b, no differences were observed amongst cell populations thus demonstrating that transfection did not damage cells and Luc reduction was indeed due to HIV editing.

To better understand how CRISPR/Cas9 editing affected HIV transcription, we transduced 293T cells with NL4-3/GFP and, 2 days later, we either left them as such (NL4-3/GFP) or transfected them with SC- or T5 gRNA-containing CRISPR/Cas9 and red fluorescent protein (mCherry)

102 plasmid, to follow edited cells (Fig.1c). Consistently with the Luc data, CRISPR/Cas9 treatment
 103 caused sharp reduction in GFP expression, so that the number of mCherry+/GFP+ cells dropped to
 104 about 10% four days after transfection (Fig. 1d and 1e). In addition, GFP was nearly undetectable
 105 by Western blot analysis at day 7 (Fig. 1f). No changes were observed after treatment with
 106 CRISPR/Cas9 combined with SC gRNA (Fig. 1a, b, d-f). The criteria used for target selection, T5
 107 and SC gRNA sequences, and CRISPR reaction conditions are provided in Supplementary
 108 Information.

109 To investigate if excision events had occurred and how cellular DNA repair had resolved the
 110 editing, we obtained individual clones of CRISPR/Cas9-transfected NL4-3/Luc cells, by limiting
 111 dilution five days post-CRISPR/Cas9 treatment. Amplification was performed using a semi-nested
 112 PCR with primers annealing upstream and downstream the T5 target site (Fig. 1g) and expecting an
 113 amplicon of roughly 536 base pairs (bp) as a result of mere joining of edited genomic DNA ends
 114 with no rearrangements in-between (Fig. 1g). As expected, PCR yielded amplicons of about 500 bp
 115 that were cloned and sequenced at random. As shown in Fig. 1h, most junctions were achieved by
 116 NHEJ and presented with deletions of DNA fragments of variable lengths.

117

118 **The excised HIV-1 provirus persists and circularizes in NL4-3/Luc-transduced**
 119 **CRISPR/Cas9-transfected 293T cells.** To investigate the fate of HIV-1 provirus after excision and
 120 determine whether the proviral fragments circularize, genomic DNA was extracted from NL4-
 121 3/Luc-transduced 293T cells at various days post-CRISPR/Cas9 and T5 or SC gRNA transfection
 122 and digested with an ATP-dependent DNA exonuclease to eliminate linear DNA fragments. The
 123 digested DNA was first checked for residual linear DNA by *β-globin* amplification (Fig. 2a) and
 124 then amplified for *gag* and *pol* (primer sequences available in Supplementary Information). As
 125 determined by agarose gel electrophoresis, HIV sequences were found in the exonuclease-digested
 126 DNA samples up to 14 days post-CRISPR/Cas9 T5 gRNA treatment (Fig. 2b). In contrast, no *gag*
 127 or *pol* sequences were found in the DNA samples treated with SC gRNA and digested with the

128 exonuclease (data not shown). Since PCR concatemer analysis was performed in a population of
129 dividing cells and the concatemers were likely to be less and less during subsequent cell mitosis, it
130 is not known whether the PCR signal disappeared because DNA molecules were eventually
131 degraded or progressively diluted out. Anyhow, these results suggest that, upon excision, the
132 provirus persists for at least a couple of weeks, most likely in circular or similar DNA exonuclease-
133 resistant forms.

134 To explore such a conclusion, we repeated the above experiment with NL4-3/Luc-transduced 293T
135 cells that were transfected with CRISPR/Cas9+T5 and then examined for the presence of circular
136 DNA molecules. To this aim, five replicas of such cells were transduced with NL4-3/Luc,
137 propagated for two weeks and treated with CRISPR/Cas9 and T5 gRNA (three replicas), SC gRNA
138 (one replica), or left untreated (one replica). Cells were propagated for 10 days in the presence of
139 Puromycin to enrich in CRISPR-transfected cells and processed to extract whole DNA. This DNA
140 was exonuclease-digested, checked for *β-globin* amplification (data not shown) and subjected to
141 rolling circle amplification (RCA) to enrich in circular DNA molecules. This technique allows
142 selective amplification of circularized DNA as concatemers, requiring digestion with a single cutter
143 to obtain full length fragments. Fig. 2c shows the RCA amplicons electrophoresed as such or after
144 digestion with *EcoRI*, which cuts pNL4-3/Luc once. Upon transfection with T5 gRNA, one sample
145 produced a smear and was not analyzed any further, while two samples contained RCA amplicons
146 of large size that, upon *EcoRI* digestion, yielded a discrete band (Fig. 2c). Of note, this predominant
147 band had a size compatible with full-length HIV-Luc genome. Conversely, normal 293T cells or
148 cells transduced with NL4-3/Luc and transfected or not with CRISPR/Cas9 + SC gRNA had
149 negligible amounts of circular DNA molecules (Fig. 2c). In all, this experiment indicates that the
150 amount of circular HIV molecules is increased in T5 gRNA-transfected cells, possibly originating
151 from the excised provirus. This experiment also demonstrates that the circularized provirus
152 persisted for a prolonged period of time and, as judged by the size of *EcoRI* fragments, did not
153 undergo large deletions.

154 To evaluate whether circularization occurred intra- or inter-molecularly, *EcoRI*-digested RCA
 155 products of approximately 11 Kb were extracted from agarose gel, divided into four replicas per
 156 fragment, diluted to 1 ng and amplified in their LTR junctions. To this aim, we designed forward
 157 (Fwd) and reverse (Rev) primers annealing to *luc/nef* and *gag* regions (Fig. 2d), respectively, and
 158 generating amplicons the size of which depended on whether circularization had occurred within a
 159 single excised provirus (intra-molecular circularization) or between two or more molecules (inter-
 160 molecular circularization). In particular, as shown in Fig. 2d, a single, circularized molecule or two
 161 HIV proviral molecules bound in sense-sense orientation would yield an amplicon of 750 bp.
 162 Conversely, two molecules bound in sense-antisense orientation would yield two amplicons. The
 163 first, obtained by extending two Fwd primers, would be 300 bp, the second, obtained with two Rev
 164 primers, would be 1200 bp (Fig. 2d). Because circular DNA generated by sense-sense inter-
 165 molecular joining has the potential to recreate full-length LTRs (Fig. 2d), we used PCR conditions
 166 privileging amplification of shorter fragments. Cells transfected with SC gRNA produced a faint
 167 band of 750 bp, as expected for a circular monomer; cells treated with T5 gRNA yielded more
 168 amplicons, two of which compatible with the estimated 750 and 300 bp (Fig. 2e). The 750-bp
 169 amplicons shown in Fig. 2e were then retrieved from agarose gel and sequenced. Most of them had
 170 In/Dels. Of note, one of them had a wild-type sequence demonstrating that intra-molecular or inter-
 171 molecular sense-sense orientation could recreate wild-type LTRs (Fig. 2f).

172

173 **The excised HIV-1 provirus can generate concatemers through inter-molecular joining.** To
 174 further confirm that circularization of excised proviruses can also occur through binding of two or
 175 more molecules in sense-sense orientation, we devised the approach shown in Fig. 3a. Using the
 176 NL4-3/Luc backbone, to prepare VSV-G pseudotyped viral vectors, we constructed two different
 177 HIV-1 molecular clones named NL4-3/Luc/Ori and NL4-3/Luc/KanR (Fig. 3b). NL4-3/Luc/Ori
 178 contained the low copy bacterial origin of replication SC101, and NL4-3/Luc/KanR encoded the
 179 Kanamycin resistance gene. The genes were cloned in the same restriction site within Δenv (Fig.

3b). This would allow to identify and select inter-molecular concatemers, which may generate *in vitro* after CRISPR/Cas9 excision, because inter-molecular concatemers between the two constructs (Ori+KanR) could be used as plasmids to transform bacteria that would only grow with Ori *and* KanR on Kanamycin-containing agar plates. The respective VSV-G-pseudotyped particles were first tested for competence for transduction. Both NL4-3/Luc/Ori and NL4-3/Luc/KanR particles had slightly reduced transduction capacity compared to NL4-3/Luc, but it was deemed sufficient to perform the subsequent experiments (Fig. 3c). As shown in Fig. 3a, 293T cells were co-transduced with NL4-3/Luc/Ori and NL4-3/Luc/KanR, then cultivated for two weeks to obtain stable transduction. Cells were cloned by limiting dilution and probed to select for double- positive clones. Screening was performed with an upstream primer annealing to pNL4-3 and downstream primers annealing to Ori or KanR. Ori and KanR primers were also designed to yield amplicons of about 220 and 280 bp, respectively, to discriminate both clones by agarose gel electrophoresis (Fig. 3d). Double-positive cell clones were then pooled, treated with CRISPR/Cas9 and T5 gRNA, or SG gRNA, to excise both NL4-3/Luc/Ori and NL4-3/Luc/KanR, and cultivated for two weeks in the presence of Puromycin to select for CRISPR/Cas9 transfectants. Whole cellular DNA was then extracted and treated with DNA exonuclease to eliminate the linear fragments (Fig. 3a). To check if the excised NL4-3/Luc/Ori and NL4-3/Luc/KanR had formed inter-molecular concatemers, which we named NL4-3/Luc/KanR/Ori, we took advantage of *KanR*, the Kanamycin resistance gene, and SC101, the bacterial origin of replication, to select and expand inter-molecular concatemers in bacterial cells. DNA exonuclease-digested cellular DNA was thus used to transform bacterial cells which were grown in the presence of Kan (Fig. 3a). From 200 ng of cellular DNA, measured before exonuclease digestion, we obtained 30-50 bacterial colonies. Two hundred ng of cellular DNA is roughly equivalent to 28,000 cells. Considering that this amount was used to transform 10^8 bacterial cells with a transformation efficiency below 10% for plasmids sizing 20 Kb, we estimate that an inter-molecular concatemer was present in about 1 out to 1,000 cells.

