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Supplemental Information

In Vivo Excision of HIV-1 Provirus by saCas9 and Multiplex Single-Guide RNAs in Animal Models

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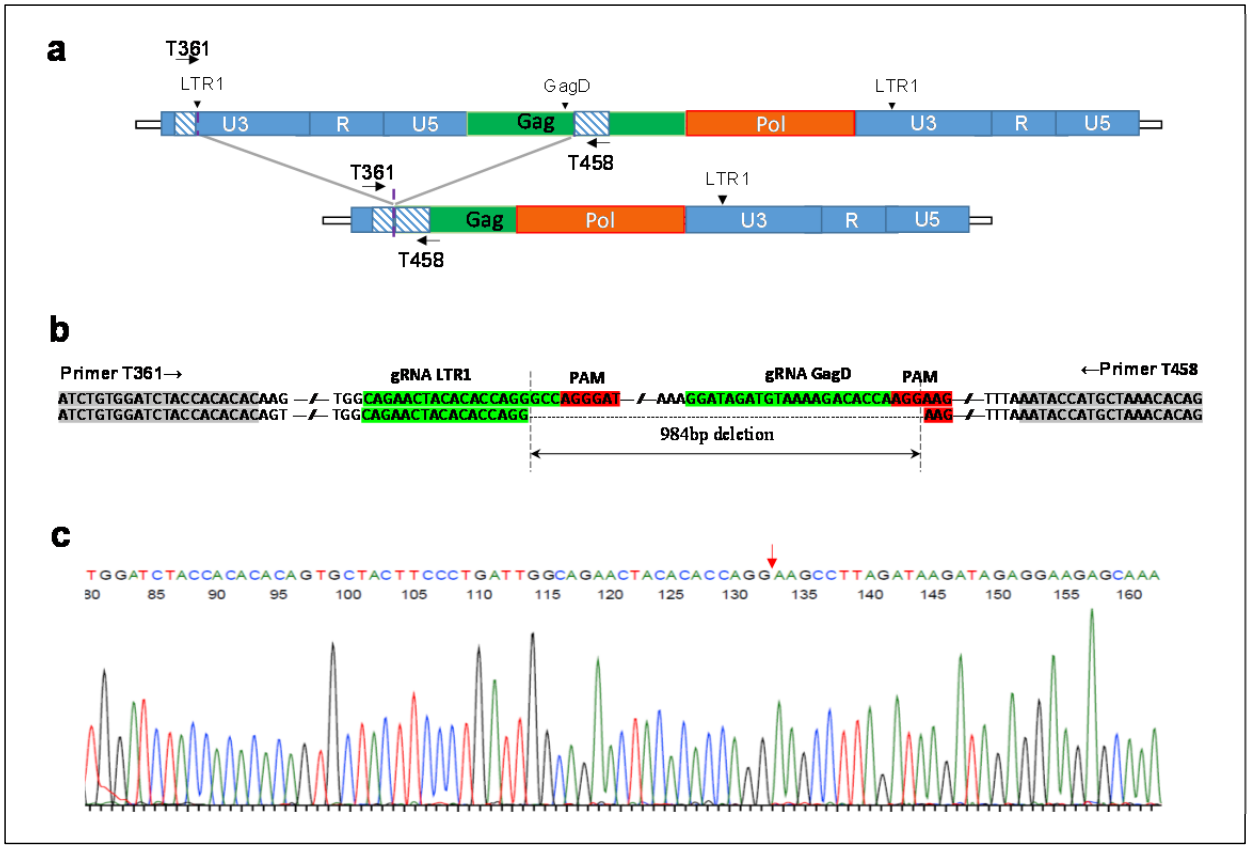

Fig. S1. Validation of the expected fragmental deletion of EcoHIV-eLuc genome using TA-cloning and Sanger sequencing analysis. (**a**) Schematics of the sgRNA target sites and PCR primer locations. (**b**) Comparative analysis of 984 nucleotide deletion after precise cleavage at the third nucleotide from PAM (red text) in LTR1 and GagD. (**c**) Representative Sanger sequence tracing showing the editing/re-ligation site between LTR1 and GagD.

a

Plasmid#	Plasmid Name	sgRNA	GC/ml	Fold	TU/ml	Fold
882	AAV-saCas9-sgRNA(TCf4)	Monoplex	3.50E+13	0.83	2E+09	8.00
924	AAV-saCas9-sgRNA(LTR1+GagD)	Duplex	4.15E+13	0.99	9.7E+08	3.88
938	AAV-saCas9-sgRNA(LTR3+PolB)	Duplex	4.30E+13	1.02	4.4E+08	1.76
962	AAV-saCas9-sgRNA(LTR1+GagD+LTR3+PolB)	Quadruplex	4.20E+13	1.00	2.5E+08	1.00

