A Single H1 Promoter Can Drive Both Guide RNA and Endonuclease Expression in the CRISPR-Cas9 System

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The RNA-guided endonuclease Cas9 (CRISPR-Cas9) genome editing system has been widely used for biomedical research and holds great potential for therapeutic applications in eukaryotes. The conventional vector-based CRISPR-Cas9 delivery system requires two different RNA polymerase promoters for expression of the guide RNA (gRNA) and Cas9 endonuclease. The large size and relative complexity of such CRISPR transgene cassettes impede their broad implementation, especially in gene therapy applications with viral vectors that have a limited packaging capacity. Here, we report the design of a single-promoter-driven CRISPR-Cas9 system that uses the dual-polymerase (Pol II and Pol III) activity of the H1 promoter. This size reduction strategy of the vector insert provides a significant titer advantage in the lentiviral vector over the regular CRISPR system.

INTRODUCTION
The bacterially derived CRISPR-Cas9 system has been harnessed for genome editing in eukaryotes and holds significant promise for biomedical research and therapeutic applications.1–4 This system is composed of two components: the Cas9 nuclease that induces double-stranded DNA breaks and a guide RNA (gRNA) that guides the Cas9 nuclease to a specific DNA target sequence.5,6

Of the many CRISPR delivery strategies, plasmids and viral vectors that carry the gRNA and Cas9 genes are the most widely used approaches, owing to their relative ease of production and efficiency of cellular transfection and transduction.5,7 In vector-based CRISPR-Cas9 systems, an RNA polymerase (Pol) III (Pol III) promoter like U6 or H1 is commonly used for gRNA production, and a Pol II promoter is used for Cas9 expression. Due to the large size of these transgene cassettes (>4.2 kb for the commonly used Streptococcus pyogenes Cas9 (SpCas9) and ~0.4 kb for the gRNA expression unit), insertion of the complete CRISPR-Cas9 system in a lentiviral vector (LV) causes a severe reduction of the vector titer.8–12 The adeno-associated virus (AAV) represents an alternative viral vector for gene therapy, but its limited packaging capacity (~4.7 kb, including the inverted terminal repeats) also restricts packaging of CRISPR-Cas transgene cassettes.13,14 Thus, there is a need for more simple and smaller CRISPR-Cas transgene constructs.

We recently reported that the human H1 Pol III promoter is also active as Pol II promoter.15 This opens the possibility to produce both the short gRNA (Pol III) and the extended Cas9 mRNA (Pol II) from a single promoter in a small transgene cassette. In the present study, we report the design of a single H1 promoter-driven CRISPR-Cas9 system. Compared with the regular dual-promoter system, this single-promoter system exhibited equally potent gene knockout activity but expressed the gRNA and Cas9 protein at a significantly reduced level, which may improve the safety of gene therapy applications. The big advantage of this new system is the reduced size of the H1-CRISPR-Cas9 cassette, which yields a profound titer advantage in a lentivirus vector production. This should enable similar CRISPR-Cas applications in other viral vectors with limited packaging capacity and facilitate difficult gene therapy applications.

RESULTS
The H1 Promoter Can Act as a Pol II Promoter for Cas9 Protein Production
We recently reported that the human H1 Pol III promoter has robust Pol II activity for expression of a translation-competent mRNA.15 Thus, the H1 promoter has the potential to simultaneously produce a non-coding regulatory RNA and a protein-encoding mRNA. Here, we tested whether a single H1 promoter can express the two components (gRNA and Cas9 nuclease) of the popular CRISPR-Cas9 system.

The widely used pX458 vector was used as backbone.7 This vector contains a Pol III (U6) promoter-driven gRNA expression cassette that is equipped with a T6 termination signal and a downstream