Five random colonies, named *A-E*, were picked and PCR checked for sense-sense inter-molecular concatemers as by Fig. 2e, and clones a-e, clearly distinguishable in the agarose gel of Fig. 3e, were sequenced at their LTR junctions (Fig. 3f). Sequence analysis confirmed that *a-d* clones were derived from excised NL4-3/Luc/Ori and NL4-3/Luc/KanR which were bound in sense-sense orientation as shown in Fig. 3g. Clones a and b contained large deletions, and clones c and d had a single nucleotide deletion and a single mutation compared to parental NL4-3 sequence, respectively. Parallel analyses performed with CRISPR/Cas9 and SC gRNA did not yield bacterial colonies (data not shown).

In all, these results demonstrate that NL4-3/Luc/KanR/Ori form spontaneously upon excision of NL4-3/Luc/Ori and NL4-3/Luc/KanR proviruses and that inter-molecular, sense-sense joining rebuilds full-length LTRs.

HIV-1 concatemers show perceptible transcriptional activity but do not lead to infectious particles. To understand whether full-length LTRs in concatemers possess functional activity, 293T cells were transfected in parallel with NL4-3/Luc/KanR/Ori concatemers, pNL4-3/Luc, and pNL4-3/Luc/KanR. Three days after transfection, cells were divided in two, and one half was treated with CRISPR/Cas9 + T5 gRNA. Treated and untreated cells were cultured for five days and then analyzed for HIV *gag* mRNA expression, and for HIV proteins. Measurement of intracellular HIV *gag* mRNA was performed by real-time reverse transcriptase (RT)-PCR in three independent experiments. Compared to pNL4-3/Luc and pNL4-3/Luc/KanR, mRNA levels of NL4-3/Luc/KanR/Ori were much lower but detectable (Fig. 4a). As regards protein expression, pNL4-3/Luc/KanR and pNL4-3/Luc produced p24 levels that exceeded the highest limit of quantification of the ELISA used. These samples were therefore diluted 1:50 and retested. In all three experiments, NL4-3/Luc/KanR/Ori yielded results above the cut-off and comparable to pNL4-3/Luc/KanR and pNL4-3/Luc samples diluted 1:50. By contrast, pNL4-3/Luc/KanR cells treated with CRISPR/Cas9 + T5 gRNA produced p24 at levels below cut-off, further demonstrating deep

HIV-1 impairment after DNA cleavage (Fig. 4b). Western blot analysis of cell lysates, performed with anti-HIV gag and IN, showed weak production of Gag and IN, confirming minimal transcriptional activity of NL4-3/Luc/KanR/Ori (Fig. 4c). Anti-Tat antibodies demonstrated Tat expression by pNL4-3/Luc and after giving Tat in *trans*, whereas no Tat can be detected after HIV concatemer transfection.

To investigate whether expression of unspliced HIV-1 mRNA was accompanied and possibly driven by expression of multiply spliced HIV-1 mRNA (msRNA), we probed the cell lysates for *tat/rev* msRNA, a marker that has been shown to reflect the ability of a cell to produce virus^{18,19}. To this aim, we used the *tat/rev* induced limiting dilution assay (TILDA), a method that discerns between latently and productively infected CD4⁺ T-lymphocytes²⁰. As shown in Fig. 4d, and in contrast to pNL4-3/Luc, NL4-3/Luc/KanR/Ori did not express *tat/rev* msRNA. This result and the low p24 intracellular content suggested negligible viral replication. To further investigate this matter, we repeated the experiment by co-transfecting cells with NL4-3/Luc/KanR/Ori or pNL4-3 and VSV-G. At day 2 post-transfection, the supernatants were collected, assayed for the presence of RT activity, p24 and infectious particles. RT activity, tested using SYBR Green PCR-enhanced RT assay (SG-PERT)²¹, was negative for HIV concatemers alone (Fig. 4e, upper panel). As discussed below, provision of Tat + Rev in *trans* restored detection of RT activity in supernatants, even if at low levels (Fig. 4e, lower panel). In contrast, p24, assayed by ELISA, yielded an absorbance value slightly above the cut-off (Fig. 4f). The presence of infectious particles was examined by using these cell-free supernatants to transduce naïve 293T cells, that were then probed for the presence of NL4-3 provirus at day 3 post-transduction. This task was performed using Xpert HIV-1 Qual, a highly sensitive qualitative real-time PCR assay approved for *in vitro* diagnostics. In contrast to supernatants from cells treated with pNL4-3/Luc, which scored clearly positive (mean threshold cycle 20.2), the three replicas of NL4-3/Luc/KanR/Ori supernatants yielded weak amplification signals and reaching the threshold cycle at the 37th cycle detection (Fig. 4g). Based on information provided by the manufacturer, experience accrued by routine use of the assay and reference

papers^{22, 23}, these values could not be scored as positive. These results confirm that inter-molecular concatemers are unable to produce infectious virions.

Cells transfected with NL4-3/Luc/KanR/Ori release HIV-1 particles following addition of exogenous HIV-1 Tat and Rev. Prompted by previous reports showing that the HIV-1 genome persists for weeks as an episome in a latent form in lymphoid and myeloid cells and can be reactivated by superinfection²⁴⁻²⁷, we tested whether this may also occur with NL4-3/Luc/KanR/Ori. Since superinfection of NL4-3/Luc/KanR/Ori transfectants with another HIV virus would yield indistinguishable virions, 293T cells were transfected with the concatemer and, three days later, transfected again with HIV *tat* or *tat + rev* plasmids to mimic superinfection. Cells were monitored for intracellular HIV RNA (Fig 4a), p24 production (Fig. 4b and f) and virion release (Fig 4e) three days after the second transfection. As shown in Fig. 4a, provision of *tat* or *tat + rev* boosted *gag* RNA expression at levels significantly higher compared to those of cells transfected with NL4-3/Luc/KanR/Ori alone. Interestingly, in all three independent experiments, *gag* mRNA values were slightly higher in the presence of *tat + rev* compared to *tat* alone, suggesting that Rev, which exports unspliced HIV mRNAs from nucleus to cytoplasm, facilitates *gag* transcription. As expected, the presence of Rev had a more relevant boosting effect at a protein level: p24 content (Fig. 4f), which increased noticeably with Tat alone, reached levels similar to NL4-3/Luc in the presence of Tat and Rev. Fig 4c also shows that p55 increased proportionally in the presence of Tat + Rev (Fig. 4c).

To ascertain whether an increment in RNA expression and protein production corresponded to release of viral particles, NL4-3/Luc/Ori /KanR and *tat*- or *tat + rev*- transfected cells were further transfected with VSV-G. Supernatants from treated cells were tested for RT activity by SG-PERT two days later. As shown in Fig. 4e, lower panel, low but detectable RT activity in the supernatants was observed suggesting that Tat + Rev, provided in *trans*, triggered release of viral particles. To evaluate whether these were also infectious, supernatants (three replicas) were used to transduce

283 293T cells and these were examined for proviral DNA by Xpert HIV-1 Qual. Samples generated
284 clearly positive amplification curves with a mean threshold cycle of 33.1 (Fig. 4g). This result
285 indicates that 293T cells were transduced and, therefore, that the viral particles released following
286 addition of Tat and Rev were infectious.

287

288 **HIV-1 IN activity facilitates persistence of the excised provirus in 293T cells.** IN is thought to
289 bind the 3' ends of the linear cDNA of HIV and mediate integration of the proviral DNA in cell
290 genome^{29,30}. Prompted by this, we hypothesized that the excised provirus mimics linear, non-
291 integrated double stranded HIV DNA and, as such, can be concatemerized, thereby reconstituting
292 two complete LTRs (Fig 2d). To address this hypothesis, we set up the experiment illustrated in Fig.
293 5a and opted to use GFP as a reporter gene. To mimic CRISPR/Cas9 cleavage, pNL-CMV-GFP,
294 differing from pNL4.3-GFP because its heterologous protein expression is driven by the CMV
295 promoter, was digested with *PmlI*, which has two blunt-ended cutting sites outside the HIV-1
296 genome. After digestion, the HIV-1 genome was extracted from agarose gel. As shown in Fig. 5a,
297 the linearized HIV-1 genome was transfected alone or together with pCMV-IN, a plasmid encoding
298 HIV-1 IN under the control of CMV promoter. Non-transfected cells and cells transfected with
299 uncut pNL-CMV-GFP served as negative and positive controls, respectively. Three days post-
300 transfection, cells were examined for GFP expression by flow cytometry and fluorescence
301 microscopy. As shown in Fig. 5c and 5d, non-transfected 293T cells showed no fluorescence,
302 whereas nearly 50% of the cells transfected with uncut pNL-CMV-GFP were fluorescent. As
303 regards cells transfected with the linearized HIV genome, provision of HIV-1 IN *in trans* increased
304 the percentage of fluorescent cells that, in three independent experiments, rose from about 3% to
305 over 20% with an increment that was statistically significant (Fig. 5c and 5d). These experiments
306 suggest that partial rescue of transcriptional capacity is facilitated by HIV-1 IN.

