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Supplemental information

Targeted intracellular delivery of Cas13 and Cas9 nucleases using bacterial toxin-based platforms

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Supplementary Information

Supplementary Figures S1 – S6

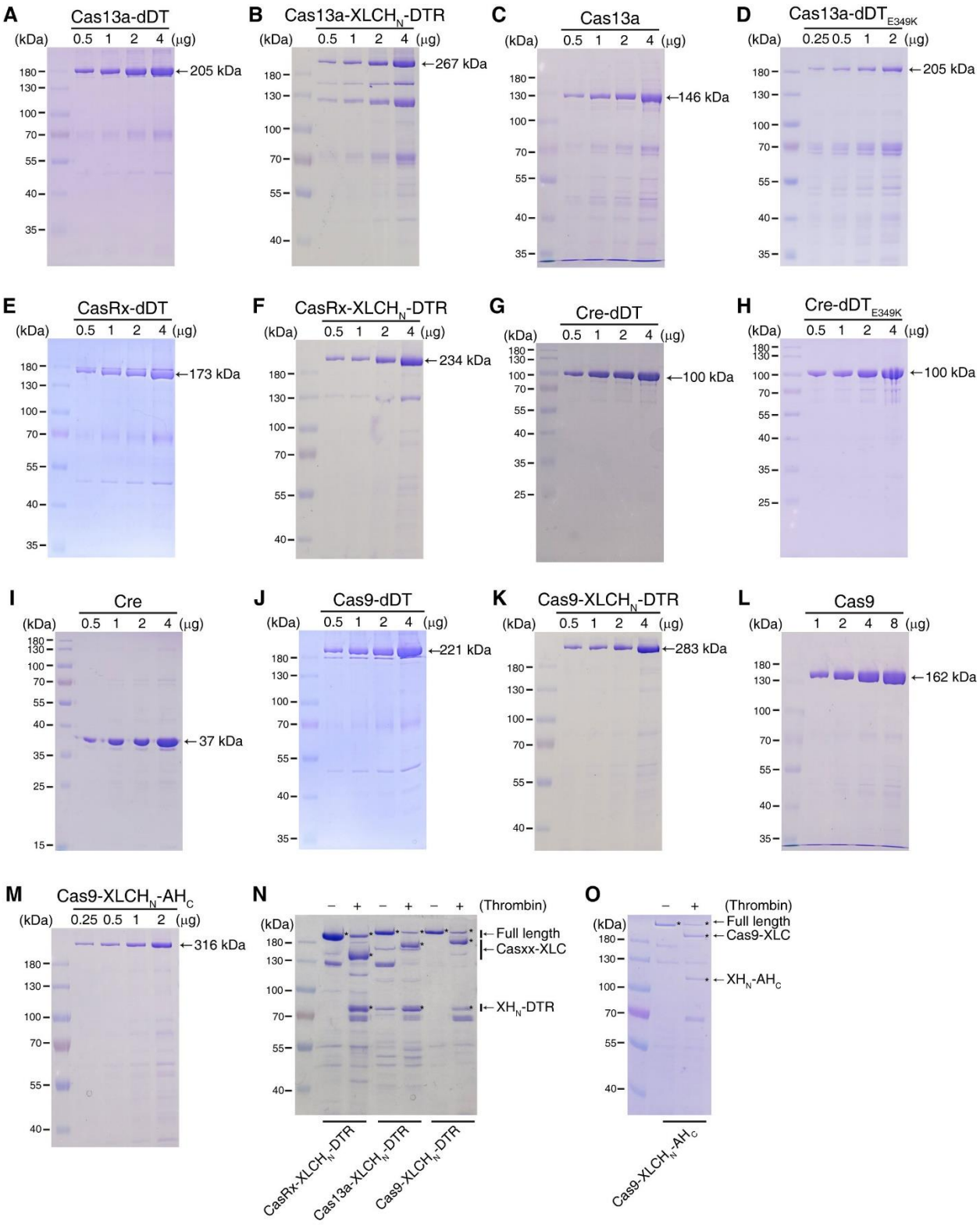
A
Cas13a-dDT
(kDa) 0.5 1 2 4 (μg)
180
130
100
70
55
40
35
←205 kDa
B
Cas13a-XLCH$_N$-DTR
(kDa) 0.5 1 2 4 (μg)
180
130
100
70
55
40
←267 kDa
C
Cas13a
(kDa) 0.5 1 2 4 (μg)
180
130
100
70
55
40
35
←146 kDa
D
Cas13a-dDT$_{E349K}$
(kDa) 0.25 0.5 1 2 (μg)
180
130
100
70
55
40
35
←205 kDa
E
CasRx-dDT
(kDa) 0.5 1 2 4 (μg)
180
130
100
70
55
40
35
←173 kDa
F
CasRx-XLCH$_N$-DTR
(kDa) 0.5 1 2 4 (μg)
180
130
100
70
55
40
←234 kDa
G
Cre-dDT
(kDa) 0.5 1 2 4 (μg)
180
130
100
70
55
40
35
25
←100 kDa
H
Cre-dDT$_{E349K}$
(kDa) 0.5 1 2 4 (μg)
180
130
100
70
55
40
35
25
←100 kDa
I
Cre
(kDa) 0.5 1 2 4 (μg)
180
130
100
70
55
40
35
25
15
←37 kDa
J
Cas9-dDT
(kDa) 0.5 1 2 4 (μg)
180
130
100
70
55
40
35
←221 kDa
K
Cas9-XLCH$_N$-DTR
(kDa) 0.5 1 2 4 (μg)
180
130
100
70
55
40
←283 kDa
L
Cas9
(kDa) 1 2 4 8 (μg)
180
130
100
70
55
40
35
←162 kDa
M
Cas9-XLCH$_N$-AH$_C$
(kDa) 0.25 0.5 1 2 (μg)
180
130
100
70
55
40
←316 kDa
N
− + − + − + (Thrombin)
(kDa)
180
130
100
70
55
40
←Full length
←Casxx-XLC
←XH$_N$-DTR
CasRx-XLCH$_N$-DTR
Cas13a-XLCH$_N$-DTR
Cas9-XLCH$_N$-DTR
O
− + (Thrombin)
(kDa)
180
130
100
70
55
40
←Full length
←Cas9-XLC
←XH$_N$-AH$_C$
Cas9-XLCH$_N$-AH$_C$

