Molecular Therapy
Nucleic Acids
Original Article

CRISPR-Cas13a Inhibits HIV-1 Infection

Lijuan Yin,[1,4] Fei Zhao,[1,4] Hong Sun,[1,4] Zhen Wang,[2] Yu Huang,[1] Weijun Zhu,[1] Fengwen Xu,[1] Shan Mei,[1] Xiaoman Liu,[1] Di Zhang,[1] Liang Wei,[1] Shan Cen,[3] Siqi Hu,[1] Chen Liang,[2] and Fei Guo[1]

[1]NHC Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, and Center for AIDS Research, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, P.R. China; [2]McGill University AIDS Centre, Lady Davis Institute, Jewish General Hospital, Montreal, QC H3T 1E2, Canada; [3]Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100005, P.R. China

CRISPR-Cas provides bacteria and archaea with immunity against invading phages and foreign plasmid DNA and has been successfully adapted for gene editing in a variety of species. The class 2 type VI CRISPR-Cas effector Cas13a targets and cleaves RNA, providing protection against RNA phages. Here we report the repurposing of CRISPR-Cas13a to inhibit human immunodeficiency virus type 1 (HIV-1) infection through targeting HIV-1 RNA and diminishing viral gene expression. We observed strong inhibition of HIV-1 infection by CRISPR-Cas13a in human cells. We showed that CRISPR-Cas13a not only diminishes the level of newly synthesized viral RNA, either from the transfected plasmid DNA or from the viral DNA, which is integrated into cellular DNA, but it also targets and destroys the viral RNA that enters cells within viral capsid, leading to strong inhibition of HIV-1 infection. Together, our results suggest that CRISPR-Cas13a provides a potential novel tool to treat viral diseases in humans.

INTRODUCTION

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) genes constitute an RNA-guided adaptive immune system that protects bacteria and archaea against viruses and foreign DNA.[1–7] The CRISPR-Cas system is divided into two major classes, which are further separated into six types (I to VI) and 33 sub-types, according to the architecture of the CRISPR array and the signature interference effector.[7] Cas13a (formerly C2c2) is a newly identified class 2, type VI CRISPR-Cas effector endonuclease.[8,9] In contrast to type II Cas9, Cas13a is a ribonuclease that catalyzes both CRISPR RNA (crRNA) maturation and RNA-guided ssRNA (single-stranded RNA) degradation in an interdependent fashion involving two separated catalytic sites.[10–14] After activation by the target RNA, surrounding RNA molecules in the solution are cleaved as well in an unspecific manner. However, this unspecific RNA degradation by Cas13a observed *in vitro* and in prokaryotic cells has not been reported in eukaryotic cells.[12,13,15] It has been demonstrated that the collateral cleavage activity of Cas13a could be used to detect specific RNA transcripts with very low copies.[16] Exploiting the promiscuous RNase activity of Cas13a upon target recognition, diagnostic tools have been developed for detection of Zika and Dengue viruses.[17,18] Recently, Qin et al.[19] developed an automated microfluidic system and a sensitive fluorometer, coupled with a fully solution-based CRISPR assay for detection of Ebola viral RNA. A rapid detection method for H7N9 influenza virus based on a CRISPR-Cas13a nanomachine has also been successfully established.[20] In addition, Chen et al.[21] generated a system to rapidly detect N1-methyladenosine (m^1A)-induced mismatch.

In addition to its utility in RNA detection, Cas13a has been expressed in cells to knock down either reporter or endogenous RNA with efficiency comparable to RNA interference (RNAi) and higher specificity, thus providing an alternative technology for the development of new therapeutics.[10,15,22,23]

During the preparation of our manuscript, researchers reported that Cas13 can be harnessed to target some RNA viruses, including lymphocytic choriomeningitis virus (LCMV), influenza A virus (IAV), vesicular stomatitis virus (VSV),[24] Dengue virus (DENV),[25] and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2),[26] supporting CRISPR-Cas13 as a powerful antiviral technology to inhibit a wide variety of RNA viruses. HIV replicates in cells using the reverse transcriptase enzyme to produce DNA from viral RNA genome. We have designed Cas13a/crRNA to target HIV-1 RNA and investigated the inhibitory effect on HIV-1 RNA expression and viral infection.

First, we observed that the *Leptotrichia buccalis* (Lbu) Cas13a efficiently knocked down EGFP mRNA in human cells. Second, we showed that Cas13a effectively diminished HIV-1 RNA expression and as a result, strongly inhibited HIV-1 infection. Lastly, we observed that Cas13a suppressed viral RNA expression from the activated latent HIV-1 DNA. Our results suggest a potential utility of Cas13a in controlling the infection of HIV-1 and other viruses.

Received 24 February 2020; accepted 26 May 2020;
https://doi.org/10.1016/j.omtn.2020.05.030.

[4]These authors contributed equally to this work.

Correspondence: Siqi Hu, NHC Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, and Center for AIDS Research, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, P.R. China.
E-mail: husiqi_2000@163.com
Correspondence: Chen Liang, McGill University AIDS Centre, Lady Davis Institute, Jewish General Hospital, Montreal, QC H3T 1E2, Canada.
E-mail: chen.liang@mcgill.ca
Correspondence: Fei Guo, NHC Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, and Center for AIDS Research, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, P.R. China.
E-mail: guofei@ipb.pumc.edu.cn

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

RESULTS

LbuCas13a Knocks Down RNA in Human Cells

First, we evaluated the ability of LbuCas13a to degrade RNA in human cells. To this end, we cloned the codon-optimized LbuCas13a into mammalian expression vectors with the FLAG tag attached to the C terminus. The vector also has the U6-driven crRNA cassette (Figure S1A). To target the EGFP sequence, we designed and constructed crRNAs complementary to sequences of three different regions of EGFP RNA (Figure S1B). We co-transfected LbuCas13a/crRNA and EGFP plasmid DNA into HEK293T cells (Figure S1C) and measured EGFP expression by western blotting, flow cytometry, and fluorescence microscopy. After 48 h of transfection, all three crRNAs caused efficient EGFP knockdown in the HEK293T cells (Figures S1D–S1G), as a result of a decrease in EGFP mRNA expression (Figures S1H and S1I). We next transfected the LbuCas13a/crRNA vector into the HEK293 cell stably expressing EGFP. Levels of EGFP were reduced by 3-fold, concomitant with a 4- to 5-fold decrease in EGFP mRNA (Figure S2). These data demonstrate that LbuCas13a, with the guide of its crRNA, is able to diminish RNA expression in HEK293T cells.

Nuclease Activity Is Required for Cas13a to Knock Down RNA in Human Cells

Cas13a contains two HEPN domains, both of which bear the nuclease active site. We created a catalytically inactive Cas13a mutant, dLbuCas13a, by mutating the histidine and arginine catalytic residues within the two HEPN domains (Figure S3A). Both wild-type (WT) and dLbuCas13a proteins express equally well in HEK293T cells (Figure S3B). As expected, when the dLbuCas13a was transfected into EGFP-expressing HEK293T cells, EGFP protein and mRNA expression were not affected, as opposed to marked reduction of EGFP as a result of the expression of WT LbuCas13a (Figures S3C–S3E). These data indicate that the nuclease activity is essential for Cas13a to knock down RNA.