307 To understand if HIV-1 IN contributes to restoration of transcriptional activity by a novel HIV
308 genome arising from the transcription of the excised and rearranged provirus, we took advantage of

309 Alu repeated sequences interspersed in human genome to set up an HIV-1 Alu-PCR assay (Fig. 5e).
 310 Briefly, this assay employed a primer annealing to Alu and another one annealing to LTR
 311 sequences, and thus selectively amplified the HIV-1 genome integrated in Alu. The second primer
 312 contained the λ t sequence tag, so that this PCR could be followed by a second one using a primer
 313 annealing to the sequence tag and another to LTR and a probe to quantitate the amplicons. HIV-1
 314 Alu-PCR assay was run with DNA extracted from pNL-CMV-GFP -transfected and untransfected
 315 293T cells (positive and negative controls, respectively), and 293T cells transfected with linearized
 316 pNL-CMV-GFP and further transfected or not with pCMV-IN. As shown by Fig. 5f, treatment with
 317 pCMV-IN increased the number of integrated pNL-CMV-GFP by about 7-fold compared to
 318 untreated cells. The increment was statistically significant. This experiment was performed in the
 319 presence of high amounts of linearized HIV-1 genome and HIV-1 IN, which could have facilitated
 320 interaction between molecules. Nonetheless, this experiment suggests that HIV-1 IN plays a role in
 321 novel integration events after gene-editing.

322

323 **The CRISPR/Cas9 excised HIV provirus persists and circularizes also in T-lymphocytes.** With
 324 the aim to monitor the fate of the excised provirus in an *in vitro* model that closely mimics natural
 325 HIV infection, we repeated some experiments using Jurkat cells, a human T-lymphoid cell line,
 326 either actively producing or latently infected by HIV-1. The former was obtained by infecting cells
 327 with HIV NL4-3 strain. The latently infected cells were J-Lat cells, clone 9.2, harboring HIV-R7/E-
 328 /GFP, a full-length integrated HIV-1 genome expressing GFP. In such cells, HIV-R7/E-/GFP is
 329 present as a single copy per cell and persists in a latent phase from which it can be reawakened by
 330 treating cells with phorbol esters, tumor necrosis factor- α (TNF- α), or exogenous Tat³³. For these
 331 features, J-Lat are widely used to study HIV latency and reactivation³⁴.

332 Due to low sensitivity of T-cells to lipofection^{35,36}, J-Lat cells were CRISPR/Cas9 transfected using
 333 the ribonucleoprotein (RNP) nucleoporation system. For this purpose, we redesigned gRNAs to
 334 optimize efficiency of transfection. The first guide, named g1, targets the U5 region of LTR and the

second, g2, binds the same R site targeted by T5 gRNA. As a negative control, we also designed a non-related gRNA (SC). To monitor formation of LTR circular molecules, we used droplet digital PCR (ddPCR) as described³⁷. Primers and probe were designed in such a way to detect *nef*-LTR junctions, i.e. LTR circle molecules (depicted as green dots in Fig. 6a), as opposed to linear excised molecules that were not amplified (grey dots). Results were obtained from cellular DNA extracted 24 hours post-CRISPR/Cas9 treatment and normalized using primers and probe targeting housekeeping EIF2C1 gene (Fig. 6a).

Because HIV excision by CRISPR/Cas9 was conceived to cure latently infected cells and most studies were directed to target these cells^{3, 4, 7, 16}, we first investigated the fate of the excised provirus in J-Lat cells. As shown in the timeline of Fig. 6b, J-Lat cells were first treated with TNF- α and, after 1h, supplemented with 10 μ M RAL, an HIV IN inhibitor. An additional hour later, we performed RNP transfection and total DNA was extracted 24 h after TNF- α addition. Fig. 6c shows the results of a typical ddPCR experiment: basal numbers of LTR circle molecules in J-Lat and J-Lat + TNF- α were below 100 copies. Similar numbers were found after treatment with CRISPR/Cas9 SC gRNA and TNF- α . In contrast, the number of HIV circular molecules increased 10-fold following TNF- α addition and CRISPR/Cas9 cleavage with g1 and g2 alone or in combination (Fig. 6c and d). These results show that not all gRNAs are equal. For reasons that were not addressed, the number of HIV circular molecules were consistently higher using g2, which targets the same LTR site as T5 (Fig. 6c). Interestingly, formation of LTR circle molecules upon CRISPR/Cas9 treatment occurred in the absence of TNF- α as well, although at a much lower concentration (Fig. 6e and f). These experiments indicate that circularization of the excised provirus also occurs in cells harboring one to very few numbers of integrated HIV genomes and circularization is enhanced by stimulating cells with TNF- α . The latter evidence implies other mechanisms, in addition to induction of HIV-1 transcription, that cause circularization of the

excised genomes³² and take place in the absence of IN. These might be cellular proteins involved in NHEJ pathway.

361

HIV IN helps reactivation of the HIV provirus in J-Lat cells. The observation that addition of TNF- α and editing promotes LTR circle molecule formation, together with our own findings (Fig 5) led us to hypothesize that the inhibition of IN might enhance CRISPR-Cas9 mediated eradication. To probe this idea, J-Lat cells, pretreated or not with 10 μ M RAL for 24h, were then transfected with RNP with g1, g2, g1+g2 or the SC control guide labelled with Atto550. This dye allows tracking of RNP-transfected cells that appear fluorescently labeled in red. Five hours post-RNP treatment, HIV-1 transcription, and associated GFP expression, was induced by TNF- α , while keeping cells under RAL treatment. HIV reactivation was quantified in edited cells by enumerating GFP⁺Atto550⁺ cells by flow cytometry, performed 24 hours post-RNP treatment (Fig. 7a). Non-transfected, non-activated J-Lat cells had no red fluorescence and traces of GFP fluorescence (<1%, Fig. 7b, grey overlay histogram in every panel). TNF- α induction caused GFP⁺ cells to increase to 61.2% (Fig. 7b, dot plot, top panel). SC gRNA treatment did not affect HIV activation, as cells were nearly 70% GFP⁺ (Fig. 7c, grey bar). Treatment with g1 or g2 reduced HIV activation by TNF- α to 40% (Fig. 7c, red bar) and 56% (Fig. 7c, blue bar) GFP⁺ cells, respectively. Again, we show that multiple targeting of LTRs has a stronger effect in reducing HIV activation compared to single targeting, since g1 + g2 proved to reduce GFP⁺ cells to 30% (Fig. 7c, green bar). A substantial percentage of cells were still GFP⁺ after RNP transfection. As shown in Fig. 7b and 7c, pretreatment with RAL reduced GFP expression over 10 times compared to RAL-untreated counterparts. Indeed, compared to 60% GFP⁺ cells observed in cell populations treated with SC gRNA and RAL, after treatment with g2, g1 and g1+g2, reduction was 91.7, 71.5 and 96%, respectively. These findings confirm the previous results on 293T cells, by demonstrating that IN plays a crucial role in relapse of viral transcription of gene editing in lymphocytes as well.

384

385 **IN and reverse transcriptase (RT) inhibition are essential for a more complete clearance of**

386 **HIV-1 provirus.** To test whether novel viral DNA could integrate in cellular genome in J-Lat cells

387 after editing, we extracted J-Lat genomic DNA 24 h post-RNP treatment and probed it by Alu-PCR

388 as described in Fig. 5e. Alu-LTR amplification was chosen so as to not amplify the single provirus

389 integrated in J-Lat 9.2 cells, which has been mapped far from Alu sequences, i.e. within PP5 gene,

390 chromosome 19, by two independent studies^{39, 40}. Alu-PCR, therefore, did not amplify the original

391 provirus but rather those that integrated back close to an Alu region. As shown in Fig. 8a, no

392 integration in Alu was detected in latent J-Lat or J-Lat treated with any gRNA. Conversely, HIV

393 activation by TNF- α increased the number of Alu-LTR elements, while integration was greatly

394 reduced by RAL. This preliminary experiment shows that J-Lat cells are a suitable model to

395 investigate integration by Alu-PCR.

396 We therefore proceeded with the strategy exposed in Fig. 8b: briefly, cells activated for 24 h with

397 TNF- α were treated with RAL or EFV, an HIV-1 RT inhibitor, for 24 h. RNP transfection with g2

398 was then carried out and, an additional 24h later, genomic DNA was extracted. Alu-LTR circles

399 were determined after normalization with β -globin DNA content. Fig. 8c shows that: i) RAL

400 inhibits integration in Alu more than EFV; ii) Combined treatment of RAL+EFV is more efficient

401 than the single drugs alone in preventing integration; iii) most importantly, transfection with g2

402 alone dramatically reduces Alu integration in the presence of RAL/EFV. More detailed analysis of

403 the differences among the g2-RNP transfected groups revealed that there is highly significant

404 difference between the integration events occurring with or without associated drug administration,

405 where RAL+EFV almost abolished reintegration in Alu.