Figure S1. Protein purification and activation. Related to Figure 1-6.

(A-M) Recombinant Cas13a-dDT (**A**), Cas13a-XLCH$_N$-DTR (**B**), Cas13a (**C**), Cas13a-dDT$_{E349K}$ (**D**), CasRx-dDT (**E**), CasRx-XLCH$_N$-DTR (**F**), Cre-dDT (**G**), Cre-dDT$_{E349K}$ (**H**), Cre (**I**), Cas9-dDT (**J**), Cas9-XLCH$_N$-DTR (**K**), Cas9 (**L**), and Cas9-XLCH$_N$-AH$_C$ (**M**) were purified as His6-tagged proteins. SDS-PAGE was used to confirm the purity. The molecular weights of all full-length protein were marked.

(N-O) Activation of Cas13a-XLCH$_N$-DTR (**N**), CasRx-XLCH$_N$-DTR (**N**), Cas9-XLCH$_N$-DTR (**N**), and Cas9-XLCH$_N$-AH$_C$ (**O**). The purified proteins were mixed with thrombin and incubated at 4 °C overnight. SDS-PAGE was run under reduction conditions (with DTT). The bands for full-length proteins and cleaved fragments were marked. Around 80~90% full-length proteins were activated.

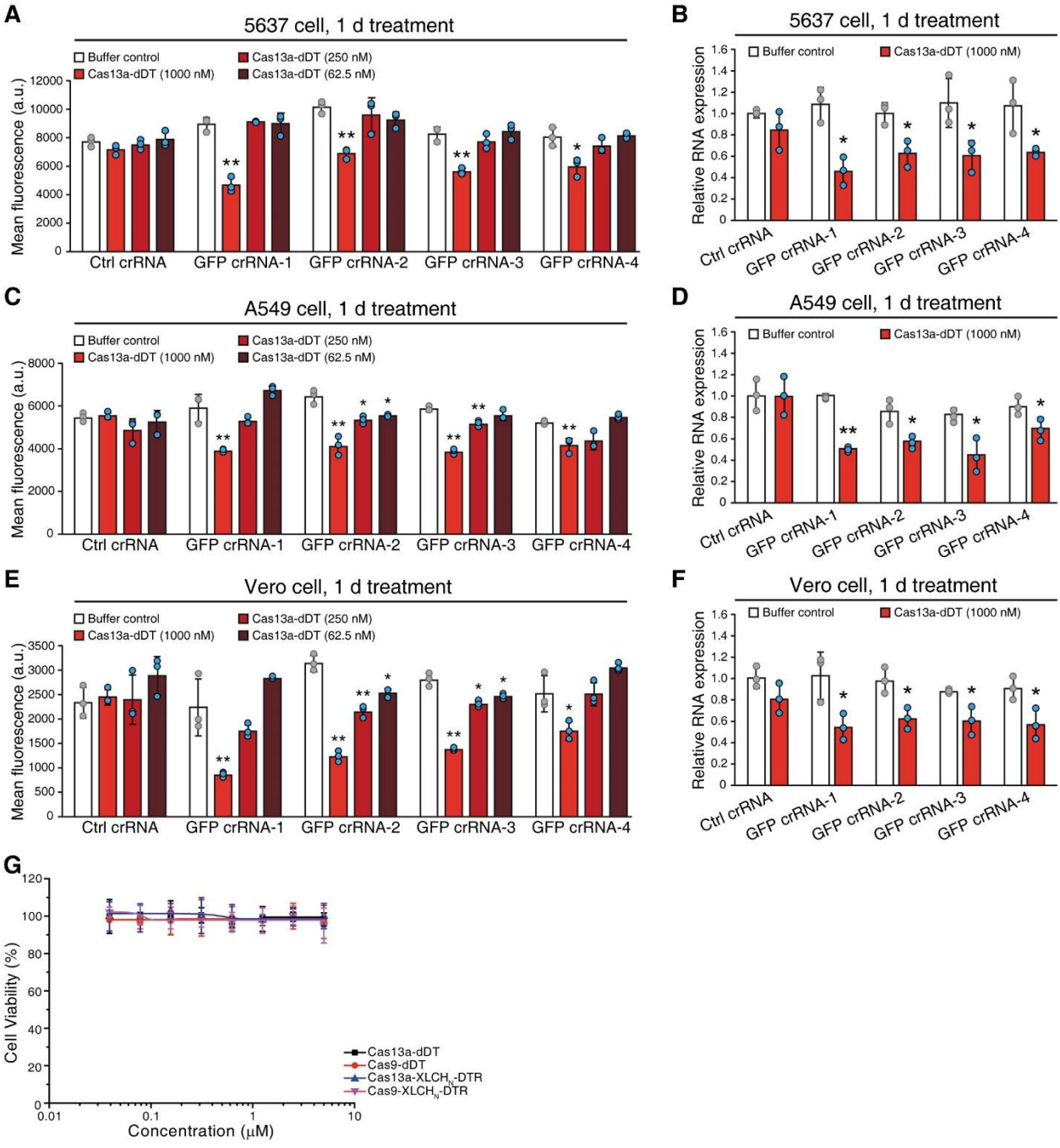
A
5637 cell, 1 d treatment
Buffer control
Cas13a-dDT (250 nM)
Cas13a-dDT (1000 nM)
Cas13a-dDT (62.5 nM)
Mean fluorescence (a.u.)
Ctrl crRNA
GFP crRNA-1
GFP crRNA-2
GFP crRNA-3
GFP crRNA-4
B
5637 cell, 1 d treatment
Buffer control
Cas13a-dDT (1000 nM)
Relative RNA expression
C
A549 cell, 1 d treatment
D
A549 cell, 1 d treatment
E
Vero cell, 1 d treatment
F
Vero cell, 1 d treatment
G
Cell Viability (%)
Concentration (μM)
Cas13a-dDT
Cas9-dDT
Cas13a-XLCH$_N$-DTR
Cas9-XLCH$_N$-DTR

Figure S2. Cas13a-dDT is validated on multiple cell lines. Related to Figure 1-2.

(A-F) GFP expressing human 5637 (**A-B**), human A549 (**C-D**), or green monkey Vero (**E-F**) cells were transfected with control (Ctrl) or GFP-targeting crRNAs and treated with different concentration of Cas13a-dDT for 24 hours. The protein level of GFP was measured by flow cytometry and quantified by MFI (**A, C, E**), the mRNA level of GFP was analyzed by qRT-PCR (**B, D, F**). Error bars indicate mean ± SD; $N = 3$ (biological replicates); *, $p < 0.05$; **, $p < 0.01$ (Student's *t*-test).

