Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo

John A. Zuris, David B. Thompson, Yilai Shu, John P. Guilinger, Jeffrey L. Bessen, Johnny H. Hu, Morgan L. Maeder, J. Keith Joung, Zheng-Yi Chen, & David R. Liu

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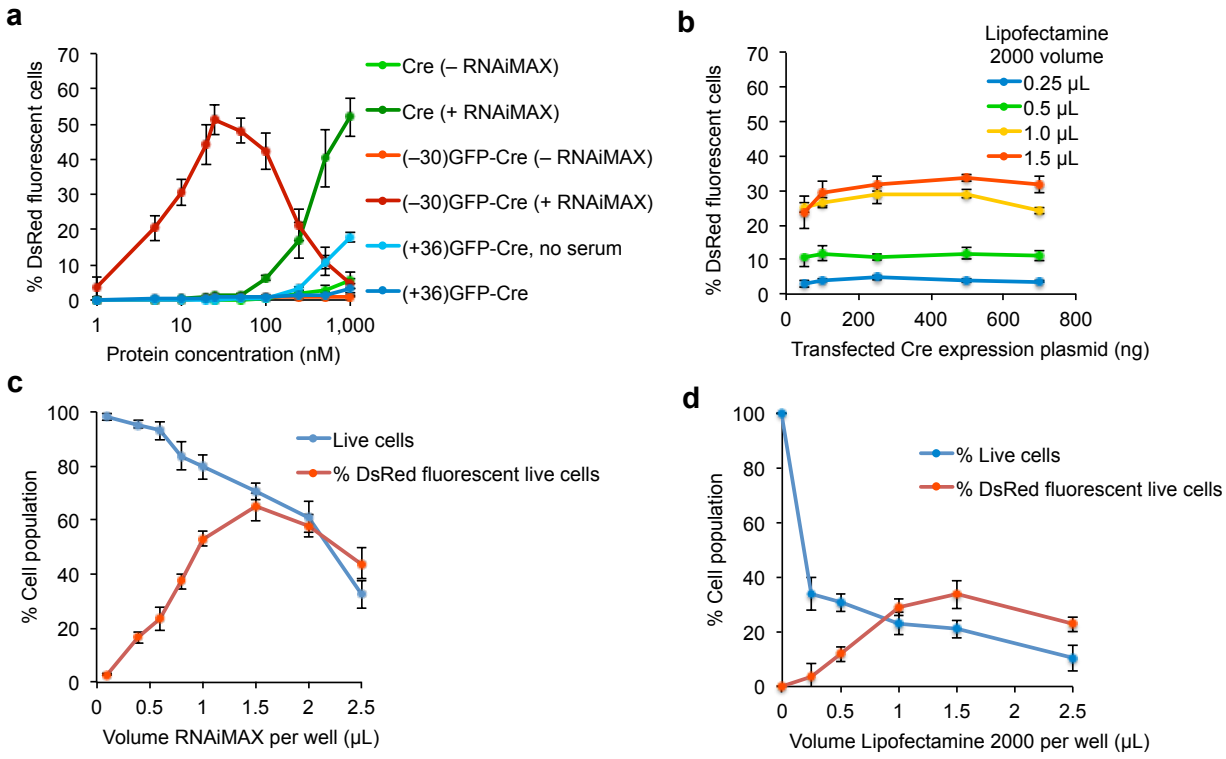
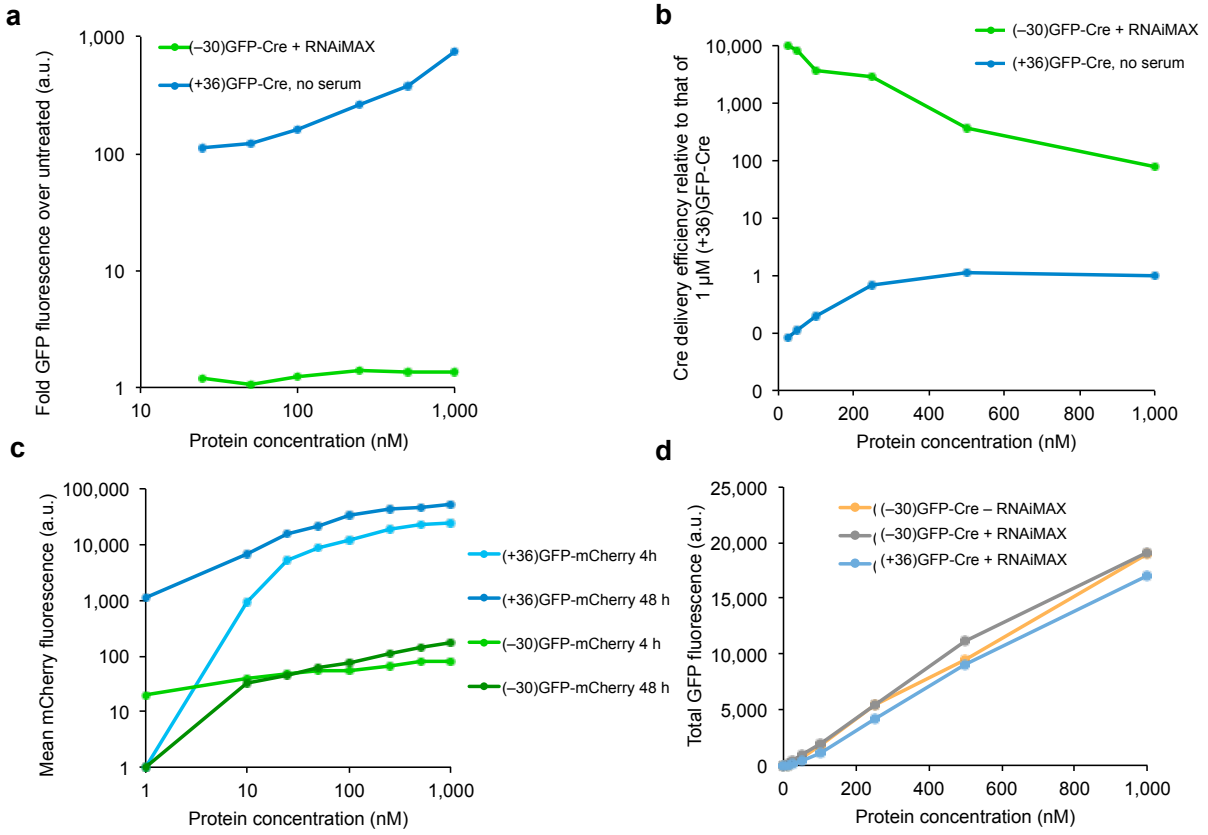
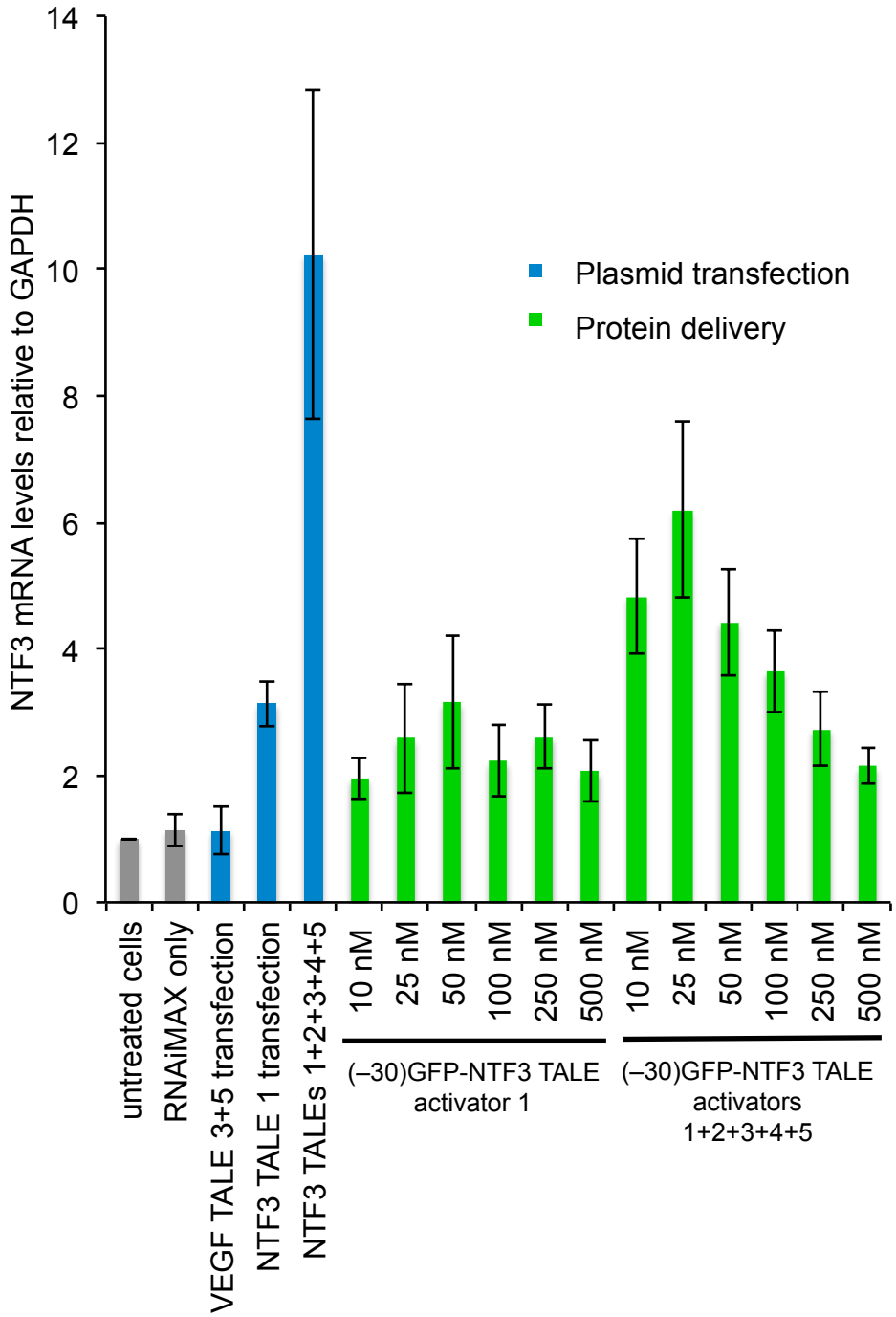
SUPPLEMENTARY FIGURES



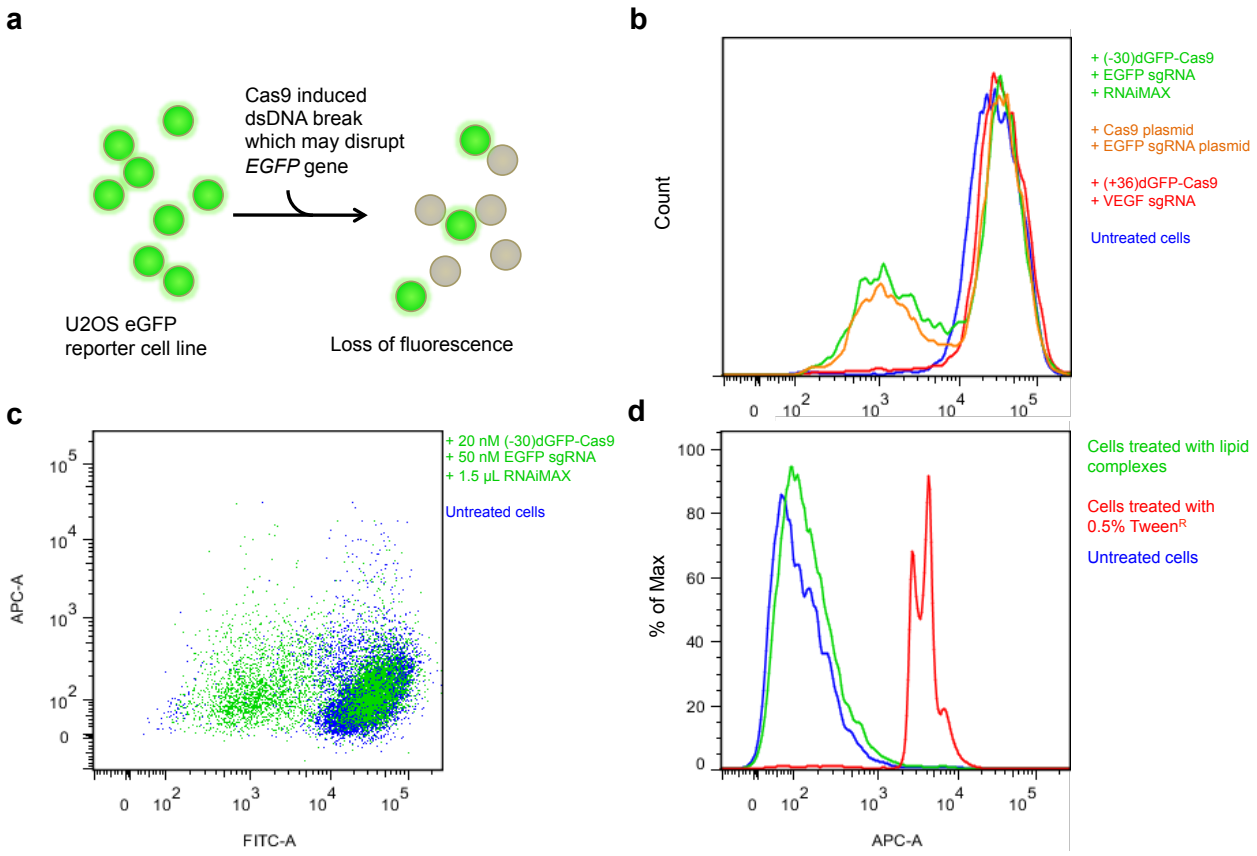
Supplementary Figure 1. Optimization of cationic lipid-mediated delivery of Cre and comparison to delivery using (+36)GFP-Cre and plasmid transfection. **(a)** Optimization of (–30)GFP-Cre delivery in BSR-TdTomato cells, a second reporter cell line used for measuring Cre recombination efficiency. (**b**) Optimization of Cre expression plasmid transfection in HeLa DsRed reporter cells by varying both plasmid dose and Lipofectamine 2000 dose and measuring the presence of DsRed fluorescent cells 48 hours after transfection by flow cytometry. Based on these results, 500 ng of Cre expression plasmid was chosen for 48-well format experiments using 275 μL of DMEM-FBS without antibiotics. (**c**) Effect of RNAiMAX dosage on (–30)GFP-Cre recombination efficiency in HeLa dsRed reporter cells and corresponding toxicity as measured by flow cytometry using the TO-PRO-3 live/dead stain (Life Technologies). (**d**) Effect of Lipofectamine 2000 dosage on transfected Cre plasmid DsRed recombination efficiency and corresponding toxicity as measured by flow cytometry using the TO-PRO-3 live/dead stain. For (**a**)-(**d**), error bars reflect s.d. from three biological replicates performed on different days.