Received 13 September 2018; accepted 26 October 2018; https://doi.org/10.1016/j.omtn.2018.10.016.
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Figure 1. Schematic of the CRISPR-Cas9 Cassettes
(A) The constructs and composition of the CRISPR-Cas9 cassettes (size in kilobases) are shown. Expression of the gRNA is executed by the Pol III promoter U6 or H1, and Cas9 expression is regulated by the Pol II activity of the CBh or Pol III H1 promoter. T6 is the Pol III termination signal. Three tandem FLAG epitope tags (3XFlag) and a nuclear localization signal of simian virus 40 (SV40 NLS) were fused to the Cas9 gene. (B) Illustration of two transcripts (gLuc1 and Cas9) expressed from the H1 promoter to drive Cas9 expression. Construct 4 (H1-gRNA-Cas9) is the truly single-promoter construct in which the H1 promoter to drive Cas9 expression. Construct 2 with the H1 promoter was somewhat less active (87.5% silencing), indicating that gLuc1 expression is limiting and that H1 is a less efficient Pol III promoter than U6, which is consistent with previous results.16,17 We used Northern blot analysis to directly measure gLuc1 expression, which confirmed that U6 generates more gLuc1 than the H1 promoter (Figure 2B). The novel construct 3 is equally efficient in Luc silencing as construct 1, indicating that Cas9 protein is functionally expressed from the H1 promoter in amounts that are at least comparable to that of the standard CBh-driven construct 3. Indeed, this was verified by western blot analysis of Cas9-Flag protein expression (Figure 2C, with quantification in Figure 2D). Most importantly, we observed reasonably good silencing activity (77.6%) with the single-promoter construct 4 (Figure 2A). Perhaps, the best comparison is with the H1-using dual-promoter construct 2. We noticed that both gLuc1 expression and Cas9 expression from the single H1 promoter are significantly reduced (Figures 2B–2D). Reduced Cas9 expression may be due to changes in the 5’ UTR that can affect the translational efficiency. We have no explanation for the observed reduction in gRNA expression. Thus, the single-promoter CRISPR-Cas system is active but needs further optimization.

Improving the CRISPR Efficiency of the Single-Promoter System by gRNA Scaffold Optimization
To improve the activity of the single-promoter CRISPR system, one can optimize the gRNA or Cas9 as a potential limiting factor. Inspection of the anti-Luc gRNA scaffold provides one possible reason for inefficient gRNA expression, as the gRNA scaffold contains a T4 stretch (Figure S2), which can act as a Pol III termination signal. In an unrelated study, we measured ~20% termination efficiency when a T4 signal is inserted in an H1 transcript,18 and a previous study already described the negative effect of this T4 stretch in the gRNA context.19 Thus, we modified the T4 stretch by mutating the fourth T to C, because T3 has no Pol III termination activity.19 Indeed, this mutant (construct 2') outperformed the original construct 2 in Luc knockdown (Figure 3A), and increased gRNA expression was confirmed by Northern blot (quantitation in Figure 3B). Extension of the duplex in the RNA scaffold was reported to improve the efficiency in a synergistic manner with the T3 mutation.19 We extended the base-paired stem of the gRNA with a single base pair (Figure S2), while avoiding the introduction of a start codon or the creation of a too-stable hairpin structure that may affect translation of the downstream Cas9 open reading frame. This stem mutant (2‘) was expressed at a slightly higher level (Figure 3B) yet resulted in improved silencing activity compared to construct 2. This may be due

To test the activity of the four anti-Luc CRISPR constructs, we transfected equimolar amounts of these plasmids into HEK293T cells together with the Luc reporter and a Renilla plasmid to control for variation in transfection efficiency. Luc activity (ratio of Firefly to Renilla) was measured in cellular extracts prepared at 2 days post-transfection. The pX458 and pX458-G control vectors resulted in unhindered Luc expression, and the former value was set at 100% (Figure 2A). We observed potent inhibition (95.9% silencing) of Luc expression for the standard construct 1 with the U6 promoter for gLuc1 expression. Construct 2 with the H1 promoter was somewhat less active (87.5% silencing), indicating that gLuc1 expression is limiting and that H1 is a less efficient Pol III promoter than U6, which is consistent with previous results.16,17 We used Northern blot analysis to directly measure gLuc1 expression, which confirmed that U6 generates more gLuc1 than the H1 promoter (Figure 2B). The novel construct 3 is equally efficient in Luc silencing as construct 1, indicating that Cas9 protein is functionally expressed from the H1 promoter in amounts that are at least comparable to that of the standard CBh-driven construct 3. Indeed, this was verified by western blot analysis of Cas9-Flag protein expression (Figure 2C, with quantification in Figure 2D). Most importantly, we observed reasonably good silencing activity (77.6%) with the single-promoter construct 4 (Figure 2A). Perhaps, the best comparison is with the H1-using dual-promoter construct 2. We noticed that both gLuc1 expression and Cas9 expression from the single H1 promoter are significantly reduced (Figures 2B–2D). Reduced Cas9 expression may be due to changes in the 5’ UTR that can affect the translational efficiency. We have no explanation for the observed reduction in gRNA expression. Thus, the single-promoter CRISPR-Cas system is active but needs further optimization.