b

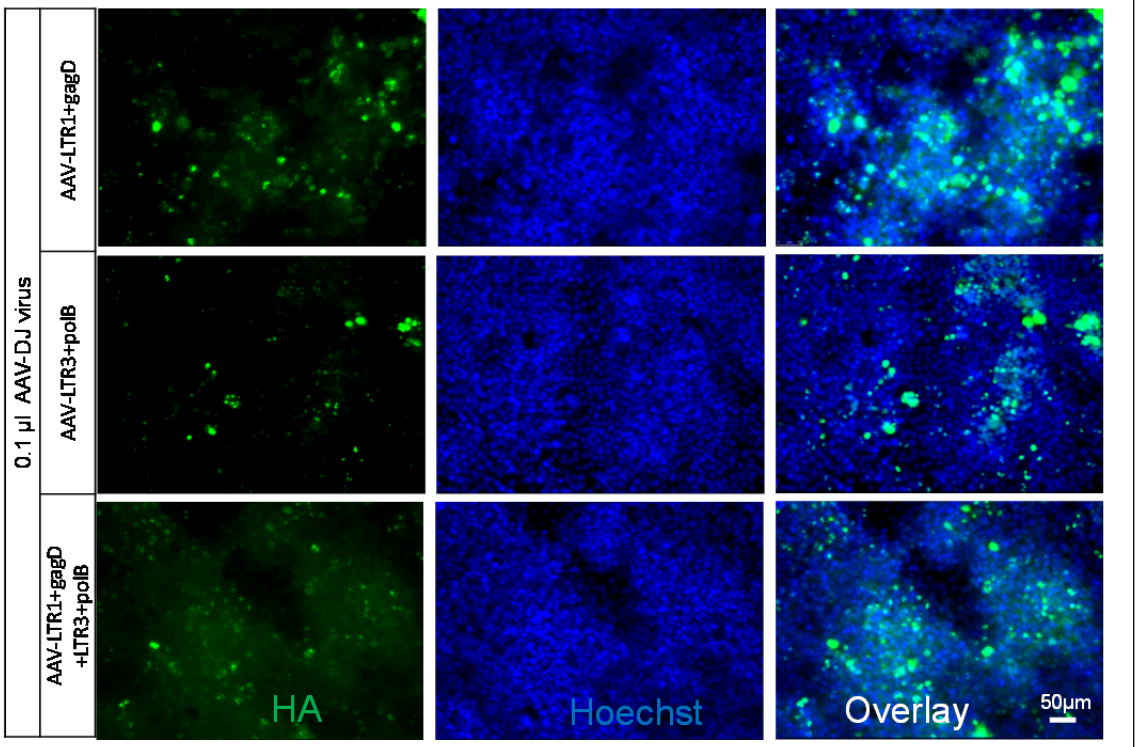

Fig. S2. Functional titer of AAV-DJ carrying multiplex sgRNAs and saCas9 in HEK293T cells. (**a**) Summary of genomic and functional titer for monoplex (plasmid 822), duplex (plasmid 924 and 938) and quadraplex (plasmid 962) sgRNAs/saCas9 AAV-DJ. (**b**) Representative images of immunofluorescent staining with anti-HA antibody at 2 days after 0.1 µl AAV-DJ virus infection in HEK293T cells plating on 96-well plate. The positive cells were counted in each of three wells and the functional titer was calculated as transduction units (TU) per ml. The genomic titer was determined by the viral genome copy number in 1 ml virus sample (GC/ml) by quantitative PCR analysis using the copy number of standard samples.

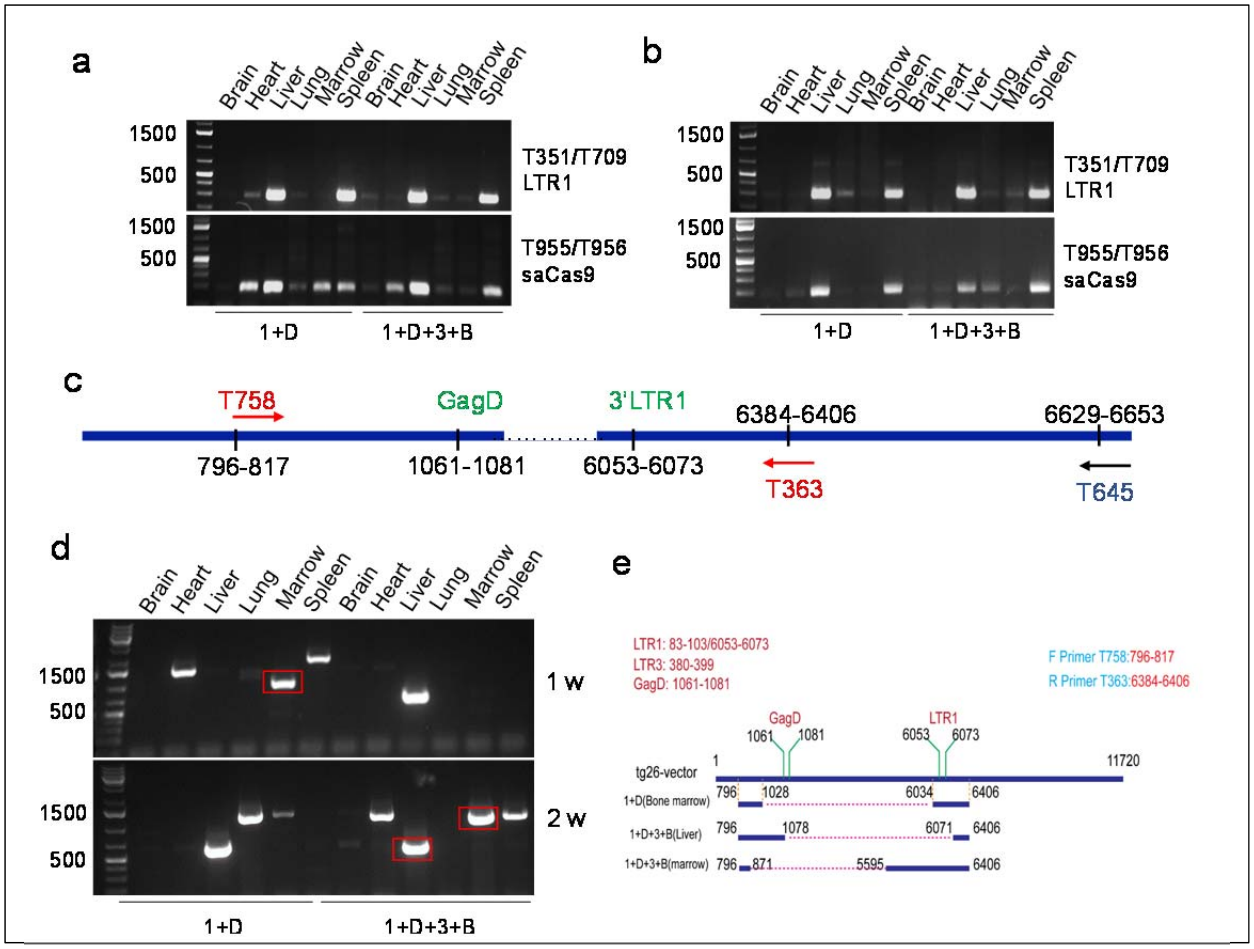

Fig. S3. Similar efficiency of transgene expression and HIV-1 genome excision in different organs/tissues of Tg26 transgenic mice infected with duplex and quadruplex sgRNAs/saCas9 AAV-DJ. (a, b) High efficiency of AAV delivery for saCas9 and representative sgRNA LTR-1 expression cassette in the liver and spleen of Tg26 mice intravenously injected with AAV-DJ virus (total 4.15-4.20 x 10^{12} GC in 100 µl PBS/mouse) via tail vein at 1 week after infection (a) and one additional injection at 1 week after the first injection. Tissue samples were collected at 1 (**a**) and 2 weeks (**b**) after first injection. (**c, d**) Nested PCR analysis identified fragmental deletion in liver, heart, bone marrow and spleen. The representative deletion fragments (red box) were verified with TA-cloning and Sanger sequencing (**e**).