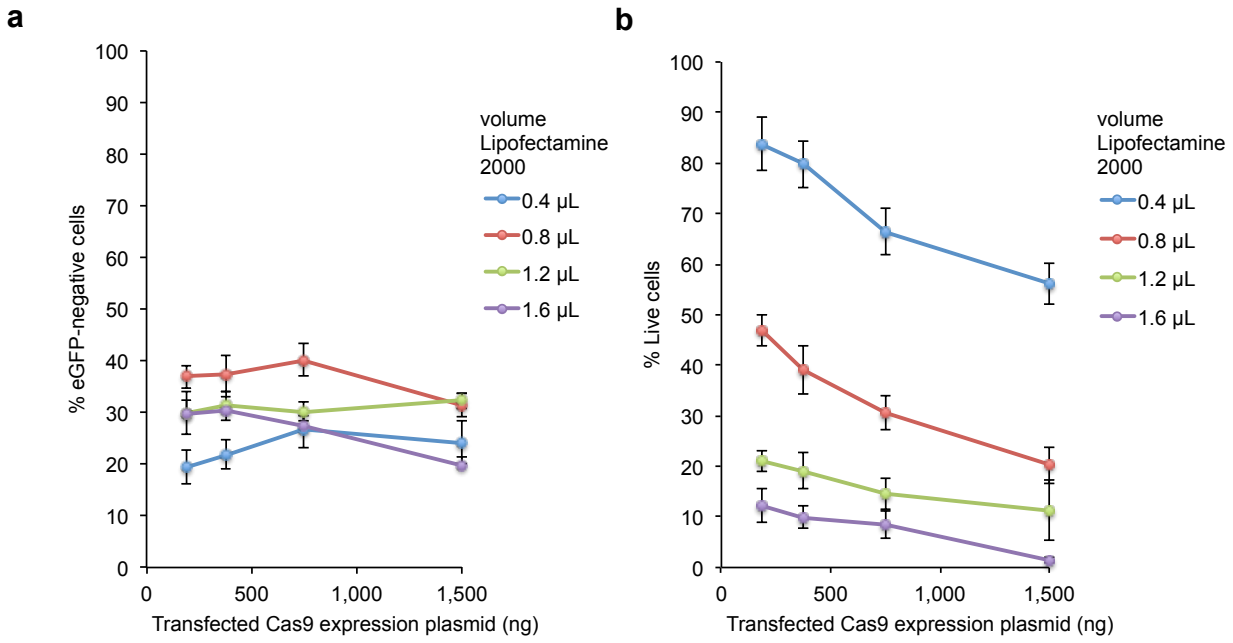
Supplementary Figure 2. Protein uptake by cationic lipid-mediated delivery compared with superpositively charged cationic protein delivery. (**a**) Quantification of GFP fluorescence from cells treated with either (–30)GFP-Cre and RNAiMAX or (+36)GFP-Cre after washing cells with PBS + heparin (20 U/mL) to remove unbound protein. (**b**) Comparison of mCherry uptake by (–30)GFP-fusion + 1.5 μM RNAiMAX treatment versus (+36)GFP fusion by measuring mean mCherry fluorescence of total cell population 48 h after treatment and washing cells with PBS + heparin. (**c**) Total cellular GFP fluorescence of (–30)GFP-Cre or (+36)GFP-Cre in the presence or absence of RNAiMAX. Data shown reflect a single biological replicate.



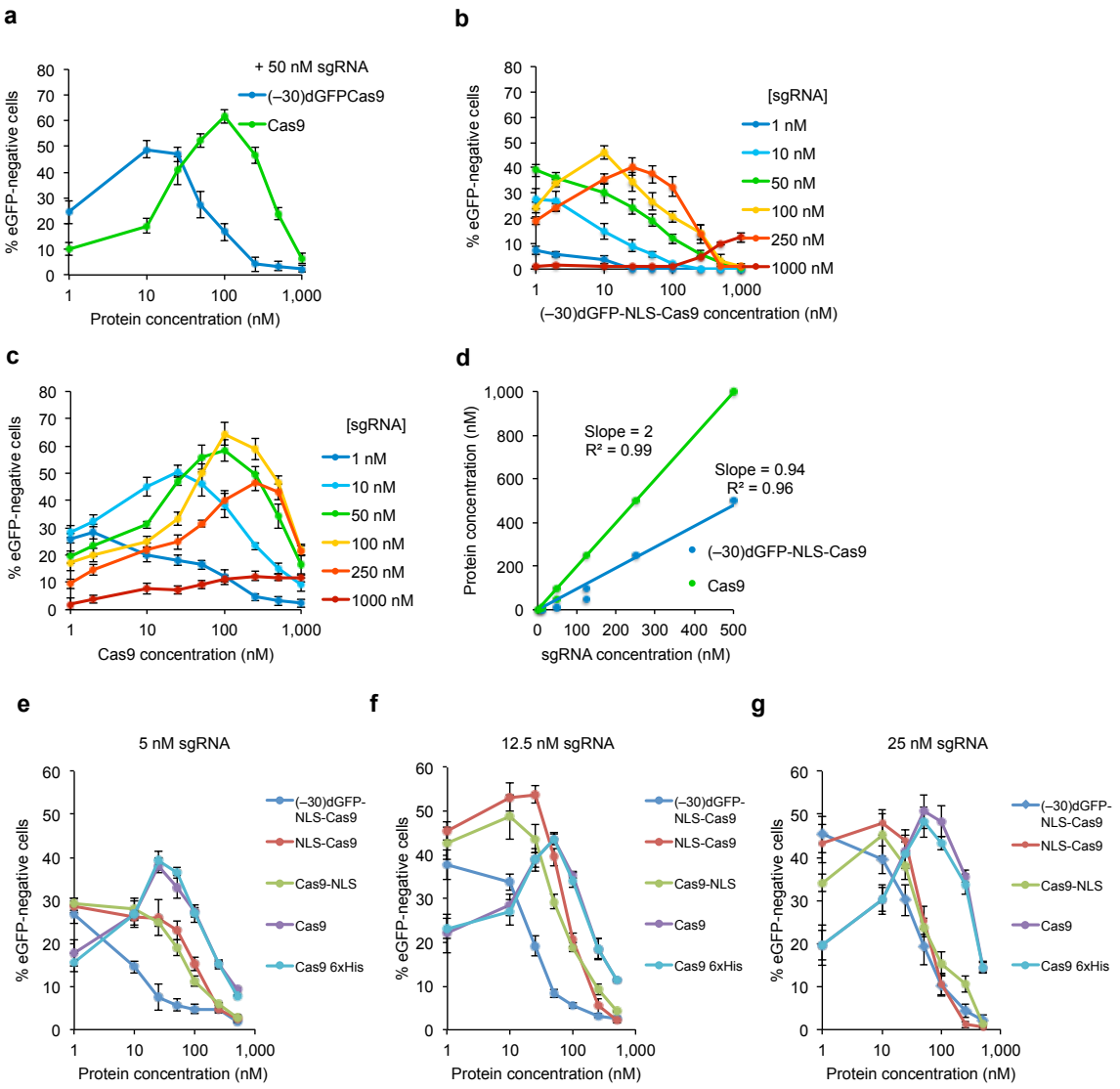
Supplementary Figure 3. Delivery optimization of TALE activators designed to target the *NTF3* gene. (**a**) HEK293T cells were treated with either NTF3 TALE plasmid by transfection of by liposomal delivery of NTF3 TALE proteins. Cells were harvested after empirically determined optimal incubation time for both treatments and analyzed by qRT-PCR for mRNA levels of NTF3. All protein-delivery and transfection experiments were performed in a 48-well plate with 275 μL DMEM-FBS without antibiotics. Error bars reflect s.d. from six biological replicates performed on different days.



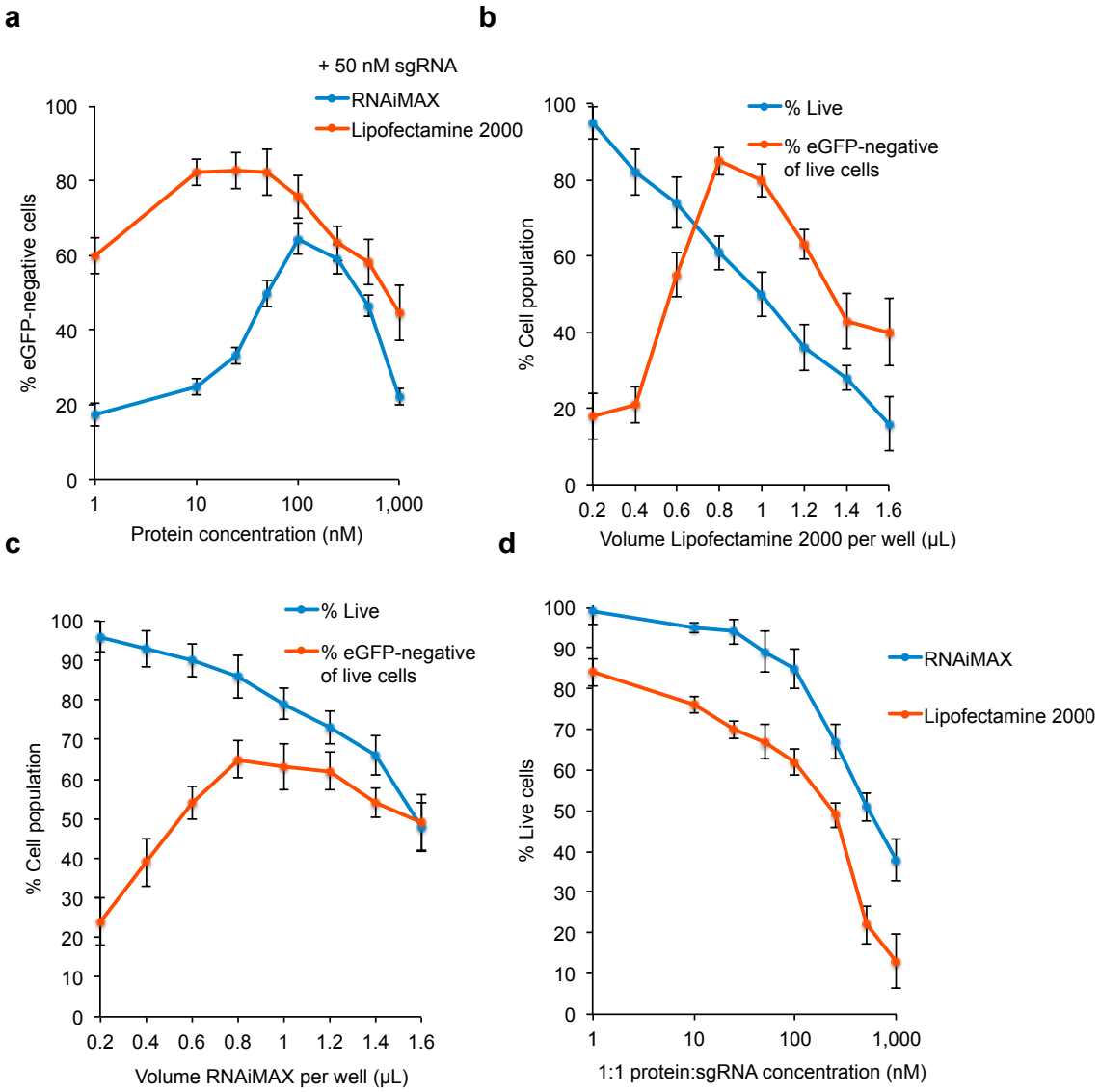
Supplementary Figure 4. Gene disruption frequency of an *EGFP* reporter gene from delivery of Cas9:sgRNA as analyzed by flow cytometry. (**a**) Schematic of EGFP disruption in U2OS cells by NHEJ induced by Cas9 double-stranded breaks. (**b**) Delivery of *EGFP*-targeting sgRNA or an off-target sgRNA complexed with (–30)dGFP-Cas9 using RNAiMAX along with a plasmid transfection positive control (orange). (**c**) Confirmation that disruption of EGFP fluorescence is not a result of cellular toxicity by treating samples with the TO-PRO-3 live/dead stain (Life Technologies, Carlsbad CA) and analyzing the resulting cells by flow cytometry. (**d**) Testing the TO-PRO-3 stain by addition of a cell permeabilizing, but not completely membrane lysing, detergent (0.5% Tween).