Inhibition of HIV-1 Production by Cas13a

We next tested whether Cas13a/crRNA is able to target and lead to degradation of HIV-1 RNA. We therefore designed crRNAs that target HIV-1 long terminal repeat (LTR), *gag*, *tat*, and *rev* regions (Figure 1A; Table S1). A crRNA targeting both yellow fluorescent Protein (YFP) and EGFP RNA (called crYFP or crEGFP) was included as a positive control. When being transfected with plasmid DNA expressing LbuCas13a and crRNA, these Cas13a/crRNA-expressing HEK293T cells showed similar viability to that of the control cells (Figure S4). We then transfected HEK293T cells with LbuCas13a/crRNA and pNL4-3-ΔE-YFP plasmid DNA. 48 h after transfection, cells were harvested and YFP expression from HIV-1 DNA was monitored by flow cytometry. The results showed 4- to 5-fold decrease in YFP expression in cells co-transfected with HIV-1 RNA-targeting crRNA (Figures 1B–1D). Overall, crRNAs targeting the LTR and the *rev* gene inhibit YFP expression more strongly than the other crRNAs tested (Figure 1E). This inhibitory effect was supported by the marked reduction in viral Gag expression in cells expressing the HIV-1-targeting Cas13a/crRNA (Figure 1F). We next extracted total RNA from Cas13a/crRNA transfected cells and performed quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to measure levels of HIV-1 RNA. A 3- to 4-fold decrease was observed with HIV-1 RNA in cells expressing HIV-1-targeting Cas13a/crRNA (Figure 1G). Lastly, we measured the production of HIV-1 particles by western blotting and observed a drastic loss of HIV-1 particles from cells expressing HIV-1-targeting Cas13a/crRNA (Figure 1H). We also performed the above experiments using the catalytically inactive Cas13a and observed no effect either on YFP expression or Gag expression (Figure S5). Together, these results demonstrate that Cas13a inhibits HIV-1 production by diminishing the expression of viral RNA and viral proteins.

Cas13a Inhibits HIV-1 Infection

We then asked whether Cas13a is able to protect cells from HIV-1 infection. To test this, we first used replication-incompetent pseudotyped lentiviruses as the target to restrict our analysis to a single round of viral infection. We first transfected HEK293T cells with plasmid DNA expressing Cas13a and crRNA. 6 h after transfection, 293T cells were infected with the HIV-$1_{NL4\text{-}3\text{-}\Delta E\text{-}YFP}$ reporter virus pseudotyped with VSV-G. The results of flow cytometry showed that YFP expression was reduced by 65%–80% in cells expressing HIV-1-targeting crRNA (Figures 2A and 2B), which correlated with the decrease in YFP RNA that was expressed from HIV-1 DNA (Figure 2C). We also observed a dose-dependent reduction in YFP expression with an increasing expression of Cas13a and crRNA (Figure S6). Furthermore, crRNAs targeting the LTR and the *rev* gene inhibited HIV-1 infection more strongly than the other crRNAs tested (Figure 2D). These defects in HIV-1 gene expression, caused by Cas13a/crRNA, were reflected in the marked decrease in the production of HIV-1 particles (Figure 2E). Because crRNAs targeting the LTR2 strongly inhibit YFP expression, we introduced consecutive single and double substitutions in the spacer (Figure S7). We then quantified YFP expression with these mismatched spacers in the HIV NL4-3 infection assay and found that Cas13a was fully tolerant to single mismatches across the spacer (Figure S7A). However, when we introduced consecutive double substitutions, we found a reduction in the protection for mismatches in the center, but not at the 5′ or 3′ end, of the crRNA (Figure S7B). We measured RNA levels of Tubulin, HPRT1, ACTIN, and GAPDH by qRT-PCR and did not observe significant Ct value changes of these genes (Figure S7C) as a result of Cas13a expression. This result is consistent with previous studies showing that Cas13 orthologs have minimal off-target effects.[10,11,27] Together, these data demonstrate that Cas13a/crRNA inhibits HIV-1 infection and that the specificity of Cas13a is more dependent on the center sequence of the spacer.

Cas13a/crRNA Impairs Multiple Steps of HIV-1 Infection

In addition to targeting and diminishing the newly synthesized HIV-1 RNA, we asked whether Cas13a/cRNA can also abrogate the viral RNA coming into cells within viral capsid. We thus extracted the total RNA from HEK293T cells, expressing Cas13a/crRNA, which had been infected with HIV-$1_{NL4\text{-}3\text{-}\Delta E\text{-}YFP}$ virus for 3 h, and performed qRT-PCR to measure the levels of HIV-1 RNA that had entered cells

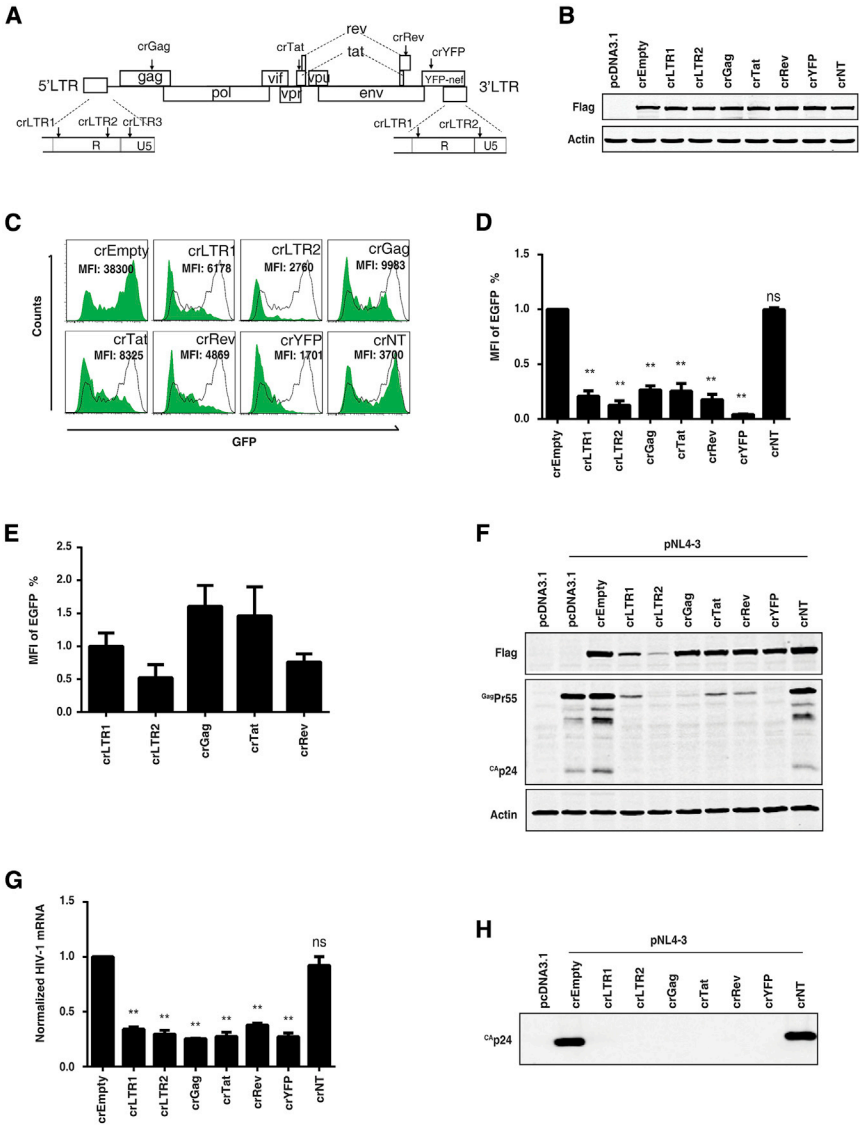

Figure 1. Cas13a/crRNA Inhibits HIV-1 Gene Expression and Virus Production from the Transfected Plasmid DNA