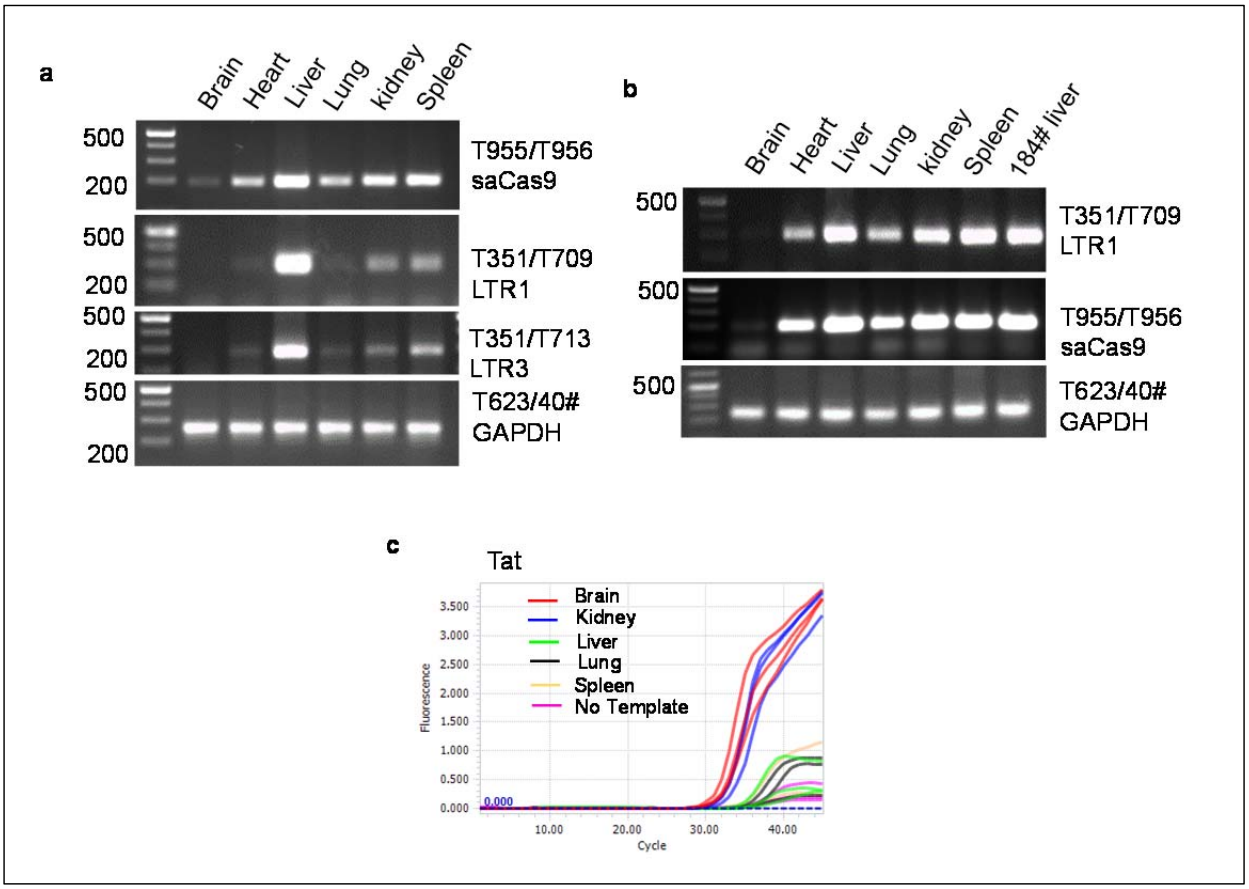

Fig. S4. Quadruplex sgRNAs/saCas9 AAV-DJ/8 induced efficient transgene transduction (a, b) and RT-qPCR analysis validated Tat transcription in various organs/tissues of HIV Tg26 transgenic mice. Tg26 mice were injected via the tail vein with purified AAV-DJ/8 virus (1.535 x 1012 GC/mouse) for once (**a**) or twice (**b**) separated by two weeks. Two weeks after last injection, mice were euthanized and their tissues were collected for genomic DNA extraction and PCR genotyping for the cDNA encoding sgRNAs and saCas9 as indicated. (**c**) Representative amplification curves showing relative Ct values for Tat transcription to validate efficiency of RT-qPCR assay.