406 These data show that RT and IN play important roles in the number of HIV-1 proviral genomes

407 integrated in Alu. Using both RAL and EFV after RNP transfection improved efficacy of HIV

408 clearance.

409

410 **The CRISPR/Cas9 excised HIV-1 provirus circularizes and accumulates also in acutely wt**
 411 **HIV-1-infected lymphocytes.** To assess whether cells acutely infected by wt HIV-1 accumulated
 412 circular HIV-1 genomes after editing, we repeated the study in Jurkat cells infected with a clinical
 413 isolate of HIV-1 at 0,05 MOI (Fig. 9a). Infected cells were treated with RAL and/or EFV for 7 days,
 414 then, CRISPR/Cas9 transfected using the same conditions described for J-Lat experiments. Two
 415 days after RNP transfection, we harvested culture supernatants and extracted total cellular DNA.
 416 Supernatants were examined for HIV genome content to determine reduction in viral replication
 417 following CRISPR/Cas9 treatment alone or supported by antiviral drugs. To evaluate whether
 418 CRISPR/Cas9 treatment increased the number of HIV-1 LTR circles in a model closely resembling
 419 natural HIV infection, cellular DNA was analyzed by ddPCR. As shown by Figures 9b and 9c,
 420 CRISPR/Cas9 transfection doubled the number of LTR circles in the absence of drugs (from 27.4 to
 421 52.5 copies/ μ l). Similarly, the number of LTR circles was significantly higher upon CRISPR/Cas9
 422 treatment in the presence of EFV, but not for RAL that was confirmed to decrease the number of
 423 circles. Interestingly, such increments, as well as absolute numbers of circle molecules, were much
 424 lower in the presence of both RAL + EFV, suggesting a synergistic effect (Fig. 9c and 9d). These
 425 findings recapitulate perfectly what observed with latently infected cells. Another striking similarity
 426 between acutely and latently infected cells is that RAL decreased the amount of LTR circles after
 427 HIV-1 ablation more than EFV, the RT inhibitor.

428 Finally, we measured the change in viral load in time in supernatants from cells treated with
 429 antiviral drugs and CRISPR/Cas9. As shown in Fig. 9d and 9e, the combination of antiviral drugs
 430 and CRISPR/Cas9 transfection performed at d9 after infection reduced HIV viral load by 3 logs.
 431 This finding confirms the antiviral potential of CRISPR/Cas9 when associated with pre- and post-
 432 treatment with antiretroviral drugs.

433

Discussion

CRISPR technology is becoming the leading gene editing tool, with increasingly expanding fields of application^{11, 41}. These include HIV therapy, where CRISPR might allow excision of the integrated HIV genome, which can be excised from cellular DNA by taking advantage of CRISPR/Cas9 site-specific cleavage^{42, 43}. The ends of cellular DNA are then joined by NHEJ⁸. This approach has proven to be effective and potent *in vitro*, whereas a number of limitations may be relevant when it is transposed *in vivo*. CRISPR/Cas9 may exhibit possible off-target activity and defiant gene rearrangement following DNA repair^{41, 44}; moreover, diversity and mutations of HIV genome may constrain target selection⁴⁵⁻⁴⁷. Lastly, site of integration and transcriptional activity of the provirus may impact on susceptibility to CRISPR cleavage⁴⁷⁻⁵⁰. *In vivo*, this scenario is further complicated by the lack of effective delivery vehicles¹⁶.

The main aim of this study was to monitor the fate of the HIV-1 provirus once it is excised, and the cellular repair mechanisms are triggered to heal the scars generated by CRISPR/Cas9. To this purpose, the experimental design was structured in two main parts. First, starting from previous observations showing that the HIV-1 provirus can be excised from 293T cell genome as in lymphoid T-cells^{19, 20}, the fate of HIV-1 provirus was first studied in 293T transduced with a replication-deficient HIV-1-based vector. Second, findings were confirmed in a human T-lymphocyte cell line that contains one copy of integrated HIV-1 per cell and is a well-established model to study latency and reactivation^{34, 35, 50}. Third, further confirmation was achieved in lymphoid cells infected with a clinical isolate of HIV-1.

One of the difficulties of CRISPR strategies is the control of the type of editing in cells and of the ensuing NHEJ process. Targeting the HIV-1 LTR alone, although allowing use of a single guide to cut out the provirus, has been repeatedly shown to rapidly lead to viral escape^{5, 6, 7}. Thus, to effectively eliminate HIV infection *in vivo*, it would be advisable to convey multiple RNA guides into a single cell and ensure chopping of the provirus. Although rapid progress in CRISPR/Cas9 technology^{41, 51} and improvement of delivery strategies^{52, 53}, will provide a way to circumvent these

drawbacks, current technologies of *in vivo* delivery do not allow to control the number and amounts of RNA guides introduced into single cells. We found that, if cut with a single RNA guide, the excised HIV-1 provirus persists in cells for a protracted period. Depending on the number of copies per cell, it may persist in a linear form, with partially restored expression activity, but HIV-1 can also be found. The excised linear provirus differs from the linear HIV-1 cDNA produced by RT¹² during viral replication *in vitro* and *in vivo* because it misses a portion of LTR at both ends. However, it may be converted into concatemeric HIV-1 cDNAs that either persist as episomes in infected cells or may be transcribed into a full viral genome that might follow the classic intasome-mediated integration pathway because, when they dimerize, they reconstitute the functional LTR⁵⁴ at both ends. HIV episomes are believed to be the result of abortive integration processes^{46, 64, 65}, deletion of sequences necessary for chromosome integration, or autointegration events occurring when an HIV cDNA integrates into another cDNA molecule⁵⁶. They are found *in vitro*^{57, 58, 59} and in patients at advanced phases of the disease^{13, 60-63}. Episomal HIV-1 genomes persist in lymphoid cells and macrophages suggesting that extrachromosomal HIV-1 and quiescent T lymphocytes are major reservoirs in infected individuals^{63, 64, 67}. In keeping, episomal HIV-1, which also forms under antiretroviral regimens, has been postulated as a marker of ongoing *de novo* infection that may trigger viral rebound after treatment interruption^{25, 68}.

Recent studies showed that episomes are neither rare nor inactive results of a dead-end process. Indeed, it has been shown that, despite the notion that integration is a prerequisite for protein expression, the HIV episome can be expressed. Expression of Tat and Nef or Tat alone by unintegrated HIV-1 activates resting T-cells and maintains persistent viral transcription in macrophages^{14, 67}. Unintegrated HIV-1 is abundant in resting, non-proliferating CD4⁺ T cells and yields *de novo* virus production following cytokine exposure of resting cells⁶⁹. Moreover, infection by HIV-1 mutants deleted of IN generated episomes producing Gag and Env *in vitro*, although they were incapable to establish production of infectious HIV-1^{7, 70}. Episomal DNA was found to express early gene mRNAs at a low level and, upon superinfection, it could also express late

486 genes, indicating that it has the full potential for transcription^{67, 70, 72}. Because of its potential for
 487 transcription, the unintegrated DNA may influence viral RNA decay consequent to therapy, and
 488 even recombine with a second incoming virus, thus contributing to the generation of viral
 489 diversity^{26, 27, 68}.

490 The results of our study support the observation that CRISPR/Cas9 gene editing is an extremely
 491 powerful technique that allows HIV excision. However, our work demonstrates that CRISPR/Cas9
 492 excised provirus persists in cells and can re-enter the replication flow. In transduced 293T cells, we
 493 found that two or more linear fragments could bind together in sense-sense orientation and express
 494 unspliced mRNA at low levels. Importantly, as described elsewhere for non-integrated HIV DNA^{24,}
 495 ^{67, 70, 72}, this viral DNA responded to exogenous Tat and Rev. Tat alone remarkably increased the
 496 level of expression of unspliced mRNA but, when tested at a protein level, Rev made the real
 497 difference, as it significantly augmented the amount of p24 protein. Further, infectious VSV-G
 498 pseudotyped virions could be detected in these supernatants. This indicates that provision of
 499 exogenous Tat and Rev also promotes release of viral particles from HIV episomes. It might be
 500 argued that transfection of Tat and Rev, chosen to facilitate measurement of concatemer products,
 501 led to much higher protein levels compared to superinfection, and that this is a rare event *in vivo*.
 502 How rare it really is remains still poorly defined. Besides superinfection by a second strain, which is
 503 indeed rare, Geldelblom and colleagues showed that the same HIV strain can reinfect the cell, a
 504 process known as co-infection²⁴.