(A) Illustration of the crRNAs that target HIV-1 genome. Positions of the nucleotide refer to the HIV-1 (NL4-3) genome. Arrows indicate location of the crRNA. (B) Western blotting to measure the expression of LbuCas13a. (C) Cas13a/crRNA diminishes HIV-1 gene expression. HEK293T cells were co-transfected with LbuCas13a/crRNA and pNL4-3-ΔE-YFP plasmid DNA. 48 h post transfection, cells were harvested and YFP expression from HIV-1 LTR promoter was monitored by flow cytometry. (D) MFI (mean fluorescence intensity) of YFP. The MFI value in cells that were treated with empty crRNA was arbitrarily set as 1. Error bars indicate SD (n = 3; *p < 0.05; **p < 0.01; Dunnett's t test). (E) MFI of YFP. The MFI value in cells that were treated with LTR1 was arbitrarily set as 1. (F) Effect of Cas13a/crRNA on HIV-1 Gag expression, measured by western blotting. (G) HIV mRNA was quantified by real-time RT-PCR. Results obtained with the empty crRNA vector were arbitrarily set as 1. Error bars indicate SD (n = 3; *p < 0.05; **p < 0.01; Dunnett's t test). (H) Cas13a/crRNA inhibits virus production. Viruses in the supernatants were harvested by ultracentrifugation and the amounts were determined by western blotting with anti-HIV-1 p24/Gag antibodies.

within viral capsid. As shown in Figure 2F, HIV-1-targeting Cas13a/crRNA caused about 50% reduction in viral RNA. This decrease led to lowered levels of the late viral DNA and the integrated viral DNA in the infected cells (Figures 2G and 2H). Furthermore, we expressed Cas13a in Jurkat cells by electroporation, and then infected cells with HIV-1 for 3 days. The results showed a significant decrease in p24 production in cells transfected with the Cas13a/crRNA plasmid DNA (Figures 3A and 3B), demonstrating that Cas13a suppresses HIV replication in $CD4^+$ T cells.

Cas13a/crRNA Diminishes RNA Expression from Latent Proviral DNA

We further tested whether Cas13a/crRNA inhibits RNA expression from the integrated DNA. Experiment was performed with HEK293 cells carrying the integrated LTR-EGFP DNA, which expresses EGFP under the control of HIV-1 LTR promoter. These LTR-EGFP reporter cells were transfected with Cas13a/crRNA, and EGFP expression was monitored by flow cytometry. A significant reduction of EGFP expression was observed with Cas13a/crRNA (Figures 4A–4C). We next measured the effect of Cas13a/crRNA on RNA expression from latent HIV-1 DNA using the JLat10.6 HIV latent cells.[28] JLat10.6 cells carry HIV-1 DNA that is transcriptionally dormant in the absence of external stimulation. Treatment with cytokines such as tumor necrosis factor alpha (TNF-α) activates viral gene expression, which can be detected by monitoring the expression of EGFP that has been inserted into HIV-1 DNA as a reporter. The results showed a significant decrease in EGFP expression in cells transfected with Cas13a/crRNA plasmid DNA (Figure 5A). Furthermore, results of qRT-PCR showed that levels of EGFP RNA were markedly diminished in cells that were transfected with HIV-1 targeting crRNAs (Figure 5B). Lastly, we measured the production of HIV-1 particles by western blotting and observed a drastic loss of HIV-1 particles from cells expressing HIV-1 targeting Cas13a/crRNA (Figure 5C). These data suggest that Cas13a/crRNA has the potential to suppress the expression of viral RNA from the latent HIV-1 DNA.

DISCUSSION

CRISPR is an immune defense mechanism that bacteria and archaea employ to resist viruses and foreign nucleic acids. The discovery of