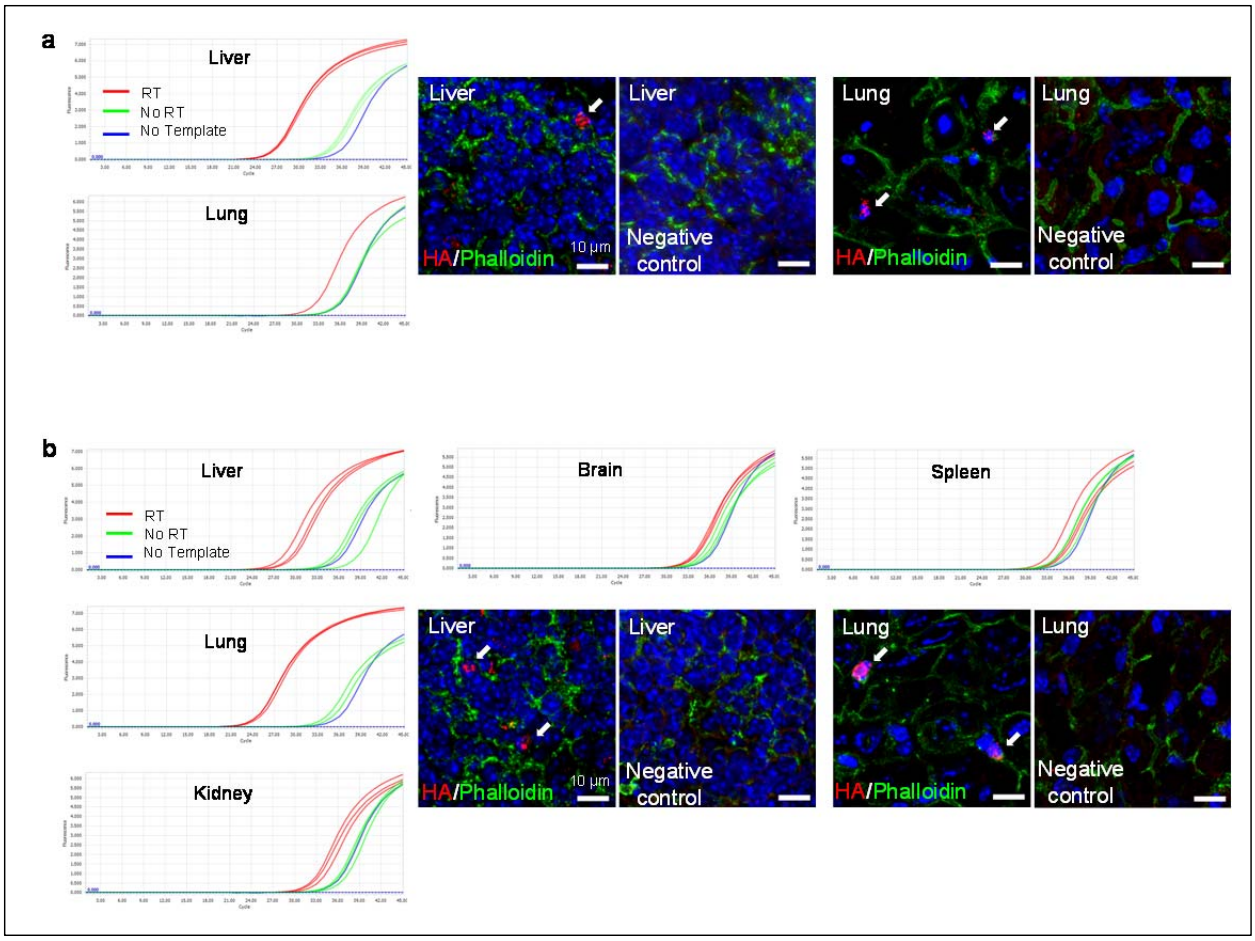

Fig. S5. The expression of saCas9 protein in the organs/tissues of Tg26 mice at 2 (a) and 4 (b) weeks after intravenous injection of quadruplex sgRNAs/saCas9 AAV-DJ/8. Total RNAs of the indicated organs/tissues were extracted with an RNeasy Mini kit and the residual genomic DNA was removed through an in-column DNase digestion with an RNase-Free DNase Set. Real-time PCR analysis of the cDNA reversely transcribed (RT) from total RNA was used to measure the expression of the transgene saCas9, which is mainly localized to the liver and lung. No RT was used as a negative control for genomic DNA contamination. No template was used as PCR control. Representative micrographs of the tissue frozen sections immunostained with rabbit anti-HA tag antibody (*red*) for detecting the saCas9 protein and Phalloidin (*green*) for F-actin in the liver and lung. Negative control only used secondary antibody. White arrows pointed the presence of saCas9-like immunoreactivity in nuclei. Scale bars=10 μm.

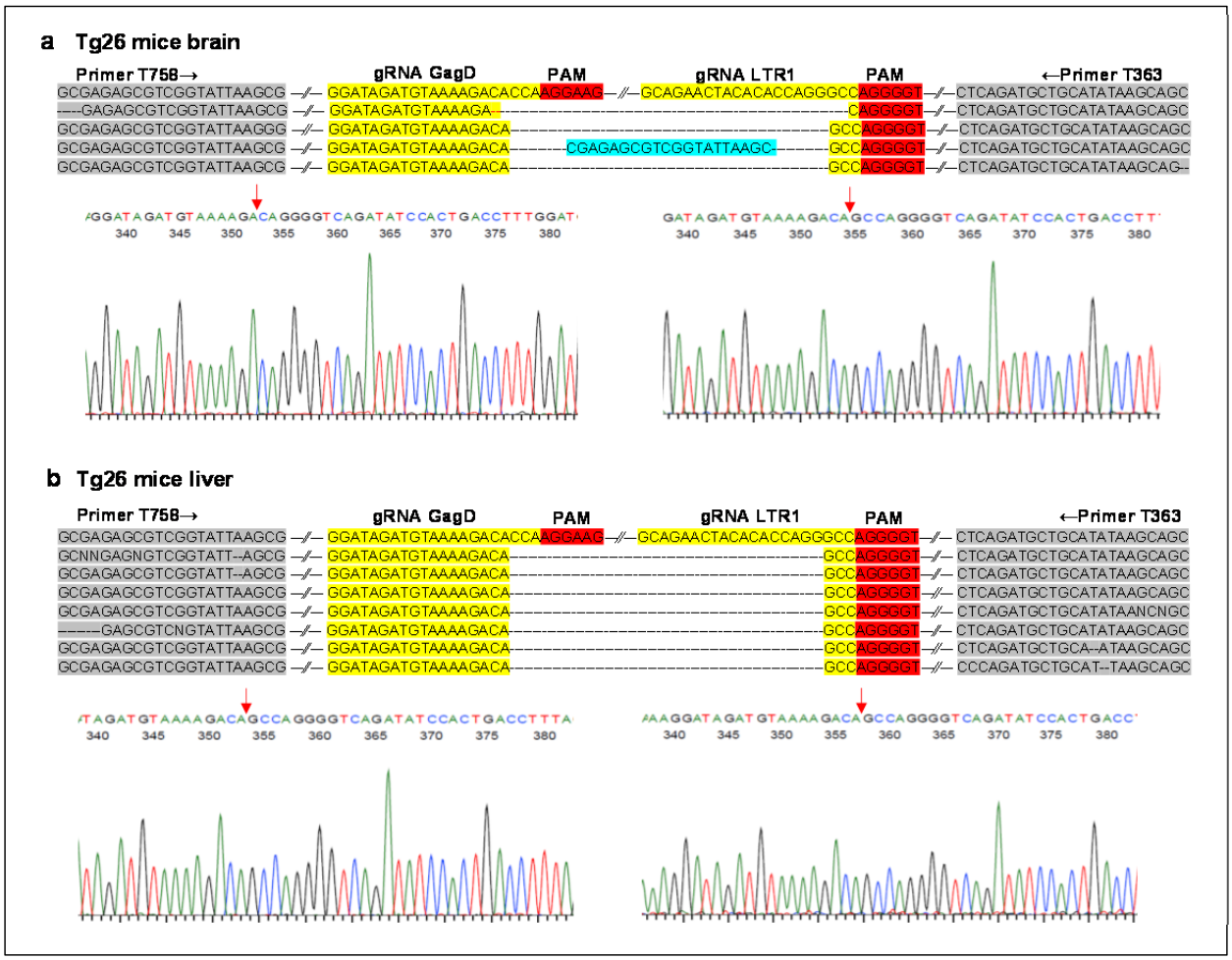

Fig. S6. Validation of the GagD to 3'-LTR1 fragmental deletion of HIV-1 genome in the brain (a) and liver (b) of Tg26 transgenic mice at 2 weeks after intravenous administration of the quadruplex sgRNAs/saCas9 AAV-DJ8. The predicted deletion of the 4992 bp fragment after cleavage at the third nucleotide from the PAM (highlighted red) was observed in most of the bacterial clones of PCR product in TA-cloning with a small insertion or deletion in a few clones. Representative Sanger sequence tracing was presented to show the cleaving/re-ligation site (red arrows) between GagD and 3'-LTR1.

