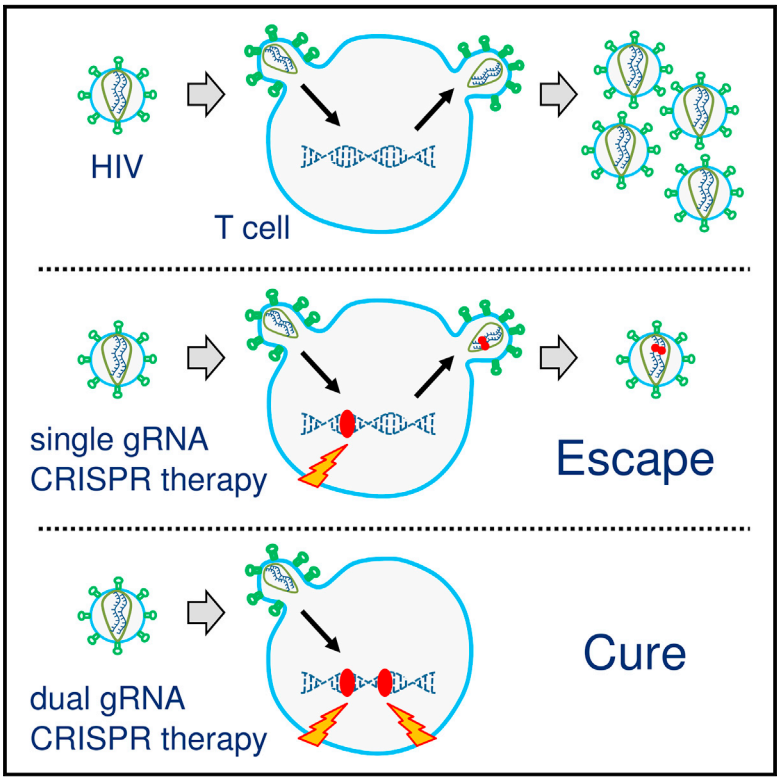
Cell Reports

Report

A Combinatorial CRISPR-Cas9 Attack on HIV-1 DNA Extinguishes All Infectious Provirus in Infected T Cell Cultures

Graphical Abstract



Authors

Gang Wang, Na Zhao, Ben Berkhout, Atze T. Das

Correspondence

b.berkhout@amc.uva.nl (B.B.), a.t.das@amc.uva.nl (A.T.D.)

In Brief

Although antiviral drug therapy is a life-saving treatment for HIV-infected individuals, a cure is never reached because the virus persists. Using CRISPR-Cas9 gene editing, Wang et al. find that complete virus inactivation can be achieved in cultured T cells, suggesting an avenue toward a functional cure.

Highlights

- Targeting HIV with CRISPR-Cas9 and dual gRNAs can durably inhibit virus replication
- Identification of dual-gRNA combinations that prevent HIV-1 escape
- Repeated Cas9 activity results in hypermutation of gRNA target sites
- HIV-infected cells can be functionally cured by dual-gRNA-guided Cas9 treatment

Wang et al., 2016, Cell Reports *17*, 2819–2826
December 13, 2016
http://dx.doi.org/10.1016/j.celrep.2016.11.057

CellPress

Cell Reports
Report

A Combinatorial CRISPR-Cas9 Attack on HIV-1 DNA Extinguishes All Infectious Provirus in Infected T Cell Cultures

Gang Wang,[1] Na Zhao,[1] Ben Berkhout,[1,*] and Atze T. Das[1,2,*]
[1]Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, the Netherlands
[2]Lead Contact
*Correspondence: b.berkhout@amc.uva.nl (B.B.), a.t.das@amc.uva.nl (A.T.D.)
http://dx.doi.org/10.1016/j.celrep.2016.11.057

SUMMARY

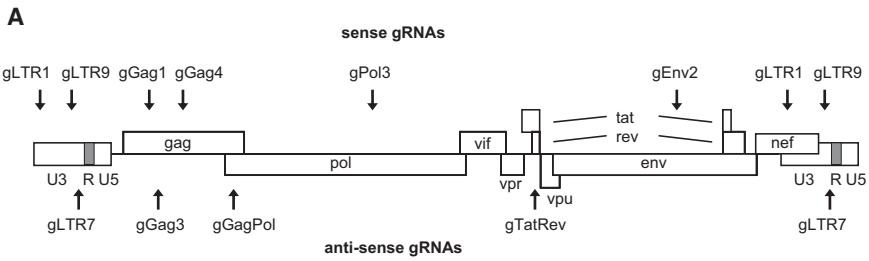
Current drug therapies effectively suppress HIV-1 replication but do not inactivate the provirus that persists in latent reservoirs. Recent studies have found that the guide RNA (gRNA)-directed CRISPR/Cas9 system can be used for sequence-specific attack on this proviral DNA. Although potent inhibition of virus replication was reported, HIV-1 can escape from a single antiviral gRNA by mutation of the target sequence. Here, we demonstrate that combinations of two antiviral gRNAs delay viral escape, and identify two gRNA combinations that durably block virus replication. When viral escape is prevented, repeated Cas9 cleavage leads to saturation of major mutations in the conserved target sequences that encode critical proteins. This hypermutation coincides with the loss of replication-competent virus as scored in sensitive co-cultures with unprotected cells, demonstrating complete virus inactivation. These results provide a proof-of-principle that HIV-1-infected cells can be functionally cured by dual-gRNA CRISPR/Cas9 treatment.

INTRODUCTION

Current drug therapies against HIV-1 effectively suppress virus replication but do not lead to a cure. The proviral DNA, the replication intermediate that is formed upon reverse transcription of the viral RNA genome and that is stably integrated into the cellular genome, persists in latent reservoirs in the human body. As a consequence, the virus always rebounds when drug treatment is interrupted. Several approaches to extinguish this reservoir have been investigated, including strategies that aim to activate the latent virus so that the infected cells will be cleared by the immune system (shock-and-kill) and approaches to permanently inactivate the proviral DNA in infected cells. The bacterial clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system that was developed into a versatile method for genome engineering in eukaryotic cells may be a powerful and specific tool for elimination of the provirus. The Cas9 endonuclease induces double-stranded breaks (DSBs) at specific DNA sequences. This sequence specificity is due to a guide RNA (gRNA) that directs Cas9 to a 20-nt complementary sequence adjacent to a 3-nt motif (protospacer adjacent motif [PAM]). Several studies demonstrated that CRISPR/Cas9 can indeed be used for a sequence-specific attack on HIV proviral DNA. This enables novel gene therapy approaches for the treatment of HIV-1-infected individuals, in which Cas9 and antiviral gRNAs are either expressed in infected cells to inactivate the integrated provirus or used to protect cells against infection.

Initially, several groups demonstrated that Cas9 and gRNAs effectively inhibit HIV-1 gene expression and short-term virus replication in T cell cultures (Ebina et al., 2013; Hu et al., 2014; Liao et al., 2015; Zhu et al., 2015). However, we and others recently demonstrated that HIV-1 can escape from this inhibition by acquisition of mutations in the gRNA target sequence that provide resistance against Cas9 attack (Liang et al., 2016; Wang et al., 2016a, 2016b; Yoder and Bundschuh, 2016). We observed very rapid escape, mostly due to insertions or deletions (indels) in the target sequence, when poorly conserved non-essential viral sequences were targeted. When highly conserved essential domains were targeted, escape was significantly delayed and mostly due to nucleotide substitutions in the target sequence. All mutations clustered around the Cas9 cleavage site. This signature implicated the non-homologous end-joining (NHEJ) pathway for DNA repair, which acts on Cas9-generated DNA breaks, in the generation of the escape mutations. Non-essential viral target sequences can accommodate the indels that frequently result from NHEJ repair, which explains the immediate escape when such regions are targeted. When conserved protein-coding sequences are targeted, only a small subset of the NHEJ-generated mutations, in particular point mutations, will support virus replication, thus explaining the significant delay in viral escape.

HIV-1 is known for its capacity to escape from most if not all types of inhibitors when applied as mono-therapy, including antiviral drugs and a sequence-specific attack on the viral RNA genome by RNAi. Also targeting of HIV-1 with zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) may—in a similar way as observed for CRISPR/Cas9 targeting—result in the generation of nuclease-resistant escape variants (De Silva Feelixge et al., 2016). However, combination of several antiviral drugs did result in successful antiretroviral therapy (ART). This success is based on two independent effects.