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Pol II (CBh) cassette for expression of the SpCas9 enzyme (Figure 1A). Based on pX458, two regular CRISPR constructs were created: pX458-G (U6-gGag-CBh-Cas9), with a gRNA that targets the HIV-1 Gag sequence; and test construct 1 (U6-gLuc1-CBh-Cas9), with a gRNA (gLuc1) against Firefly luciferase (Luc). Construct 2 is similar to construct 1, but the U6 promoter was exchanged for the dual active H1 promoter. Construct 3 is the first novel design with the regular U6-gLuc1 expression cassette but with the H1 promoter to drive Cas9 expression. Construct 4 (H1-gRNA-Cas9) is the truly single-promoter construct in which the H1 promoter is designed to drive the expression of both the promoter-proximal gLuc1 and the distal Cas9 gene. We plotted the size of the different cassettes (Figure 1A), indicating that the single-promoter construct 4 is 0.91 kb shorter than the regular dual-promoter construct 1, which could be a significant advantage in certain viral vector systems. The two transcripts (gLuc1 and Cas9) expressed from the H1 promoter in construct 4 are illustrated in Figure 1B.

To test the activity of the four anti-Luc CRISPR constructs, we transfected equimolar amounts of these plasmids into HEK293T cells together with the Luc reporter and a Renilla plasmid to control for variation in transfection efficiency. Luc activity (ratio of Firefly to Renilla) was measured in cellular extracts prepared at 2 days post-transfection. The pX458 and pX458-G control vectors resulted in unhindered Luc expression, and the former value was set at 100% (Figure 2A). We observed potent inhibition (95.9% silencing) of Luc expression for the standard construct 1 with the U6 promoter for gLuc1 expression. Construct 2 with the H1 promoter was somewhat less active (87.5% silencing), indicating that gLuc1 expression is limiting and that H1 is a less efficient Pol III promoter than U6, which is consistent with previous results.16,17 We used Northern blot analysis to directly measure gLuc1 expression, which confirmed that U6 generates more gLuc1 than the H1 promoter (Figure 2B). The novel construct 3 is equally efficient in Luc silencing as construct 1, indicating that Cas9 protein is functionally expressed from the H1 promoter in amounts that are at least comparable to that of the standard CBh-driven construct 3. Indeed, this was verified by western blot analysis of Cas9-Flag protein expression (Figure 2C, with quantification in Figure 2D). Most importantly, we observed reasonably good silencing activity (77.6%) with the single-promoter construct 4 (Figure 2A). Perhaps, the best comparison is with the H1-using dual-promoter construct 2. We noticed that both gLuc1 expression and Cas9 expression from the single H1 promoter are significantly reduced (Figures 2B–2D). Reduced Cas9 expression may be due to changes in the 5’ UTR that can affect the translational efficiency. We have no explanation for the observed reduction in gRNA expression. Thus, the single-promoter CRISPR-Cas system is active but needs further optimization.

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to the modified gRNA structure, which was actually reported to enhance the binding to Cas9 protein. This latter increase was not as pronounced as that seen for the 2T mutant (Figure 3A). When these two mutations were combined in 2TS, no improved silencing activity was observed when compared to construct 2T, which may indicate that maximal silencing was already reached in this experimental system.

We next moved the TS optimizations into the single-promoter construct 4 (Figure S3). This 4TS variant showed significantly improved silencing activity compared to that of construct 4 (Figure 3A). We also measured a ~2-fold increased gRNA production for 4TS (Figure 3B), whereas Cas9 expression was not affected (Figure S4). In conclusion, we were able to construct an active CRISPR-Cas9 system with a single H1 promoter through the use of an optimized gRNA scaffold.

The Optimized Single-Promoter System versus the Standard Dual-Promoter System

We next compared the standard dual-promoter system (construct 1) with the optimized single-promoter system (construct 4TS) for gLuc1 and two additional anti-Luc gRNAs (gLuc2 and gLuc3). Potent silencing activity was observed when compared to construct 2T, which may indicate that maximal silencing was already reached in this experimental system.
inhibition of Luc expression was observed for the three standard constructs 1 with each of the gRNAs (Figure 4A). The single-promoter construct 4TS exhibited similar, but slightly reduced, silencing efficiency. This reduced activity correlates with reduced gRNA expression (3- to 7-fold) (Figure 4B; Figure S5) and reduced Cas9 expression (5- to 11-fold) (Figure 4B; Figure S5).