505 Interestingly, the fate of excised provirus was influenced by the number of provirus copies per cell.
 506 Indeed, RT activity increased the amount of HIV-1 provirus transcribed from RNA in activated J-
 507 Lat cells, possibly raising the chances of dimer/concatemer formation and reintegration (Fig. 8).
 508 Concatemer formation reconstituting 2 functional LTRs was detected after CRISPR in 293T cells.
 509 This might be due to the fact that multiple copies of provirus are present, a condition not so rare and
 510 that has been observed *in vivo*⁸². For example, when HIV-1 infection spreads through virological
 511 synapses, i.e. adhesive structures between infected and uninfected cells, multiple copies of HIV-1

are transmitted to the engaged uninfected cells^{81, 83-86}. This phenomenon occurs in experimental models and in humans⁸⁷⁻⁸⁹, and has been linked to reduced sensitivity to antiretrovirals^{82, 85, 90}. To understand what happens in a less artificial system, we repeated the experiments in J-Lat cells, an HIV-1 infected T-cell line harboring one HIV copy per cell, which persists in a latent state, and, finally, in Jurkat cells infected with a clinical isolate of HIV-1. As in 293T cells, excision through HIV-LTRs determined a sharp and statistically significant increment in episome numbers. In J-Lat cells we found that, in the absence of inhibition of RT and IN, the integration events are more abundant. IN seems to exert a critical role in rescuing proviral DNA, a fact that is not surprising; IN takes part in various steps of HIV replication^{29, 30}. Integration itself is a complex, multistep process catalyzed by IN that inserts the viral DNA ends into the cellular DNA strands. Integration creates a 2-nucleotide gap in the viral genome³⁰ that is then repaired by cellular DNA repair machinery after integration⁷³. Screening of knockout libraries showed that NHEJ plays a chief role in this process, and depletion of some cellular proteins involved in this pathway decreases provirus integration and viral infectivity⁷⁴⁻⁷⁷. It has also been demonstrated that some NHEJ proteins bind IN and this complex recruits the catalytic subunit of DNA-dependent protein kinase which triggers a cascade of phosphorylation and fills the nucleotide gap⁷⁸⁻⁸⁰. From our results, it can be speculated that activation of NHEJ by CRISPR/Cas9 facilitates circularization of excised proviral DNA. Although this mechanism was not thoroughly investigated, in both cellular models studied, circularization and reactivation could be enhanced or prevented by adding exogenous IN or inhibiting it, respectively. No matter what cells were used, IN played a crucial role in circularization and HIV persistence, as demonstrated by the reduction in LTR circle molecule formation and integration in cells treated with the IN inhibitor RAL. Interestingly, in a recent paper, Dash and colleagues treated HIV-1 infected humanized mice with a long-acting slow-effective release antiviral therapy for 4 weeks and three weeks later with CRISPR/Cas9 to excise the provirus. The drug regimen included an IN inhibitor. Five out of 7 mice showed rebound of viremia at levels comparable to animals that

538 received no CRISPR/Cas9 treatment. The Authors did not investigate why this occurred but
539 speculated that, in the 5 animals, the virus rearranged to reinstate competence for replication⁴. In the
540 light of our results, it would be informative to perform CRISPR/Cas9 treatment in the context of
541 antiretroviral treatment that includes RAL, to shed light on the role of IN and RT in shaping the fate
542 of the excised provirus *in vivo*. We did not observe any difference between LTR circles in J-Lat
543 cells before or after TNF- α administration, showing that activation alone cannot increase the
544 number of circles detected (Fig. 6d).

545 Interestingly, circles are indeed present also in Jurkat cells, no matter whether the virus is
546 productively replicating or dormant. These data show that circles do form without activation, even
547 if at an obviously much lower number. These data suggest that cutting DNA, thereby activating
548 NHEJ, brings to the formation of LTR circles. Activation determines amplification of this
549 phenomenon by the combined activity of RT and IN (Fig. 6 and 8).

550 In conclusion, we provide evidence that if the HIV-1 genome is excised as a single fragment, it
551 persists and reorganizes in concatemers giving the virus a second chance to express itself and
552 rebuild an infectious form. Most concatemers and episomes are likely to be lost during subsequent
553 cell mitosis and have limited persistence in dividing cells but are nonetheless a warning note. This
554 work stresses the importance of CRISPR/Cas9 strategy in the cure of HIV, and should be a stimulus
555 to 1) implement the efficacy of delivery systems and CRISPR/Cas9 strategies *in vivo* to achieve
556 cleavage of HIV genome in multiple sites and in all cells, no matter where the provirus is integrated
557 and how many copies are present within the cell; 2) prolong antiretroviral treatment to allow
558 excised provirus to be eliminated.

559

560 **Materials and Methods**

561

562 **Cell cultures and plasmids.** Human 293T cell line was purchased from American Type Culture
563 Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented
564 with 10% fetal bovine serum (FBS), 2 mM L-glutamine and antibiotics (penicillin, streptomycin) at
565 37°C and 5% CO₂. The Jurkat T-cell line was purchased from ATCC and grown in RPMI 1640
566 supplemented with 10% FBS, 2 mM L-glutamine and antibiotics at 37°C and 5% CO₂ and infected
567 with a clinical isolate of HIV-1 at a multiplicity of infection (MOI) of 0.05. The human T-lymphoid
568 J-Lat cell line was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID. J-
569 Lat cells were produced by transducing Jurkat cells with HIV-R7/E-/GFP at low multiplicity of
570 infection (MOI) in such a way to generate clones containing one copy of integrated HIV per cell.
571 Cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine and antibiotics
572 at 37°C and 5% CO₂. J-Lat HIV-R7/E-/GFP, which is full length HIV-1 genome with a non-
573 functional Env due to a frameshift, and GFP in place of the Nef gene, generates incomplete virions.
574 HIV-R7/E-/GFP is activated for transcription and expresses GFP by treating J-Lat with TPA, TNF-
575 α, or exogenous Tat³⁴. Activation was performed by supplementing J-Lat cells culture medium with
576 10 nM TNF-α.

577 pNL4-3/Luc (https://www.aidsreagent.org/pdfs/ds3418_010.pdf Cat n: 3418) and pNL4-3/GFP
578 (https://www.aidsreagent.org/11100_003.pdf, Cat n:11100) were obtained through the AIDS
579 reagent program. The first is an HIV-1 NL4-3 luciferase reporter vector that contains defective Nef,
580 Env and Vpr; it is competent for a single round of replication. It can only produce infectious virus
581 after cotransfection with env expression vector. The second is also derived from pNL4-3 but carries
582 enhanced green fluorescent protein (EGFP) in the *env* open reading frame. This vector expresses an
583 endoplasmic reticulum (ER)-retained truncated Env-EGFP fusion protein. For linearization
584 experiments (Fig. 5) we used plasmid pNL-EGFP/CMV/WPREdU3 (pNL-CMV-GFP) (Addgene,
585 MA, USA). pNL4-3/Luc/Ori and pNL4-3/Luc/Kan were produced by molecular cloning into pNL4-

3/Luc. Both SC101, the low copy bacterial origin of replication, and *KanR* were extracted from pSF_CMV-SC101 (#OG13, Oxford Genetics, Oxford, UK) following digestion with *SwaI* (SC101) and *PmeI* (*KanR*). Fragments were cloned in *BseII* site of pNL4-3 *env*.

Digestion of host and linear DNA. Host and proviral DNA were extracted from cells using a standard phenol/chloroform method. Briefly, we added one volume of phenol:chloroform:isoamyl alcohol (25:24:1) per sample and vortexed for 20 seconds, then centrifuged for 5 min at $16,000 \times g$. The aqueous phase containing total DNA was purified by ethanol precipitation. Three μg of purified DNA were treated with 2 μl of Plasmid-Safe ATP-dependent exonuclease (Epicentre, Madison, USA) at 37°C for 30 minutes and then heat-inactivated by a 30-min incubation at 70°C .

RCA. Following Plasmid-Safe ATP-dependent exonuclease treatment, circular DNA molecules were amplified with random examers and TempliPhi DNA polymerase (Merck KGaA, Darmstadt, Germany) following manufacturer's instruction. Briefly, the reaction mixture containing 10 ng DNA was incubated at 30°C for 6 h. The reaction was then blocked by heat inactivation at 65°C for 5 min.

CRISPR/Cas9 design and transfection. T5 (TTAGACCAGATCTGAGCCT), the CRISPR/Cas9 gRNA targeting the LTR R region and used in 293T cells, was cloned into pSpCas9-2A-Puro (Cat. No. 62988, Addgene, MA, USA) or in pU6-Cas9-T2A-mCherry (Cat. No. 64324, Addgene) following a standard protocol⁹². Transfection of DNA plasmids into 293T cells was performed with standard calcium phosphate method. J-Lat cells were transfected with CRISPR/Cas9 RNP and tracrRNA Atto550 (IDT, Coralville, Iowa) by electroporation (NEON Electroporation System, Thermo Fisher, Massachusetts, USA) using the following parameters: 1400 V, width 10 ms, 3 pulses.