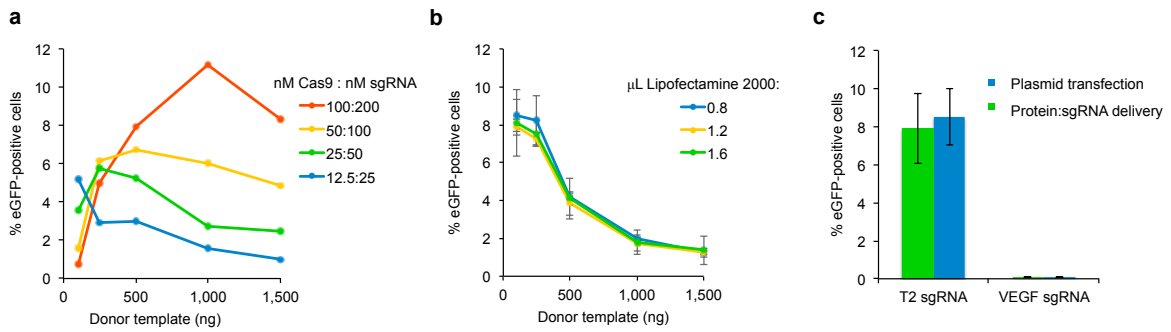
Supplementary Figure 5. Optimization of Cas9 plasmid transfection conditions and measurement of cellular toxicity at different doses of Lipofectamine 2000. (**a**) Optimization of transfection efficiency for Cas9 expression plasmid in U2OS EGFP reporter cell line was performed by varying both the amount of Cas9 plasmid and the dose of Lipofectamine 2000. Input sgRNA expression plasmid was held constant at 250 ng input DNA for all treatments. All treatments were performed in a 48-well plate with 275 μL DMEM-FBS without antibiotics. After 48 hours, cells were assayed for loss of EGFP by flow cytometry. (**b**) Measuring toxicity of various Cas9 plasmid/Lipofectamine 2000 transfection conditions after 48 hours using TO-PRO-3 live/dead stain and quantifying cellular toxicity by flow cytometry. From (**a**) and (**b**) a Cas9 plasmid dose of 750 ng and a Lipofectamine 2000 dose of 0.8 μL were chosen as plasmid transfection conditions that resulted in maximal gene disruption for the remaining studies in this work. For (**a**) and (**b**), error bars reflect s.d. from three biological replicates performed on different days.



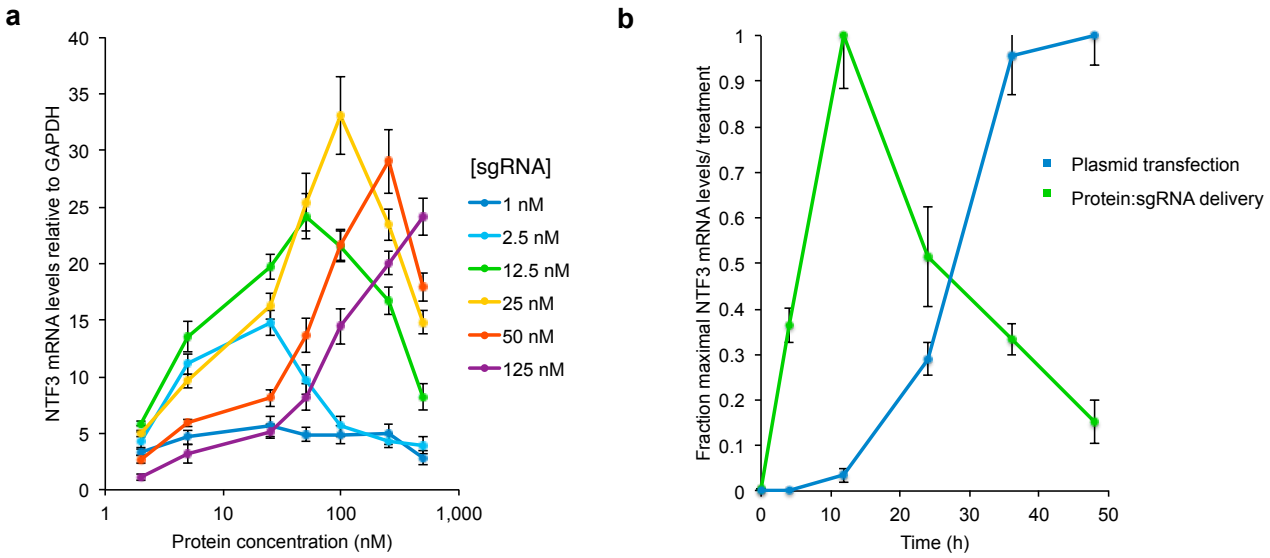
Supplementary Figure 6. Optimization of Cas9:sgRNA-mediated gene disruption. (**a**) Cationic lipid-mediated delivery efficiency of two tested constructs showing that the more anionic (–30)dGFP-NLS-Cas9 facilitates more efficient delivery at low protein and sgRNA concentrations compared with native Cas9. (**b**) Delivery optimization of (–30)dGFP-NLS-Cas9 and (**c**) Cas9 function of protein and sgRNA concentration. (**d**) Optimal sgRNA to protein ratio for RNAiMAX-mediated delivery of (–30)dGFP-NLS-Cas9 and native Cas9. All experiments were performed in a 48-well plate using a volume of 275 μL DMEM-FBS without antibiotics and EGFP gene disruption was measured by flow cytometry. (**e-g**) Effect of an N– or C–terminal NLS, an N–terminal (–30)dGFP fusion, or a C–terminal His-tag on functional Cas9 delivery as a function of both sgRNA and Cas9 concentration. *EGFP* gene disruption in U2OS EGFP reporter cell line was measured at three fixed sgRNA concentrations: (**e**) 5 nM, (**f**) 12.5 nM, and (**g**) 25 nM, along with varying protein concentrations show in the graphs. Delivery was performed using 0.8 μL RNAiMAX in 48-well format using 275 μL DMEM-FBS without antibiotics and assayed by flow cytometry 48 hours later for loss of EGFP fluorescence signal. Error bars reflect s.d. from three biological replicates performed on different days.



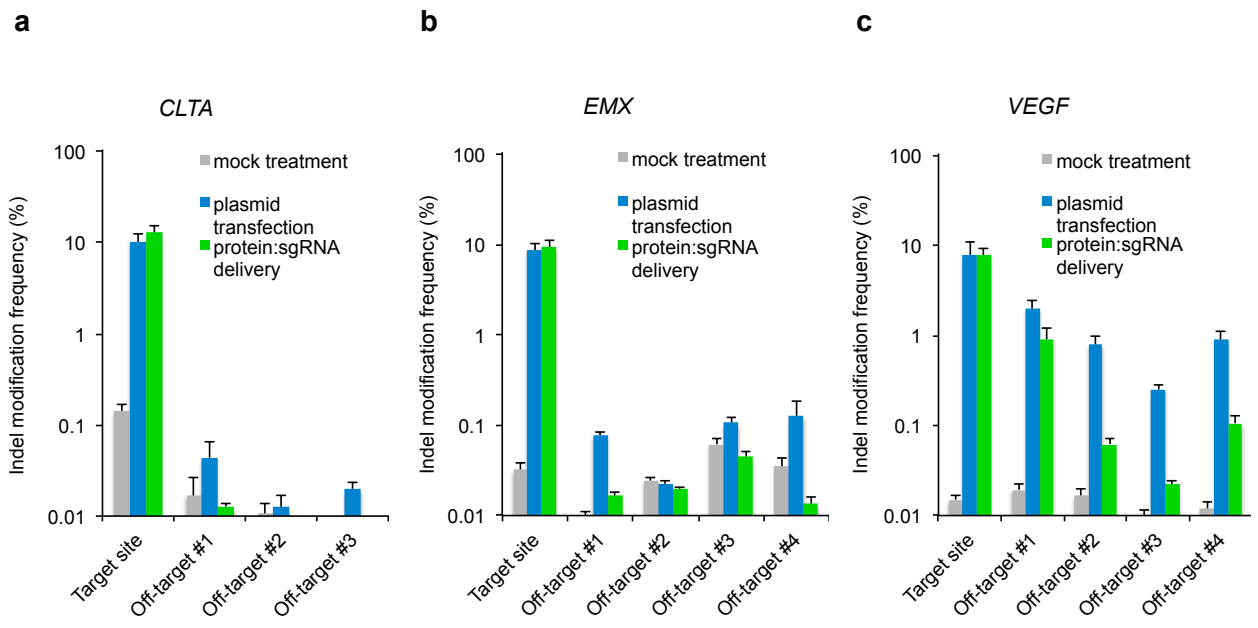
Supplementary Figure 7. Effect of RNAiMAX and Lipofectamine 2000 on Cas9:sgRNA delivery efficiency and cellular toxicity. (**a**) *EGFP* gene disruption at different Cas9 protein concentrations and a constant dose of 50 nM EGFP sgRNA in U2OS EGFP reporter cells treated with either 0.8 μL of RNAiMAX or 0.8 μL Lipofectamine 2000. After 16 hours, media was removed and fresh media was added to cells until end point of assay 48 hours post protein delivery treatment. The live cell population was determined by flow cytometry using TO-PRO-3 live/dead stain. (**b**) Toxicity profile for Cas9:sgRNA delivery to U2OS cells as a function of Lipofectamine 2000 dose. (**c**) Toxicity profile for U2OS cells as a function of RNAiMAX dose. (**d**) Cellular toxicity for a broad range of Cas9:sgRNA treatments using 1:1 protein:sgRNA delivery conditions at optimal doses of RNAiMAX or Lipofectamine 2000 by TO-PRO-3 live/dead stain and flow cytometry. Dose of RNAiMAX and Lipofectamine 2000 were both 0.8 μL in a volume of 275 μL in a 48-well plate format. For (**a**)-(**d**), error bars reflect s.d. from three biological replicates performed on different days.