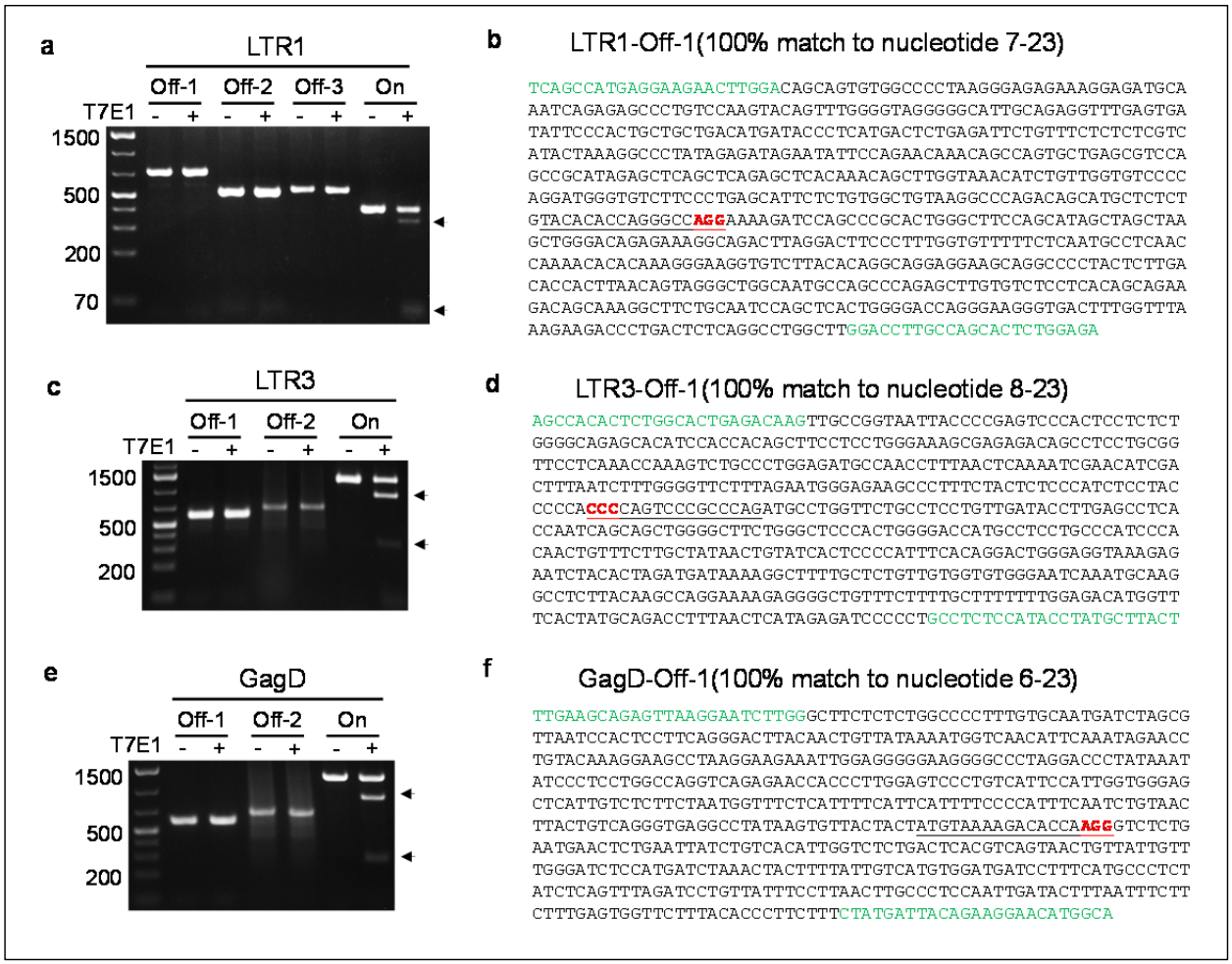

Figure S7. T7E1 mismatch cleavage assay showing no off-target effects in the liver tissue of Tg26 mice receiving a single intravenous injection of quadruplex sgRNAs/saCas9 AAV-DJ8. Representatives of the most potential off-target sites predicted for LTR1 (**a,b**), LTR3 (**c,d**) and GagD (**e,f**) were examined by T7E1 mismatch cleavage assay using the PCR product amplified from the genomic DNA extracted from the liver tissue of Tg26 mice at 4 weeks after sgRNAs/saCas9 treatment. The on-target PCR products were used as positive controls. Arrows indicated the InDel mutation patterns for the positive control PCR products generated with primer pairs T361/T363 for LTR1 (**a**), T361/T458 for LTR3 (**c**) and GagD (**e**). (**b, d, f**) A representative sequence of PCR product encompassing the predicted potential off-target sites in mouse genomic DNA. The PAM sequence is highlighted in red and the PCR primers are highlighted in green.