Guides 1 and 2 (g1 and g2) were designed using IDT algorithm and as follows: g1 -/A1TR1/rUrGrArCrArUrCrGrArGrCrUrUrUrCrUrArCrArArGrUrUrUrUrArGrArGrCrUrArUrGrCrU/A1TR

612 2/; g2 –/A1TR1/
613 rArCrUrCrArArGrGrCrArArGrCrUrUrUrArUrUrGrGrUrUrUrArGrArGrCrUrArUrGrCrU/ALT
614 R2/; both gRNAs target LTR, g2 the same R site as T5, g1 targets U5.
615
616 **293T cell transduction.** Transduction of 293T cells was performed with VSV-G-pseudotyped
617 particles. These were produced by transfecting 293T cells with pNL4-3 or its derivatives described
618 above and VSV-G plasmid. Generated particles were harvested at day 2 or 3 post-transfection and
619 titrated as described elsewhere⁹³. Transduction was performed using MOI 5 to transduce 2×10^5
620 293T cells. Cloning of transduced 293T cells was performed in 96-well plates at the indicated days
621 post-transduction.
622
623 **ddPCR.** For ddPCR analysis, total DNA from 5×10^5 J-Lat cells was purified using Qiagen blood
624 mini kit (Qiagen, Hilden, Germany) and quantitated spectrophotometrically. Aliquots of 2.5 µg
625 were then *Bse*II digested (Thermo Fisher, MA, USA) to fragment the genomic DNA without
626 cutting the LTR junctions. Restriction products were column-purified using PCR Clean Up kit
627 (Qiagen) and 2.5 ng of purified DNA were amplified by ddPCR using the following primers: LTR
628 circles: Fwd -AACTAGGGAACCCACTGCTTAAG; Rev TCCACAGATCAAGGATATCTTGT;
629 Probe (FAM) – ACACTACTTGAAGCACTCAAGGC. Reaction was performed as follows: 10 min
630 at 95°C denaturation, 40 cycles of 95°C for 30 s, and 60°C for 60 s, followed by 98°C for 10 min.
631 After completion of PCR cycling, reactions were placed in a QX200 instrument (Bio-Rad, Milan,
632 Italy) and droplets analyzed according to manufacturer's instructions. Normalization was performed
633 targeting the EIF2C1 gene, using a premade probe solution (Bio-Rad) following manufactures'
634 instruction.
635
636 **Concatemer isolation, characterization and sequencing.** Selection of clones double positive for
637 pNL4-3/Luc/Ori and pNL4-3/Luc/Kan was performed by PCR amplification using the following

638 primers: Env Fwd – GACACAATCACACTCCCA; Kan Rev - AATAGCCTCTCCACCCAA; Ori
 639 Rev – TGTGGTGCTATCTGACTT. Selected clones were then transfected with T5 gRNAspCas9
 640 plasmid (Cat. No. 459 Addgene) and cultivated in the presence of Puromycin (3 µg/ml) to select for
 641 the transfected cells. Following DNA extraction and digestion with ATP-dependent exonuclease,
 642 50-200 ng of total DNA, quantitated before exonuclease digestion, were used to transform 10⁸
 643 ultracompetent Stbl2 *E. coli* cells (Invitrogen, Carlsbad, CA, USA). Transformants were seeded in
 644 Luria Bertani (LB) agar plates supplemented with Kanamycin (50 µg/ml). Bacterial colonies were
 645 picked and screened to eliminate spurious clones containing residual pNL4-3/Luc/Kan, which also
 646 harbors the Ampicillin resistance gene in the plasmid vector. To this aim, colonies were split and
 647 seeded in two LB broth cultures containing Kanamycin or Ampicillin. Clones growing only in
 648 Kanamycin medium were expanded and concatemers extracted and purified using MAXI prep
 649 (Qiagen). Recovered DNA was tested by PCR to confirm the LTR junction, using the following
 650 primers: Fwd - AACTAGGGAACCCACTGCTTAG; Rev -
 651 GACAAGATATCCTTGATCTGTGGA. Positive clones were sequenced in LTR junctions by cycle
 652 sequencing.

653

654 **Biological activity of excised proviruses and concatemers.** Analyses were focused on the
 655 detection and quantitation of HIV mRNAs and proteins and performed on cell lysates obtained by
 656 transfecting 1 × 10⁵ 293T cells with 0.5-1.0 µg of pNL4-3 derivatives. The same analyses and
 657 infectivity as the released virions were assessed with 1 × 10⁵ 293T transfected with 0.5-1.0 µg
 658 pNL4-3 derivatives and 0.1 µg VSV-G plasmid. Total cellular RNA was extracted using Maxwell
 659 16 LEV simply RNA extractor (Promega, Madison, USA). Total proteins were extracted using
 660 RIPA buffer (0.22% Beta glycerophosphate, 10% Tergitol-NP40, 0.18% Sodium orthovanadate, 5%
 661 Sodium deoxycholate, 0.38% EGTA, 1% SDS, 6.1% Tris, 0.29% EDTA, 8.8% Sodium chloride,
 662 1.12% Sodium pyrophosphate decahydrate).

663

664 **Measurement of HIV RNA and HIV DNA.** Intracellular and supernatant HIV RNA were
 665 quantitated using COBAS AmpliPrep and COBAS-6800 (Roche, Milan, Italy), respectively. Both
 666 platforms and tests are routinely used at the Virology Unit, Pisa University Hospital, are certified
 667 for *in vitro* diagnostics and detect up to 20 HIV RNA copies/ml. HIV mRNA was detected and
 668 quantitated using TILDA²⁰. Briefly, total RNA extracted as above was reverse transcribed at 50°C
 669 for 15 min, denatured at 95°C for 2 min and amplified for 24 cycles (95°C 15 seconds, 60°C, 4 min)
 670 on T100 PCR instrument (Biorad, Hercules, CA, USA). At the end of this process, samples were
 671 diluted to 50 µl with Tris-EDTA buffer and 1 µl of sample was used as template for a second *tat/rev*
 672 real-time PCR reaction. Primer sequences and details to calculate mean and standard deviation of
 673 ΔCT (threshold cycle) are provided elsewhere²⁰. Proviral DNA in 293T cells was assayed with
 674 Xpert HIV-1 Qual, manufactured by Cepheid (Milan, Italy) and certified for *in vitro* diagnostics.
 675 This assay has a sensitivity of 278 copies/ml in whole blood^{22, 23}. Before Xpert HIV-1 Qual
 676 analysis, genomic DNA extracted from 293T cells was RNase treated to eliminate contaminating
 677 cellular RNA.

678

679 **HIV-1 integration assay (Alu-PCR).** HIV-1 integration was examined in 293T cells and J-Lat cells
 680 by isolating genomic DNA from 1×10^6 cells using the DNeasy Tissue Kit (Qiagen). Alu-LTR
 681 sequences were amplified during first round PCR from 100 ng of total genomic DNA using the
 682 following primers: LM667 5'-
 683 ATGCCACGTAAGCGAACTCTGGCTAACTAGGGAACCCACTG; Alu1 5'-
 684 TCCCAGCTACTGGGGAGGCTGAGG. Amplification was performed for 16 cycles performed as
 685 follows: denaturation for 3 min at 95°C, 16 cycles of 95°C for 30 s, and 60°C for 60 s, followed by
 686 72°C for 60 s. First round PCR is followed by a second round real-time PCR using a 1:500 dilution
 687 of first PCR mixture together with the Alu-specific primer λT (5'-ATGCCACGTAAGCGAACT),
 688 U5 LTR primer LR (5'-TCCCACTGACTAAAAGGGTCTGA) and probe ZXF-P81 (5'-FAM-

689 TGTGACTCTGGTAACTAGAGATCCCTCAGACCC-TAMRA). Real-time PCR amplifications
690 were performed on a CFX96 machine (Bio-Rad). Results were normalized using the single-copy
691 Lamin B2 gene that was quantified by real-time PCR (Fwd 5'- CCCCAGGGAGTAGGTTGTGA;
692 Rev 5'- 5'-TGTTATTTGAGAAAAGCCCAAAGAC).

693

694 **Western blot and HIV protein detection.** Total proteins were extracted by direct lysis of samples
695 using RIPA buffer. Extracted proteins were titrated using the Bradford assay and then analyzed by
696 ELISA or Western blot. Capsid p24 was measured in cell extracts and supernatants using
697 SimpleStep ELISA (ABCAM, Cambridge, UK) and ADVIA Centaur HIV Ag/Ab Combo ELISA
698 (Siemens Healthcare Diagnostics, NY, USA), respectively. RT activity in supernatants was
699 determined by SG-PERT and as described by Vermeire and colleagues⁹⁴. Western blot analysis was
700 performed using 20 µg RIPA-extracted proteins and using a mixture of antibodies anti-Tat, anti-
701 Gag, and anti-IN (ab42359, ab63917 and ab66645, AbCam, Cambridge, UK) or anti-GFP
702 (ab183734, Abcam), or anti-actin (ab179467, Abcam).

703

704 **Flow cytometry.** Fluorescent cells were measured using Attune NTX (Thermoscientific, USA) or
705 FACScan (Becton-Dickinson, Florence, Italy). Cells were analyzed 2-3 days post-transfection or as
706 indicated, following detachment from well plates by Trypsin treatment and pelleting by
707 centrifugation at 300 × g for 5 min. Data were analyzed using FSC Express 4 software (DeNovo
708 Software, Glendale, CA).