(G) HeLa cells were exposed to a series of concentrations of the indicated proteins for 2 days and cell viability was measured using MTT assays. Error bars indicate mean ± SD, N = 3 (biological replicates).

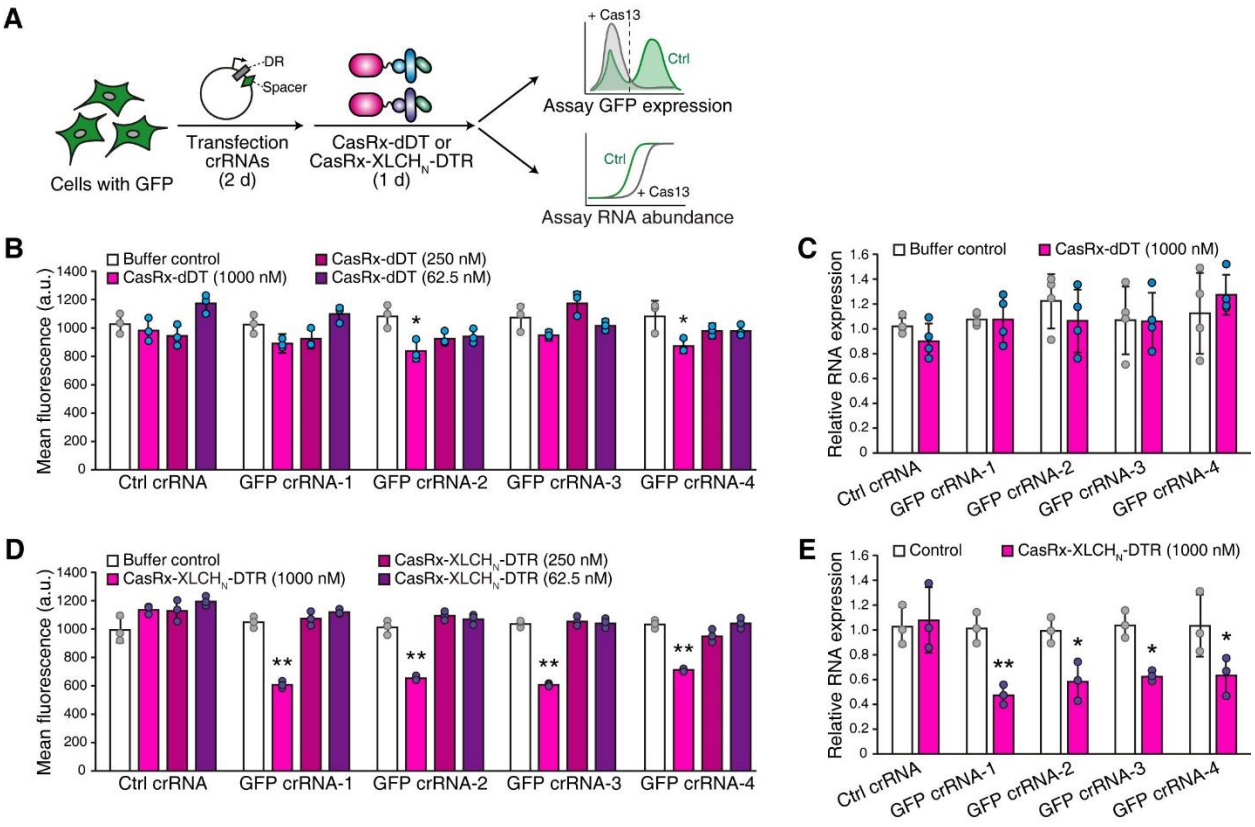

Figure S3. CasRx can be delivered using DT-based and BoNT/X-based systems. Related to Figure 1-2.

(A) Schematic of the CasRx delivery experiment. HeLa+GFP cells were transfected with Ctrl or GFP-targeting crRNAs for 48 hours, followed by treatment with different concentrations of CasRx-dDT or activated CasRx-XLCH$_N$-DTR for 24 hours. GFP expression at the protein level and the mRNA level were analyzed by flow cytometry and qRT-PCR, respectively. DR, direct repeat.