To test the dosage effect of the CRISPR-Cas9 component, constructs 1 and 4TS encoding gLuc1 were titrated for Luc knockdown. A clear dosage-dependent effect was apparent for both constructs (Figure 4C). The constructs performed similarly at all concentrations, but silencing activity that was a bit reduced was scored for 4TS at low concentrations.

We next wanted to test the general applicability of the novel single-promoter design. To do so, we transfected constructs 1 and 4TS into cell types other than 293T cells. Very similar activity profiles were apparent in HeLa cells (Figure S6A) and HCT116 cells (Figure S6B), indicating that the single-promoter construct 4TS is functional in diverse cellular contexts.

Chromosomal DNA Targeting by the Single-Promoter CRISPR System

To check whether the optimized single-promoter design 4TS is also effective in CRISPR applications on chromosomal DNA templates, we used the same gRNAs (gLuc1, gLuc2, and gLuc3) to target the chromosomally integrated Luc reporter in HeLa X1/6 cells. Equimolar amounts of the standard 1 and novel 4TS CRISPR constructs were transfected into these cells, together with the Renilla control plasmid and pCMV-rtTA, to allow doxycycline-inducible Luc transcription. Luc knockdown by the single-promoter constructs 4TS is similar to that of the standard construct 1 (Figure 5A), which confirmed the results measured with the episomal Luc reporter plasmid (Figure 4A). These results indicate that the single-promoter 4TS system fulfills the genome editing tasks as efficient as the standard dual-promoter system.

To confirm CRISPR-Cas9-induced breakage of chromosomal Luc DNA and the introduction of insertion or deletion (indel) mutations by means of non-homologous end joining (NHEJ)-mediated DNA repair, we performed the surveyor nuclease assay for the two gLuc1 constructs (1 and 4TS), with the empty pX458 vector as negative control. Both constructs yield a similar amount of cleavage products of the expected size, indicating comparable cleavage efficiency (Figure 5B). Thus, the single-promoter 4TS system can be used for efficient genome editing.

The 4TS CRISPR System Ensures a High LV Titer

Due to the large size of CRISPR-Cas9 cassettes, introduction in the LV delivery system usually yields a low vector titer. Previous reports indicated that an increasing insert length correlates with decreased LV titer. Compared with the standard CRISPR-Cas9 expression system, the single-promoter 4TS system has a smaller size and simplified structure. To evaluate whether the new design can improve the LV titer, the vectors (LV-1 and LV-4TS) were created (Figure 6A). The same molar amount of LV DNA was used for transfection and LV production. The vector production was measured by CA-p24 ELISA before and after LV concentration (Figure 6B). The LV-1 and LV-4TS
and Renilla. This reporter cell line was transduced at MOIs of 0.1, this, we established a stable HEK293T cell line that expresses Luc gene editing when it is integrated in a chromosomal context. To do this, we evaluated the transduction titers on SupT1 cells treated with the same amount of concentrated LV and analyzed the GFP-positive cells by flow cytometry. LV-1 yields a much lower percentage of GFP-positive cells than LV-4TS (Figure 6C), indicating a much higher transduction efficiency of the latter. We also performed the LV transduction by means of viral vectors. Collectively, we demonstrated that the integrated LV-4TS cassette is active for efficient gene editing.

DISCUSSION

In this study, we report that the dual-polymerase active H1 promoter can be used for the simultaneous expression of a gRNA and the Cas9 protein of the CRISPR-Cas9 system. This single H1 system exhibits similar knockdown activity as the standard dual-promoter system across different cell lines, but its shorter size is beneficial for the transduction by means of viral vectors.

The H1 system produces significantly less gRNA and Cas9 nuclease, which may, in fact, improve the vector safety, as increased cellular gRNA and Cas9 expression correlates with increased off-target effects. A change in gRNA and Cas9 levels is likely to have a non-linear effect on on-target/off-target editing efficiency. For example, a 5-fold decrease of the CRISPR plasmid dose in a transfection experiment triggered a 7-fold increase in specificity, at the sacrifice of a modest ~2-fold decrease in on-target efficiency. Therefore, it is advisable that the actual gRNA and Cas9 expression levels are fine-tuned for gene therapy applications. However, the single H1 promoter-driven CRISPR-Cas9 system may reduce off-target effects and, consequently, increase the safety of gene therapy vectors.