B

gRNA I	gRNA II	distance (bp)
gLTR7	gLTR9	22 (2x), 8810
	gLTR1	322 (2x), 9110
	gGag1	988, 8145
	gTatRev	5590, 3542

gRNA I	gRNA II	distance (bp)
gGag1	gGag3	91
	gGag4	438
	gGagPol	888
	gPol3	2796
	gTatRev	4602
	gEnv2	6452

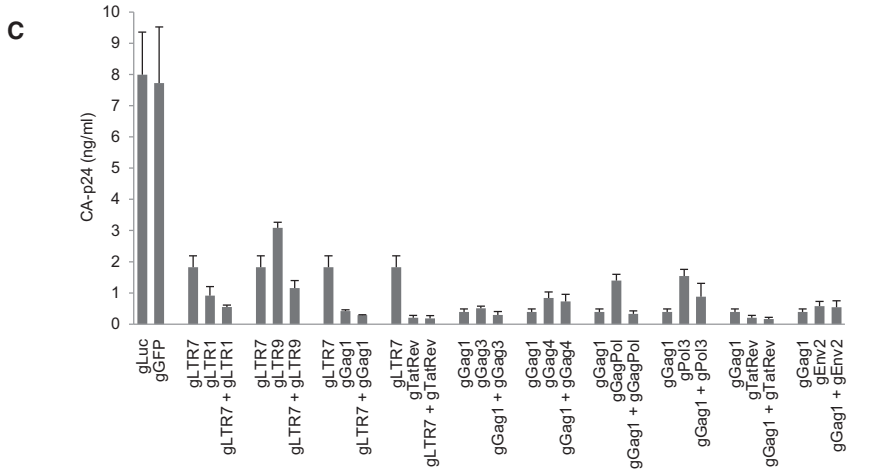


Figure 1. Targeting HIV-1 DNA with Single and Dual gRNAs

(A) The position of the gRNAs targeting the sense and antisense strand are indicated above and below the HIV-1 proviral DNA, respectively.

(B) Tested dual-gRNA combinations (gRNA I and gRNA II) with the distance between the target sites.

(C) The efficiency of the single and dual gRNAs to silence HIV-1 DNA was tested in 293T cells transfected with plasmids expressing HIV-1 LAI, Cas9, and single or dual gRNAs. To quantify viral gene expression, the CA-p24 level was measured in the culture supernatant at 2 days after transfection. Average values (±SEM) of four experiments are shown.

First, multiple drugs can provide additive or even synergistic inhibition. Second and more importantly, combination therapy will increase the genetic threshold for the acquisition of drug resistance as more mutations are required. Similarly, combinatorial RNAi attack was shown to durably inhibit virus replication and prevent viral escape (ter Brake et al., 2006). We therefore set out to test whether combination of two gRNAs that target conserved HIV-1 domains can permanently block virus replication in infected cells and prevent virus escape in long-term cultures. We indeed identified durable gRNA combinations, which allowed us to test whether one can functionally cure HIV-1-infected cells through permanent inactivation of all integrated provirus. We demonstrate that such complete sterilization can be accomplished in a robust T cell infection model.

RESULTS

Efficient Targeting of HIV-1 DNA with Single and Dual gRNAs

The CRISPR design web tool (Hsu et al., 2013) was used to select ten gRNAs that target either the sense or antisense strand of the HIV-1 DNA genome (subtype B LAI isolate) with a high predicted on-target activity (Figure 1A; Table 1). Three gRNAs targeted the long-terminal repeat (LTR) sequences that are present at both the 5′ and 3′ end of the viral DNA, and seven gRNAs targeted protein-encoding sequences present at a single position. Seven of these gRNAs were validated in our previous study that compared targeting of conserved and non-conserved viral sequences (Wang et al., 2016a). We now preferentially target highly conserved HIV-1 sequences (Shannon entropy < 0.2, except for gLTR1 and gLTR9) to avoid rapid NHEJ-mediated escape.

The gRNAs were tested individually and when combined in pairs (Figure 1B). In a first set, gLTR7 was combined with a second gRNA targeting either the LTR (gLTR1 or gLTR9) or protein-encoding sequences (gGag1 or gTatRev). These combinations target HIV-1 DNA at four and three positions, respectively. A second set was based on gGag1, which was combined with another gRNA that targets protein-encoding sequences (gGag3, gGag4, gGagPol, gPol3, gTatRev, or gEnv2). The latter combinations always targeted the HIV-1 DNA at two positions.

To compare the HIV-1 DNA cleavage efficiency, 293T cells were transiently transfected with plasmids expressing HIV-1 LAI, Cas9, and single or dual HIV-targeting gRNAs. We included several control gRNAs that target non-viral sequences (luciferase, GFP). HIV-1 gene expression was quantified by measuring viral capsid protein (CA-p24) production at 2 days after transfection. A high CA-p24 level was observed for the control gRNAs (Figure 1C), which was significantly reduced for all single antiviral gRNAs due to Cas9/gRNA-induced cleavage of the transfected HIV-1 plasmid. No reduction in CA-p24 production was observed in control experiments with only Cas9 or gRNA (data not shown). Most dual gRNA combinations resulted in a similarly low or further reduced CA-p24 level as measured with the corresponding single gRNAs. We did not detect a

Table 1. Selected gRNAs Targeting HIV-1

Name	Position in HIV-1 LAI DNA	Target Sequence + PAM[a]	Orientation	Predicted On-Target Activity (%)[b]	Conservation[c]
gLTR1	79–101, 9211–9233	ATTGGCAGAACTACACACCAGGG	sense	78	0.20
gLTR9	379–401, 9511–9533	GGCGTGGCCTGGGCGGGACTGGG	sense	50	0.48
gLTR7	413–435, 9544–9566	CCCTCAGATGCTGCATATAAGCA	antisense	60	0.09
gGag1	1389–1411	GTTAAAAGAGACCATCAATGAGG	sense	64	0.15
gGag3	1480–1502	CCAAGGGGAAGTGACATAGCAGG	antisense	67	0.07
gGag4	1827–1849	GATGACAGCATGTCAGGGAGTGG	sense	44	0.09
gGagPol	2288–2310	CCCTCAGATCACTCTTTGGCAAC	antisense	66	0.07
gPol3	4185–4207	GCATGGGTACCAGCACACAAAGG	sense	64	0.13
gTatRev	6002–6024	CCTATGGCAGGAAGAAGCGGAGA	antisense	54	0.11
gEnv2	7841–7863	GGAGCAGCAGGAAGCACTATGGG	sense	69	0.07

[a]PAM sequence is underlined.
[b]The on-target activity, computed as 100% minus a weighted sum of off-target hit scores in the human genome (hg19), was calculated using the CRISPR design web tool from crispr.mit.edu.
[c]The Shannon entropy was calculated to estimate the variation in the gRNA target sequence among the HIV-1 isolates (group M) described in the HIV Database 2014 (https://hiv.lanl.gov/; only the complete viral sequences were included). The entropy can vary from 0 to 1.5, with an invariant sequence having a score of 0.

correlation between the CA-p24 level and the actual number of target sites in HIV-1 DNA (ranging from two to four), which may be due to the robust inhibition caused by the single gRNAs.

Dual gRNAs Prevent HIV-1 Breakthrough Replication More Effectively than Single gRNAs

We next tested the capacity of the single and dual gRNAs to inhibit a spreading HIV-1 infection in SupT1 T cells stably transduced with Cas9- and gRNA-expressing lentiviral vectors. Importantly, none of the tested gRNAs target the HIV-based lentiviral vectors used for transduction. For each gRNA and gRNA-combination, four cell cultures were infected with HIV-1 LAI (1 ng of CA-p24) and virus replication was monitored for 60 days. In control non-transduced SupT1 cells and Cas9-only transduced cells, efficient virus replication resulted in a rapid increase in the CA-p24 level (Figure 2A) and the appearance of large virus-induced syncytia and cell death around day 8 after infection. Virus replication was significantly inhibited by a single gRNA, but we eventually scored an increase in CA-p24 (shown for gGag1 and gTatRev cultures in Figure 2A), which coincided with syncytia formation. This breakthrough replication likely represents viral escape as we described previously (Wang et al., 2016a). We did not detect any virus replication in a single gPol3 and three of the four gGag4 cultures in the 60-day follow-up period. To estimate the time required for viral breakthrough, we averaged the moment at which large syncytia and massive cell death were observed in the four cultures. When no virus replication was detected, we scored 60 days. For cells expressing a single gRNA targeting protein-encoding sequences, this breakthrough time varied from 18 days (gEnv2) to 51 days (gGag4; see Figure 2B). Virus replication was also inhibited in cells expressing the LTR-targeting gLTR7 or gLTR9 (breakthrough around 16 days), but no significant virus block was apparent in gLTR1 cells (replication apparent around day 8). Except for gLTR9, these results are in agreement with our previous report (Wang et al., 2016a) that demonstrated a significant delay in breakthrough virus replication only when highly conserved HIV-1 sequences are targeted (Shannon entropy < 0.2), whereas rapid virus escape was scored when less conserved domains were targeted (e.g., gLTR1). The less conserved gLTR9 target sequence encodes binding sites for the Sp1 transcription factor, which likely restricts the escape options and can explain the observed delay in breakthrough replication.