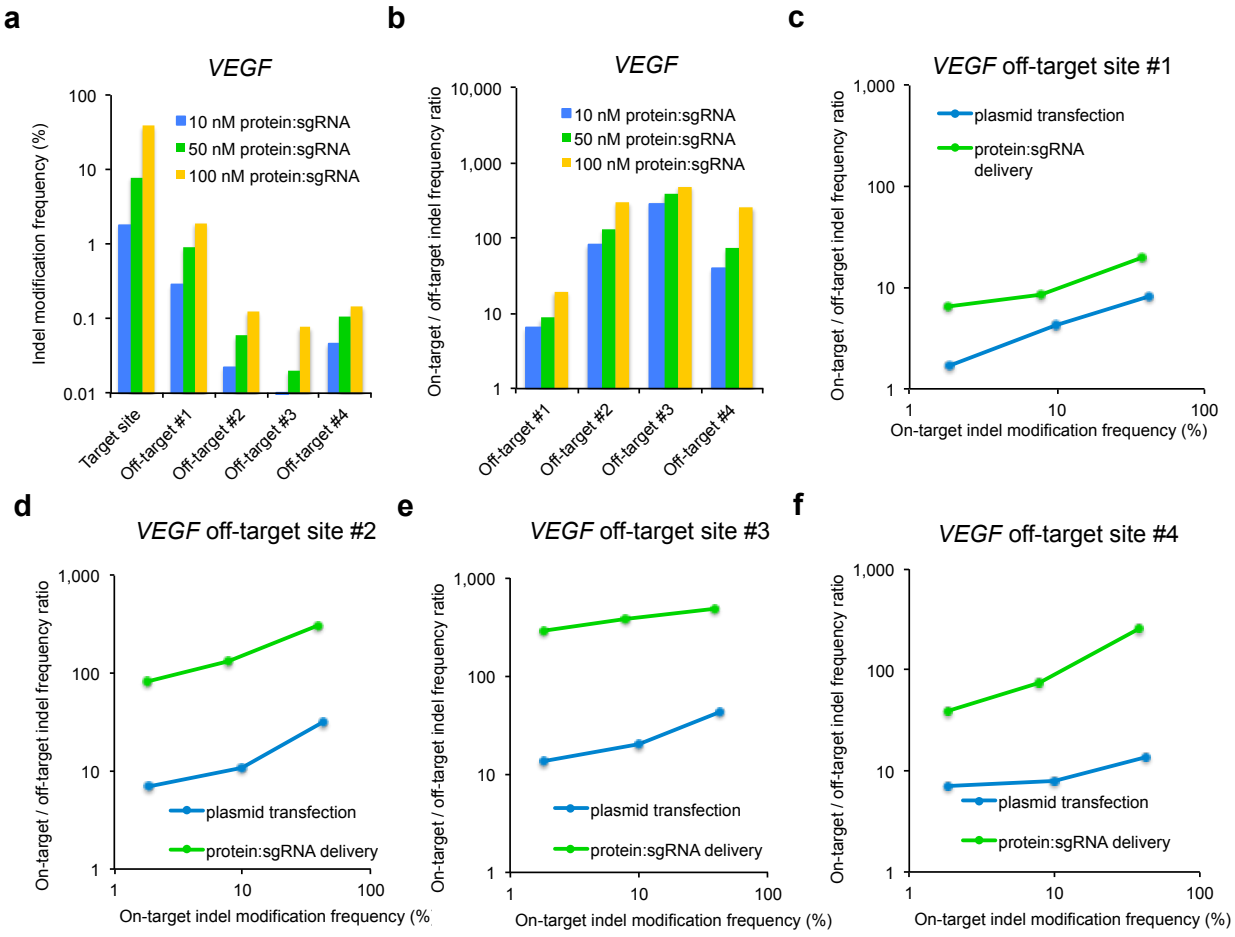
Supplementary Figure 8. Optimization and comparison of homology-directed repair (HDR) efficiency for Cas9:sgRNA delivery by cationic lipids and plasmid transfection (**a**) Cas9:sgRNA protein delivery optimization of HDR efficiency in a reporter cell line that expresses EGFP upon repair of a disrupted *EGFP* reporter gene[11] using cationic lipid-mediated protein delivery, a 2:1 ratio of T2 sgRNA:Cas9 protein, 1 μL Lipofectamine 2000, and variable amounts of ssODN donor template (**Supplementary Notes**) performed as a single treatment. (**b**) Optimization of plasmid transfection-mediated HDR using 700 ng Cas9 plasmid and 250 ng sgRNA plasmid with variable doses of Lipofectamine 2000 and ssODN donor template. (**c**) HDR efficiency comparison of cationic lipid-mediated protein:sgRNA delivery and plasmid DNA transfection at optimized conditions for both techniques using on-target (T2) and non-target (VEGF) sgRNAs. For (**b-c**), error bars reflect s.d. of three independent biological replicates performed on different days.



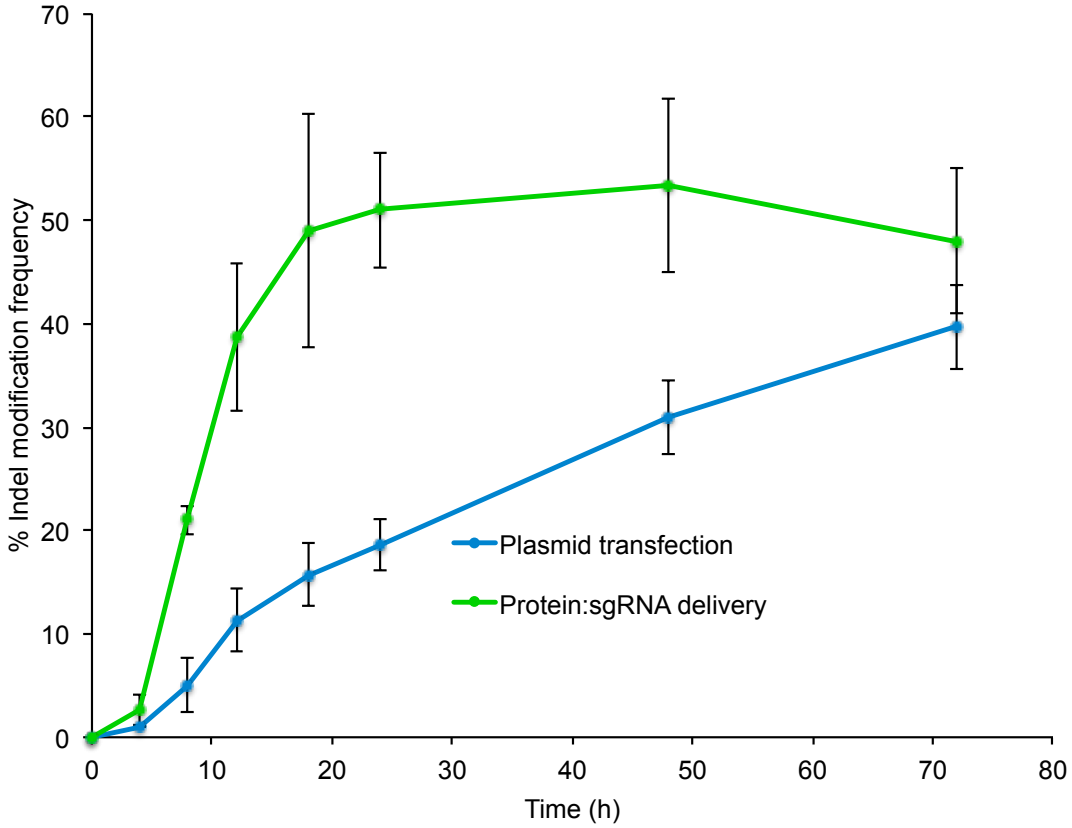
Supplementary Figure 9. Optimization of dCas9-VP64 delivery targeting the *NTF3* gene at varying concentrations of protein and sgRNA. (**a**) HEK293T cells were treated with dCas9-VP64 activator at varying protein concentrations and a mixture of all six *NTF3*–targeting sgRNAs for 12 hours using 0.8 μL RNAiMAX in 275 μL DMEM-FBS without antibiotics in a 48-well plate format. NTF3 mRNA levels were determined by qRT-PCR and normalized to those of GAPDH. Total sgRNA concentrations are listed (each sgRNA is present at one-sixth of the listed total concentration). (**b**) Time course for *NTF3* gene activation by protein:sgRNA delivery and plasmid transfection. NTF3 mRNA levels were measured at several time points using all six sgRNAs either from expression plasmids (in the case of the dCas9-VP64 activator plasmid transfection treatment), or as *in vitro* transcribed sgRNAs complexed with 100 nM dCas9-VP64 activator and cationic lipids (in the case of protein:sgRNA delivery). For (**a**) and (**b**), error bars reflect s.d. from six biological replicates performed on different days.



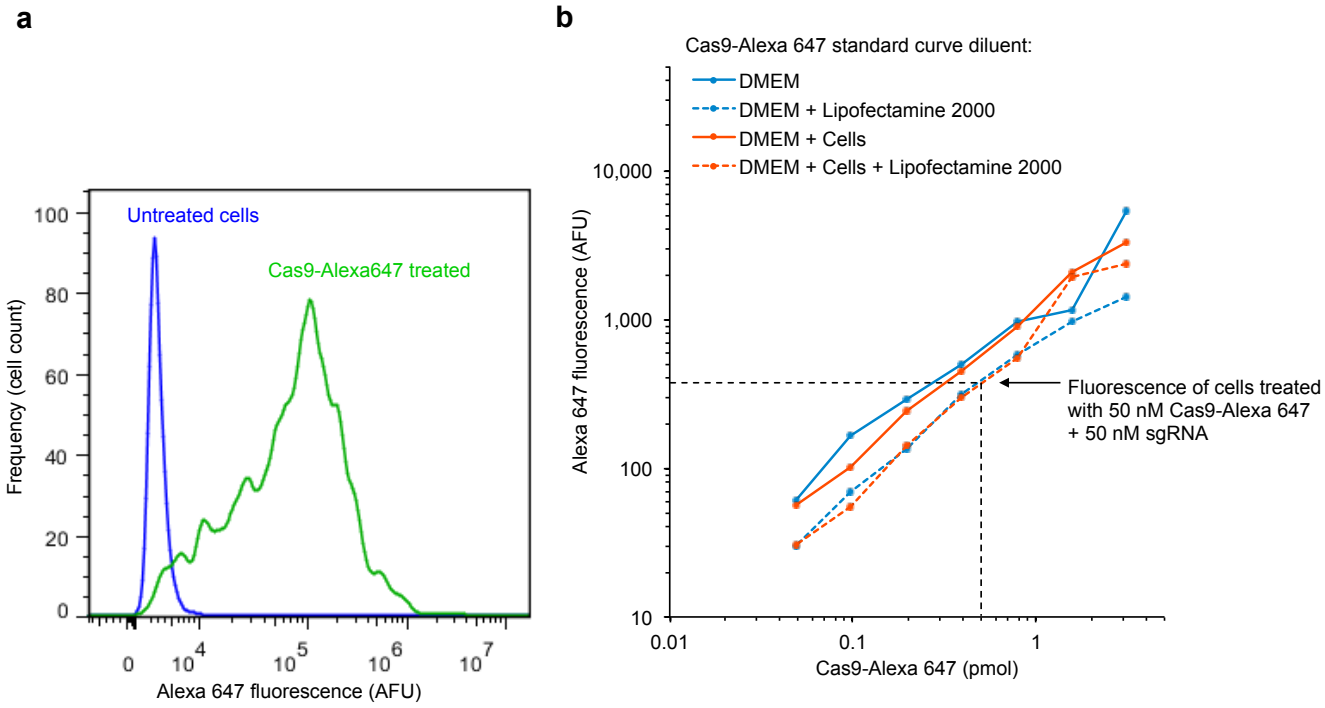
Supplementary Figure 10. Indel frequencies, measured by high-throughput sequencing, of several human genes treated either by a mock treatment, by transfection of Cas9 plasmid and sgRNA linear DNA PCR product, or by cationic lipid-mediated protein:sgRNA delivery. Mock treatment involved cationic lipid-mediated protein:sgRNA delivery of *EGFP*-targeting sgRNA instead of one of the three human gene-targeting sgRNAs. (a) On-target and off-target indel frequencies for the *CLTA* gene. (**b**) On-target and off-target indel frequencies for the *EMX* gene. (**c**) On-target and off-target indel frequencies for the *VEGF* gene. Each on- and off-target sample was sequenced once with > 10,000 sequences analyzed per on-target sample and an average of > 111,000 sequences analyzed per off-target sample (**Supplementary Table 2**). For (**a**)-(**c**), error bars reflect s.d. from three biological replicates performed on different days.