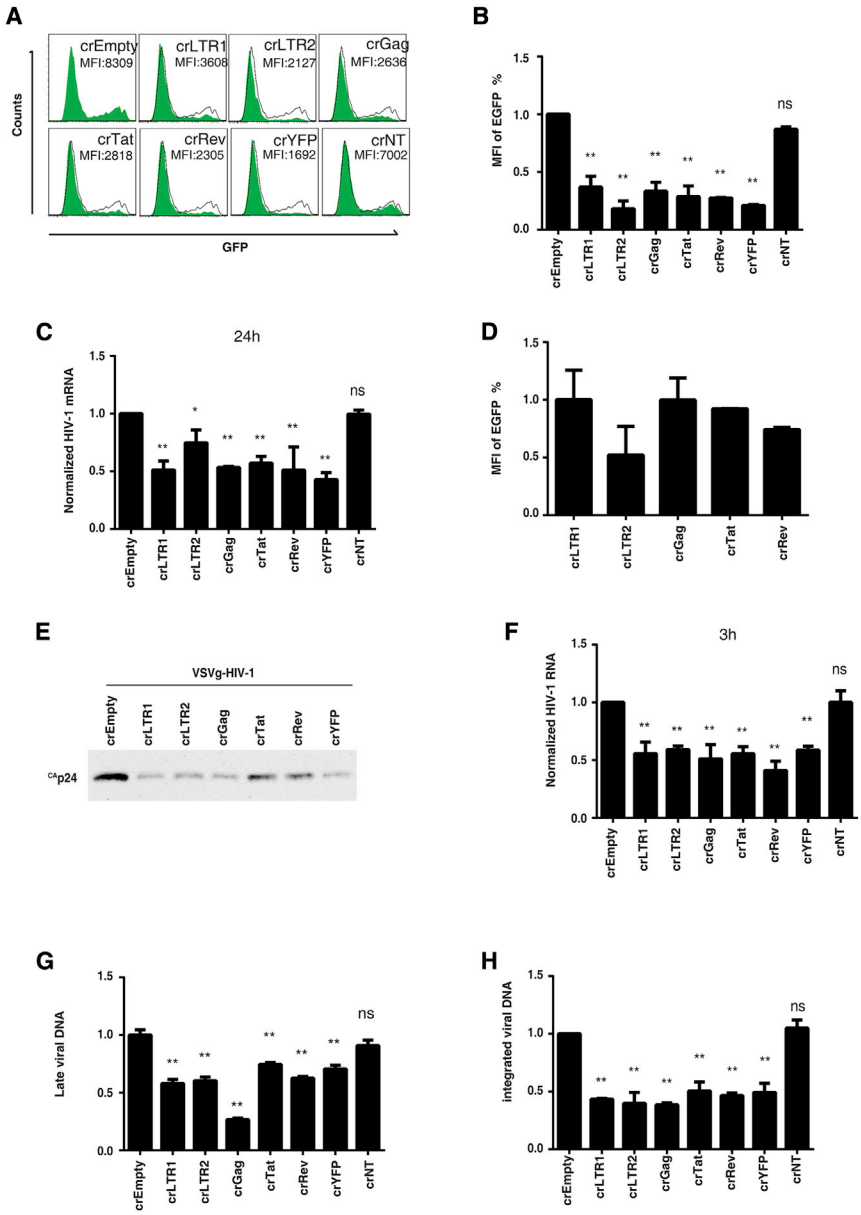

Figure 2. Cas13a/crRNA Inhibits HIV-1 Infection

(A and B) HEK293T cells were transfected with the Cas13a/crRNA plasmid DNA. 6 h after transfection, cells were infected with HIV-1 (carrying YFP reporter) for 36 h. YFP expression from the infected HIV-1 was measured by flow cytometry. Representative histograms are shown in (A). Values of YFP MFI are shown in (B). YFP MFI in cells that were treated with empty crRNA was arbitrarily set as 1. Error bars indicate SD (n = 3; *p < 0.05; **p < 0.01; Dunnett's t test). (C) HIV-1 RNA was quantified by real-time RT-PCR. Results obtained with the empty crRNA vector were arbitrarily set as 1. Error bars indicate SD (n = 3; *p < 0.05; **p < 0.01; Dunnett's t test). (D) YFP MFI in cells that were treated with LTR1 crRNA was arbitrarily set as 1. Error bars indicate SD. (E) Cas13a/crRNA impairs virus production. Viruses in the supernatants were harvested by ultracentrifugation, followed by western blotting to measure levels of HIV-1 Gag protein. (F) Cas13a/crRNA attacks the HIV-1 RNA entering into cells. HEK293T cells were transfected with Cas13a/crRNA and infected with HIV-1. 3 h after infection, real-time RT-PCR was performed to measure levels of HIV-1 RNA that entered into cells within viral capsid. Results of three independent infections are shown with the value of control set as 1. Error bars indicate SD (*p < 0.05; **p < 0.01; Dunnett's t test). (G) Cas13/crRNA diminishes the production of HIV-1 DNA. Real-time PCR was performed to determine the levels of HIV-1 late reverse transcription products using the (gag-U5) primers. Results of three independent infections are shown, with the data of the control set as 1. Error bars indicate SD (*p < 0.05; **p < 0.01; Dunnett's t test). (H) Cas13a/crRNA reduces the levels of integrated HIV-1 DNA. Values in cells that were treated with empty crRNA vector were arbitrarily set as 1. Error bars indicate SD (n = 3; *p < 0.05; **p < 0.01; Dunnett's t test).

Cas13a provides a new tool to protect species other than bacteria and archaea against virus infections. Indeed, researchers have programmed and expressed Cas13a/crRNA in plants in an attempt to inhibit TMV (Tobacco mosaic virus), SRBSDV (Southern rice black-streaked dwarf virus), and RSMV (Rice stripe mosaic virus). The results demonstrate that Cas13a functions in plants by successfully targeting and degrading viral RNA and conferring resistance to these RNA viruses.[29] A separate study also showed that Cas13a inhibits the replication of Potyviruses in plants.[22] Aman et al.[22] used this approach to create resistance against Turnip mosaic virus (TuMV) in Nicotiana benthamiana. Recently, Freije et al.[24] demonstrated that Cas13 is a potent antiviral against mammalian RNA viruses.

RNAi was first discovered in plants, which was quickly developed into a tool to control gene expression by diminishing RNA production. RNAi can be delivered in multiple forms of small RNA duplex, including microRNA (miRNA), small interfering RNA (siRNA), Dicer substrate RNA (DsiRNA), or short hairpin RNA (shRNA).[30] Not surprisingly, many studies have demonstrated inhibition of HIV-1 replication by RNAi targeting conserved regions of HIV-1 genome, including *gag*, *pol*, *vif*, and *env*.[31–34] RNAi has also been used to diminish the expression of HIV-1 co-receptor CCR5, to protect lymphocytes from infection by CCR5 tropic HIV-1.[35] HIV-1 does escape from RNAi, either by mutating the targeted viral sequence or changing viral sequences outside the RNAi targets.[36–39] In any case, a proper combination of shRNA has shown sustainable suppression of HIV replication.[40–43] Multiple sets of different crRNAs will enhance the efficiency of Cas13a-mediated RNA cleavage.

In this study, we have shown that Cas13a can inhibit HIV-1 infection through targeting HIV-1 RNA and diminishing viral gene expression.

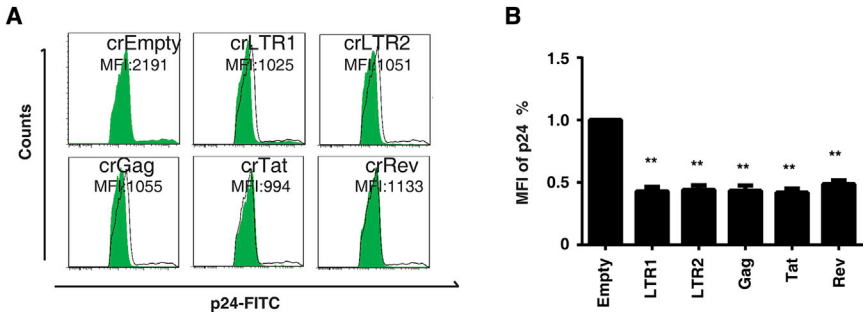

Figure 3. Cas13a/crRNA Inhibits HIV-1 Infection in Jurkat Cells

(A and B) Jurkat cells were transfected with the Cas13a/crRNA plasmid DNA. 6 h after transfection, cells were infected with HIV-1 for 72 h. p24 expression from the infected HIV-1 was measured by flow cytometry. Cells were first gated by FSC/SSC to exclude debris, FLAG+/p24+ cells were gated for p24 expression analysis. Representative histograms are shown in (A). Values of p24 MFI are shown in (B). p24 MFI in cells that were treated with empty crRNA was arbitrarily set as 1. Error bars indicate SD (n = 3; *p < 0.05; **p < 0.01; Dunnett's t test).

This suggests that Cas13a has the potential to effectively control viral infection through the RNAi mechanism. We also demonstrate that Rev-targeting crRNA inhibits HIV-1 infection most efficiently. Coincidentally, the Rev DNA is also vulnerable to Cas9 cleavage and inhibition,[44] thus may represent a common target for different CRISPR effectors. Cas13a not only diminishes the level of newly synthesized viral RNA, either from the transfected plasmid DNA or from the viral DNA that is integrated into cellular DNA, but it also targets and destroys the viral RNA that enters cells within viral capsid. By targeting viral RNA at the early and late stages of HIV-1 infection, Cas13a is expected to generate strong resistance to HIV-1 infection. We also observed that Cas13a suppresses the expression of viral RNA from the activated latent HIV-1 DNA, suggesting a potential utility of Cas13a in "locking" HIV-1 latency toward a functional cure.

Previous studies have shown that newly transcribed HIV-1 mRNA in the cytoplasm is sensitive to siRNA degradation, but the incoming HIV-1 RNA genome is not targeted by RNAi, probably because of viral protection against the RNAi machinery.[45,46] In contrast, our data demonstrate that CRISPR-Cas13a readily destroys the incoming HIV-1 RNA. Another advantage of Cas13a over siRNA, miRNA, and shRNA is that the activity of Cas13a does not depend on the cellular RNAi machinery. Thus, Cas13a can effectively inhibit viruses that have evolved strategies impairing cellular RNAi pathways.[47–50] Similar to other CRISPR effectors whose specificity depends on the matching of guide RNA (gRNA) to the target DNA or RNA, Cas13a may also exert off-target effects by cleaving RNA molecules that do not completely match the crRNA sequence. This possibility is supported by our data showing that Cas13a cleavage is not affected by single point mutations in the target RNA. This issue may worsen if the activated Cas13a exhibits non-specific RNA cleavage in human cells. However, up-to-date, Cas13 orthologs have shown minimal off-target effects in several studies.[10,11,27] Previous studies did not observe mutations within the crRNA target site or see evidence of elevated mutation rates in the secreted LCMV viral populations.[24] Nonetheless, given that Cas13a does not modify DNA sequence, cells may have a chance to tolerate and recover from the off-target effect of Cas13a, in contrast to the possible permanent damage in cellular DNA by Cas9. In the meantime, delivering the exogenous Cas13a and other CRISPR effectors poses a great challenge to edit host genes *in vivo*, whereas siRNA and other small RNA-based therapy can readily take advantage the endogenous RNAi machinery. We also recognize that Cas13a targets RNA, is not able to cleave and eliminate HIV DNA, and thus theoretically cannot achieve HIV cure.