In most cells expressing two gRNAs, HIV-1 replication was inhibited more durably than in cells expressing one of the corresponding gRNAs and breakthrough virus replication was further delayed or even prevented (Figures 2A and 2B). In gLTR7+gLTR1-expressing cells, the level of inhibition was similar to that obtained for gLTR7 alone, which is likely due to relatively poor gLTR1-mediated inhibition. In seven dual-gRNA cells, no virus replication and thus also no virus escape was observed in 60-day follow-up. When these dual-gRNA cells were challenged with a higher HIV-1 input (6 and 12 ng instead of 1 ng of CA-p24; Figures 2C and 2D) and followed for a prolonged period (up to 114 and 128 days, respectively), breakthrough virus replication was frequently triggered. However, virus replication was always significantly delayed when compared with unhindered replication in control SupT1 and SupT1-Cas9 cells. Two gRNA combinations (gGag1+gTatRev and gGag1+gEnv2) exhibited a more durable antiviral effect. None of the gGag1+gTatRev cultures and only one of the eight gGag1+gEnv2 cultures demonstrated breakthrough virus replication. The average time required for breakthrough replication in the dual-gRNA SupT1 cells did not correlate with the potency of inhibition of HIV-1 gene expression in transiently transfected 293T cells (Figure 3A), nor with the number of gRNA target sites (not shown). We also did not observe a clear correlation when the breakthrough time was plotted against the level of conservation of the gRNA target sequences (i.e., sum of the Shannon entropies; not shown), which is likely due to the fact that nearly all selected gRNAs target highly conserved HIV-1 sequences. Nevertheless, the gLTR7 combinations with gLTR1 and gLTR9, which target less conserved domains, were less effective in preventing virus breakthrough in all experiments (Figures 2B–2D).

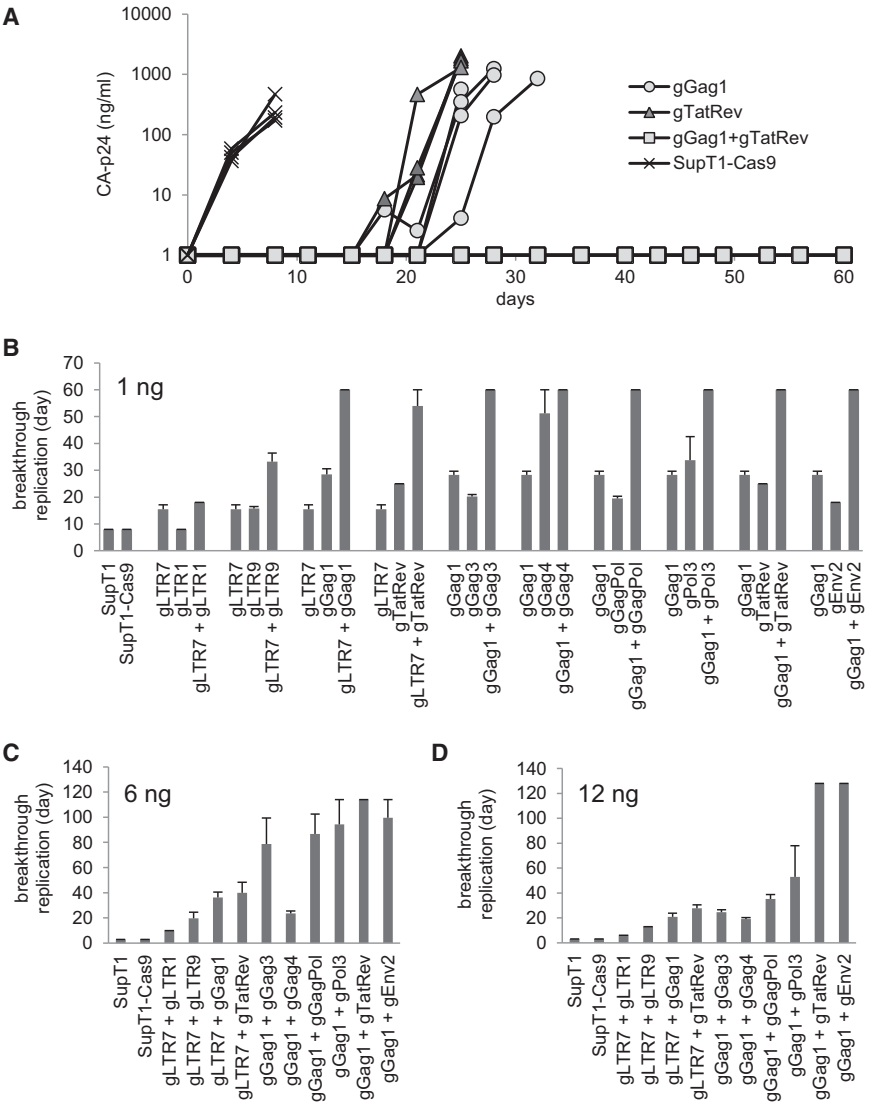


Figure 2. HIV-1 Replication in Cas9- and gRNA-Expressing T Cells

SupT1 T cells stably transduced with lentiviral vectors expressing Cas9 and single or dual gRNAs were infected with HIV-1 LAI virus and cultured for 60 (A and B), 114 (C), or 128 (D) days. (A) Virus replication in gGag1, gTatRev, gGag1+gTatRev, and control SupT1-Cas9 cells infected with 1 ng CA-p24 HIV-1 was monitored by measuring the CA-p24 level in the culture supernatant.

(B–D) The day at which massive virus-induced syncytia were observed (reflecting breakthrough virus replication) was scored for four cell cultures infected with 1 (B), 6 (C), or 12 (D) ng CA-p24 HIV-1. When no virus replication was detected, we scored 60, 114, and 128 days, respectively. The average values ± SEM are shown. SupT1, control non-transduced cells.

Surprisingly, for gRNA combinations against protein-coding sequences, we observed that a larger distance between the two Cas9/gRNA cleavage sites further delayed or even prevented breakthrough replication (Figure 3B).

Monitoring of Escape Mutations in the HIV-1 Targets

We previously demonstrated that breakthrough HIV-1 replication in single-gRNA protected cells was due to acquisition of escape mutations in the gRNA target. Sequence analysis of breakthrough viruses in the dual-gRNA cell cultures revealed mutations in both gRNA target sites (Figure S1). As previously observed when targeting HIV-1 with a single gRNA (Wang et al., 2016a), we predominantly observed deletions and insertions in LTR targets. In protein-coding sequences, we observed only nucleotide substitutions and 3-nt insertions that do not abrogate the open reading frame. All mutations clustered around the Cas9 cleavage site at 3 nt from the PAM, which confirms that these mutations are due to NHEJ DNA repair. Remarkably, we observed mutations in the gGag1 but not the gGag4 target sequence of gGag1+gGag4 breakthrough viruses. Although gGag4 obviously can delay replication, this gRNA may not be a very strong inhibitor and eventual breakthrough replication of the wild-type (WT) virus may occur. Similar breakthrough replication of WT virus has previously been described with moderately active RNAi inhibitors (ter Brake et al., 2008).

Accumulation of Mutations in the Proviral Genome

The gGag1+gTatRev and gGag1+gEnv2 combinations inhibited HIV-1 replication very efficiently, and viral escape was blocked in all or 11 of the 12 challenged cultures, respectively (combined results in Figures 2B–2D). To investigate the extent of Cas9-induced mutations in these cultures with complete virus control, we sequenced the gRNA-target sites of the integrated proviral DNA in protected cell cultures at 110 days after infection with the highest virus input (12 ng of CA-p24). The gRNA targets were individually amplified by PCR and multiple DNA fragments were analyzed by TA cloning and sequencing (Figures S2 and S3). In gGag1+gTatRev cultures, we observed mutations in 95% of gGag1 and 100% of gTatRev sites (genotype test in Figure 4B). Similarly, we observed mutation of 97% of gGag1 and 100% of gEnv2 targets in the gGag1+gEnv2 cultures. We scored mostly indels that consistently clustered around the Cas9 cleavage site (Figures S2 and S3), which is the pattern typical for NHEJ repair following Cas9-mediated cleavage.

As nearly all target sites were mutated at this late time (only 3% to 5% WT gGag1 and no WT gTatRev and gEnv sites at day 110; Figure 4B), we decided to also analyze early culture samples collected at day 12 to study the mutation kinetics (Figures S2 and

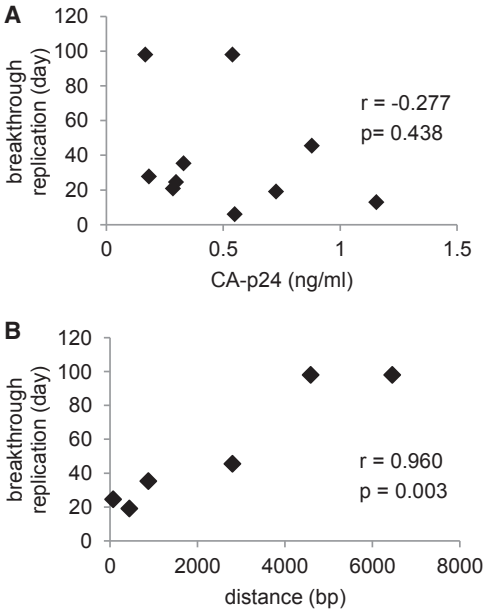


Figure 3. Dual-gRNA-Mediated Inhibition of Breakthrough Replication Does Not Correlate with the Efficiency to Silence HIV-1 DNA but Correlates with Target Site Distance

Correlation between the time required for breakthrough replication in the infected dual-gRNA SupT1 cells (data from Figure 2D) and (A) the level of HIV-1 gene expression measured in transiently transfected 293T cells (data from Figure 1C) and (B) the distance between the target sites (for dual gRNAs targeting protein-coding domains; distance shown in right panel in Figure 1B). The Pearson correlation coefficient (r) and p value (two-tailed) are shown.