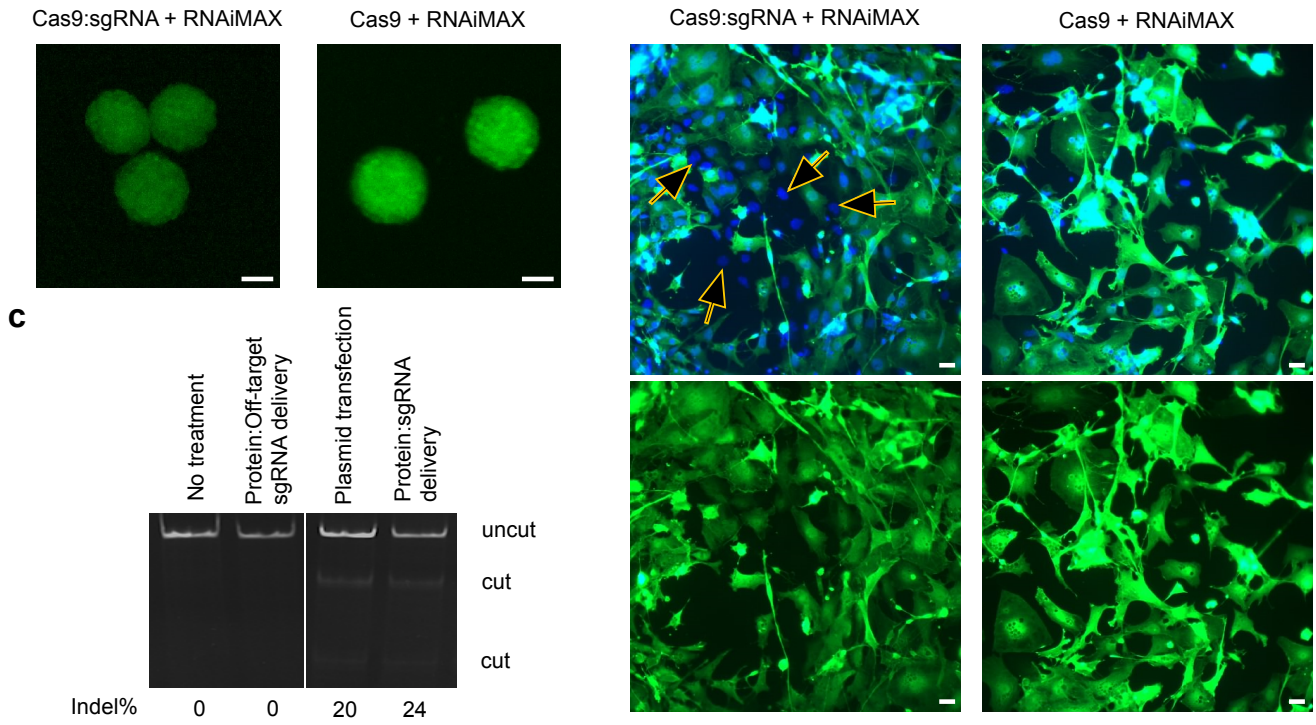
Supplementary Figure 11. Concentration dependence of on-target and off-target indel modification frequencies for Cas9 plasmid transfection or lipid-mediated protein:sgRNA delivery. (**a**) Indel modification frequencies measured by high-throughput sequencing for *VEGF* on- and off-target sites at varying doses of Cas9:sgRNA. (**b**) On-target:off-target specificity ratio at different Cas9:sgRNA concentrations. (**c**) Comparison of on-target:off target specificity ratio for protein delivery and plasmid transfection at *VEGF* off-target site #1 as a function of on-target indel modification frequency at a range of modification frequencies for both treatments (~1% to ~40 % indel modification frequency). (**d, e, f**) Same as (**c**) for VEGF off-target sites #2, #3, and #4. Each on- and off-target sample was sequenced once with > 10,000 sequences analyzed per on-target sample and an average of > 111,000 sequences analyzed per off-target sample. All data shown were from a single biological replicate.



Supplementary Figure 12. Time course of Cas9 nuclease activity from protein:sgRNA delivery and plasmid transfection. U2OS EGFP reporter cells were treated with either 50 nM Cas9 protein and 50 nM sgRNA and 0.8 μL Lipofectamine 2000 in 275 μL DMEM-FBS without antibiotics, or transfected with 750 ng Cas9 expression plasmid and 250 ng EGFP sgRNA expression plasmid for 2 hours. Media was either removed and samples collected after another 2 hours, or replaced with fresh DMEM-FBS without delivery agents and collected at later time points, as shown. Samples were analyzed for indels in the *EGFP* gene using a Surveyor T7E1 cleavage assay. Bands were quantified by ImageJ software. Error bars reflect s.d. from three biological replicates performed on different days.



Supplementary Figure 13. Quantification of Cas9 protein uptake into U2OS EGFP reporter cells. (**a**) Flow cytometry plots showing Alexa647 fluorescence of cells treated with 50 nM Alexa647-conjugated Cas9 and 50 nM EGFP sgRNA, or of untreated cells. (**b**) U2OS EGFP reporter cells were treated with 50 nM Alexa647-conjugated Cas9 protein, 50 nM EGFP sgRNA, and 0.8 μL of Lipofectamine 2000. After a 4-hour incubation at 37 °C, cells were washed extensively with PBS containing 20 U/mL of heparin to remove electrostatically-bound cationic lipid complexes, and then trypsinized. In a plate reader (Tecan M1000 Pro) with fluorescence excitation at 650 nm and emission at 665 nm, wells each containing 10,000 Cas9-Alexa647-treated cells were measured for whole population fluorescence. Standard curves were established by measuring the fluorescence of known quantities of Cas9-Alexa647 in either DMEM containing 10% FBS, or in a suspension of trypsinized U2OS cells at 10,000 cells per well, with protein either diluted directly, or pre-complexed with 0.8 μL Lipofectamine 2000 then diluted. A two-fold serial dilution starting from 50 pmol to 0.048 pmols was performed to generate the standard curve samples. Values for 0.048 pmol to 3.125 pmol are shown. The intersection of the dotted black lines shows the measured total Alexa647 fluorescence of 10,000 cells treated with 50 nM Alexa647-conjugated Cas9 and 50 nM EGFP sgRNA and washed as described above. 50 nM Cas9-Alexa647-treated cells showed a total cell-associated Alexa647-labeled protein signal of 0.5 pmol per well. This quantity represents 4% of the input protein in the Cas9-Alexa647:sgRNA treatment, and corresponds to $(6.02 \times 10^{23}) * 5.0 \times 10^{-13}$ moles Cas9-Alexa647 / 10,000 cells per well = 3×10^{7} molecules of Cas9-Alexa647 per cell. Assuming a total protein content per cell of roughly 7.9×10^{9} molecules (estimate from *Molecular Cell Biology*, Section 1.2, 4th edition), internalized Cas9-Alexa647 represented 0.4% of total cellular protein. All values shown are the average of three technical replicates.



Supplementary Figure 14. Delivery of Cas9 nuclease to mouse embryonic stem cells. Delivery of Cas9 endonuclease to mouse embryonic stem cells. (**a**) Floating spheres treated with 100 nM Cas9 protein, and 0.8 μL Lipofectamine 2000 but no sgRNA (control) retained strong GFP fluorescence (right), while those treated with 100 nM Cas9:sgRNA and 0.8 μL Lipofectamine 2000 exhibited decreased GFP fluorescence under identical imaging conditions (left). Scale bars are 100 μm. (**b**) After cell attachment, virtually all control progenitor cells were GFP positive (right panels). Cas9:sgRNA treatment led to significant reduction in GFP expression (left panels) and many progenitor cells showed complete GFP knockdown (arrows) after cell attachment. Scale bars are 20 μm. **(c)** T7EI assay on stem cells harvested after imaging confirm cleavage of GFP reporter. Similar gene target modification efficiencies were observed from cationic lipid-mediated Cas9:sgRNA delivery (24%) and from co-transfection of Cas9 and EGFP sgRNA plasmids (20%).

Supplementary Figure 15. Genome modification induced by cationic lipid-mediated protein delivery of Cas9 nuclease and sgRNA at endogenous loci *in vivo*. Approximately 10 days after injection of Cas9:sgRNA protein into Atoh1-GFP mice under identical conditions described in **Fig. 6d**, ~15 mg of mouse hair cell tissue was dissected. 150 ng of isolated genomic DNA was prepared for high-throughput sequencing. (**a**) Representative examples of genomic DNA sequences at the *EGFP* on-target locus that are modified following cationic lipid-mediated delivery of Cas9 and EGFP sgRNA in Atoh1-GFP mouse hair cells. For each example shown, the unmodified genomic site is the first sequence, followed by the most abundant eight sequences containing deletions and three sequences containing insertions. The numbers before each sequence indicate sequencing counts. The sgRNA target sites are bold and underlined in green. Insertions and deletions are shown in red. PAM site is shown in blue. (**b**) Identical analysis as in (**a**) for *EMX* on-target site in Atoh1-GFP mouse hair cells. Indels shown here for both the *EGFP* and *EMX* genomic loci are from a single biological replicate chosen from a representative set of sequenced samples all showing similar indel profiles.

EMX_On	GAGTCCGAGCAGAAGAAGAAGGG
EMX_Off1	GAGgCCGAGCAGAAGAAagACGG
EMX_Off2	GAGTCCtAGCAGgAGAAGAAGaG
EMX_Off3	GAGTCtaAGCAGAAGAAGAAGaG
EMX_Off4	GAGTtaGAGCAGAAGAAGAAAGG
VEGF_On	GGGTGGGGGGAGTTTGCTCCTGG
VEGF_Off1	GGaTGGaGGGAGTTTGCTCCTGG
VEGF_Off2	GGGaGGGtGGAGTTTGCTCCTGG
VEGF_Off3	cGGgGGaGGGAGTTTGCTCCTGG
VEGF_Off4	GGGgaGGGGaAGTTTGCTCCTGG
CLTA_On	GCAGATGTAGTGTTTCCACAGGG
CLTA_Off1	aCAtATGTAGTaTTTCCACAGGG
CLTA_Off2	cCAGATGTAGTaTTcCCACAGGG
CLTA_Off3	ctAGATGaAGTGcTTCCACATGG

Supplementary Table 1. On-target and known off-target substrates of Cas9:sgRNAs that target sites in *EMX*, *VEGF*, and *CLTA*. List of genomic on-target and off-targets sites for *EMX*, *VEGF*, and *CLTA* are shown with mutations from the on-target sequence shown in lower case and red. PAMs are shown in blue.

CLTA Sites	**Mock treatment**	**Plasmid transfection**	**Protein:sgRNA delivery**
CLTA_On			
Indels	14	1228	1498
Total	10000	10000	10000
Modified (%)	0.140	12.280	14.980
P-value		<1.0E-300	<1.0E-300
On:off specificity	1	1	1
CLTA_Off1			
Indels	7	29	14
Total	41518	205204	125370
Modified (%)	0.017	0.014	0.011
P-value		6.6E-01	4.5E-01
On:off specificity		869	1341
CLTA_Off2			
Indels	5	11	8
Total	25338	83944	54409
Modified (%)	0.020	0.013	0.015
P-value		5.5E-01	5.7E-01
On:off specificity		937	1019
CLTA_Off3			
Indels	6	22	8
Total	41643	189886	76863
Modified (%)	0.014	0.012	0.010
P-value		6.2E-01	5.8E-01
On:off specificity		1060	1439

EMX Sites	**Mock treatment**	**Plasmid transfection**	**Protein:sgRNA delivery**
EMX_On			
Indels	3	930	1140
Total	10000	10000	10000
Modified (%)		0.030	9.300
P-value		1.6E-264	<1.0E-300
On:off specificity	1	1	1
EMX_Off1			
Indels	0	6	6
Total	24623	90935	100778
Modified (%)		<0.002	0.007
P-value		3.5E-01	6.1E-01
On:off specificity		1409	1915
EMX_Off2			
Indels	16	53	38
Total	36061	204068	130084
Modified (%)		0.044	0.026
P-value		6.4E-02	1.8E-01
On:off specificity		358	390
EMX_Off3			
Indels	20	147	44
Total	32575	157848	110878
Modified (%)		0.061	0.093
P-value		8.1E-02	1.3E-01
On:off specificity		100	287

EMX_Off4			
Indels	16	141	23
Total	45548	86586	73451
Modified (%)		0.035	0.163
P-value		2.8E-12	7.4E-01
On:off specificity		57	364
VEGF Sites	**Mock treatment**	**Plasmid transfection**	**Protein:sgRNA delivery**
VEGF_On			
Indels	1	989	785
Total		10000	10000
Modified (%)	0.010	9.890	7.850
P-value		1.5E-285	5.7E-228
On:off specificity	1	1	1
VEGF_Off1			
Indels	4	4240	602
Total		38625	184554
Modified (%)	0.010	2.297	0.394
P-value		<1.0E-300	3.7E-52
On:off specificity		4	20
VEGF_Off2			
Indels	5	727	18
Total		30301	79164
Modified (%)	0.017	0.918	<0.002
P-value		4.7E-93	1.3E-04
On:off specificity		11	3925
VEGF_Off3			
Indels	2	536	21
Total		26379	110902
Modified (%)	0.008	0.483	0.022
P-value		2.0E-46	2.0E-01
On:off specificity		20	352
VEGF_Off4			
Indels	0	1531	45
Total		26012	122403

Supplementary Table 2. Indel frequencies, P values, and on-target:off-target cleavage specificity ratios for *EMX*, *CLTA*, and *VEGF* on-target sites and 11 known off-target sites. Total: total number of sequence counts; only the first 10,000 sequences were analyzed for the on-target site sequences. Modified: number of indels divided by total number of sequences as percentages. Upper limits of potential modification were calculated for sites with no observed indels by assuming there is less than one indel then dividing by the total sequence count to arrive at an upper limit modification percentage, or taking the theoretical limit of detection (1/49,500; see **Supplementary Results** above), whichever value was larger. P-values: for mock treatment, Cas9 plasmid transfection, and liposomal Cas9 protein:sgRNA delivery, P-values were calculated as previously reported[4] using a two-sided Fisher's exact test between each *CLTA*-targeted treatment sample (either DNA transfection or protein:sgRNA delivery) versus the control sample (mock treatment) treated with Cas9 protein and an sgRNA targeting *EGFP*. On:off specificity is the ratio of on-target to off-target genomic modification frequency for each site. (**b**) Experimental and analytic methods as in (**a**) applied to *EMX* target sites.