(B-E) Quantification of GFP expression. HeLa+GFP cells were transfected with Ctrl or GFP-targeting crRNAs and treated with different concentrations of CasRx-dDT (**B-C**) or activated CasRx-XLCH$_N$-DTR (**D-E**) for 24 hours. GFP protein was measured by flow cytometry and quantified by MFI (**B, D**), and GFP mRNA was analyzed by qRT-PCR (**C, E**). CasRx cannot be delivered by DT system but can be delivered BoNT/X system at 1 μM concentration. Error bars indicate mean ± SD; $N = 3$ (biological replicates); *, $p < 0.05$; **, $p < 0.01$ (Student's *t*-test).

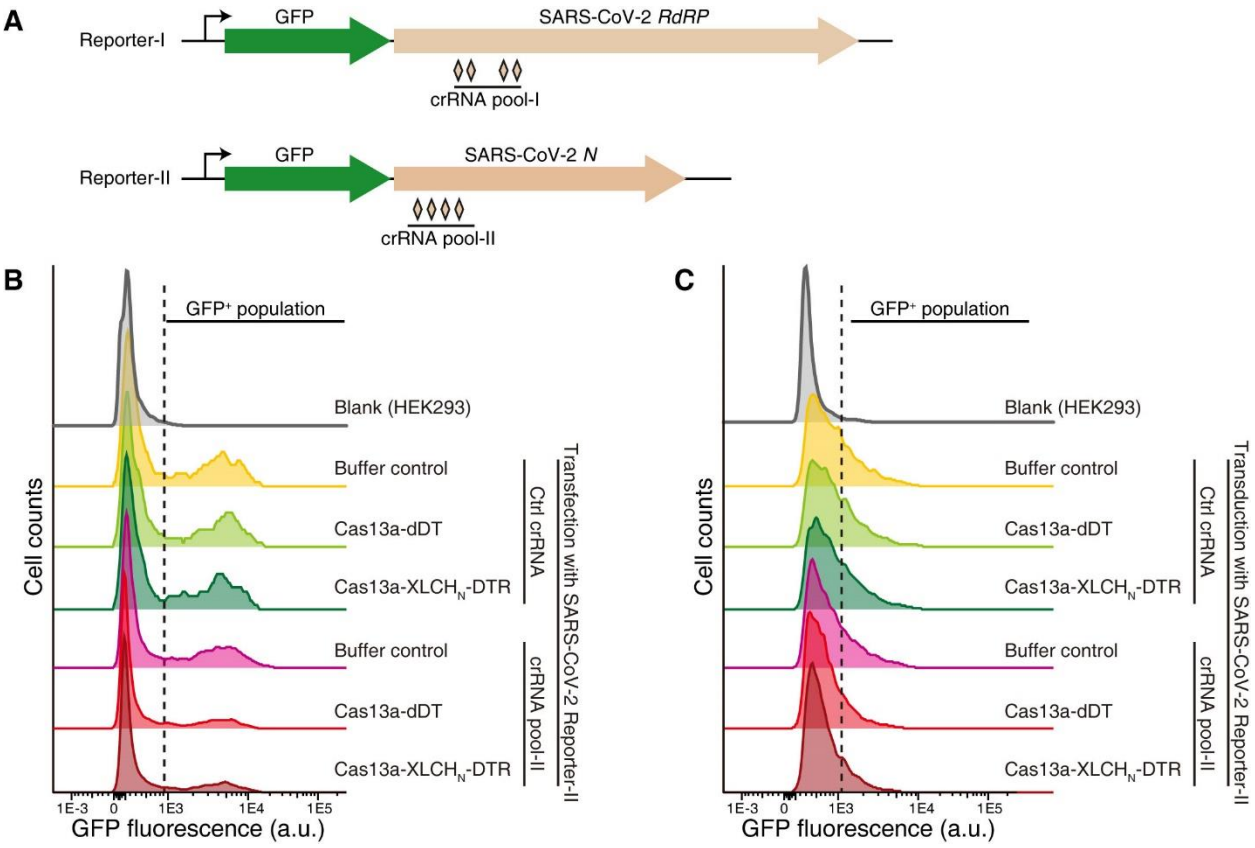

Figure S4. Cas13a inhibits SARS-CoV-2 reporters. Related to Figure 4.