This new single H1-gRNA-Cas9 system provides a substantial size advantage over the standard two-promoter system. This is an important feature for therapeutic viral vectors that have a limited genome packaging capacity, such as the popular LV and AAV vectors. We demonstrated that the new LV-H1-gRNA-Cas9 system generates a superior vector with a high transduction titer compared to the standard LV-CRISPR system, which carries two promoter cassettes. The in vivo delivery efficiency of AAV vectors has been hindered by the large size of the SpCas9 protein. Ran et al. recently demonstrated the importance of smaller vector inserts by substituting SpCas9 for the shorter Staphylococcus aureus Cas9 (SuCas9) variant. This novel single H1 promoter system can realize a further gain in vector efficiency.

Lastly, we wanted to test whether this novel LV-4TS cassette enables gene editing when it is integrated in a chromosomal context. To do this, we established a stable HEK293T cell line that expresses Luc and Renilla. This reporter cell line was transduced at MOIs of 0.1, 1, and 5, with LVs based on LV-1 and LV-4TS that express anti-Luc gLuc1 (Figure 6A). An LV without gLuc1 was included in parallel as control. Transduced (GFP-positive) cells were sorted by flow cytometry and maintained for 3 weeks. The dual-luciferase reporter assay was performed at the indicated time points, and the relative Luc activity (Luc:Renilla) was calculated (Figure 6E). Luc knockout manner was increased over time for LV-1 and LV-4TS, compared to the control. LV-1 was a bit more active than LV-4TS at 6 days post-transduction, but very similar knockout efficiencies were measured at later time points (70%–80% Luc knockout efficiency at 16 days post-transduction). These results were independent of the MOI used. Collectively, we demonstrated that the integrated LV-4TS construct is active for efficient gene editing.

vectors produce a similar amount of vector particles, and ~60-fold concentration was achieved by the lentivirus concentration kit. We next evaluated the transduction titers on SupT1 cells treated with the same amount of concentrated LV and analyzed the GFP-positive cells by flow cytometry. LV-1 yields a much lower percentage of GFP-positive cells than LV-4TS (Figure 6C), indicating a much higher transduction efficiency of the latter. We also performed the LV transduction with 3- or 9-fold dilution of the LV stock. As illustrated by the percentage of GFP-positive cells in Figure 6D, LV-4TS yields a ~10-fold higher titer than that of LV-1. Thus, the single-promoter 4TS system provides a profound titer advantage over the standard system.

Figure 5. Targeting a Chromosomal Luciferase Gene

(A) Equimolar amounts of CRISPR constructs and Renilla plasmid were transfected into HeLa X1/6 cells. The rtTA-expressing plasmid is co-transfected for doxycycline-inducible luciferase expression. The luciferase activity was determined 2 days post-transfection. The data represent the mean ± SD (n = 3). (B) Detection of indels by the surveyor nuclease assay. The arrows indicate the truncated DNA fragments due to CRISPR-Cas9-induced indels in the chromosomal Luc gene.

This new single H1-gRNA-Cas9 system provides a substantial size advantage over the standard two-promoter system. This is an important feature for therapeutic viral vectors that have a limited genome packaging capacity, such as the popular LV and AAV vectors. We demonstrated that the new LV-H1-gRNA-Cas9 system generates a superior vector with a high transduction titer compared to the standard LV-CRISPR system, which carries two promoter cassettes. The in vivo delivery efficiency of AAV vectors has been hindered by the large size of the SpCas9 protein. Ran et al. recently demonstrated the importance of smaller vector inserts by substituting SpCas9 for the shorter Staphylococcus aureus Cas9 (SuCas9) variant. The novel single H1 promoter system can realize a further gain in vector efficiency.

This novel H1 system may have some additional unique features. For instance, the Pol III promoter is constitutively and highly active in all cell types, and the same may be true for the Pol II activity. Thus, the new H1 system can likely be applied for robust transgene expression in all cell types. On the negative side, ubiquitous activity of the H1 promoter would not be compatible with cell- or tissue-specific
transgene applications. In addition, the new H1 system does not allow temporal regulation of the CRISPR-Cas activity. These two reasons limit the potential therapeutic use of the new H1 system. The dual-polymerase active H1 promoter can be valuable in totally different biological studies and applications that require the combined expression of a short RNA and protein. The former can, e.g., be a short hairpin RNA (shRNA) to induce the RNAi pathway, and the latter can be any therapeutic protein.