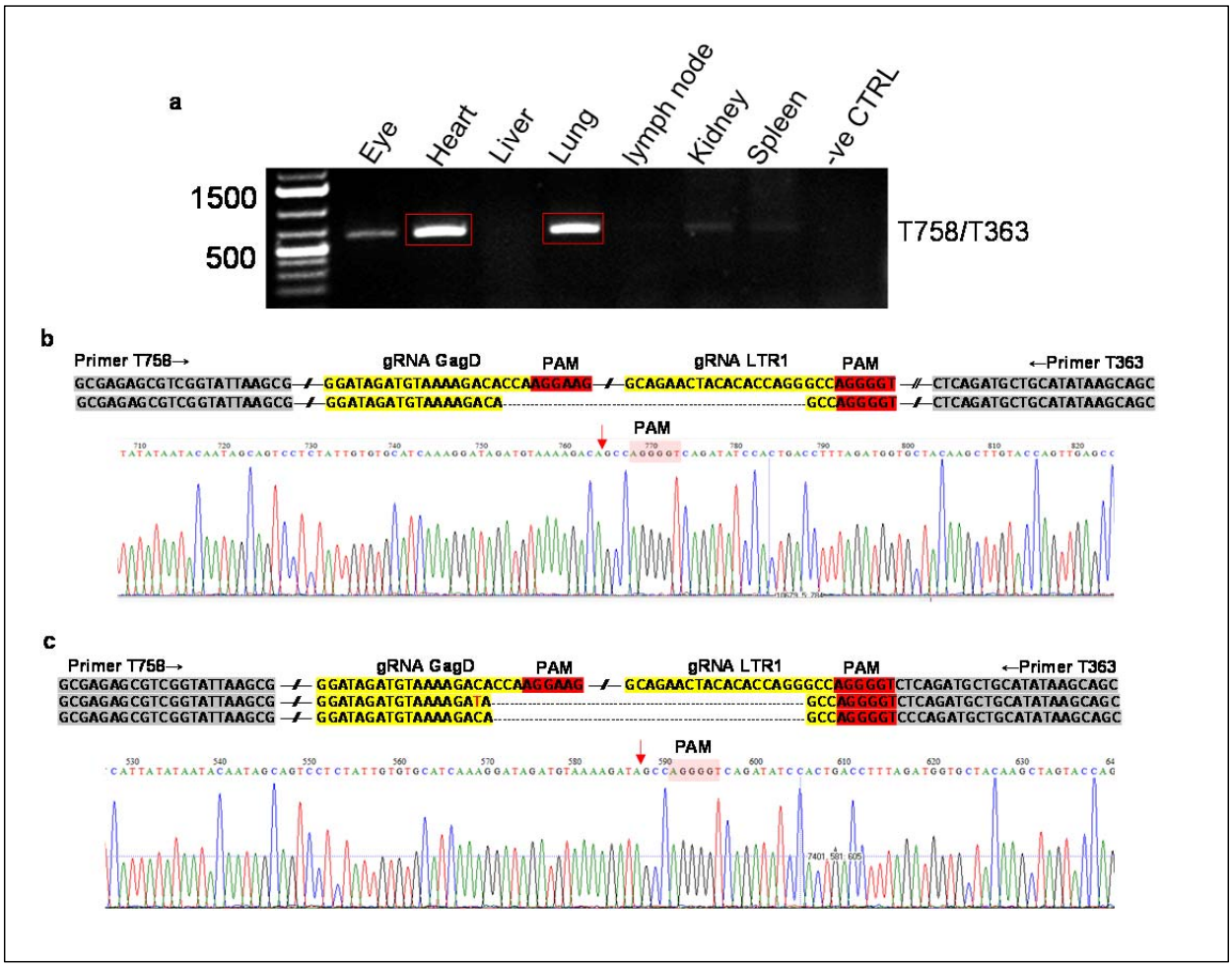

Fig. S8. Excision of EcoHIV-eLuc by the quadruplex sgRNAs/saCas9 in the heart and lung tissues of NCr nude mice. The mice received an administration of EcoHIV-eLuc via retro-orbital injection at the right eye and then another injection of AAV-DJ8 carrying quadruplex sgRNA/saCas9 via the same injection route. Mice were sacrificed 2 weeks after the injection. (**a**) PCR genotyping with primers T758/T363 amplified the expected fragment with a deletion between GagD and 3'-LTR1. The deletion fragments in heart and lung indicated by red color frame were extracted from the gel for TA-cloning and Sanger sequencing. (**b, c**) Representative Sanger sequence showing the expected cleaving/re-ligation site (red arrows) between GagD and 3'-LTR1. The deletion occurred exactly at the third nucleotides from the PAM (highlighted red) in heart (**b**) and lung (**c**).

5’-LTR1+GagD	uncut	T872/T759
	cut	T361/T946
	internal	T457/T458
GagD+3’-LTR1	uncut	T873/T874
	cut	T758/T535
	internal	T872/T759
GagD+3’-LTR3	uncut	T873/T874
	cut	T758/T363
	internal	T872/T759

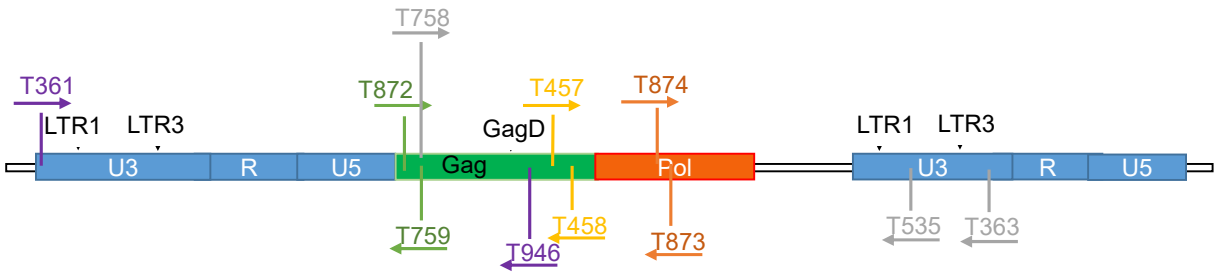

Fig. S9. The strategy of qPCR for detecting HIV excision efficiency. The table showing all qPCR primer pairs for indicating uncut, cut and internal EcoHIV DNA level, and the diagram showing all primers’ location.