(A) Schematic of the SARS-CoV-2 reporters. Reporters were built by fusing GFP with SARS-CoV-2 fragments, RNA-dependent RNA polymerase (*RdRP*) for Reporter-I and Nucleocapsid (*N*) for Reporter-II, respectively. For each fragment, four crRNAs were designed to target different regions and mixed to produce crRNA pool-I and pool-II.

(B-C) Representative flow cytometry histograms for SARS-CoV-2 reporter-II, which were over-expressed in HEK293 cells by transient transfection (**B**) or lentiviral transduction (**C**). Cells were transfected with crRNAs, followed by treatment with 1 μM Cas13a-dDT or activated Cas13a-XLCH$_N$-DTR. The GFP$^+$ population was significantly reduced when co-treatment with crRNA pools and Cas13 proteins. Representative histograms from one of three independent experiments are shown.

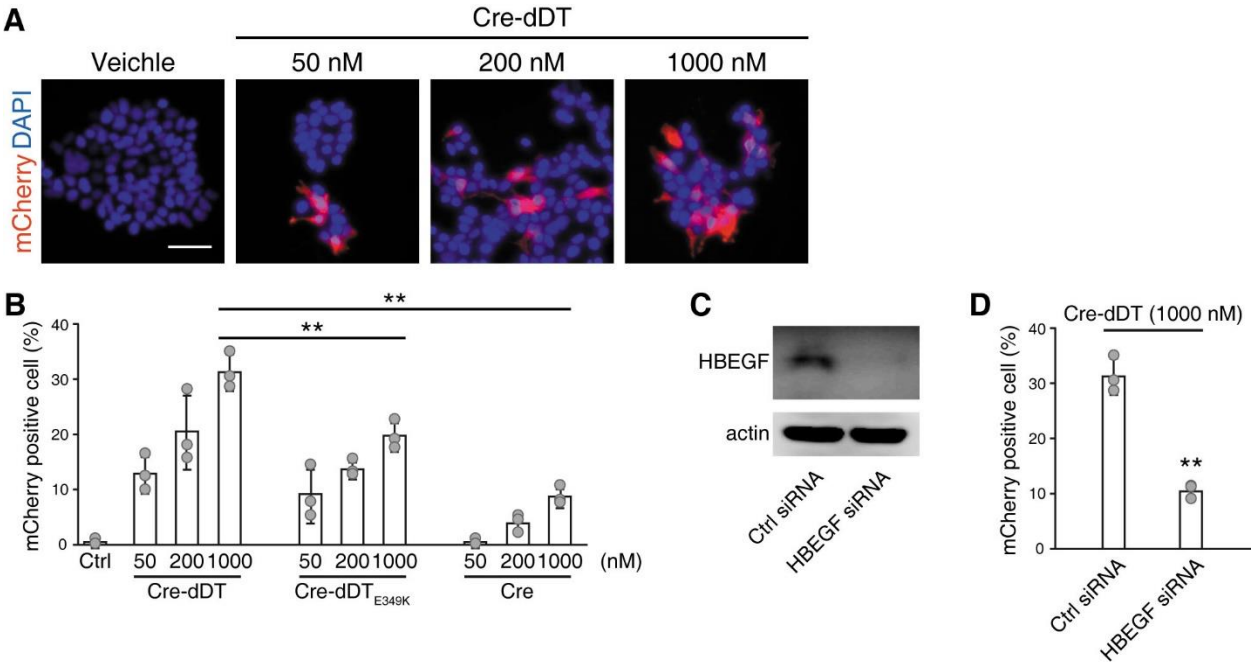

Figure S5. Delivery of Cre recombinase into cells. Related to Figure 5.

(A) Cre reporter cells (HEK293 stably expressing reporter; Cre-mediated DNA recombination can turn on mCherry expression) treated with 50, 200, or 1000 nM Cre-dDT for 48 h. The mCherry signal was analyzed under fluorescence microscope. The representative images were from one of three independent experiments. Nuclei were labeled with DAPI. Scale bar, 25 μm.