**MATERIALS AND METHODS**

**Plasmid Construction**

Plasmid pX458 (Addgene, #48138) was kindly donated by Feng Zhang and used for expression of the gRNA and the human codon-optimized SpCas9 protein. Complementary oligonucleotides (Tables S1 and S2) encoding the gRNAs targeting either the HIV-1 Gag sequence or the Firefly reporter sequence were annealed and ligated into pX458. DNA fragments encoding H1-gRNAs were synthesized by Integrated DNA Technologies (IDT) and cloned into pX458 by Gibson cloning.
according to a standard protocol (New England Biolabs). The H1 promoter was cloned into pX458 using the XbaI and AgeI sites to create construct 3. The sequence for the H1 promoter is provided in Figure S1. For LV-1 and LV-4T5 construction, the lentCas9-Blast (Addgene, #52962) was used as backbone. The U6-gRNA-CBh and H1-gRNA fragments were amplified with primers containing NheI and XbaI restriction enzyme sites, which were used for ligation into lentCas9-Blast. The two resulting vectors were ligated to the pAE-GFP-EF1α fragment at the Nhel site. The pAE-GFP-EF1α fragment was synthesized by IDT. To make the LV expressing Luc (LV-Luc), the Luc gene of the pGL3 plasmid was PCR-amplified and cloned into LentCas9-blast (Addgene, #48138) using the XbaI and BamHI sites. The LV-Renilla construct was created by amplifying the Renilla luciferase gene from the pRL plasmid and cloning the digested fragment into an LV backbone (Addgene, #84740), using the Nhel and BamHI restriction sites. All constructs were verified by sequencing using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems).

Cell Culture
HEK293T cells, HeLa cells, HCT116 cells, and HeLa X1/6 cells were cultured in DMEM (Life Technologies, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/mL), and streptomycin (100 µg/mL) in a humidified chamber at 37°C and 5% CO2. SupT1 T cells (ATCC CRL-1942) were grown in RPMI (GIBCO BRL, Carlsbad, CA, USA) supplemented with L-glutamine, 1% FCS, penicillin (30 U/mL), and streptomycin (30 µg/mL).

Dual-Reporter Luciferase Assays
HEK293T, HeLa, and HCT116 cells were seeded into 12-well plates to reach ~80% confluency for transfection. For evaluating the anti-Luc activity of respective CRISPR-Cas9 systems, equimolar amounts (32 fmol) of the CRISPR construct (equivalent of 200 ng pX458), 200 ng pGL3-plasmid control, and 4 ng pRL plasmid were co-transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For the titration experiment in Figure 4C, construct 1 or 4T5 encoding gLuc1 was co-transfected with 200 ng pGL3-control plasmid and 4 ng pRL into HEK293T cells using Lipofectamine 2000. Two days post-transfection, luciferase activity was determined with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The ratio of Firefly to Renilla was calculated to represent the relative luciferase activity in the presence of each CRISPR-Cas9 system. Three independent experiments were performed. The resulting values were corrected for between-session variation.

Chromosomal Luciferase Targeting
1.5 × 10^5 HeLa X1/6 cells were seeded in 12-well plates 1 day prior to transfection. Doxycycline (1 µg/mL) was added to the medium. Equimolar amounts of CRISPR constructs (equivalent of 200 ng pX458) were co-transfected with 40 ng pCMV-rtTA plasmid and 4 ng pRL plasmid. Luciferase activity was measured at 2 days post-transfection.

gRNA Detection by Northern Blot
Equimolar amounts of CRISPR constructs (equivalent of 2 µg pX458) were transfected into 6 × 10^5 HEK293T cells in 6-well plates using Lipofectamine 2000 (Invitrogen). Cellular RNA was extracted 2 days post-transfection, and 5 µg total RNA was separated in a 15% denaturing polyacrylamide gel (Precast Novex TBE Gel, Life Technologies) and then electro-transferred to a positively charged nylon membrane (Boehringer Mannheim). Locked nucleic acid (LNA) oligonucleotides pLuc1 or pSca (Table S1) were 5′ end-labeled with the KinaseMax Kit (Ambion) in the presence of 1 µL [γ-32P]-ATP (0.37 MBq/µL, PerkinElmer). The membrane was incubated overnight at 42°C with the labeled LNA oligonucleotides. The blot was washed twice for 5 min at 42°C with 2 × SSC/0.1% SDS and twice for 5 min at 42°C with 0.1 × SSC/0.1% SDS. The signals were exposed by the Typhoon FLA 9500 (GE Healthcare), and densitometric analyses were performed using ImageJ software. Two independent transfections and Northern blots were performed.