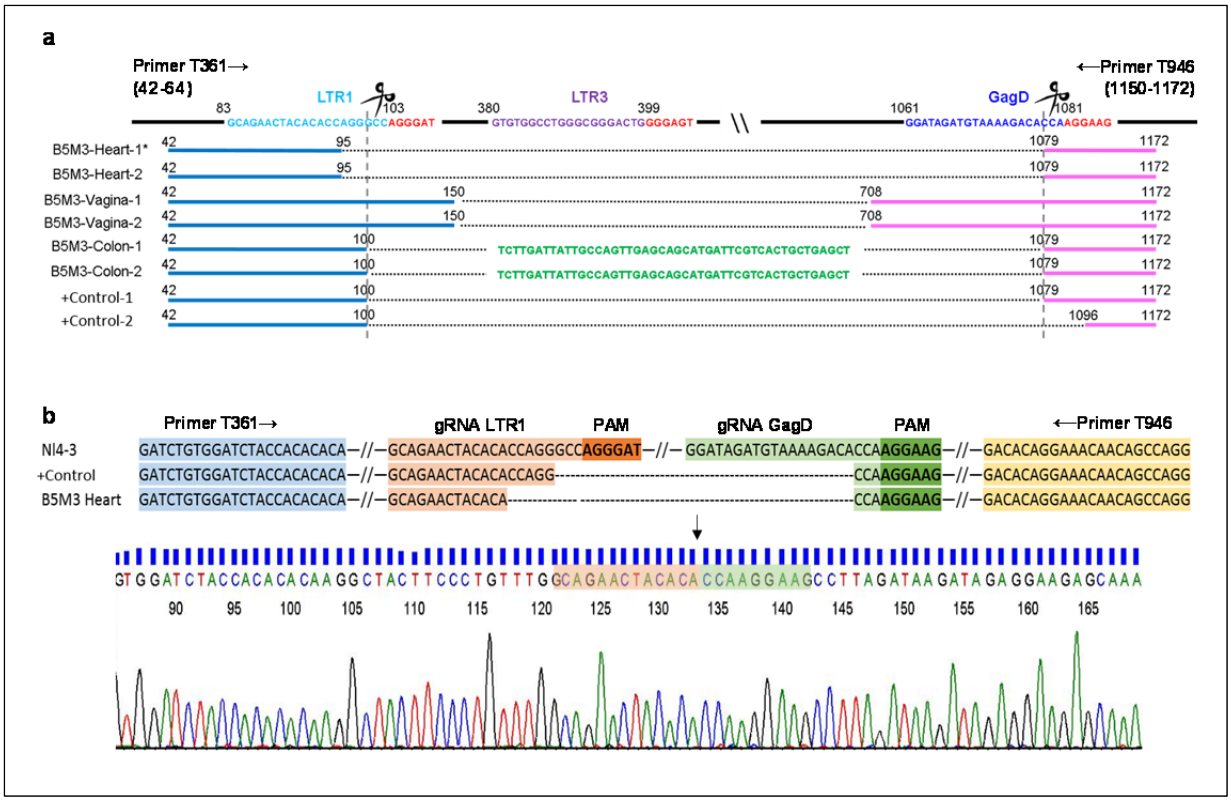

Fig. S10. Validation of the fragmental deletion of HIV-1 proviral DNA between 5'-LTR1 and GagD sites in organs/tissues of humanized BLT mice after saCas9/sgRNA genome editing. (a) Schematics of the junction sequence and unexpected sequence inserted in between the predicted cleavage sites. Nested PCR products using primer T361/T946 (the template from the first round PCR with the primer T361/T458) were extracted from the gel for TA cloning and 2-5 clones were sequenced from each sample. The precise cleavage site at the third nucleotide from the PAM (highlighted red) is indicated by the scissors. The predicted deletion between the 5'-LTR1 and GagD as well as various additional insertions (green) or deletions (black dot line) were identified. The blue and pink solid bar indicate the cleaved residual sequence. The numbers above the fragments indicated the start and the end of the nucleotide sites. TA clones with stars are selected to show detail sequence and tracing. (b) Representative Sanger sequencing tracing of clone B5M3-heart-1 shows the cleaving/relegation site (indicated by an arrowhead) between 5'-LTR1 and GagD. The conjunctive sequence between the 5'-LTR1 and GagD is highlighted with orange and green, respectively. The primer T361 and T946 sequence is highlighted with blue and yellow, respectively.