S3). This revealed a significant percentage of WT sequences: 38% gGag1 and 25% gTatRev sites in the gGag1+gTatRev culture and 38% gGag1 in the gGag1+gEnv2 culture, but no WT gEnv2 sites were scored (Figure 4B). Thus, a large fraction of the proviral gGag1 and gTatRev sites maintain a WT sequence early but are mutated at a later time. These results indicate progressive target site mutation due to continuous Cas9/gRNA action. In contrast, all gEnv2 sites were mutated early after infection, which indicates a very high activity of this gRNA. Taken together, we observed 25% WT sequences at day 12, which was reduced to 2% at day 110 (Figure 4C). Also the frequency of 1- and 2-nt substitutions decreased significantly (from 9% to 1%), whereas the frequencies of $\geq$3-nt substitutions (from 0% to 3%) and indels (from 66% to 94%) increased significantly. This shift from minor to major mutations over time indicates continued Cas9/gRNA recognition of minor mutants.

At 110 days after infection, we observed mostly indels that either shift the open reading frame or delete/insert codons in highly conserved and essential HIV-1 protein-encoding sequences, which likely inactivate virus replication. The integrated proviral DNA was also PCR amplified with an outer primer set to detect truncated proviruses resulting from cleavage at two targets and subsequent ligation with loss of the intervening fragment. DNA fragments with the anticipated size were successfully detected in the gGag1+gTatRev and gGag1+gEnv2 cultures. Sequencing of the PCR product confirmed deletion of the region between the two Cas9/gRNA sites, and we observed the additional removal of several adjacent base pairs in most sequenced fragments (data not shown), which is in agreement with NHEJ involvement (Ebina et al., 2013; Hu et al., 2014). These severely truncated HIV-1 genomes, missing the complete Pol gene and additional essential sequences, do not encode replication-competent virus.

No Rescue of Replication-Competent Virus upon Long-Term Dual-gRNA Inhibition

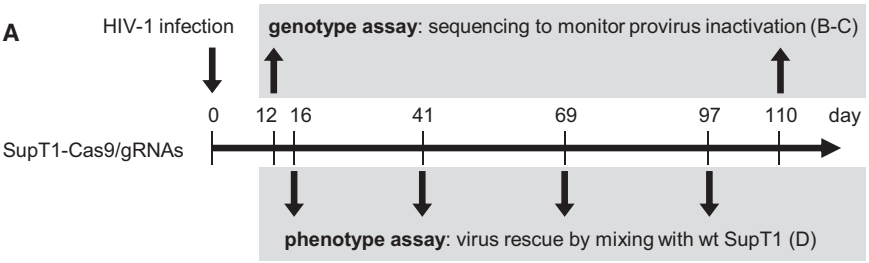
Considering the complete or nearly complete mutation of the proviral target sites in the gGag1+gTatRev and gGag1+gEnv2 cell cultures, we wondered whether we could have cured these cultures of infectious virus. As a sensitive phenotypic screen for the presence of replication-competent virus, we mixed the original cultures at several times after infection with unprotected SupT1 cells (Figure 4A). These cell mixtures were subsequently cultured for up to 30 days to monitor the spread of replication-competent viruses. We tested the original cultures infected with the highest amount of HIV-1 that did not show any sign of virus replication in the 128-day follow-up period (12 ng of CA-p24 infection described in Figure 2D) and monitored the formation of virus-induced syncytia to score virus rescue.

For gGag1+gTatRev, replicating virus could be rescued in co-cultures initiated with samples taken at day 16, but not at later times (Figure 4D). For gGag1+gEnv2, virus replication could be rescued up to 69 days, but not at later times, suggesting the loss of any replication-competent virus. For the positive co-cultures, we noticed that the period needed for viral appearance increased over time, indicating a gradual loss of replication-competent virus (Figure S4). Most importantly, these antiviral gRNAs seem able to fully extinguish all infectious virus over time. Similar rescue of replication-competent virus at early but not late times was possible for the gGag1+gEnv2 cultures infected with an intermediate amount of virus (6 ng of CA-p24 infection described in Figure 2C), whereas virus rescue from similarly infected gGag1+gTatRev cultures failed for all time samples tested (data not shown). The complete virus inactivation measured in this phenotype test is in good agreement with the nearly complete mutational inactivation of both proviral target sites in late samples (genotype test in Figure 4B).

DISCUSSION

In this study, we demonstrate that dual-gRNA-guided CRISPR/Cas9 attack on HIV-1 DNA can efficiently inhibit viral gene expression and replication. We identified gRNA pairs that permanently blocked virus replication in infected T cell cultures. To score the extent of the gRNA/Cas9-induced mutations in these cultures, we sequenced the integrated proviral genomes and performed sensitive co-cultures to detect any residual replication-competent virus. Both target sites had been severely mutated. Although some wild-type sequences and minor point mutations were observed initially, these disappeared over time at the expense of major target site mutations, mostly indels that clustered around the Cas9 cleavage site as expected for

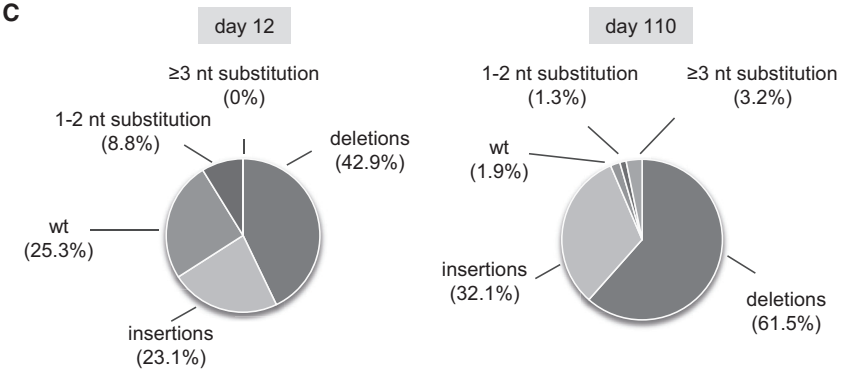
A



B

gRNAs	target region	wild-type sequence frequency (%)	
		day 12	day 110
gGag1 + gTatRev	gGag1	8 / 21 (38.1)	2 / 38 (5.3)
	gTatRev	7 / 28 (25.0)	0 / 39 (0.0)
gGag1 + gEnv2	gGag1	8 / 21 (38.1)	1 / 37 (2.7)
	gEnv2	0 / 21 (0.0)	0 / 43 (0.0)

C



D

gRNAs	culture	virus rescue			
		day 16	day 41	day 69	day 97
gGag1 + gTatRev	1	+	-	-	-
	2	+	-	-	-
	3	+	-	-	-
	4	+	-	-	-
gGag1 + gEnv2	1	+	+	+	-
	2	+	+	-	-
	3	+	+	+	-
	4	+	+	+	-

Figure 4. Genotypic and Phenotypic Analysis of Cas9/gRNA-Targeted HIV-1 Provirus

(A) Timeline of the genotype and phenotype assays performed for the gGag1+gTatRev and gGag1+gEnv2 protected SupT1 cultures that had been infected with HIV-1 LAI (12 ng of CA-p24 infections from Figure 2D).

(B and C) For the genotype assay, the intracellular proviral DNA was isolated at 12 and 110 days after infection. The gRNA target regions were PCR amplified and sequenced upon TA cloning. Sequences of the individual TA cloned sites are shown in Figures S2 and S3. (B) The frequency of the WT target sequence in individual TA clones is shown for each gRNA target site. (C) The frequency of the WT sequence, nucleotide insertions, deletions, and substitutions observed for all analyzed gRNA target sites is shown. Sequences with both an insertion/deletion and nucleotide substitutions were scored as insertion/deletion.

(D) For the phenotype assay, infected cells isolated at different times (16–97 days) were mixed with an equal amount of non-transduced SupT1 cells and cultured for 30 days. Syncytia formation in these cultures was monitored to detect rescue of replication-competent virus. +, syncytia detectable within the 30-day follow-up period; −, no syncytia detectable.