Western Blot Analysis
DNA transfection was performed as described for the Northern blot analysis. Two days after transfection, the cells were lysed in RIPA buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Thermo Fisher Scientific) according to the manufacturer’s protocol. The isolated total cellular protein was electrophoresed on a 4%–12% Tris-Glycine gel (Thermo Fisher Scientific) and transferred to a polyvinylidene fluoride (PVDF) membrane. Cas9 protein was detected using an anti-Flag antibody (F1804, Sigma) according to the manufacturer’s instructions. The β-actin protein served as loading control. The signals were visualized by ImageQuant LAS 4000 (GE Healthcare) using the SuperSignal Detection Kit (Pierce).

Surveyor Nuclease Assay
Two days after transfection of CRISPR-Cas9 constructs into HeLa X1/6 cells, the genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN). The genomic region (912 bp) around the designed cleavage site was amplified using S1-Fw and S1-Rev primers (Table S2). The PCR fragments were purified and subjected to surveyor nuclease using the Surveyor Mutation Detection Kit (Integrated DNA Technologies) according to the manufacturer’s instructions. The products were visualized on a 2% agarose gel by ethidium bromide staining.

Lentivirus Production and Transduction
Lentivirus was produced as described previously.25 One day prior to transfection, HEK293T cells were seeded in T75 square-centimeter flasks to reach a confluency of ~80%. The same molar amount of LV-1 and LV-4T5 constructs was transfected together with a fixed amount of packaging plasmids pSYNGP, pRSV-rev, and pVSV-g, using Lipofectamine 2000. After transfection, the medium was replaced with 10 mL OptiMEM (Invitrogen). The supernatant was collected 48 hr post-transfection, filtered (0.45 µm), and concentrated to 200 µL using the Lenti-X Concentrator Kit (TaKaRa). 20 µL
supernatant before and after concentration was collected for CA-p24 ELISA. To determine the vector titer, SupT1 cells (5 × 10^5) were transduced with 13.3, 40, or 120 μL concentrated lentivirus. Three days after transduction, cells were analyzed by flow cytometry for GFP expression.

**Generation of Stable Luc Reporter HEK293T Cell Lines**

To generate stable Luc reporter cell lines via LV transduction, HEK293T cells were infected with vectors based on plasmid LV-Luc and subsequently treated with blasticidin (5 μg/mL) for 10 days. To generate stable Luc and Renilla cells, the HEK293T-Luc cells were infected with viral vectors based on the LV-Renilla plasmid and subsequently treated with puromycin (1 μg/mL) for 1 week. Lentivirus was produced as described earlier.

**Luc Knockout by an Integrated CRISPR Cassette**

One day prior to transduction, 1 × 10^5 dual-reporter HEK293T cells were seeded into a single well of a 48-well plate. The cells were transduced with LV derived from LV-1, LV4-T5, and a control LV at MOIs of 0.1, 1, and 5. The control LV lacked the gLuc1 expression cassette. Three days after transduction, GFP-positive cells were selected by flow cytometry. To ensure the effective removal of GFP-negative cells that may mask the real knockout effect, we performed two additional sortings at 9 and 12 days post-transduction. The dual-reporter luciferase assay was performed 6, 11, and 16 days post-transduction, and the relative luciferase activity (Luc:Renilla) was determined. The Luc:Renilla activity scored for the control LV transduced with a MOI of 0.1 was arbitrarily set at 100%.

**Statistical Analysis**

Student's t test (two-tailed, assuming both populations have the same standard variation) was used to compare two groups of independent experiments. The results were generated by GraphPad Prism v7 software.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and two tables and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.10.016.

**AUTHOR CONTRIBUTIONS**

Z.G., E.H.-C., and B.B. designed the experiments. Z.G. and B.B. drafted the manuscript. Z.G. conducted the experiments, and all authors analyzed the data.

**CONFLICTS OF INTEREST**

The authors declare no competing interests.

**ACKNOWLEDGMENTS**

This research was supported by the AIDS Fonds (HIV– P-37102). Z.G. is supported by a scholarship from the China Scholarship Council (CSC). We thank Yi Zheng for the kind donation of reagents.

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