Cas9, now together with Cas13a, are able to target and inhibit both DNA and RNA viruses. Cas9 has been successfully used by many research groups to inhibit DNA viruses and retroviruses (reviewed in Ebrahimi et al.[51]). During the preparation of our manuscript, Cas13 has been tested to inhibit several RNA viruses, LCMV, IAV, VSV,[24] DENV,[25] and SARS-Cov-2.[26] Our study further showed that, by targeting and cleaving HIV-1 RNA, Cas13a can inhibit HIV-1 infection. This opens the possibility of using both Cas9 and Cas13a to achieve better control of DNA viruses. The *in vivo* delivery of CRISPR system remains a great challenge. It is hopeful that with a new CRISPR system to be discovered and more specific and more efficient CRISPR effectors to be engineered, CRISPR will give rise to effective therapeutics to treat viral diseases in humans.

MATERIALS AND METHODS

Plasmid Design and Construction

The hLbuCas13a-crRNA plasmid contains two expression cassettes, hLbuCas13a and the chimeric guide RNA. The human codon-optimized Lbu Cas13a-expressing cassette was synthesized and cloned between *Nhe* I and *Bam*H I sites in the pcDNA3.1 (+) vector. The crRNA-expressing cassette was synthesized and cloned between *Nhe* I and *Mlu* I sites. The *Bsm*B I site was inserted into the crRNA expressing cassette for cloning gRNA. The gRNA sequences are shown in Table S1. The LTR-TAT-IRES-GFP-P2A-puro (LTGP) vector, which expresses Tat and EGFP proteins under the control of an LTR promoter, was constructed by inserting the TAT-IRES-EGFP DNA fragment into the lentiV2 vector between the *Not* I and *Bam*H I sites. HIV-$1_{NL4\text{-}3\text{-}\Delta E\text{-}YFP}$ viral DNA was kindly provided by Dr. David Levy.[52] The infectious HIV-1 DNA clones NL4-3 was obtained from the NIH AIDS Reagent Program.

Cell Culture and Transfection

The human embryonic kidney 293T (HEK293T) cells and HEK293 cells were maintained in the Dulbecco's modified Eagle's medium (DMEM; Invitrogen). The HEK293 EGFP cell line was generated by transfecting the EGFP plasmid, followed by selection with G418 (0.75 mg/mL). The HEK293 LTR-EGFP cell line was engineered by transfecting HEK293 cells with the LTGP vector, which expresses Tat and EGFP proteins under the control of an LTR promoter,

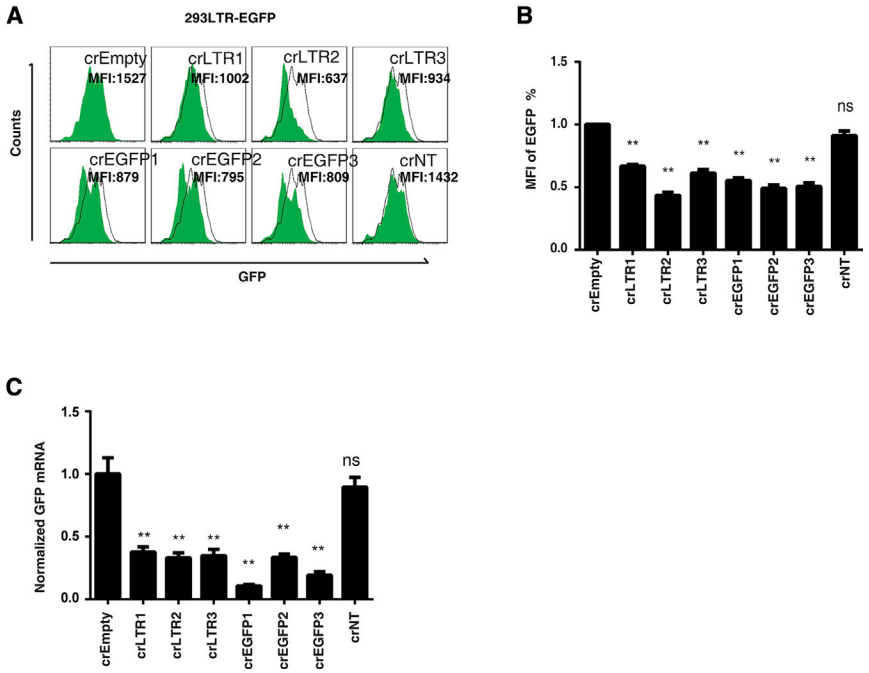

Figure 4. Cas13a/crRNA Impairs EGFP Expression from DNA that Is Integrated into Cellular DNA

(A and B) Cas13a/crRNA was transfected into HEK293T cells that bear the stably integrated LTR-EGFP. EGFP expression was measured by flow cytometry. Representative histograms are shown in (A). Values of YFP MFI are shown in (B). YFP MFI in cells that were treated with empty crRNA was arbitrarily set as 1. Error bars indicate SD (n = 3; *p < 0.05; **p < 0.01; Dunnett's t test). (C) EGFP mRNA was quantified by real-time RT-PCR. Results obtained with the empty crRNA vector were arbitrarily set as 1. Error bars indicate SD (n = 3; *p < 0.05; **p < 0.01; Dunnett's t test)

followed by selection with puromycin (0.8 μg/mL). Jurkat and JLat 10.6 (J-Lat Full Length Clone 10.6) cells were maintained in RPMI 1640 medium. All cells were supplemented with 10% fetal bovine serum (FBS; Life Technologies), 100 U/mL penicillin and 100 mg/mL streptomycin (Solarbio, P1400) at 37°C with 5% CO_2. JLat10.6 cells express EGFP and produce non-infectious viral particles after treatment with TNF-α (Pepro Tech, 300-01A).[44] HEK293T cells were seeded into plates 16 h prior to transfection. Cells were transfected with PEI (Sigma, 408727) according to the manufacturer's instructions. Jurkat and JLat 10.6 cells were transfected with hCas13a/crRNA by Neon (Life Technologies) according to the instructions. Briefly, Jurkat and and JLat 10.6 cells (2×10^5) were mixed with 2 μg of plasmid DNA in 10 μL of resuspension buffer R and electroporated at pulse voltage of 1325 V for 10 ms, 3 times. After transfection, Jurkat cells were infected with HIV-1 virus for 72 h and analyzed by flow cytometry. JLat 10.6 cells were cultured in complete growth medium with no antibiotics for 24 h. Then, the cells were treated with TNF-α (10 ng/mL) for 16 h and analyzed by flow cytometry.[44]

HIV-1 Pseudovirus Preparation and Infection

Virus stocks were generated by co-transfection of HEK293T cells with 8 μg HIV-$1_{NL4\text{-}3\text{-}\Delta E\text{-}YFP}$ and 2 μg VSV-G plasmids in 10 cm plates using PEI. 2 days after transfection, the supernatants were collected and filtered (0.45 μm). After Cas13a/crRNA transfection, HEK293T cells were infected with VSV-G pseudotyped virus. 36 h later, cells were harvested and analyzed by flow cytometry for YFP expression.