709

710 **Statistical analysis.** GraphPad Prism software V5.03 (GraphPad Software, Inc., USA) was used for
711 statistical analysis. Data were analyzed using the Student's *t*-test. Differences between groups were
712 considered statistically significant at values of $p < 0.05$. All results, including flow cytometry and
713 ddPCR and Alu-PCR data, were obtained from at least three independent experiments and
714 expressed as mean ± standard error of the mean (SEM). ddPCR and Alu-PCR results were analyzed

715 using one-way ANOVA, ** $p < 0.01$, n.s. not significant. Flow cytometry assay results were
 716 analyzed using the Student's *t*-test, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.1$.

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728 **Author contributions**

729 ML, EM, OT, GA, and MP conceived and designed the experiments; ML, PQ, GM, SC, SP
730 performed and analyzed the experiments; FM, MS, GF, OT, JLH supervised analyses, and provided
731 reagents and resources, MP supervised experiments and provided funding; ML, GF and MP wrote
732 the manuscript with input from all other authors.

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735 **Conflicts of interest**

736 The authors declare that they have no conflict of interest.

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- 946

947 Legend to figures

948

949 **Fig. 1 CRISPR/Cas9 efficiently cleaves the HIV-1 provirus in 293T cells transduced with NL4-**
950 **3/GFP or NL4-3/Luc. a,** 293T cells that had been transduced with NL4-3/Luc were transfected
951 with CRISPR/Cas9 and either scrambled (SC) or HIV-1 specific (T5) gRNAs and analyzed for Luc
952 expression at day 0, 1, 2, 3, 4. Decrease in Luc expression in T5 gRNA-treated cells compared to
953 untreated or SC gRNA-treated cells reached statistical significance at day 2 ($p < 0.001$). Standard
954 deviation (SD) was calculated from three independent experiments. **b,** Cell viability was assessed
955 by WST-8 48 hours after CRISPR/Cas9 transfection and showed no significant difference between
956 cells transfected with NL4-3/Luc alone or in combination with Cas9+SC or +T5 gRNAs, and
957 untreated cells. Shown are the means of three independent experiments with 6 technical replicates
958 ($n = 3$; $m = 6$) and SD. Statistical analyses were performed using the Student's T test. **c,** Schematic
959 of the flow cytometry analysis of GFP expression by 293T cells either not transduced or that had
960 been previously transduced with NL4-3/GFP pseudotyped with VSV-G, and left as such (NL4-
961 3/GFP) or then transfected with the SC- or T5 gRNA-containing CRISPR/Cas9 mCherry plasmid.
962 **d,** Flow cytometry analysis of cells described in **c** was performed at day 4 post-transfection,
963 showing that T5 significantly decreased, but did not ablate, GFP expression by the provirus. **e,**
964 Statistical analysis of the values obtained in **d**. Shown are the means of three independent
965 experiments with biological triplicates ($n = 3$; $m = 3$) and the SD. Statistical analyses were
966 performed using the Student's T test ($*** = p < 0.001$). **f,** Western blot analysis of GFP protein levels
967 at day 7 in lysates from 293T cells first transduced with NL4-3/GFP then either left as such (-) or
968 transfected with Cas9+SC or +T5 gRNAs. A control of cells transfected with a GFP coding plasmid
969 (pcDNA3/GFP) is included. **g,** Localization of primers used to amplify the DNA fragments
970 encompassing the CRISPR/Cas9 T5 cleavage sites. **h,** Sequence data of four amplicons sequenced
971 at the LTR junctions and blasted against pNL4-3.

972

Fig. 2 The excised HIV-1 provirus persists and circularizes after CRISPR/Cas9-transfection.

a, PCR analysis of *β-globin* gene in genomic DNA samples from NL4-3/Luc-transduced 293T cells extracted at day 2, 6, 10, 14 and 18 post CRISPR/Cas9 transfection, before (-) and after (+) ATP-dependent DNA exonuclease digestion. M: Molecular weight marker. **b**, PCR analysis of *gag* and *pol* sequences of genomic DNA treated as in **a** with ATP-dependent DNA exonuclease digestion. **c**, Rolling circle amplification (RCA) of DNA samples extracted from NL4-3/Luc-transduced at day 10 post-CRISPR/Cas9 and T5 or SC gRNAs transfection. This technique allows selective amplification of circularized DNA as concatemers, requiring digestion with a single cutter to obtain full length fragments. RCA amplicons were electrophoresed as such (-) or after *EcoRI* digestion (+), which cuts NL4-3/Luc once in *pol*. 293 T were used as a negative control. **d**, Schematic of circular molecules of HIV-1 provirus formed by a single molecule or two molecules bound together in sense-to-sense or sense-to-antisense orientation and size of the amplicons of interest written in red and generated by the primers indicated. **e**, PCR fragments obtained from RCA amplicons from 7 different experiments (#RCA1-7) using the primers shown in Fig. 2d. **f**, Alignment of LTR sequencing of the 750-bp fragments obtained from #RCA1-7 and retrieved from the agarose gel of Fig. 2e. The red sequences indicate the T5 gRNA annealing site. Dashes indicate base deletions and blue letters denote nucleotide mismatches compared to wild-type pNL4-3 sequence.

Fig. 3 HIV intermolecular concatemers can be isolated in large scale. a, Schematic of the approach devised to select and identify inter-molecular concatemers. 293T cells were transduced at 5 MOI with both HIV-Ori and HIV-KanR. A week later, their HIV-1 provirus was excised by CRISPR/Cas9 + T5 gRNA and the edited cells were selected by Puromycin. Then, their genomic DNA was extracted, digested by ATP-dependent exonuclease to eliminate linear DNA and used to transform bacteria. The recombinant bacteria were then selected on Kanamycin-containing agar plates, where only intermolecular joining brought about growing colonies. **b**, Graphic map of NL4-3/Luc/Ori and NL4-3/Luc/KanR as obtained by inserting the low-copy bacterial origin of

999 replication SC101 (Ori) or Kanamycin resistance gene (*KanR*) in the pNL4-3/Luc backbone. **c**,
 1000 Evaluation of transducing ability of lentiviral vectors as determined by luciferase activity of 293T
 1001 cells transduced with VSV-G-pseudotyped NL4-3/Luc/Ori and NL4-3/Luc/*KanR* as compared to
 1002 NL4-3/Luc, NL4-3/GFP and non-transduced 293T cells. **d**, Screening of 11 cell clones double-
 1003 positive for NL4-3/Luc/Ori and NL4-3/Luc/*KanR* obtained by limiting dilution. For PCR, primers
 1004 annealing to SC101 or *KanR* and pNL4-3 were used. **e**, PCR amplification of the LTR junctions
 1005 from NL4-3/Luc/*KanR*/Ori double-positive cell clones a-e. **f**, Sequence analysis of a-e amplicons
 1006 obtained in **e**. The red sequences indicate the T5 gRNA annealing site. Dashes indicate base
 1007 deletions and green letters denote nucleotide mismatches compared to wild-type pNL4-3 LTR
 1008 sequence. **g**, Schematic of inter-molecular concatemers bound in sense-sense orientation, as
 1009 determined by nucleotide sequencing.

1010

1011 **Fig. 4 Without Tat and Rev, the concatemers are not transcribed.** Analysis of HIV mRNA and
 1012 protein production by NL4-3/Luc/*KanR*/Ori transfected alone or with Tat + Rev provided *in trans*.
 1013 **a**, qRT-PCR quantitation of *gag* mRNA expression by cells transfected with pNL4-3/Luc or pNL4-
 1014 3/Luc/*KanR* or pNL4-3/Luc/*KanR*/Ori alone or after transfection of Tat alone or Tat + Rev. Cells
 1015 were lysed 3 days after transfection. Increment in *gag* mRNA expression following Tat or Tat +
 1016 Rev provided *in trans* reached statistical significance ($p=0.0013$). **b**, Determination of p24 content
 1017 by ELISA in lysates of cells transfected with pNL4-3/Luc or pNL4-3/Luc/*KanR*, as such or after
 1018 1:50 dilution, or with NL4-3/Luc/*KanR*/Ori before and after CRISPR/Cas9+T5 treatment.
 1019 Production of p24 with pNL4-3/Luc and pNL4-3/Luc/*KanR* was comparable but significantly
 1020 decreased following pNL4-3/Luc/*KanR* treatment with CRISPR/Cas9+T5. Isolated concatemers
 1021 exhibited reduced but significant transcriptional activity. **c**, Western blot analysis performed on
 1022 lysates of cells transfected with pNL4-3/Luc or NL4-3/Luc/*KanR*/Ori, either with Tat + Rev or
 1023 alone, or not transfected 293T cells. Detection with antibodies anti-Tat, anti-p24, and anti-IN
 1024 demonstrated low production of the three proteins by NL4-3/Luc/*KanR*/Ori that markedly increased

after addition of Tat + Rev. **d**, TILDA, a method that detects multiply spliced RNA (msRNA), did not indicate production of msRNA by NL4-3/Luc/KanR/Ori-transfected cells. **e**, SG-PERT, which measures RT activity in supernatants as quantified by 10-fold dilutions of mammalian murine leukemia virus (MMLV) RT, was used to determine RT activity in supernatants of cells transfected with NL4-3/Luc/KanR/Ori or pNL4-3/Luc (upper panel) or NL4-3/Luc/KanR/Ori with or without Tat + Rev (lower panel). **f**, Determination of p24 content as in **b** and after transfection with Tat or Tat + Rev. **g**, Analysis of proviral DNA content of 293T cells cultivated in the presence of supernatants from cells transfected with pNL4-3/GFP, or NL4-3/Luc/KanR/Ori, alone or combined with Tat+Rev, or control 293T cells, and, for all, VSV-G provided *in trans*. This analysis, performed with HIV-Qual assay, demonstrated that addition of Tat + Rev, compared to NL4-3/Luc/KanR/Ori alone, yields increased HIV viral particle production. Data were collected from three independent experiments with four replicates each and analyzed by Student's T test (***) = $p < 0.001$).