(B) The percentage of mCherry positive cells was shown as a bar-chart. Cre-dDT$_{E349K}$ and Cre were analyzed in parallel. Error bars indicate mean ± SD; $N = 3$ (biological replicates); **, $p < 0.01$ (Student's *t*-test).

(C-D) Delivery of Cre-dDT is dependent on HBEGF receptor. The HBEGF-targeting or non-targeting control siRNAs were transfected into reporter cells and cell lysates were analyzed by immunoblot detecting HBEGF and actin (**C**). Cells were then treated with Cre-dDT (1 μM) for 48 h. The percentage of mCherry-positive cells shown as a bar-chart (**D**). Error bars indicate mean ± SD; $N = 3$ (biological replicates); **, $p < 0.01$ (Student's *t*-test).

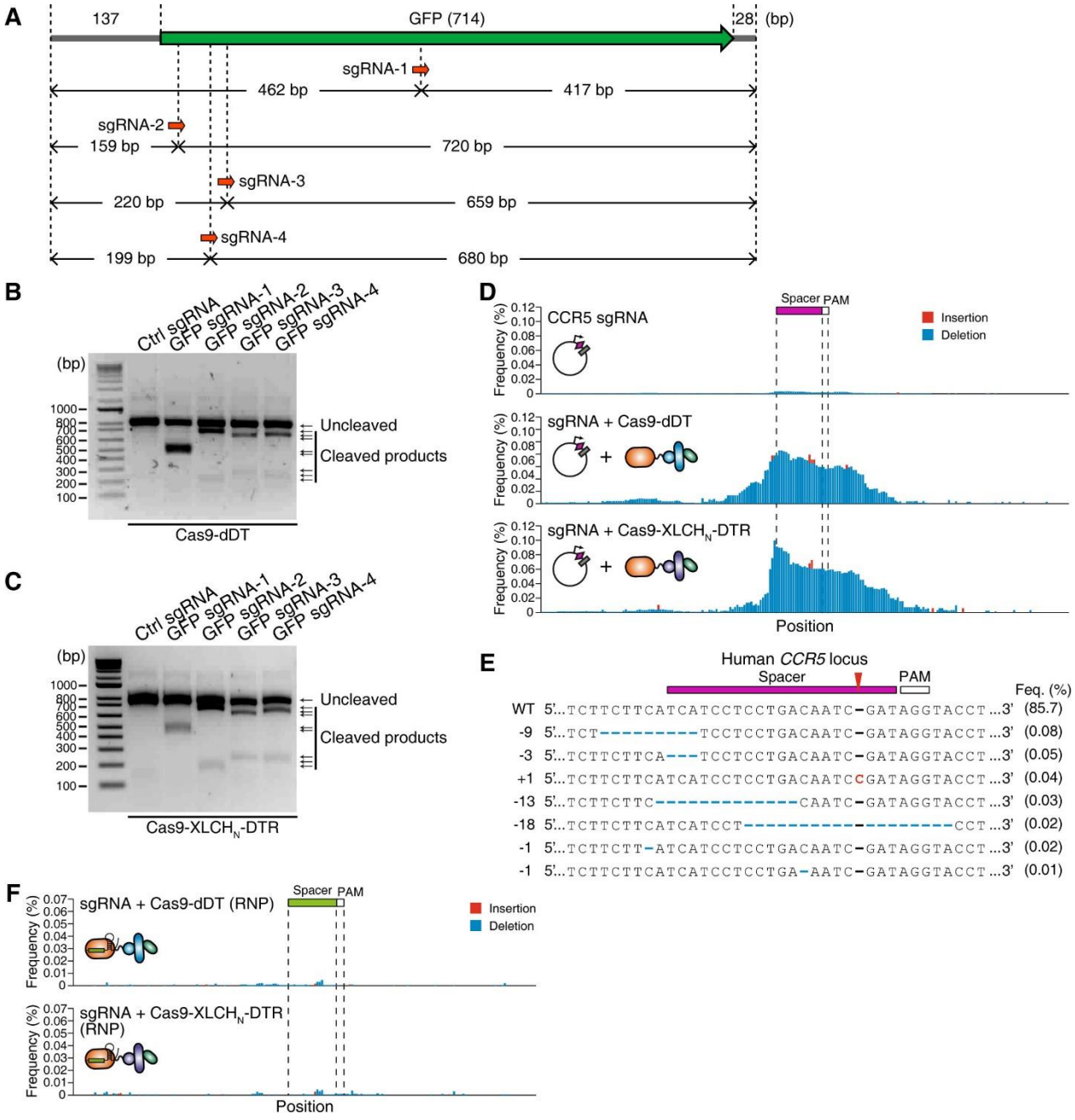
A
137
GFP (714)
28 (bp)
sgRNA-1
462 bp
417 bp
sgRNA-2
159 bp
720 bp
sgRNA-3
220 bp
659 bp
sgRNA-4
199 bp
680 bp
B
Ctrl sgRNA
GFP sgRNA-1
GFP sgRNA-2
GFP sgRNA-3
GFP sgRNA-4
(bp)
1000
800
700
600
500
400
300
200
100
Uncleaved
Cleaved products