Cas9 cleavage followed by NHEJ DNA repair. This gradual saturation of major mutations coincided with the total loss of replication-competent virus, demonstrating complete virus inactivation. These results indicate that HIV-1-infected cells can be functionally cured by dual-gRNA CRISPR/Cas9 treatment without viral escape.

The observed loss of minor mutations (single- and double-nucleotide substitutions) and appearance of major mutations (indels and multiple nucleotide substitutions) over time indicates continuous Cas recognition of mildly mutated targets, which are thus re-cleaved and hypermutated by subsequent NHEJ repair. These results are in agreement with reports demonstrating that targets with one or two point mutations can still be cleaved by Cas9/gRNA, albeit with reduced efficiency (Fu et al., 2013; Hsu et al., 2013). Likewise, a recent study demonstrated that 1-bp insertions and 1- to 2-bp deletions are dominant early Cas9/gRNA products, but larger deletions appeared later (van Overbeek et al., 2016). Thus, continuous Cas surveillance eventually results in more grossly mutated proviruses, which are less likely to be replication competent. This means that the likelihood of a complete cure increases over time, leaving these cells with inactivated proviruses ("viral grave yard").

The gGag1+gTatRev and gGag1+gEnv2 inhibitor pairs permanently blocked virus replication in all or nearly all infected T cell cultures, respectively, whereas the virus consistently escaped from the corresponding single gRNAs. Prolonged virus inhibition was also observed for most other gRNA pairs compared to the corresponding single gRNAs, but virus escape did occur

eventually. Escape was due to mutations in both target sites that prevent Cas9/gRNA binding and cleavage, but that apparently do not inactivate virus replication. Whereas HIV-1 escape from antiviral drugs and RNAi is predominantly due to mutations introduced during the error-prone reverse-transcription process and escape from combinations of inhibitors requires the accumulation of several mutations during multiple rounds of virus replication, CRISPR/Cas9 escape mutations are generated instantaneously upon Cas9 cleavage by subsequent NHEJ repair of the proviral DNA, and thus even in the absence of virus replication (Wang et al., 2016a, 2016b). When dual gRNAs are applied, both target sites will be promptly mutated in a large fraction of the proviruses. Thus, the first step of virus evolution, that is, the generation of genetic variants, is not a bottleneck for CRISPR/Cas9 escape. However, most mutations will inactivate the virus, especially when conserved essential viral sequences are targeted. Therefore, the second step of evolution, the selection of resistant and replication-competent virus variants, remains a major bottleneck, which explains why dual gRNAs targeting two conserved regions strongly delay or even prevent viral escape.

For gRNA combinations targeting protein-coding sequences, it seemed that virus escape was further delayed when the distance between the targets was increased (Figure 3B). This result cannot easily be explained. In fact, one may have expected the opposite effect because simultaneous Cas9 cleavage at two targets in a mammalian genome, with subsequent deletion of the intervening fragment, was reported to occur more frequently for smaller distances (Canver et al., 2014), although other studies could not reproduce this effect (Chen et al., 2014; He et al., 2015; Xiao et al., 2013). Simultaneous cleavage and fragment deletion in HIV-1 would create a defective provirus and prevent viral escape. If deletion would occur less frequently for larger distances, one would expect a shorter instead of longer delay in viral escape. Although we could detect such truncated proviral DNA products after dual-gRNA attack by PCR analysis, we also identified full-length proviral DNA with mutations at both target sites. Thus, the efficiency of dual cleavage and fragment deletion may be rather low. In agreement with this, Kaminski et al. (2016) demonstrated that a double LTR-attack resulted in incomplete provirus excision in primary $CD4^+$ T cells.

The gGag1+gTatRev combination yielded rapid, robust, and durable HIV-1 inhibition without virus escape and functionally cured the infected cells. Several factors likely contributed to the therapeutic potency of this gRNA combination. First, both gRNAs are potent inhibitors of viral gene expression (Figure 1C) and replication (Figure 2A). Second, both gRNAs target sequences encoding essential structural and regulatory proteins (Gag and the overlapping Tat and Rev frames, respectively), which restricts the options to generate escape virus variants. Most NHEJ-mutated target sequences will provide resistance against Cas9 but are likely incompatible with virus replication. This is certainly true for all indels that shift the open reading frame, but also in-frame indels that insert or remove one or several codons (nucleotide triplets) will likely diminish or abolish virus replication. Only minor sequence variants, in particular nucleotide substitutions that represent silent codon changes, are likely to support virus replication, although this possibility is restricted for the overlapping Tat/Rev codons. Anyhow, although such minor HIV-1 mutants may initially spread in the culture, they will soon be inactivated by a subsequent round of Cas9-NHEJ action.

The potent gGag1+gTatRev antiviral combination seems ideal for pre-clinical testing and further development of a CRISPR/Cas9-based gene therapy for the treatment of HIV-1-infected individuals. However, we realize that major issues need to be addressed in order to develop a safe and effective in vivo gene therapy. This includes the choice of target cell (hematopoietic stem cells or mature T cells), the type of gene delivery vector used (e.g., integrating lentiviral vector seems suitable for an ex vivo gene therapy), and safety aspects, much like we discussed previously for an RNAi-based gene therapy (Herrera-Carrillo and Berkhout, 2015). Another important question concerns the efficacy against different HIV-1 strains. Both gRNAs target highly conserved viral sequences and minor mismatches are tolerated for Cas9 cleavage, but the activity spectrum should be experimentally demonstrated against other viral strains and subtypes. Potential off-target effects induced by the dual-gRNA/Cas9 treatment should also be investigated, and it may be necessary to develop expression systems for regulated or transient expression of the antiviral transgene. Nevertheless, we here demonstrate the proof-of-principle that a dual-gRNA-guided CRISPR/Cas9 system can trigger a functional cure of HIV-1-infected cells.

EXPERIMENTAL PROCEDURES

Plasmids

The lentiviral vector pLenti-SpBsmBI-sgRNA-Hygro (Addgene; 62205) used for the expression of gRNA I was a gift from Rene Maehr (Pham et al., 2016). The lentiviral vectors LentiGuide-Puro (Addgene; 52963) used for the expression of gRNA II and LentiCas9-Blast (Addgene; 52962) containing the human codon-optimized *Streptococcus pyogenes* Cas9-expression cassette were gifts from Feng Zhang (Sanjana et al., 2014). Oligonucleotides encoding HIV-1 targeting gRNAs and control gRNAs targeting the firefly luciferase and EGFP gene (Liao et al., 2015) were ligated into the BsmB1 site of the pLenti-SpBsmBI-sgRNA-Hygro and LentiGuide-Puro vectors. The plasmid pLAI encodes the HIV-1 subtype B isolate LAI (Peden et al., 1991).

Cell Culture, Transfection, and Transduction

Human embryonic kidney 293T cells and SupT1 T cells were cultured as described previously (Das et al., 2011). For the Cas9/gRNA activity assay, 293T cells (at 90% confluence in 2-cm^2 wells) were transiently transfected with 140 ng of pLAI, 350 ng of LentiCas9-Blast, 350 ng of LentiGuide-Puro, and/or 350 ng of pLenti-SpBsmBI-sgRNA-Hygro plasmids using Lipofectamine 2000 (Invitrogen). For the production of an HIV-1 LAI virus stock, 293T cells (75-cm^2 flask) were transfected with 32 μg of pLAI, and after 48 hr, the virus-containing supernatant was centrifuged at 1,500 × *g* for 5 min, filtered (0.45 μm), aliquoted, and stored at −80°C. Virus production was measured by CA-p24 ELISA (Jeeninga et al., 2006).

Lentiviral vectors were produced and titrated as previously described (ter Brake et al., 2006). Briefly, the vectors were produced by transfection of 293T cells with the lentiviral vector plasmid and packaging plasmids pSYNGP, pRSV-rev, and pVSV-g using Lipofectamine 2000. After transfection, the medium was replaced with OptiMEM (Invitrogen), and the cells were cultured for 48 hr. The lentiviral vector containing supernatant was centrifuged, filtered (0.45 μm), aliquoted, and stored at −80°C. SupT1 cells (2×10^5 cells in 1 ml of culture medium) were transduced with an equal amount of LentiCas9-Blast virus particles (30 ng of CA-p24) and cultured with 1 μg/mL blasticidin for 1 week to select SupT1-Cas9 cells. These cells were subsequently transduced with pLenti-SpBsmBI-sgRNA-Hygro (gRNA I) or LentiGuide-Puro (gRNA II) virus particles and cultured with 500 μg/mL hygromycin B or 1 μg/mL puromycin,

respectively, for 2 weeks to select cells expressing Cas9 and a single gRNA. To produce SupT1 cells expressing Cas9 and two gRNAs, the SupT1-Cas9 cells were first infected with the pLenti-SpBsmBI-sgRNA-Hygro (gRNA I) virus followed by hygromycin B selection and subsequently with the LentiGuide-Puro (gRNA II) virus followed by puromycin selection.