(**c**) Experimental and analytic methods as in (**a**) applied to *VEGF* target sites. Indel numbers in the mock treatment control were subtracted from both plasmid transfection and protein:sgRNA delivery indel numbers for determining total #indels and for calculating on-target:off-target ratios in **Fig. 5** in the main text and also for **Supplementary Fig. 9**.

SUPPLEMENTARY RESULTS

Comparison of recombination efficiency and cellular toxicity for liposomal protein delivery of (–30)GFP-Cre versus optimized plasmid DNA transfection

We optimized plasmid transfection of HeLa reporter cells across a range of plasmid and Lipofectamine 2000 doses, and found that transfection efficiency in this cell line yielded a maximum of 33% DsRed fluorescent cells (**Supplementary Fig. 1b**). These findings suggest that cationic lipid-based (–30)GFP-Cre protein delivery can result in more functional Cre recombinase activity than well-established plasmid DNA transfection methods.

As nucleic acid transfection by cationic lipids is to known to induce cellular toxicity,[1] we also characterized the toxicity of cationic lipid-mediated (–30)GFP-Cre protein delivery and compared the results with those of plasmid transfection methods (**Supplementary Figs. 1b, and c**). Cells undergoing protein delivery or plasmid transfection were analyzed for cell survival by flow cytometry using the TO-PRO-3 live/dead cell stain (Life Technologies). While increasing the amount of RNAiMAX predictably increased toxicity (**Supplementary Fig. 1b**), the use of 1.5 μL RNAiMAX per 275 μL sample maximized recombination efficiency (> 50% DsRed-positive live cells) while inducing minimal cell toxicity (> 80% live cells, **Supplementary Fig. 1c**). In contrast, all efficacious plasmid DNA delivery conditions tested exhibited much greater toxicity (**Supplementary Fig. 1d**), with fewer than 40% of cells surviving plasmid transfection under any condition that resulted in > 5% DsRed-positive live cells. These results indicate that optimized cationic lipid-mediated delivery of anionic Cre recombinase achieves substantially greater delivered Cre activity with much lower toxicity than optimized plasmid DNA transfection.

Evaluation of different liposomal formulations on (–30)GFP-Cre delivery

While RNAiMAX remained the most effective functional delivery agent for (–30)GFP-Cre, other cationic lipid formulations also resulted in potent delivery. Lipofectamine 2000 and Lipofectamine LTX (Life Technologies), two plasmid transfection reagents based on cationic lipid formulations,[2] and SAINT-Red (Synvolux Therapeutics), an siRNA delivery formulation containing a synthetic pyridinium-based cationic lipid, all resulted in strong functional (–30)GFP-Cre delivery over a range of concentrations (**Fig 2e**). In contrast, we did not observe strong delivery with the cationic lipid DOTAP (Roche Diagnostics) or the peptide-based nucleic acid delivery agent EZ-PLEX (Ascension Bio) (**Fig. 2e**). These observations collectively indicate that several, but not all, cationic lipid formulations are able to complex with and deliver negatively charged proteins into human cells.

Functional protein delivery efficacy and protein uptake of (–30)GFP-Cre + cationic lipids

To determine if the higher potency of liposome-mediated (–30)GFP-Cre delivery compared with that of cationic protein delivery arises from more total protein uptake by cells or from a higher fraction of functional, non-endosomal protein molecules taken up by the cells, we used flow cytometry to measure GFP fluorescence of cells treated with either (+36)GFP-Cre or liposomal (–30)GFP-Cre under their respective optimal Cre delivery conditions. Cellular GFP fluorescence reports the total endocytosed (–30)GFP-Cre or (+36)GFP-Cre, regardless of endosomal or non-endosomal localization.[3] Lipid-mediated protein delivery resulted in surprisingly small increases in total protein uptake (**Supplementary Fig. 2a**), despite the high efficiency of lipid-mediated functional Cre delivery. While (+36)GFP-Cre treatment increased cellular GFP fluorescence by up to three orders of magnitude in a dose-dependent manner (**Supplementary Fig. 2a**), consistent with previous reports,[3,4] liposomal (–30)GFP-Cre treatment induced at most 5-fold increases in cellular GFP fluorescence (**Supplementary Fig. 2a**). Comparison of cellular fluorescence and recombination efficiency reveals that lipid-mediated functional delivery of (–30)GFP-Cre resulted in ~100-fold less Cre protein in the cell yet required ~1,000-fold less material (**Fig. 2c**) than delivery of (+36)GFP-Cre to achieve comparable recombination efficiency.

To test if complexation of anionic (–30)GFP with cationic lipids interferes with GFP fluorescence and thus masks the true amount of cargo that enters the cell we fused mCherry, which is fluorescent but not highly anionic, to either (–30)GFP or (+36)GFP and delivered both protein fusions to HeLa cells. After washing away protein that may have adhered to cell surface but did not enter the cell with PBS + heparin (20 U/mL), we analyzed cells by flow cytometry for mCherry fluorescence 4 hours and 24 hours after treatment. We observed that lipid-mediated delivery of (–30)GFP-fused mCherry results in only slight increases in cellular mCherry fluorescence, whereas mCherry fluorescence upon delivery of (+36)GFP-mCherry was generally ≥ 100-fold higher (**Supplementary Fig. 2b**) suggesting that fusion to (–30)GFP does not cause substantial amounts of protein cargo to enter the cell. Moreover, addition of lipids to (–30)GFP-Cre did not measurably alter the GFP fluorescence signal (**Supplementary Fig. 2c**), despite the fact that cationic lipids and anionic (–30)GFP clearly interact. Taken together, these results suggest that the unusually high potency of lipid-mediated delivery of anionic proteins does not arise from unusually high protein uptake in each cell, but rather from post-endocytosis processes that likely include avoidance of protein degradation and endosomal escape into the cytoplasm.

Delivery efficacy of various anionic proteins fused to Cre

We observed that both VP64 and 3x FLAG enhance functional delivery of Cre recombinase with cationic lipids, though not as effectively as (–30)GFP, likely due to their lower overall negative charge (**Fig. 2f**). To further probe the relationship between net anionic charge and protein delivery efficiency, we generated two new anionic GFP-Cre fusions of comparable charge as 3xFLAG-Cre and VP64-Cre using (–7)GFP and (–20)GFP, respectively. The (–7)GFP-Cre and (–20)GFP-Cre fusions showed nearly identical protein delivery efficacy as their like-charged anionic peptide-tagged counterparts (**Fig. 2f**), suggesting that the efficacy of delivery by cationic lipids is predominantly a function of the total negative charge, and not the distribution or density of charged residues.

Protein delivery of (–30)dGFP-NLS-Cas9 versus native Cas9

Comparison of gene disruption efficiency arising from the cationic lipid-mediated delivery of (–30)dGFP-NLS-Cas9:sgRNA versus Cas9:sgRNA revealed that at low doses (–30)dGFP-NLS-Cas9 results in more efficient gene disruption than native Cas9 (**Supplementary Fig. 6a**), but is outperformed by native Cas9 at higher concentrations, as well as at the respective optimal protein:sgRNA dose of either protein (**Supplementary Figs. 6b-c**). These results further establish that sgRNA can supply sufficient negative charge to support cationic lipid-based delivery of complexed Cas9 protein. We also observed that, while overall less protein was required for optimal delivery of (–30)dGFP-NLS-Cas9 than Cas9, a higher sgRNA:protein ratio was required for maximal (–30)dGFP-NLS-Cas9-mediated EGFP gene disruption than for native Cas9-mediated gene disruption (**Supplementary Fig. 6d**). We speculate that more equivalents of sgRNA are needed to complex with (–30)dGFP-NLS-Cas9 since fused (–30)dGFP may electrostatically interfere with Cas9:sgRNA complexation. As the ideal protein dose for (–30)dGFP-NLS-Cas9 mediated *EGFP* gene disruption is 10-fold lower than that of wild-type Cas9, our results also suggest that (–30)dGFP-Cas9 may form complexes with cationic liposomes more effectively than Cas9 due to its higher overall negative charge, but may interfere with sgRNA interactions, necessitating more sgRNA per protein.

Effect of NLS on functional Cas9 protein delivery

We generated and tested NLS-Cas9 and Cas9-NLS proteins, and observed that Cas9, NLS-Cas9, and Cas9-NLS all result in higher efficiency of *EGFP* disruption than (–30)dGFP-NLS-Cas9 at 25 nM or higher concentrations (**Supplementary Figs. 6e-g**). We therefore speculate that the lower

overall performance of (–30)dGFP-NLS-Cas9 is due to the lower activity of the fusion compared to that of Cas9 constructs lacking (–30)dGFP. While the (–30)dGFP fusion appears to improve complexation and delivery at lower protein doses (**Supplementary Figs. 6e-g**), the reduction in activity due to the presence of the large anionic fusion partner to Cas9 compromises its overall performance.

Comparison of Lipofectamine 2000 and RNAiMAX for Cas9 delivery efficiency and toxicity

We tested Cas9:sgRNA delivery with cationic lipid formulations other than RNAiMAX. *EGFP* disruption with Lipofectamine 2000 was notably more efficient than with RNAiMAX, resulting in up to 80% Cas9-mediated gene disruption (**Supplementary Fig. 7a**), and maintaining high efficiency (60% gene disruption) even at 1 nM protein (**Supplementary Fig. 7a**). However, due to the somewhat higher toxicity of Lipofectamine 2000 (**Supplementary Fig. 7b**) for protein:sgRNA delivery compared to that of RNAiMAX (**Supplementary Fig. 7c**) under cell culture conditions, we continued to use RNAiMAX for subsequent cell culture studies. We also observed that increasing the dosage of Cas9:sgRNA increased toxicity at constant amounts of either RNAiMAX or Lipofectamine 2000 (**Supplementary Fig. 7d**).

Functional delivery of Cas9 nickases and dCas9 activators

Next we tested if cationic lipid-based protein delivery could be extended to deliver other Cas9-derived genome engineering tools such as Cas9 nickases[5] and Cas9-based transcriptional activators.[6] We measured gene disruption efficiency in U2OS EGFP reporter cells resulting from delivery of Cas9 D10A nickase, either by cotransfection of nickase and appropriate paired EGFP-targeting sgRNA plasmids, or as 100 nM purified protein complexed with pairs of EGFP sgRNAs (50 nM each) using RNAiMAX (**Fig. 4d**). Both plasmid and cationic lipid-mediated protein:RNA delivery of dual Cas9 nickases resulted in EGFP disruption with similar efficiencies (**Fig. 4d**) only in the presence of sgRNA pairs targeting opposite strands, (sgRNA pairs g1+g5, and g3+g7), but not with sgRNA pairs targeting the same strand (sgRNA pair g5+g7) (**Fig 4d**), consistent with previous reports of Cas9 nickase cleavage requirements.[7]

We also compared *NTF3* transcriptional activation efficiency in HEK293T cells resulting from either plasmid transfection or direct protein:sgRNA complex delivery of dCas9 fused to a VP64 activation domain.[6] Delivery of dCas9-VP64 activators either by plasmid transfection or RNAiMAX-mediated protein delivery resulted in strong (≥ ~10-fold) activation of *NTF3* transcription (**Fig. 4e and**

Supplementary Fig. 9a). Transcriptional activation levels resulting from plasmid transfection were more potent than activation resulting from protein delivery at optimal assay times for each delivery method (**Fig. 4e**), potentially due to the sustained expression both Cas9 activator protein and sgRNA from the plasmids compared to the transient, single dose of purified protein and sgRNA (**Supplementary Fig. 9b**). Nevertheless, these results collectively indicate that both Cas9 nickases and Cas9 transcriptional activators can also be delivered effectively by cationic lipid-mediated protein:sgRNA complex delivery.

Cas9:sgRNA delivery modifies genomes with greater specificity than DNA transfection across a range of different on-target modification efficiencies

We tested whether the observed increase in specificity for Cas9 protein delivery holds at different cleavage efficiencies, focusing on the *VEGF* on-target and its four known off-target sites. We tuned Cas9-mediated on-target modification rates over a broad range by scaling the amount of Cas9:sgRNA delivered by plasmid transfection and liposomal protein delivery, resulting in conditions that yield low (~1%), moderate (~10%), and high (~40%) on-target DNA modification. We observed that across all levels of on-target modification, Cas9:sgRNA delivery always resulted in substantially (typically ~10-fold) higher on:off-target modification ratios than comparable Cas9 plasmid DNA transfections (**Supplementary Fig. 11**)**.** This increase in specificity can likely be explained by the transient nature of the delivered protein:sgRNA complexes (**Supplementary Fig. 12**) as well as the quality of the sgRNA complexed with the Cas9 protein compared to that of the endogenously produced sgRNA transcripts. There is the potential for degraded or otherwise modified sgRNAs to interact with the Cas9 protein and allow it to mediate unintended and unpredictable genome modifications. We also note that RNA pol III transcription has an error rate of ~10^{-5}, while published T7 RNAP error rates may be up to 10-times lower. In a given 20-base spacer target sequence, there would be one incorrect version per every 5,000 transcripts versus one in every 50,000 for our pre-complex sgRNAs. Such differences may further account for the observed increases in specificity.