Cas9-dDT
C
Ctrl sgRNA
GFP sgRNA-1
GFP sgRNA-2
GFP sgRNA-3
GFP sgRNA-4
(bp)
1000
800
700
600
500
400
300
200
100
Uncleaved
Cleaved products
Cas9-XLCH$_N$-DTR
D
CCR5 sgRNA
Spacer
PAM
Insertion
Deletion
Frequency (%)
0.12
0.10
0.08
0.06
0.04
0.02
0
sgRNA + Cas9-dDT
sgRNA + Cas9-XLCH$_N$-DTR
Position
E
Human *CCR5* locus
Spacer
PAM
Feq. (%)
WT 5'...TCTTCTTCATCATCCTCCTGACAATC-GATAGGTACCT...3' (85.7)
-9 5'...TCT----------TCCTCCTGACAATC-GATAGGTACCT...3' (0.08)
-3 5'...TCTTCTTCA---TCCTCCTGACAATC-GATAGGTACCT...3' (0.05)
+1 5'...TCTTCTTCATCATCCTCCTGACAATCCGATAGGTACCT...3' (0.04)
-13 5'...TCTTCTTC----------CAATC-GATAGGTACCT...3' (0.03)
-18 5'...TCTTCTTCATCATCCT----------CCT...3' (0.02)
-1 5'...TCTTCTT-ATCATCCTCCTGACAATC-GATAGGTACCT...3' (0.02)
-1 5'...TCTTCTTCATCATCCTCCTGA-AATC-GATAGGTACCT...3' (0.01)
F
sgRNA + Cas9-dDT (RNP)
Spacer
PAM
Insertion
Deletion
Frequency (%)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
sgRNA + Cas9-XLCH$_N$-DTR (RNP)
Position