HIV-1 Infection and Provirus Analysis

Transduced and control SupT1 T cells (2×10^5 cells in 1 ml of culture medium) were infected with an equal amount of HIV-1 LAI virus corresponding to 1, 6, or 12 ng of CA-p24. Cells were passaged twice a week. Virus spread was monitored by measuring the CA-p24 production in the culture supernatant and scoring the formation of syncytia every 3 or 4 days. To analyze the gRNA-targeted proviral sequence in cultures that did not demonstrate breakthrough virus replication, infected cells were harvested by centrifugation, and the cellular DNA with the integrated provirus was isolated with the QIAGEN DNAeasy kit. The gRNA target regions were amplified by PCR (primers listed in Table S1). The PCR product was cloned in a TA cloning vector, and the sequence of multiple cloned fragments was analyzed. Furthermore, to demonstrate the presence or absence of replication-competent proviruses in these non-breakthrough cultures, the infected cells were mixed with an equal amount of control (non-transduced) SupT1 cells, and the formation of virus-induced syncytia was monitored during co-culturing of the cells for up to 30 days.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.11.057.

AUTHOR CONTRIBUTIONS

G.W., N.Z, B.B., and A.T.D. designed the experiments and wrote the manuscript. G.W. and N.Z. performed the experiments.

ACKNOWLEDGMENTS

G. Wang is recipient of a fellowship of the China Scholarship Council (CSC). We thank Y. Zheng for technical assistance.

Received: October 7, 2016
Revised: November 7, 2016
Accepted: November 18, 2016
Published: December 13, 2016

REFERENCES

Canver, M.C., Bauer, D.E., Dass, A., Yien, Y.Y., Chung, J., Masuda, T., Maeda, T., Paw, B.H., and Orkin, S.H. (2014). Characterization of genomic deletion efficiency mediated by clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 nuclease system in mammalian cells. J. Biol. Chem. *289*, 21312–21324.

Chen, X., Xu, F., Zhu, C., Ji, J., Zhou, X., Feng, X., and Guang, S. (2014). Dual sgRNA-directed gene knockout using CRISPR/Cas9 technology in *Caenorhabditis elegans*. Sci. Rep. *4*, 7581.

Das, A.T., Harwig, A., and Berkhout, B. (2011). The HIV-1 Tat protein has a versatile role in activating viral transcription. J. Virol. *85*, 9506–9516.

De Silva Feelixge, H.S., Stone, D., Pietz, H.L., Roychoudhury, P., Greninger, A.L., Schiffer, J.T., Aubert, M., and Jerome, K.R. (2016). Detection of treatment-resistant infectious HIV after genome-directed antiviral endonuclease therapy. Antiviral Res. *126*, 90–98.

Ebina, H., Misawa, N., Kanemura, Y., and Koyanagi, Y. (2013). Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. Sci. Rep. *3*, 2510.

Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K., and Sander, J.D. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat. Biotechnol. *31*, 822–826.

He, Z., Proudfoot, C., Mileham, A.J., McLaren, D.G., Whitelaw, C.B.A., and Lillico, S.G. (2015). Highly efficient targeted chromosome deletions using CRISPR/Cas9. Biotechnol. Bioeng. *112*, 1060–1064.

Herrera-Carrillo, E., and Berkhout, B. (2015). Gene therapy strategies to block HIV-1 replication by RNA interference. Adv. Exp. Med. Biol. *848*, 71–95.

Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O., et al. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. Nat. Biotechnol. *31*, 827–832.

Hu, W., Kaminski, R., Yang, F., Zhang, Y., Cosentino, L., Li, F., Luo, B., Alvarez-Carbonell, D., Garcia-Mesa, Y., Karn, J., et al. (2014). RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. Proc. Natl. Acad. Sci. USA *111*, 11461–11466.

Jeeninga, R.E., Jan, B., van den Berg, H., and Berkhout, B. (2006). Construction of doxycyline-dependent mini-HIV-1 variants for the development of a virotherapy against leukemias. Retrovirology *3*, 64.

Kaminski, R., Chen, Y., Fischer, T., Tedaldi, E., Napoli, A., Zhang, Y., Karn, J., Hu, W., and Khalili, K. (2016). Elimination of HIV-1 genomes from human T-lymphoid cells by CRISPR/Cas9 gene editing. Sci. Rep. *6*, 22555.

Liang, C., Wainberg, M.A., Das, A.T., and Berkhout, B. (2016). CRISPR/Cas9: a double-edged sword when used to combat HIV infection. Retrovirology *13*, 37.

Liao, H.K., Gu, Y., Diaz, A., Marlett, J., Takahashi, Y., Li, M., Suzuki, K., Xu, R., Hishida, T., Chang, C.J., et al. (2015). Use of the CRISPR/Cas9 system as an intracellular defense against HIV-1 infection in human cells. Nat. Commun. *6*, 6413.

Peden, K., Emerman, M., and Montagnier, L. (1991). Changes in growth properties on passage in tissue culture of viruses derived from infectious molecular clones of HIV-1LAI, HIV-1MAL, and HIV-1ELI. Virology *185*, 661–672.

Pham, H., Kearns, N.A., and Maehr, R. (2016). Transcriptional regulation with CRISPR/Cas9 effectors in mammalian cells. Methods Mol. Biol. *1358*, 43–57.

Sanjana, N.E., Shalem, O., and Zhang, F. (2014). Improved vectors and genome-wide libraries for CRISPR screening. Nat. Methods *11*, 783–784.

ter Brake, O., Konstantinova, P., Ceylan, M., and Berkhout, B. (2006). Silencing of HIV-1 with RNA interference: a multiple shRNA approach. Mol. Ther. *14*, 883–892.

ter Brake, O., 't Hooft, K., Liu, Y.P., Centlivre, M., von Eije, K.J., and Berkhout, B. (2008). Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. Mol. Ther. *16*, 557–564.

van Overbeek, M., Capurso, D., Carter, M.M., Thompson, M.S., Frias, E., Russ, C., Reece-Hoyes, J.S., Nye, C., Gradia, S., Vidal, B., et al. (2016). DNA repair profiling reveals nonrandom outcomes at Cas9-mediated breaks. Mol. Cell *63*, 633–646.

Wang, G., Zhao, N., Berkhout, B., and Das, A.T. (2016a). CRISPR-Cas9 can inhibit HIV-1 replication but NHEJ repair facilitates virus escape. Mol. Ther. *24*, 522–526.

Wang, Z., Pan, Q., Gendron, P., Zhu, W., Guo, F., Cen, S., Wainberg, M.A., and Liang, C. (2016b). CRISPR/Cas9-derived mutations both inhibit HIV-1 replication and accelerate viral escape. Cell Rep. *15*, 481–489.

Xiao, A., Wang, Z., Hu, Y., Wu, Y., Luo, Z., Yang, Z., Zu, Y., Li, W., Huang, P., Tong, X., et al. (2013). Chromosomal deletions and inversions mediated by TALENs and CRISPR/Cas in zebrafish. Nucleic Acids Res. *41*, e141.

Yoder, K.E., and Bundschuh, R. (2016). Host double strand break repair generates HIV-1 strains resistant to CRISPR/Cas9. Sci. Rep. *6*, 29530.

Zhu, W., Lei, R., Le Duff, Y., Li, J., Guo, F., Wainberg, M.A., and Liang, C. (2015). The CRISPR/Cas9 system inactivates latent HIV-1 proviral DNA. Retrovirology *12*, 22.

Cell Reports, Volume *17*

Supplemental Information

A Combinatorial CRISPR-Cas9 Attack on HIV-1 DNA Extinguishes All Infectious Provirus in Infected T Cell Cultures

Gang Wang, Na Zhao, Ben Berkhout, and Atze T. Das

Table S1. Primer sets used for sequencing of gRNA target regions, related to Figures 2 and 4

gRNA target	sense primer	antisense primer
gLTR1/7/9	CAGCATCTCGAGACCTGGAAAAACAT	GCCACCTGACGTCTAAGAAACCATT
gGag1	TAAACACAGTGGGGGGACATCAAG	AATCTGGGTTCGCATTTTGGACCA
gGag3	TAAACACAGTGGGGGGACATCAAG	AATCTGGGTTCGCATTTTGGACCA
gGag4	TAAACACAGTGGGGGGACATCAAG	CTATGTGCCCTTCTTTGCCACAATT
gGagPol	TCAGAGCAGACCAGAGCCAACAG	CCAATCTGAGTCAACAGATTTCTTCC
gPol3	ATAGTAACAGACTCACAATATGCA	AGGTTAAAATCACTAGCCATTGCTCTCC
gTatRev	ATATCAAGCAGGACATAACAAGG	CTATGATTACTATGGACCACACA
gEnv2	GCACCCACCAAGGCAAAGAGAAGAGTGG	CAACCCCAAATCCCCAGGAGCTGTTGATCC