Flow Cytometry

Cells were detached using Trypsin-EDTA (0.25%; Life Technologies), re-suspended in complete growth media. Cells were washed with phosphate-buffered saline (PBS; Hyclone, SH30256.01) and suspended in PBS containing 1% paraformaldehyde (Solarbio, P1110). Cell membrane was permeabilized with 0.2% Triton X-100 (Bio-Rad). hCas13a-FLAG was detected by 647 conjugate FLAG antibody (Cell Signaling Biotechnology, 3916s). p24 was detected by fluorescein isothiocyanate (FITC) Anti-HIV-1 p24 antibody (Abcam, ab20569). EGFP, p24, and hCas13a expression were determined using the FACSCanto II and CELLQuest software.

Western Blotting

Western blotting was performed as previously described.[53] In briefly, 36 h post infection of HEK293T cells with the $HIV_{NL4\text{-}3\text{-}\Delta E\text{-}YFP}$ virus, which had been transfected with hCas13a/crRNA plasmid, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA). Equal amounts of cell lysates were separated in SDS-12% PAGE (WB1103, Beijing Biotides Biotechnology). Proteins were transferred onto nitrocellulose membranes (Whatmann). The membranes were probed with anti-CA-p24 antibody (Sino Biological, 11695-V08E), anti-FLAG antibody (Sigma, F3165), anti-actin antibody (Proteintech, 60008-1-Ig), or anti-GFP antibody (Proteintech, 66002-1-Ig), followed by incubation with IRDye secondary antibodies (1:20,000). Protein bands were visualized on a Li-Cor Odyssey instrument.

Quantitative Real-Time RT-PCR

Total RNA was extracted from cells with the QIAamp RNeasy Plus Mini Kit (QIAGEN, 74134), following the manufacturer's instructions. Reverse transcription was performed with the SuperScript II Reverse Transcriptase (Invitrogen, 18064071). The primer sequences were provided in Table S2 or obtained from Sino Biological (Tubulin, HP101479; HPRT1, HP100005; ACTIN, HP100001). Real-time PCR was conducted with the PowerUp SYBR Green Master Mix (Applied Biosystems, A25742) in accordance with the manufacturer's instructions.

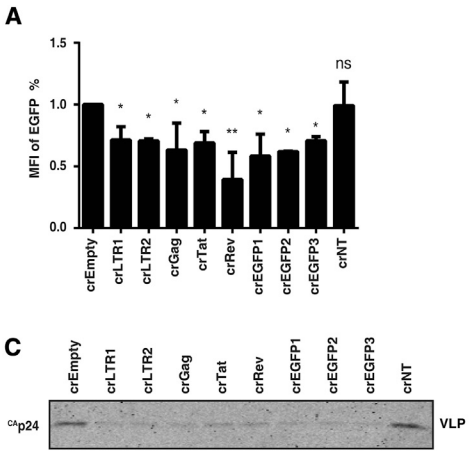

Figure 5. Cas13a/crRNA Inhibits HIV-1 Gene Expression and Virus Production from Latent HIV-1

(A) JLat10.6 cells were transfected with Cas13a/crRNA. EGFP expression was measured by flow cytometry. Results obtained with the empty crRNA vector were arbitrarily set as 1. YFP MFI in cells that were treated with empty crRNA was arbitrarily set as 1. Error bars indicate SD (n = 3; *p < 0.05; **p < 0.01; Dunnett's t test). (B) Levels of HIV-1 RNA were measured using TaqMan PCR in JLat10.6 cells transfected with Cas13a/crRNA. YFP MFI in cells that were treated with empty crRNA was arbitrarily set as 1. Error bars indicate SD (n = 3; *p < 0.05; **p < 0.01; Dunnett's t test). (C) Levels of viruses in the supernatants from JLat10.6 cells were determined by western blotting with anti-HIV-1 p24/Gag protein.

Viral RNA Quantification and Measuring Viral cDNA Synthesis

Levels of HIV RNA and late viral reverse transcription products, as well as the levels of integrated viral DNA, were measured by real-time PCR as previously reported.[28,54–57] Briefly, equal amounts of DNase-treated virions (100 ng CA-p24) were used to infect 5×10^6 293T cells that had been transfected with hCas13a/crRNA plasmids. Following a 2-h incubation at 4°C, cells with bound virus were washed twice with PBS, and maintained with fresh DMEM medium. At 3 h post-infection, cells were collected and cellular RNA was extracted with the QIAamp RNeasy Plus Mini Kit (QIAGEN, 74134). The viral RNA was reverse transcribed with SuperScript III First-Strand (Invitrogen, 1903729) and quantitated by the Light Cycler Instrument (BIO-RAD CFX96) using HIV RNA primers. At 24 h post-infection, cells were collected and cellular DNA was extracted with the QIAamp DNA mini kit (QIAGEN, 51304). To measure the integrated viral DNA, we subjected equal amounts of extracted cellular DNA to the real-time PCR. The primer sequences used to detect each product were shown in Table S2. Real-time PCR was conducted with the Taqman Universal PCR Master Mix (Applied Biosystems, 4440040) in accordance with the manufacturer's instructions.

Determination of Viral Capsid Protein (^{CA}p24) in Supernatant

Cells were transfected with pNL4-3-ΔE-YFP plasmid DNA or infected with HIV-$1_{NL4\text{-}3\text{-}\Delta E\text{-}YFP}$ for 6 h. Cells were then washed twice with PBS to remove unbound virus and maintained with fresh complete medium for 48 h. Culture supernatants were clarified by passing through the 0.45 μm filter, and viruses in the supernatants were harvested by ultracentrifugation at 35,000 rpm for 1 h (SW41Ti, Beckman Coulter). Virus amounts were then determined by western blotting with anti-HIV-1 p24/Gag antibodies.

Cell Viability Assay

Cell viability was examined as previously described.[58] 293T cells were cultured on 96-well plates at a concentration of 1×10^3 cells per well, and then transfected with plasmid DNA using Lipofectamine (Invitrogen). Cell proliferation was determined by adding 10 μL cell counting kit-8 (96992, Sigma) to each well. After 3 h, optical density (OD) value was measured using a microplate reader (Multiskan FC, Thermo Fisher) at 450 nm.

Statistical Analysis

All experiments were performed three or more times independently under similar conditions. All data were plotted as mean values, with variation as SD. Statistical significance was calculated by Dunnett's t test. p values of statistical significance are represented as **p < 0.01, *p < 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.05.030.

AUTHOR CONTRIBUTIONS

S.H., C.L., S.C., and F.G. conceived the project. L.Y., F.Z., H.S., S.H., W.Z., Y.H., F.X., S.M., X.L., D.Z., and L.W. performed the experiments. All authors contributed to experimental design and data analysis. S.H., C.L., and F.G. composed the manuscript. All authors reviewed the manuscript and discussed the work.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGMENTS

This study was supported by funds from the Ministry of Science and Technology of the People's Republic of China (2018ZX10301408-003 to F.G., 2018ZX10731101-001-018 to F.X.); the National Key Plan for Scientific Research and Development of China (2016YFD0500307 to F.G.); the National Natural Science Foundation of China (81371808 and 81528012 to F.G., 81702451 to S.M., and 81401673 to F.X.); CAMS Innovation Fund for Medical Sciences (CIFMS 2018-I2M-3-004 to F.G. and CIFMS 2017-I2M-1-014 to F.X.); the Canadian Institutes of Health Research (CCI-132561 to C.L.); the CAMS general fund (2019-RC-HL-012 to S.H.); and the PUMC Youth Fund/

Fundamental Research Funds for the Central Universities (3332018202 to S.H.). All relevant data are within the paper and Supplemental Information files.