Figure S6. Cas9, but not Cas9 RNP, can be delivered into cells using DT-based and BoNT/X-based systems. Related to Figure 5-6.

(A) Schematic of the substrate and sgRNAs. A DNA substrate (contains GFP gene) and four well-established GFP-targeting sgRNAs were produced. The expected size of substrate and cleaved products are shown. bp, base pair.

(B-C) In vitro cleavage assay. 250 ng purified Cas9-dDT (**B**) or Cas9-XLCH$_N$-DTR (**C**) were pre-mixed with 50 ng sgRNA at room temperature for 5 min to form RNP. 400 ng DNA substrate was added to the RNP and the mixture was incubated at 37 °C for 1 h. Agarose gel (1%) electrophoresis was used to analyze the cleaved products. Representative gel images from one of three independent experiments are shown.

(D) Genome editing for *CCR5* locus. HEK293 cells were transfected with *CCR5*-targeting sgRNA and treated with 1 μM Cas9-dDT or activated Cas9-XLCH$_N$-DTR. The amplicon sequence of the sgRNA targeting region was plotted on the x-axis, and the frequency of insertion (red) and deletion (blue) at individual position were plotted on the y-axis. The position of spacer and PAM (protospacer adjacent motif) were marked. Representative histograms from one of three independent experiments are shown.

(E) Representative genotypes of Cas9-dDT edited human *CCR5* locus in HEK293 cells. The -9 bp mutation is the most frequent genotype.

(F) Cas9 RNP cannot be delivered. RNP was generated by mixing protein and sgRNA (5:1, w/w) at room temperature for 5 min. HeLa cells were treated with 1 μM RNP for two days. No significant InDel peaks can be identified. Representative histograms from one of three independent experiments are shown.