Figure S1. Escape mutations in gRNA target sites, related to Figure 2. The gRNA target regions in breakthrough viruses obtained in cultures of HIV-1 on different SupT1-Cas9-dual gRNA (gRNA I and II) cells were sequenced. For each gRNA, the wild-type HIV-1 nucleotide sequence is shown on top. If applicable, protein codon triplets are boxed in grey and the translated amino acid sequence is shown on the right. The PAM sequence is boxed and the arrowhead indicates the Cas9 cleavage site at position -3. Nucleotide and amino acid substitutions, insertions and deletions (Δ) are indicated.

gRNA I	gRNA II	culture	gRNA I target sequence		gRNA II target sequence		
gLTR7	gLTR1		C C C T C A G A T G C T G C A T A T A A G C A		A T T G G C A G A A C T A C A C A C C A G G G	aa Nef	
		1-3	· · · · · · A · · · · · · · · · · · · · · · ·		· · · · · Δ Δ Δ Δ Δ Δ Δ Δ Δ Δ Δ Δ G T T C · ·	Y F P D W Q N Y T P G P G V · · · · · Δ Δ Δ Δ V R · · ·	
	gLTR9		C C C T C A G A T G C T G C A T A T A A G C A		G G C G T G G C C T G G G C G G G A C T G G G		
		1-3	· · · · · · A · · · · · · · · · · · · · · · ·		· · · · · · · · · · · · · · · · · · G · · · ·		
	gGag1		C C C T C A G A T G C T G C A T A T A A G C A		G T T A A A A G A G A C C A T C A A T G A G G	aa Gag	
		6-2	· · · · · · A · · · · G · · · · · · · · · · · ·		· · · · · · · · · · · · · · · · A · · · · · ·	M L K E T I N E E · · · · · · · · ·	
		6-4	· · · · · Δ · · · · · · · · · · · · · · · · ·		· A ·	· · · · · · · · ·	
	gTatRev		C C C T C A G A T G C T G C A T A T A A G C A		C C T A T G G C A G G A A G A A A G C G G A G A	aa Tat	aa Rev
		1-3	· · · · · · · G G · · · · · · · · · · · · · · ·		· · · · · · · A C G · · · · · · · · · · · · · ·	S Y G R K K R R · · · · R · · · ·	M A G R S G D · D · · · · · ·
		6-4	· · · · · · · G · · · · · · · · · · · · · · · ·		· · · · · · · A C G · · · · · · · · · · · · · ·	· · · · R · · · ·	· D · · · · · ·
		12-3	· · · · · · A · · · G · · · · · · · · · · · · ·		· · · · · · · A C G · · · · · · · · · · · · · ·	· · · · R · · · ·	· D · · · · · ·
gGag1	gGag3		G T T A A A A G A G A C C A T C A A T G A G G	aa Gag	C C A A G G G G A A G T G A C A T A G C A G G	aa Gag	
		6-1	· · · · · · · · · · · · · · · · G · · · · · ·	M L K E T I N E E · · · · · · D · ·	· · · · · A · · · · · · · · · · · · · · · · ·	P R G S D I A G · · · · · · · ·	
		6-2	· · · · · · · · · · · · · · · · C · · · · · ·	· · · · · · H · ·	· · · · · A · · G · · · · · · · · · · · · · ·	· · · · · · · ·	
		12-4	· · · · · · · · · · · · · · · · A · · · · · ·	· · · · · · · · ·	· · · · C · · · · · · · · · · · · · · · · · ·	· T · · · · · ·	
	gGag4		G T T A A A A G A G A C C A T C A A T G A G G	M L K E T I N E E	G A T G A C A G C A T G T C A G G G A G T G G	aa Gag	
		6-1	· · · · · · · · · · · G · · · · · · · · · · · ·	· · · · · · · · ·	· ·	M M T A C Q G V G · · · · · · · · ·	
		6-2	· A · ·	· · · · · · · · ·	· ·	· · · · · · · · ·	
		6-3	· · · · · · · · · · · · · · · · C · · · · · ·	· · · · · · H · ·	· ·	· · · · · · · · ·	
		6-4	· · · · · · · · · · · · · · · · C · · · · · ·	· · · · · · H · ·	· ·	· · · · · · · · ·	
	gGagPol		G T T A A A A G A G A C C A T C A A T G A G G	M L K E T I N E E	C C C T C A G A T C A C T C T T T G G C A A C	aa Gag	aa Pol
		6-4	· · · · · · · · · · · · · · · · T C · · · · · ·	· · · · · · H · ·	· · · · · · · G · · · · · · · · · · · · · · · ·	S L R S L F G N · · · · · · · ·	F P Q I T L W Q · · · V · · · ·
		12-1	· · · · · · · · · · · · · · · · G G · · · · · ·	· · · · · · G · ·	· · · · · · A · · · · · · · · · · · · · · · · ·	· · K · · · · ·	· · · · · · · ·
		12-4	· · · · · · · · · · · · · · · · C · · · · · ·	· · · · · · H · ·	· · · · A C · · · · · · · · · · · · · · · · · ·	· · · · · · · ·	· · T · · · · ·
	gPol3		G T T A A A A G A G A C C A T C A A T G A G G	M L K E T I N E E	G C A T G G G T A C C A G C A C A C A A A G G	aa Pol	
		12-2	· · · · · · · · · · · · · · · · A · · · · · ·	· · · · · · · · ·	· · · · · · · · · · · · · G · · · · · · · · ·	A W V P A H K G · · · · · · · ·	
	gEnv2		G T T A A A A G A G A C C A T C A A T G A G G	M L K E T I N E E	G G A G C A G C A G G A A G C A C T A T G G G	aa Env	
		6-2	· · · · · · · · · · · · · · · · G · C · · · · ·	· · · · · · D · ·	· · · · · · · · · · · · · · · · · · G · · · · ·	G A A G S T M G · · · · · · · ·	

Figure S2. Sequence analysis of HIV-1 proviral DNA in gGag1+gTatRev-protected SupT1 cells, related to Figure 4. Cellular DNA was isolated at 12 and 110 days after HIV-1 infection. The gRNA-targeted region was amplified by PCR and TA cloned. Multiple TA clones (17 to 28 for each culture) were sequenced. Sequences were aligned to the wild-type HIV-1 sequence (ref, reference sequence). The gRNA target and PAM sequence are underlined. The blue arrowhead indicates the Cas9 cleavage site. Mutations are shown in red (wt, wild-type sequence; -x/+x, x nt deleted/inserted; ns, non-silent amino acid substitution).