REFERENCES

1. Deveau, H., Garneau, J.E., and Moineau, S. (2010). CRISPR/Cas system and its role in phage-bacteria interactions. Annu. Rev. Microbiol. *64*, 475–493.
2. Garneau, J.E., Dupuis, M.E., Villion, M., Romero, D.A., Barrangou, R., Boyaval, P., Fremaux, C., Horvath, P., Magadán, A.H., and Moineau, S. (2010). The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature *468*, 67–71.
3. Horvath, P., and Barrangou, R. (2010). CRISPR/Cas, the immune system of bacteria and archaea. Science *327*, 167–170.
4. Makarova, K.S., Haft, D.H., Barrangou, R., Brouns, S.J., Charpentier, E., Horvath, P., Moineau, S., Mojica, F.J., Wolf, Y.I., Yakunin, A.F., et al. (2011). Evolution and classification of the CRISPR-Cas systems. Nat. Rev. Microbiol. *9*, 467–477.
5. Makarova, K.S., Wolf, Y.I., and Koonin, E.V. (2013). The basic building blocks and evolution of CRISPR-CAS systems. Biochem. Soc. Trans. *41*, 1392–1400.
6. Shmakov, S., Abudayyeh, O.O., Makarova, K.S., Wolf, Y.I., Gootenberg, J.S., Semenova, E., Minakhin, L., Joung, J., Konermann, S., Severinov, K., et al. (2015). Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems. Mol. Cell *60*, 385–397.
7. Koonin, E.V., Makarova, K.S., and Zhang, F. (2017). Diversity, classification and evolution of CRISPR-Cas systems. Curr. Opin. Microbiol. *37*, 67–78.
8. Abudayyeh, O.O., Gootenberg, J.S., Konermann, S., Joung, J., Slaymaker, I.M., Cox, D.B., Shmakov, S., Makarova, K.S., Semenova, E., Minakhin, L., et al. (2016). C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science *353*, aaf5573.
9. East-Seletsky, A., O'Connell, M.R., Knight, S.C., Burstein, D., Cate, J.H., Tjian, R., and Doudna, J.A. (2016). Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. Nature *538*, 270–273.
10. Abudayyeh, O.O., Gootenberg, J.S., Essletzbichler, P., Han, S., Joung, J., Belanto, J.J., Verdine, V., Cox, D.B.T., Kellner, M.J., Regev, A., et al. (2017). RNA targeting with CRISPR-Cas13. Nature *550*, 280–284.
11. Cox, D.B.T., Gootenberg, J.S., Abudayyeh, O.O., Franklin, B., Kellner, M.J., Joung, J., and Zhang, F. (2017). RNA editing with CRISPR-Cas13. Science *358*, 1019–1027.
12. Liu, L., Li, X., Ma, J., Li, Z., You, L., Wang, J., Wang, M., Zhang, X., and Wang, Y. (2017). The Molecular Architecture for RNA-Guided RNA Cleavage by Cas13a. Cell *170*, 714–726.
13. Liu, L., Li, X., Wang, J., Wang, M., Chen, P., Yin, M., Li, J., Sheng, G., and Wang, Y. (2017). Two Distant Catalytic Sites Are Responsible for C2c2 RNase Activities. Cell *168*, 121–134.
14. Tambe, A., East-Seletsky, A., Knott, G.J., Doudna, J.A., and O'Connell, M.R. (2018). RNA Binding and HEPN-Nuclease Activation Are Decoupled in CRISPR-Cas13a. Cell Rep. *24*, 1025–1036.
15. Wolter, F., and Puchta, H. (2018). The CRISPR/Cas revolution reaches the RNA world: Cas13, a new Swiss Army knife for plant biologists. Plant J. *94*, 767–775.
16. East-Seletsky, A., O'Connell, M.R., Burstein, D., Knott, G.J., and Doudna, J.A. (2017). RNA Targeting by Functionally Orthogonal Type VI-A CRISPR-Cas Enzymes. Mol. Cell *66*, 373–383.
17. Gootenberg, J.S., Abudayyeh, O.O., Lee, J.W., Essletzbichler, P., Dy, A.J., Joung, J., Verdine, V., Donghia, N., Daringer, N.M., Freije, C.A., et al. (2017). Nucleic acid detection with CRISPR-Cas13a/C2c2. Science *356*, 438–442.
18. Myhrvold, C., Freije, C.A., Gootenberg, J.S., Abudayyeh, O.O., Metsky, H.C., Durbin, A.F., Kellner, M.J., Tan, A.L., Paul, L.M., Parham, L.A., et al. (2018). Field-deployable viral diagnostics using CRISPR-Cas13. Science *360*, 444–448.
19. Qin, P., Park, M., Alfson, K.J., Tamhankar, M., Carrion, R., Patterson, J.L., Griffiths, A., He, Q., Yildiz, A., Mathies, R., and Du, K. (2019). Rapid and Fully Microfluidic Ebola Virus Detection with CRISPR-Cas13a. ACS Sens. *4*, 1048–1054.
20. Liu, Y., Xu, H., Liu, C., Peng, L., Khan, H., Cui, L., Huang, R., Wu, C., Shen, S., Wang, S., et al. (2019). CRISPR-Cas13a Nanomachine Based Simple Technology for Avian Influenza A (H7N9) Virus On-Site Detection. J. Biomed. Nanotechnol. *15*, 790–798.
21. Chen, Y., Yang, S., Peng, S., Li, W., Wu, F., Yao, Q., Wang, F., Weng, X., and Zhou, X. (2019). *N*1-Methyladenosine detection with CRISPR-Cas13a/C2c2. Chem. Sci. (Camb.) *10*, 2975–2979.
22. Aman, R., Ali, Z., Butt, H., Mahas, A., Aljedaani, F., Khan, M.Z., Ding, S., and Mahfouz, M. (2018). RNA virus interference via CRISPR/Cas13a system in plants. Genome Biol. *19*, 1.
23. Zhao, X., Liu, L., Lang, J., Cheng, K., Wang, Y., Li, X., Shi, J., Wang, Y., and Nie, G. (2018). A CRISPR-Cas13a system for efficient and specific therapeutic targeting of mutant KRAS for pancreatic cancer treatment. Cancer Lett. *431*, 171–181.
24. Freije, C.A., Myhrvold, C., Boehm, C.K., Lin, A.E., Welch, N.L., Carter, A., Metsky, H.C., Luo, C.Y., Abudayyeh, O.O., Gootenberg, J.S., et al. (2019). Programmable Inhibition and Detection of RNA Viruses Using Cas13. Mol. Cell *76*, 826–837.
25. Li, H., Wang, S., Dong, X., Li, Q., Li, M., Li, J., Guo, Y., Jin, X., Zhou, Y., Song, H., and Kou, Z. (2020). CRISPR-Cas13a Cleavage of Dengue Virus NS3 Gene Efficiently Inhibits Viral Replication. Mol. Ther. Nucleic Acids *19*, 1460–1469.
26. Abbott, T.R., Dhamdhere, G., Liu, Y., Lin, X., Goudy, L., Zeng, L., Chemparathy, A., Chmura, S., Heaton, N.S., Debs, R., et al. (2020). Development of CRISPR as an Antiviral Strategy to Combat SARS-CoV-2 and Influenza. Cell *181*, 865–876.
27. Konermann, S., Lotfy, P., Brideau, N.J., Oki, J., Shokhirev, M.N., and Hsu, P.D. (2018). Transcriptome Engineering with RNA-Targeting Type VI-D CRISPR Effectors. Cell *173*, 665–676.
28. Jordan, A., Bisgrove, D., and Verdin, E. (2003). HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. EMBO J. *22*, 1868–1877.
29. Zhang, T., Zhao, Y., Ye, J., Cao, X., Xu, C., Chen, B., An, H., Jiao, Y., Zhang, F., Yang, X., and Zhou, G. (2019). Establishing CRISPR/Cas13a immune system conferring RNA virus resistance in both dicot and monocot plants. Plant Biotechnol. J. *17*, 1185–1187.
30. Rettig, G.R., and Behlke, M.A. (2012). Progress toward in vivo use of siRNAs-II. Mol. Ther. *20*, 483–512.
31. Cave, E., Weinberg, M.S., Cilliers, T., Carmona, S., Morris, L., and Arbuthnot, P. (2006). Silencing of HIV-1 subtype C primary isolates by expressed small hairpin RNAs targeted to gag. AIDS Res. Hum. Retroviruses *22*, 401–410.
32. Morris, K.V., Chung, C.H., Witke, W., and Looney, D.J. (2005). Inhibition of HIV-1 replication by siRNA targeting conserved regions of gag/pol. RNA Biol. *2*, 17–20.
33. Lee, S.K., Dykxhoorn, D.M., Kumar, P., Ranjbar, S., Song, E., Maliszewski, L.E., François-Bongarçon, V., Goldfeld, A., Swamy, N.M., Lieberman, J., and Shankar, P. (2005). Lentiviral delivery of short hairpin RNAs protects CD4 T cells from multiple clades and primary isolates of HIV. Blood *106*, 818–826.
34. McIntyre, G.J., Groneman, J.L., Yu, Y.H., Tran, A., and Applegate, T.L. (2011). Multiple shRNA combinations for near-complete coverage of all HIV-1 strains. AIDS Res. Ther. *8*, 1.
35. Qin, X.F., An, D.S., Chen, I.S., and Baltimore, D. (2003). Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc. Natl. Acad. Sci. USA *100*, 183–188.
36. Shah, P.S., Pham, N.P., and Schaffer, D.V. (2012). HIV develops indirect cross-resistance to combinatorial RNAi targeting two distinct and spatially distant sites. Mol. Ther. *20*, 840–848.
37. Berkhout, B. (2009). Toward a durable anti-HIV gene therapy based on RNA interference. Ann. N Y Acad. Sci. *1175*, 3–14.
38. von Eije, K.J., ter Brake, O., and Berkhout, B. (2008). Human immunodeficiency virus type 1 escape is restricted when conserved genome sequences are targeted by RNA interference. J. Virol. *82*, 2895–2903.
39. Herrera-Carrillo, E., Liu, Y.P., and Berkhout, B. (2014). The impact of unprotected T cells in RNAi-based gene therapy for HIV-AIDS. Mol. Ther. *22*, 596–606.
40. Scherer, L., Rossi, J.J., and Weinberg, M.S. (2007). Progress and prospects: RNA-based therapies for treatment of HIV infection. Gene Ther. *14*, 1057–1064.
41. Lee, N.S., and Rossi, J.J. (2004). Control of HIV-1 replication by RNA interference. Virus Res. *102*, 53–58.