Time course of gene disruption from Cas9:sgRNA delivery versus plasmid DNA transfection

The remarkable increases in Cas9 specificity for protein:sgRNA delivery is likely a result of the transient nature of the delivered protein that was directly observed with both TALE-activator and dCas9-activator delivery (**Supplementary Figs. 3b, 9b**). We performed a time course experiment that measured indel modification rate by Surveyor assay from protein:sgRNA or plasmid DNA delivery

over the course of 72 hours post-treatment (**Supplementary Fig. 12**). Whereas indel formation in U2OS EGFP reporter cells following Cas9 plasmid transfection continued to increase 72 hours after DNA delivery, protein:sgRNA delivery leads to near-maximal indel modification between 12 and 24 hours after treatment (**Supplementary Fig. 12**)**.** Together, these results suggest that protein:sgRNA delivery rapidly achieves a transient dose of Cas9:sgRNA activity that mediates efficient genome modification and is degraded before off-target modifications can accumulate to the extent that arises from long-term expression.

Quantification of total Cas9 protein uptake into cells

Finally, we quantitated the amount of protein internalized by cells using our cationic lipid-based protein delivery approach. We labeled Cas9 protein with Alexa 647 and delivered it to U2OS cells at 50 nM with 100 nM sgRNA. After 4 hours, cells were washed extensively to remove bound protein and trypsinized. Cellular Alexa 647 fluorescence was measured and compared to that of a standard curve of known Cas9-Alexa 647 amounts in the presence of an identical composition of media, cells, and lipid. Nearly all treated cells were found to have internalized the Cas9-Alexa 647 protein (**Supplementary Fig. 13a**), and 4% of the total protein used in the treatment was internalized by cells (**Supplementary Fig. 13b**). Comparison with the standard curve suggests that ~$3x10^7$ molecules of Cas9-Alexa 647 entered each cell, corresponding to 0.4% of total cellular protein.[8] We note, however, that the majority of this protein is likely sequestered within endosomes and may not be immediately available to effect genome modification.[4,9]

Delivery of Cas9:sgRNA into mouse embryonic stem cells

The rapid, potent, and transient cationic lipid-mediated delivery of Cas9:sgRNA to effect genome editing could be especially useful in stem cells, where Cas9 off-target activity over the course of multiple cell divisions could lead to both unwanted mutations, and mosaicism. To test the effectiveness of Cas9:sgRNA delivery in stem cells, we treated mouse embryonic stem cells expressing Tau-EGFP[10] with Cas9 and an *EGFP*-targeting sgRNA. Under standard stem-cell culture conditions, EGFP-positive floating spheres were formed. We treated these floating spheres with Cas9:sgRNA complexed with Lipofectamine 2000, or with Cas9 and Lipofectamine 2000 without sgRNA as a control. Three days post-treatment, we observed a reduction in GFP fluorescence in the Cas9:sgRNA-treated spheres compared to the control samples (**Supplementary Fig. 14a**). The treated spheres were dissociated, and the cells were allowed to attach to a laminin-coated dish and differentiate into

progenitor cells. Immunohistochemistry using an anti-GFP antibody confirmed knockdown of EGFP expression in the cells of Cas9:sgRNA treated samples, with many nuclei lacking any apparent EGFP. In contrast, all cells derived from control spheres were EGFP positive (**Supplementary Fig. 14b**). Genomic DNA harvested from Cas9:sgRNA-treated cells was subjected to T7EI assay, resulting in clear evidence of indels at the Tau-EGFP locus (**Supplementary Fig. 14c**). From this assay we calculated an indel frequency of 24% from cationic lipid-mediated Cas9:sgRNA delivery and 20% from DNA transfection. No target modification was detected in control samples lacking Cas9:sgRNA or containing Cas9 and an unrelated gRNA. These findings demonstrate that cationic lipid-mediated Cas9:sgRNA delivery can effect efficient gene disruption in mouse embryonic stem cells.

Sensitivity limit of off-target cleavage assays

The sensitivity of the high-throughput sequencing method for detecting genomic off-target cleavage is limited by the amount genomic DNA (gDNA) input into the PCR amplification of each genomic target site. A 1 ng sample of human gDNA represents only ~330 unique genomes, and thus only ~330 unique copies of each genomic site are present. PCR amplification for each genomic target was performed on a total of 150 ng of input gDNA, which provides amplicons derived from at most 50,000, unique gDNA copies, respectively. Therefore, the high-throughput sequencing assay cannot detect rare genome modification events that occur at a frequency of less than 1 in 50,000 (0.002%). This limit is noted in **Supplementary Table 2**.

SUPPLEMENTARY NOTES

DNA Sequence-Processing Algorithms. All scripts were written in bash and described in detail previously.[3] Scripts are available upon request.

List of upstream and downstream flanking sequences for each genomic target site.

Target Site	Downstream genomic sequence	Upstream genomic sequence
EMX_On	GGCCTGCTTCGTGGCAATGC	ACCTGGGCCAGGGAGGGAGG
EMX_Off1	CTCACTTAGACTTTCTCTCC	CTCGGAGTCTAGCTCCTGCA
EMX_Off2	TGGCCCCAGTCTCTCTTCTA	CAGCCTCTGAACAGCTCCCG
EMX_Off3	TGACTTGGCCTTTGTAGGAA	GAGGCTACTGAAACATAAGT
EMX_Off4	TGCTACCTGTACATCTGCAC	CATCAATGATTGGGCATTTC
VEG_On	ACTCCAGTCCCAAATATGTA	ACTAGGGGGCGCTCGGCCAC
VEG_Off1	CTGAGTCAACTGTAAGCATT	GGCCAGGTGCAGTGATTCAT
VEG_Off2	TCGTGTCATCTTGTTTGTGC	GGCAGAGCCCAGCGGACACT
VEG_Off3	CAAGGTGAGCCTGGGTCTGT	ATCACTGCCCAAGAAGTGCA
VEG_Off4	TTGTAGGATGTTTAGCAGCA	ACTTGCTCTCTTTAGAGAAC
CLT2_On	CTCAAGCAGGCCCCGCTGGT	TTTTGGACCAAACCTTTTTG
CLT2_Off1	TGAGGTTATTTGTCCATTGT	TAAGGGGAGTATTTACACCA
CLT2_Off2	TCAAGAGCAGAAAATGTGAC	CTTGCAGGGACCTTCTGATT
CLT2_Off3	TGTGTGTAGGACTAAACTCT	GATAGCAGTATGACCTTGGG
EGFP	AGCGTGTCCGGCGAGGGCGA	AGCGTGTCCGGCGAGGGCGA
MusEMX	CAGAATCGGAGGACAAAATACAAAC	ACGAAGCAGGCCAACGGGGAGGACA

Oligonucleotides used in this study

All oligonucleotides were purchased from Integrated DNA Technologies.

Primers used for generating PCR products to serve as substrates for T7 transcription of sgRNAs. T7_gRNA-Rev was used in all cases. DNA template used was EGFP sgRNA plasmid as noted in Methods section. NTF3 and VEGF sgRNAs for dCas9-VP64 activator experiments were reported previously[4]. The T2 sgRNA target was previously reported[11].

T7_EGFP1-Fwd	TAA TAC GAC TCA CTA TA GGGCACGGGCAGCTTGCCGG
T7-GFP g1-Fwd	TAA TAC GAC TCA CTA TA GGCCTCGAACTTCACCTCGGCG GAAAGGACGAAACACC
T7-GFP g5-Fwd	TAA TAC GAC TCA CTA TA GGCTGAAGGGCATCGACTTCA GAAAGGACGAAACACC
T7-GFP g3-Fwd	TAA TAC GAC TCA CTA TA GGCAGCTCGATGCGGTTCACCA GAAAGGACGAAACACC
T7-GFP g7-Fwd	TAA TAC GAC TCA CTA TA GGCAAGGAGGACGGCAACATCC GAAAGGACGAAACACC
T7-EMX-Fwd	TAA TAC GAC TCA CTA TA GGAGTCCGAGCAGAAGAAGAA GAAAGGACGAAACACC
T7-VEG-Fwd	TAA TAC GAC TCA CTA TA GGGGTGGGGGGAGTTTGCTCC GAAAGGACGAAACACC
T7-CLT2-Fwd	TAA TAC GAC TCA CTA TA GGCAGATGTAGTGTTTCCACA GAAAGGACGAAACACC
T7-T2 HDR-Fwd	TAA TAC GAC TCA CTA TA GGGGCCACTAGGGACAGGAT GAAAGGACGAAACACC
T7_gRNA-Rev	AAAAAAAGCACCGACTCGGTG

Sequence of single-stranded oligonucleotide donor template (ssODN) used in HDR studies.

CGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGA
GCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGA
GGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCA
ACATCCTGGGGCACAAGCTGG

Primers for generating linear DNA PCR product for transfection. PCR extension at (72 °C, 3 min) on plasmid containing U6 promoter as template with PCR_sgRNA-fwd1, PCR_sgRNA-rev2 and appropriate PCR_sgRNA primers listed below.

PCR_gRNA-fwd1	CTGTACAAAAAAGCAGGCTTTA
PCR_gRNA-rev2	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGG ACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

PCR-G-GFP1	GAAAGGACGAAACACC GGCCTCGAACTTCACCTCGGCGGTTTTAGAGCTAGAAATAGCAA
PCR-G-GFP3	GAAAGGACGAAACACC GGCAGCTCGATGCGGTTCACCAGTTTTAGAGCTAGAAATAGCAA
PCR-G-GFP5	GAAAGGACGAAACACC GGCTGAAGGGCATCGACTTCAGTTTTAGAGCTAGAAATAGCAA
PCR-G-GFP7	GAAAGGACGAAACACC GGCAAGGAGGACGGCAACATCCGTTTTAGAGCTAGAAATAGCAA
PCR-G-CLT2	GAAAGGACGAAACACC GGCAGATGTAGTGTTTCCACAGTTTTAGAGCTAGAAATAGCAA
PCR-G-EMX	GAAAGGACGAAACACC GGAGTCCGAGCAGAAGAAGAAGTTTTAGAGCTAGAAATAGCAA
PCR-G-VEG	GAAAGGACGAAACACC GGGGTGGGGGGAGTTTGCTCCGTTTTAGAGCTAGAAATAGCAA

Primers for performing T7 endonuclease I DNA cleavage assay.

Survey_GFP-fwd	TACGGCAAGCTGACCCTGAA
Survey_GFP-rev	GTCCATGCCGAGAGTGATCC
Survey_CLTA-fwd	GCCAGGGGCTGTTATCTTGG
Survey_CLTA-rev	ATGCACAGAAGCACAGGTTGA
Survey_EMX-fwd	CTGTGTCCTCTTCCTGCCCT
Survey_EMX-rev	CTCTCCGAGGAGAAGGCCAA
Survey_VEGF-fwd	CCACACAGCTTCCCGTTCTC
Survey_VEGF-rev	GAGAGCCGTTCCCTCTTTGC

Primers for high-throughput sequencing of on-target and off-target sites in human genome.