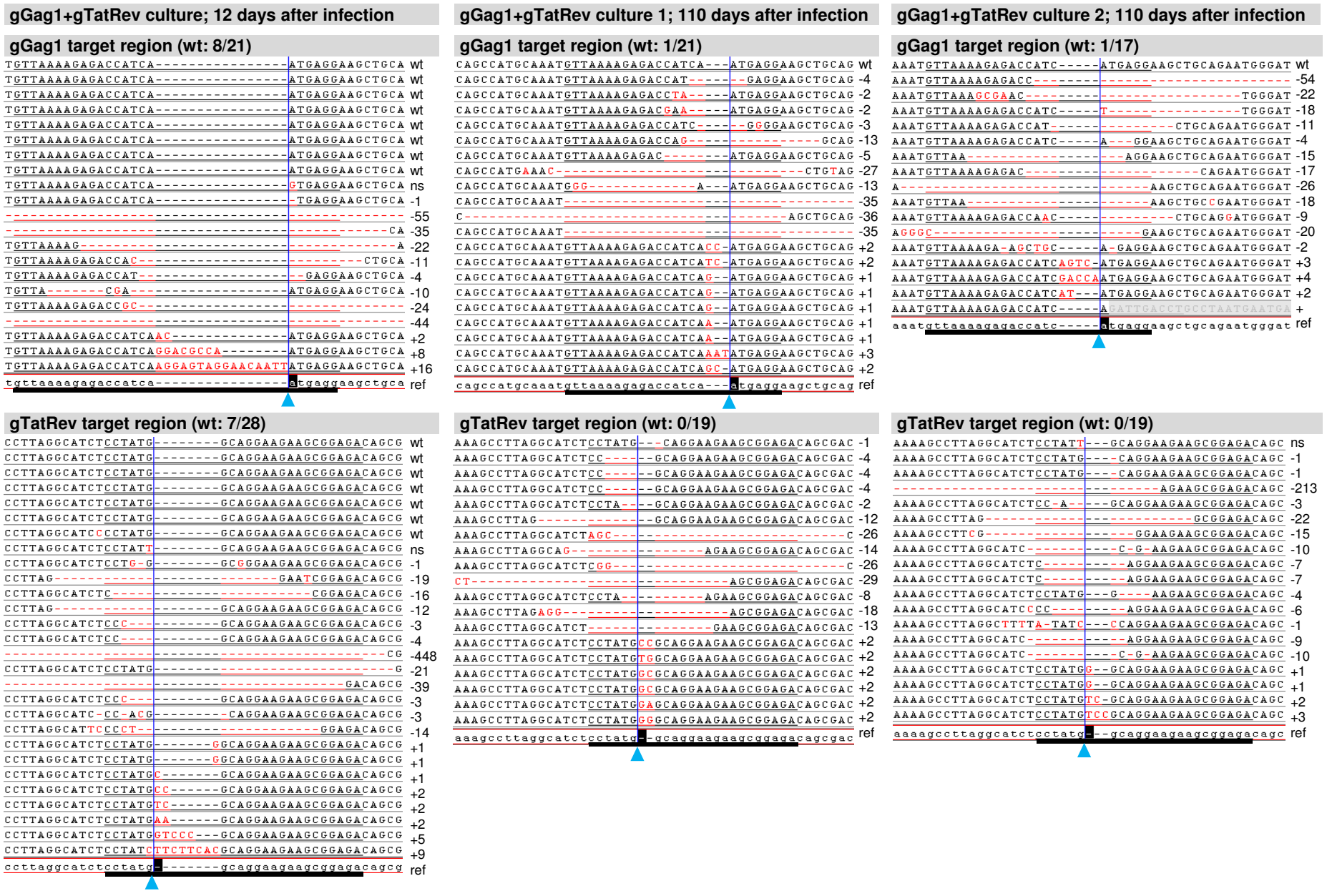
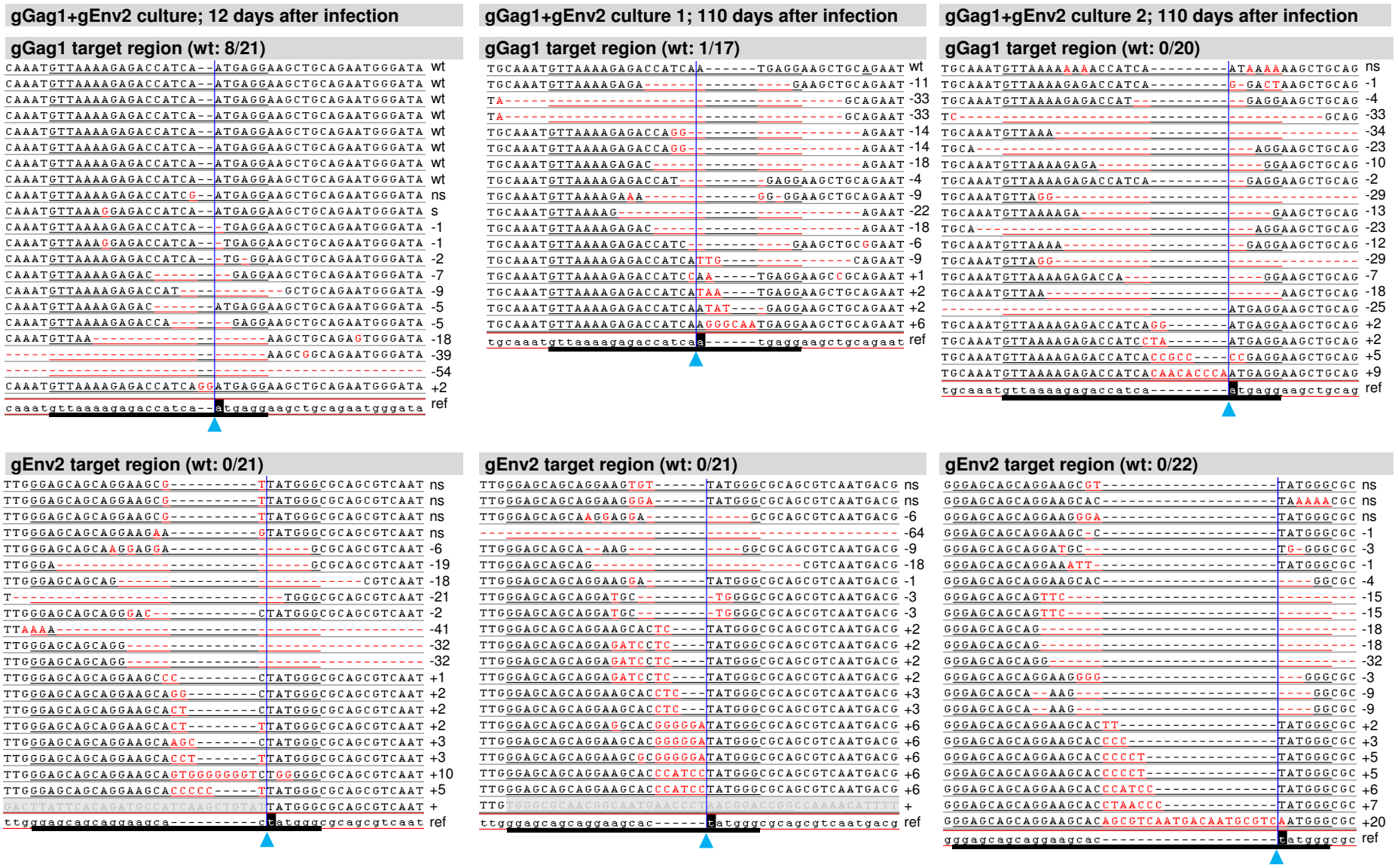


Figure S3. Sequence analysis of HIV-1 proviral DNA in gGag1+gEnv2-protected SupT1 cells, related to Figure 4. Cellular DNA was isolated at 12 and 110 days after HIV-1 infection. The gRNA-targeted region was amplified by PCR and TA cloned. Multiple TA clones (17 to 22 for each culture) were sequenced. Sequences were aligned to the wild-type HIV-1 sequence (ref, reference sequence). The gRNA target and PAM sequence are underlined. The blue arrowhead indicates the Cas9 cleavage site. Mutations are shown in red (wt, wild-type sequence; -x/+x, x nt deleted/inserted; ns, non-silent amino acid substitution).



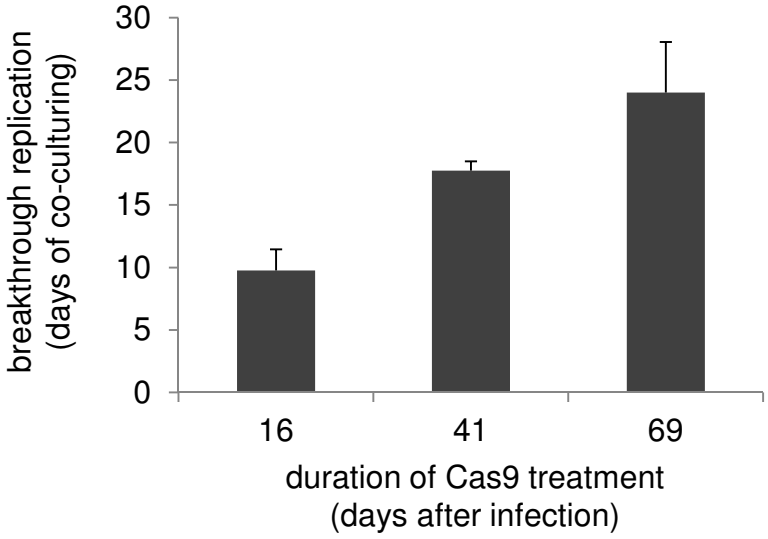


Figure S4. Time required to rescue replication-competent virus from gGag1+gEnv2 protected T cell cultures, related to Figure 4.
SupT1-gGag1+gEnv2 cells infected with 12 ng HIV-1 and cultured for different times (16, 41 and 69 days) were mixed with an equal amount of non-transduced SupT1 cells and cultured for up to 30 days (phenotype assay shown in Figure 4D). The day at which virus-induced syncytia were observed in the co-culture, indicating rescue of replication-competent virus, was scored and the average value (± SEM) is shown for the co-cultures that demonstrated virus replication (n=4 for the day 16 and 41 samples; n=3 for the day 69 samples).

Supplemental Experimental Procedures, related to Figure S1

Sequence analysis of escape variants. To analyze the gRNA-targeted proviral sequence in breakthrough virus cultures, cell-free virus was isolated by centrifugation of the culture medium at the peak of infection, when massive syncytia were observed, and passaged to fresh matching transduced cells. When syncytia were apparent in the newly infected cells, cellular DNA containing the integrated provirus was isolated for PCR and sequencing analysis, as previously described (Konstantinova et al., 2006). PCR fragments including the gRNA target regions were directly sequenced (PCR and sequencing primers listed in Table S1). Because this population sequencing yielded ambiguous sequences for the gLTR7+gLTR1 and gLTR7+gLTR9 cells, the PCR fragments obtained for these gRNA combinations were cloned in a TA cloning vector, followed by sequencing of the insert in 15 TA clones to identify the dominant target region sequence.

Reference

Konstantinova, P., de Haan, P., Das, A.T., and Berkhout, B. (2006). Hairpin-induced tRNA-mediated (HITME) recombination in HIV-1. Nucl. Acids Res. *34*, 2206-2218.