42. Li, M.J., Kim, J., Li, S., Zaia, J., Yee, J.K., Anderson, J., Akkina, R., and Rossi, J.J. (2005). Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. Mol. Ther. *12*, 900–909.

43. Unwalla, H.J., Li, H.T., Bahner, I., Li, M.J., Kohn, D., and Rossi, J.J. (2006). Novel Pol II fusion promoter directs human immunodeficiency virus type 1-inducible coexpression of a short hairpin RNA and protein. J. Virol. *80*, 1863–1873.

44. 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.

45. Westerhout, E.M., ter Brake, O., and Berkhout, B. (2006). The virion-associated incoming HIV-1 RNA genome is not targeted by RNA interference. Retrovirology *3*, 57.

46. Gao, Y., Lobritz, M.A., Roth, J., Abreha, M., Nelson, K.N., Nankya, I., Moore-Dudley, D.M., Abraha, A., Gerson, S.L., and Arts, E.J. (2008). Targets of small interfering RNA restriction during human immunodeficiency virus type 1 replication. J. Virol. *82*, 2938–2951.

47. Bobbin, M.L., Burnett, J.C., and Rossi, J.J. (2015). RNA interference approaches for treatment of HIV-1 infection. Genome Med. *7*, 50.

48. Qiu, Y., Xu, Y., Zhang, Y., Zhou, H., Deng, Y.Q., Li, X.F., Miao, M., Zhang, Q., Zhong, B., Hu, Y., et al. (2017). Human Virus-Derived Small RNAs Can Confer Antiviral Immunity in Mammals. Immunity *46*, 992–1004.

49. Li, F., and Wang, A. (2019). RNA-Targeted Antiviral Immunity: More Than Just RNA Silencing. Trends Microbiol. *27*, 792–805.

50. Cullen, B.R. (2017). RNA Interference in Mammals: The Virus Strikes Back. Immunity *46*, 970–972.

51. Ebrahimi, S., Teimoori, A., Khanbabaei, H., and Tabasi, M. (2019). Harnessing CRISPR/Cas 9 System for manipulation of DNA virus genome. Rev. Med. Virol. *29*, e2009.

52. Levy, D.N., Aldrovandi, G.M., Kutsch, O., and Shaw, G.M. (2004). Dynamics of HIV-1 recombination in its natural target cells. Proc. Natl. Acad. Sci. USA *101*, 4204–4209.

53. Hu, S., Li, J., Xu, F., Mei, S., Le Duff, Y., Yin, L., Pang, X., Cen, S., Jin, Q., Liang, C., and Guo, F. (2015). SAMHD1 Inhibits LINE-1 Retrotransposition by Promoting Stress Granule Formation. PLoS Genet. *11*, e1005367.

54. Butler, S.L., Hansen, M.S., and Bushman, F.D. (2001). A quantitative assay for HIV DNA integration in vivo. Nat. Med. *7*, 631–634.

55. Brussel, A., and Sonigo, P. (2003). Analysis of early human immunodeficiency virus type 1 DNA synthesis by use of a new sensitive assay for quantifying integrated provirus. J. Virol. *77*, 10119–10124.

56. Liu, Z., Pan, Q., Ding, S., Qian, J., Xu, F., Zhou, J., Cen, S., Guo, F., and Liang, C. (2013). The interferon-inducible MxB protein inhibits HIV-1 infection. Cell Host Microbe *14*, 398–410.

57. Guo, F., Cen, S., Niu, M., Saadatmand, J., and Kleiman, L. (2006). Inhibition of $tRNA_3(Lys)$-primed reverse transcription by human APOBEC3G during human immunodeficiency virus type 1 replication. J. Virol. *80*, 11710–11722.

58. Yin, L., Hu, S., Mei, S., Sun, H., Xu, F., Li, J., Zhu, W., Liu, X., Zhao, F., Zhang, D., et al. (2018). CRISPR/Cas9 Inhibits Multiple Steps of HIV-1 Infection. Hum. Gene Ther. *29*, 1264–1276.