Fig. 5 HIV IN contributes to circularization and integration of excised provirus. **a**, Schematic workflow of the experiment. The HIV provirus was obtained from pNL-CMV-GFP by digestion with *PmiI*, gel purified and electroporated into 293T cells, either linearized, alone or in association to pCMV-IN, or as a closed plasmid. **b**, Agarose gel electrophoresis of pNL-CMV-GFP digested with *PmiI*. The upper band denotes the linearized HIV provirus that was extracted, purified and used for transfection as described in **a**. **c**, Percentage of GFP+ 293T cells after transfection with pNL-CMV-GFP, linearized, with IN (+) or without IN (-), or closed, as determined by flow cytometry. Bars indicate SD as determined from three independent experiments. The percentage of fluorescent cells transfected with the linear provirus and pCMV-IN was significantly higher than for cells transfected with the linear provirus alone, as determined by Student's T test ($p < 0.001$). **d**, Fluorescence microscopy and flow cytometry analyses of the cells plotted in the histogram of Fig. 5c. Increment in cell fluorescence in the presence of HIV IN suggests that IN contributes to rescue

transcriptional capacity of the linearized provirus. **e**, Schematic of Alu-PCR reaction. This reaction was used to understand if the provirus integrates into host cell DNA. The first round creates a tagged Alu-HIV-LTR fragment that is subsequently reamplified and detected by a specific probe. **f**, Histogram plot of the Alu-PCR reaction showing that the amount of provirus integrated back is significantly increased in the presence of HIV IN compared to the same without HIV IN. Statistical analysis was performed by one-way ANOVA and Bonferroni post-hoc test ($p < 0.001$).

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Fig. 6 Circularization of the excised provirus is facilitated if cleaved out in the presence of HIV IN also in latently infected lymphocytes. a, Schematic illustration of J-Lat treatment, localization of g1 and g2 guides, and detection of LTR circles by ddPCR. J-Lat cells were exposed to TNF- α to activate HIV expression (and viral protein production) and then transfected with g1, g2, g1 + g2 or SC and incubated for 24 h as such, or with RAL. ddPCR was performed on genomic DNA digested with *Bse*II using primers for HIV *nef* and LTR, that allow amplification of circles only. **b**, Timeline of drug addition, RNP transfection and total DNA extraction. J-Lat cells were treated with 10 ng/ml of TNF- α and 1 h later RAL, 10 μ M, was added. RNP transfection was done after 2 h and, 12 h later, total DNA was extracted for ddPCR. **c**, A typical ddPCR experiment is shown, where *nef*-LTR junction concentration is determined. Results demonstrate that the number of LTR circle molecules significantly increased in cells treated with TNF- α . RAL treatment causes considerable decrease in LTR circle formation. Poisson error bars (confidence interval 95%) of ddPCR values are shown, as determined automatically by the software after every ddPCR run. **d**, Histogram plot of the average number of LTR circle molecules from 3 independent experiments (black circles), performed as described in **b**. Statistical analysis of ddPCR values, performed using single-way Anova (red asterisks) and Tukey's multiple comparison test (black asterisks), shows that RAL pre-treatment decreased LTR circle molecule formation (***) $p < 0.001$. **e**, The experiment described in **b** was performed without TNF- α or RAL treatment. A typical ddPCR experiment is shown as in **c**. Results demonstrate that LTR circle molecules are formed also in the absence of HIV

1077 activation. **f**, Histogram plot representation of 2 independent experiments as in **e**. Statistical analysis
1078 was performed using single-way Anova (* $p < 0.1$).

1079

1080 **Fig. 7 RAL pretreatment prevents reactivation of HIV after RNP transfection. a**, Schematic
1081 workflow of the experiment: J-Lat cells, pretreated or not with 10 μ M RAL, were transfected with
1082 Atto550-RNP containing g1, g2, g1+ g2, or the SC control guide. This allowed tracking of RNP-
1083 transfected cells that became fluorescently labelled in red. Five hours post-RNP treatment, HIV-1
1084 transcription, and associated GFP expression, was induced by treating cells with TNF- α . HIV
1085 reactivation was quantified in RNP-treated cells by flow cytometry performed 24 hours post-RNP
1086 treatment. **b**, Flow cytometry analysis of J-Lat cells untransfected, transfected with g1, g2, g1 + g2,
1087 or SC, in the presence or not of RAL. All cells were activated with TNF- α 5 h after transfection.
1088 Ungated events are analyzed. The grey histogram in the overlays shows untreated, latent J-Lat
1089 cells. RNP transfection combined with RAL abolished HIV activation. **c**, Histogram plot of panels
1090 in Fig. 7b. The number of GFP⁺ cells is significantly lower upon RNP treatment with g1, g2 and g1
1091 + g2 compared to SC gRNA. Combined treatment with g1 + g2 or with g2 alone is the most and the
1092 least effective, respectively, in reducing activated, GFP⁺ cells. Pretreatment with RAL together
1093 with RNP transfection dramatically reduces induction of HIV-1 transcription.

1094

1095 **Fig. 8 HIV-1 is reintegrated by IN and amplified by RT. a**, Alu-PCR, performed as in Fig. 5,
1096 was carried out to detect the HIV-1 provirus integrated in proximity of Alu sequences of J-Lat cells.
1097 Alu-LTR copies are detected after activation with TNF- α and much less if treated with RAL. Latent
1098 cells treated with RNPs did not exhibit integration in Alu sites. **b**, Schematic workflow of the
1099 experiment shown in Fig. 8c and d. Cells were activated for 24 h with TNF- α , then treated with
1100 RAL or EFV or RAL + EFV for 24 h. RNP transfection with g2 was then carried out and the
1101 genomic DNA extracted 24 h later. Alu-LTR molecules were determined after normalization with

1102 β -globin DNA content. **c**, Histogram representation of Alu-PCR. RAL inhibits integration in Alu
1103 more than EFV alone, while combined treatment is more efficient than the single drugs alone.
1104 Guide g2 transfection greatly decreases Alu integration, whether alone or in the presence of
1105 RAL/EFV. **d**, Statistical analysis of Alu-LTR content of g2 RNP transfected cells alone. Single or
1106 combined treatments with RAL and/or EFV decreases efficiency of HIV reintegration. Data were
1107 obtained from three independent experiments and three biological replicates. Data were analyzed
1108 using One-way Anova (* $p<0.1$, *** $p<0.001$).

1109

1110 **Fig. 9 Cas9 proviral ablation increases the LTR circles of HIV-1.** **a**, schematic illustration of the
1111 experimental procedure: 4×10^6 Jurkat cells were infected with an HIV-1 clinical isolate. 24 h after
1112 the infection, cells were treated for 7 days with RAL 10 μ M, EFV 100 nm and RAL 10 μ M + EFV
1113 100 nM. Then, cells were electroporated with Cas9 g2 RNP and processed for ddPCR. **b**, ddPCR
1114 shows an increase in LTR circles in Jurkat cells transfected with g2 RNP; RAL decreases the
1115 amount of LTR circles in both experimental groups (transfected/not transfected). Y axis = LTR
1116 circles concentration expressed as copies/ μ l. **c**, Statistical analysis performed on ddPCR reads. One-
1117 Way Anova with post-hoc Tukey test was performed, experiments are expressed as mean $\pm$ SD (*
1118 $p<0.1$, ** $p<0.01$, *** $p<0.001$). **d**, RT-PCR of viral RNA extracted from supernatants of cells
1119 described in **a** at 0, 2, 9 and 12 days from HIV infection. Y axis: copies of HIV-1 genomes/ μ l
1120 supernatant. **e**, Statistical analysis performed on RT-PCR reads at day 12. One-Way Anova with
1121 post-hoc Tukey test was performed, experiments are expressed as mean $\pm$ SD (* $p<0.1$, ** $p<0.01$,
1122 *** $p<0.001$).

f

Construct	Sequence
NL4-3	CCCTGGAAAGTCCCAGCGAAAGTCCCTTGTAGCAAGCTCGAT
NL4-3/Luc/Ori	CCCTGGAAAGTCCCAGCGAAAGTCCCTTGTAGCAAGCTCGAT
NL4-3/Luc/KanR	CCCTGGAAAGTCCCAGCGAAAGTCCCTTGTAGCAAGCTCGAT
NL4-3/Luc/KanR/Ori clones	a CCCTGGAAAGTCCCAGCG-----AGCTCGAT
	b CCCTGGAAAGTCCCAGCG-----
	c CCCTGGAAAGTCCCAGCGAAAGTCCCTT-TAGCAAGCTCGAT
	d CCCTGGAAAGTCCCAGCGAAAGTCCCTTGTAGCAAGCTCGAT

a

b

c

d