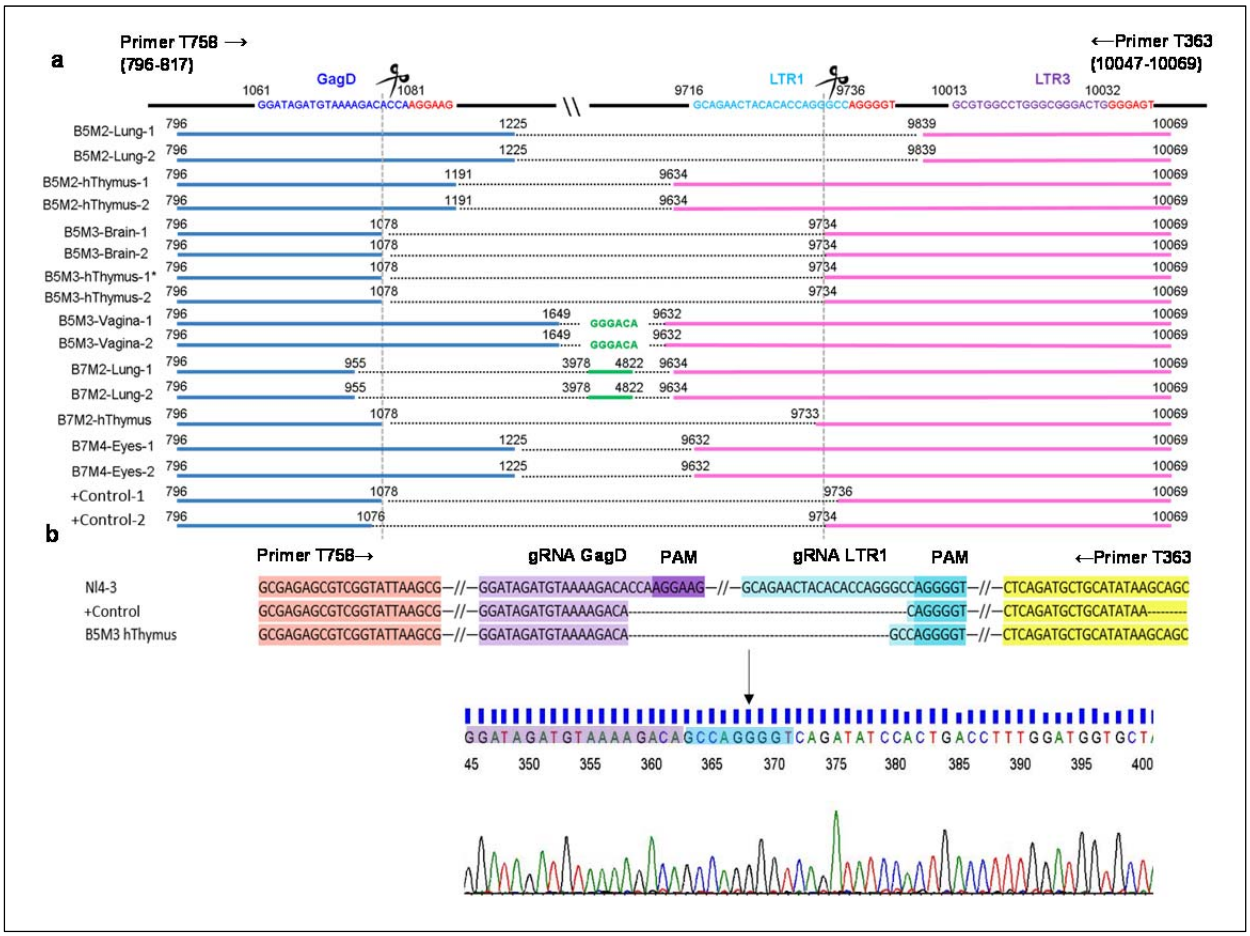

Fig. S11. Validation of the fragmental deletion of HIV-1 genome between GagD and 3'-LTR1 sites in the organs/tissues of humanized BLT mice after genome-editing. (a) Nested PCR product using primer T758/T363 after the first round PCR amplified with the primer T758/T458. PAM sequence is highlighted in red. The scissors indicate the precise cleavage site at the third nucleotide from the PAM. The predicted deletion between GagD and 3'-LTR1 is displayed along with the additional deletions or the unexpected insertions highlighted in green. The black dot line indicate the deleted portion. The blue and pink solid bar indicates the locations of the cleavage sites. The numbers above the fragments indicated the start and the end of the nucleotide sites. TA clones with stars are selected to show detail sequence. The lower fragment from hThymus tissue of mouse B5M3 indicated with green arrow in Fig. 7 was identified nonspecific by sequencing while the upper band was the cleaved residual sequence. (b) Representative Sanger sequence tracing of clone B5M3-hThymus-1 showing the cleaving/relegation site indicated by arrowheads between the GagD and 3'-LTR1. The cleaved sequence of GagD and 3'-LTR1 which were then joined together after deletion. The cleavage sites are highlighted with purple and blue respectively. The primer T758 and T363 sequence is highlighted with red and yellow respectively.

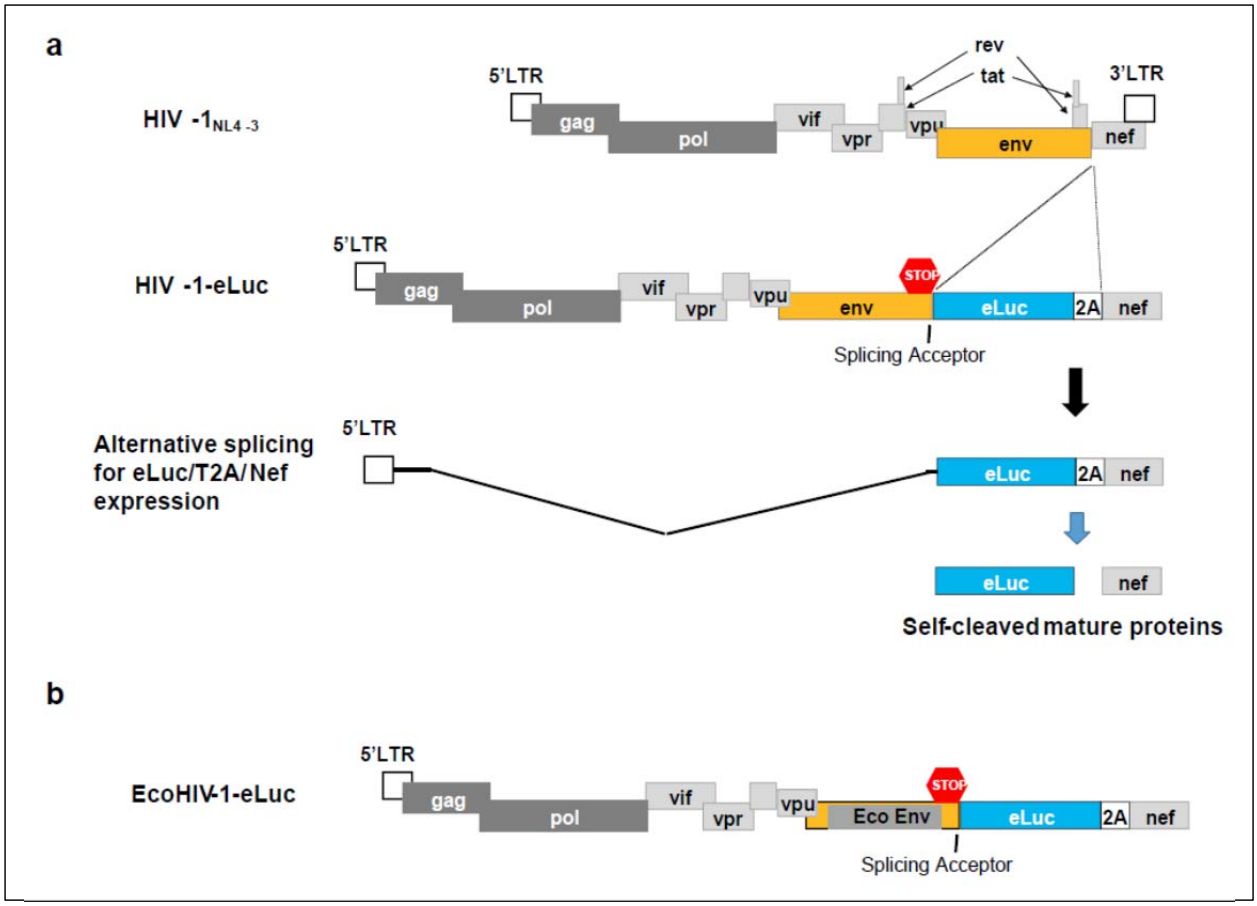

Fig. S12. Schematics of HIV$_{NL-BaL}$-eLuc and EcoHIV-eLuc. (**a**). To retain the expression of intact HIV-1 Nef for pathogenesis and early HIV infection, a P2A peptide, a self-cleaving peptide which can cleave between genes upstream and downstream, and a portion of 5'Nef were cloned at the 3' end of each reporter in frame. The cleavage site of 2A peptide is precise and well defined, in such case, only one additional amino acid at the N-terminus of Nef was expected. (**b**) In EcoHIV-eLuc, the HIV gp120 is replaced with gp80 from ecotropic murine leukemia virus to infect only mouse cells rather than human cells.