HTS_EMX_ON-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCT CCTCCCCATTGGCCTGCTTC
HTS_EMX_Off1-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCT TCGTCCTGCTCTCACTTAGAC
HTS_EMX_Off2-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCT TTTTGTGGCTTGGCCCCAGT
HTS_EMX_Off3-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCT TGCAGTCTCATGACTTGGCCT
HTS_EMX_Off4-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCT TTCTGAGGGCTGCTACCTGT
HTS_VEGF_ON-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCT ACATGAAGCAACTCCAGTCCCA
HTS_VEGF_Off1-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCT AGCAGACCCACTGAGTCAACTG
HTS_VEGF_Off2-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCT CCCGCCACAGTCGTGTCAT
HTS_VEGF_Off3-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCT CGCCCCGGTACAAGGTGA
HTS_VEGF_Off4-	CACTCTTTCCCTACACGACGCTCTTCCGATCT

fwd	GTACCGTACATTGTAGGATGTTT
HTS_CLTA2_ON-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCT CCTCATCTCCCTCAAGCAGGC
HTS_CLTA2_Off1-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCT ATTCTGCTCTTGAGGTTATTTGT
HTS_CLTA2_Off2-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCT CACCTCTGCCTCAAGAGCAGAAAA
HTS_CLTA2_Off3-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCT TGTGTGTGTGTGTGTGTAGGACT
HTS_EMX_ON-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT TCATCTGTGCCCCTCCCTCC
HTS_EMX_Off1-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT CGAGAAGGAGGTGCAGGAG
HTS_EMX_Off2-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT CGGGAGCTGTTCAGAGGCTG
HTS_EMX_Off3-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT CTCACCTGGGCGAGAAAGGT
HTS_EMX_Off4-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT AAAACTCAAAGAAATGCCCAATCA
HTS_VEFG_ON-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT AGACGCTGCTCGCTCCATTC
HTS_VEGF_Off1-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT ACAGGCATGAATCACTGCACCT
HTS_VEGF_Off2-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT GCGGCAACTTCAGACAACCGA
HTS_VEGF_Off3-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT GACCCAGGGGCACCAGTT
HTS_VEGF_Off4-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT CTGCCTTCATTGCTTAAAAGTGGAT
HTS_CLTA2_ON-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT ACAGTTGAAGGAAGGAAACATGC
HTS_CLTA2_Off1-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT GCTGCATTTGCCCATTTCCA
HTS_CLTA2_Off2-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT GTTGGGGGAGGAGGAGCTTAT
HTS_CLTA2_Off3-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT CTAAGAGCTATAAGGGCAAATGACT
HTS_EGFP-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCTNNNN ACGTAAACGGCCACAAGTTC
HTS_EGFP-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT GTCGTCCTTGAAGAAGATGGTG
HTS_MusEMX_ON-fwd	CACTCTTTCCCTACACGACGCTCTTCCGATCT CCAGGTGAAGGTGTGGTTCCAG
HTS_MusEMX_ON-rev	GGAGTTCAGACGTGTGCTCTTCCGATCT CCCCTAGTCATTGGAGGTGAC

Amino acid sequences of proteins used in this study

(+36)GFP-Cre-6xHis:

MGASKGERLFRGKVPILVELKGDVNGHKFSVRGKGKGDATRGKLTLKFICTTGKLPVPWPTL
VTTLTYGVQCFSRYPKHMKRHDFFKSAMPKGYVQERTISFKKDGKYKTRAEVKFEGRTLVN
RIKLKGRDFKEKGNILGHKLRYNFNSHKVYITADKRKNGIKAKFKIRHNVKDGSVQLADHYQ
QNTPIGRGPVLLPRNHYLSTRSKLSKDPKEKRDHMVLLEFVTAAGIKHGRDERYKTGGSGGS
GGSGGSGGSGGSGGSGGSGGTASNLLTVHQNLPALPVDATSDEVRKNLMDMFRDRQAFSEH
TWKMLLSVCRSWAAWCKLNNRKWFPAEPEDVRDYLLYLQARGLAVKTIQQHLGQLNMLHR
RSGLPRPSDSNAVSLVMRRIRKENVDAGERAKQALAFERTDFDQVRSLMENSDRCQDIRNLA
FLGIAYNTLLRIAEIARIRVKDISRTDGGRMLIHIGRTKTLVSTAGVEKALSLGVTKLVERWISV
SGVADDPNNYLFCRVRKNGVAAPSATSQLSTRALEGIFEATHRLIYGAKDDSGQRYLAWSGH
SARVGAARDMARAGVSIPEIMQAGGWTNVNIVMNYIRNLDSETGAMVRLLEDGDGGSHHHH
HH

(–7)GFP-Cre-6xHis:

MGASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTGKLPVPWPTL
VTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGDTLVNR
IELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFKIRHNVEDGSVQLADHYQQN
TPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYKTGGSGGSGG
SGGSGGSGGSGGSGGSGGTASNLLTVHQNLPALPVDATSDEVRKNLMDMFRDRQAFSEHTW
KMLLSVCRSWAAWCKLNNRKWFPAEPEDVRDYLLYLQARGLAVKTIQQHLGQLNMLHRRS
GLPRPSDSNAVSLVMRRIRKENVDAGERAKQALAFERTDFDQVRSLMENSDRCQDIRNLAFL
GIAYNTLLRIAEIARIRVKDISRTDGGRMLIHIGRTKTLVSTAGVEKALSLGVTKLVERWISVSG
VADDPNNYLFCRVRKNGVAAPSATSQLSTRALEGIFEATHRLIYGAKDDSGQRYLAWSGHSA
RVGAARDMARAGVSIPEIMQAGGWTNVNIVMNYIRNLDSETGAMVRLLEDGDGGSHHHHH
H

(–20)GFP-Cre-6xHis:

MGASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTGKLPVPWPTL
VTTLTYGVQCFSRYPDHMDQHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGDTLVNR
IELKGIDFKEDGNILGHKLEYNFNSHDVYITADKQENGIKAEFEIRHNVEDGSVQLADHYQQN
TPIGDGPVLLPDDHYLSTESALSKDPNEDRDHMVLLEFVTAAGIDHGMDELYKTGGSGGSGG
SGGSGGSGGSGGSGGSGGTASNLLTVHQNLPALPVDATSDEVRKNLMDMFRDRQAFSEHTW
KMLLSVCRSWAAWCKLNNRKWFPAEPEDVRDYLLYLQARGLAVKTIQQHLGQLNMLHRRS
GLPRPSDSNAVSLVMRRIRKENVDAGERAKQALAFERTDFDQVRSLMENSDRCQDIRNLAFL
GIAYNTLLRIAEIARIRVKDISRTDGGRMLIHIGRTKTLVSTAGVEKALSLGVTKLVERWISVSG
VADDPNNYLFCRVRKNGVAAPSATSQLSTRALEGIFEATHRLIYGAKDDSGQRYLAWSGHSA
RVGAARDMARAGVSIPEIMQAGGWTNVNIVMNYIRNLDSETGAMVRLLEDGDGGSHHHHH
H

(–30)GFP-Cre-6xHis:

MGASKGEELFDGVVPILVELDGDVNGHEFSVRGEGEGDATEGELTLKFICTTGELPVPWPTLV
TTLTYGVQCFSDYPDHMDQHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGDTLVNRI

ELKGIDFKEDGNILGHKLEYNFNSHDVYITADKQENGIKAEFEIRHNVEDGSVQLADHYQQNT
PIGDGPVLLPDDHYLSTESALSKDPNEDRDHMVLLEFVTAAGIDHGMDELYKTGGSGGSGGS
GGSGGSGGSGGSGGSGGTASNLLTVHQNLPALPVDATSDEVRKNLMDMFRDRQAFSEHTWK
MLLSVCRSWAAWCKLNNRKWFPAEPEDVRDYLLYLQARGLAVKTIQQHLGQLNMLHRRSG
LPRPSDSNAVSLVMRRIRKENVDAGERAKQALAFERTDFDQVRSLMENSDRCQDIRNLAFLGI
AYNTLLRIAEIARIRVKDISRTDGGRMLIHIGRTKTLVSTAGVEKALSLGVTKLVERWISVSGV
ADDPNNYLFCRVRKNGVAAPSATSQLSTRALEGIFEATHRLIYGAKDDSGQRYLAWSGHSAR
VGAARDMARAGVSIPEIMQAGGWTNVNIVMNYIRNLDSETGAMVRLLEDGDGGSHHHHHH

Cre-6xHis:

MASNLLTVHQNLPALPVDATSDEVRKNLMDMFRDRQAFSEHTWKMLLSVCRSWAAWCKLN
NRKWFPAEPEDVRDYLLYLQARGLAVKTIQQHLGQLNMLHRRSGLPRPSDSNAVSLVMRRIR
KENVDAGERAKQALAFERTDFDQVRSLMENSDRCQDIRNLAFLGIAYNTLLRIAEIARIRVKDI
SRTDGGRMLIHIGRTKTLVSTAGVEKALSLGVTKLVERWISVSGVADDPNNYLFCRVRKNGV
AAPSATSQLSTRALEGIFEATHRLIYGAKDDSGQRYLAWSGHSARVGAARDMARAGVSIPEIM
QAGGWTNVNIVMNYIRNLDSETGAMVRLLEDGDGGSHHHHHH

Cas9:
MDKKYSIGLAIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATR
LKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVA
YHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQ
TYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSN
FDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLS
ASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEK
MDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFR
IPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPK
HSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKI
ECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLK
TYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHD
DSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIE
MARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMY
VDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQ
LLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDK
LIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYG
DYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWD
KGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSP
TVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKY
SLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHK
HYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFD
TTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGD

Cas9-6xHis:
MDKKYSIGLAIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATR
LKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVA
YHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQ
TYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSN
FDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLS

ASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEK
MDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFR
IPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPK
HSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKI
ECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLK
TYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHD
DSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIE
MARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMY
VDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQ
LLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDK
LIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYG
DYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWD
KGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSP
TVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKY
SLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHK
HYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFD
TTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDHHHHHH

NLS-Cas9-6xHis:
MPKKKRKVMDKKYSIGLAIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFD
SGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPI
FGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDV
DKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSL
GLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVN
TEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEF
YKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDN
REKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDK
NLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVK
QLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFE
DREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFA
NRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVM
GRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLY
YLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVV
KKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSR
MNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKK
YPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIE
TNGETGEIVWDKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDW
DPKKYGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKE
VKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDN
EQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTN
LGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDHHHHHH

Cas9-NLS-6xHis:
MDKKYSIGLAIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATR
LKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVA
YHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQ
TYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSN
FDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLS

ASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEK
MDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFR
IPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPK
HSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKI
ECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLK
TYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHD
DSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIE
MARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMY
VDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQ
LLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDK
LIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYG
DYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWD
KGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSP
TVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKY
SLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHK
HYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFD
TTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDPKKKRKVMDKHHHHHH

(+36)dGFP-NLS-Cas9-6xHis **(Y67S)**:

MGASKGERLFRGKVPILVELKGDVNGHKFSVRGKGKGDATRGKLTLKFICTTGKLPVPWPTL
VTTLT**S**GVQCFSRYPKHMKRHDFFKSAMPKGYVQERTISFKKDGKYKTRAEVKFEGRTLVNR
IKLKGRDFKEKGNILGHKLRYNFNSHKVYITADKRKNGIKAKFKIRHNVKDGSVQLADHYQQ
NTPIGRGPVLLPRNHYLSTRSKLSKDPKEKRDHMVLLEFVTAAGIKHGRDERYKTGGSGGSG
GSGGSGGSGGSGGSGGSGGTALALPKKKRKVMDKKYSIGLDIGTNSVGWAVITDEYKVPSKK
FKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVD
DSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLAL
AHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRL
ENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQ
YADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKE
IFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQI
HLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNF
EEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFL
SGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDK
DFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLI
NGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSP
AIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQ
ILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKV
LTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIK
RQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNY
HHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIM
NFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSK
ESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIME
RSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYV
NFLYLASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNK
HRDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDL
SQLGGDHHHHHH

(–30)dGFP-NLS-Cas9-6xHis **(Y67S)**:

MGASKGEELFDGVVPILVELDGDVNGHEFSVRGEGEGDATEGELTLKFICTTGELPVPWPTLV
TTLT**S**GVQCFSDYPDHMDQHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGDTLVNRI
ELKGIDFKEDGNILGHKLEYNFNSHDVYITADKQENGIKAEFEIRHNVEDGSVQLADHYQQNT
PIGDGPVLLPDDHYLSTESALSKDPNEDRDHMVLLEFVTAAGIDHGMDELYKTGGSGGSGGS
GGSGGSGGSGGSGGSGGTALALPKKKRKVMDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFK
VLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDS
FFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAH
MIKFRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLEN
LIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYA
DLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFF
DQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHL
GELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEE
VVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSG
EQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDF
LDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLING
IRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAI
KKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQIL
KEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLT
RSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKR
QLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYH
HAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMN
FFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKE
SILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMER
SSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNF
LYLASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHR
DKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQ
LGGDHHHHHH

dCas9-VP64-6xHis (**D10A and H840A**):

MDKKYSIGL**A**IGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATR
LKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVA
YHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQ
TYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSN
FDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLS
ASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEK
MDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFR
IPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPK
HSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKI
ECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLK
TYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHD
DSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIE
MARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMY
VDQELDINRLSDYDVD**A**IVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQ

LLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDK
LIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYG
DYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWD
KGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSP
TVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKY
SLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHK
HYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFD
TTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDGSPKKKRKVSSDYKDHDGDYKDH
DIDYKDDDDKAAGGGGSGRADALDDFDLDMLGSDALDDFDLDMLGSDALDDFDLDMLGSD
ALDDFDLDMLHHHHHH

Cas9 nickase (**D10A**):
MDKKYSIGL**A**IGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATR
LKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVA
YHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQ
TYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSN
FDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLS
ASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEK
MDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFR
IPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPK
HSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKI
ECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLK
TYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHD
DSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIE
MARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMY
VDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQ
LLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDK
LIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYG
DYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWD
KGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSP
TVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKY
SLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHK
HYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFD
TTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDHHHHHH

DNA sequences of all protein constructs used in this study.

(–7)GFP-Cre-6xHis:

ATGGGTGCTAGCAAAGGTGAAGAGCTGTTTACGGGTGTAGTACCGATCTTAGTGGAATTA
GACGGCGACGTGAACGGTCACAAATTTAGCGTGCGCGGCGAAGGCGAAGGTGACGCTAC
CAATGGTAAATTGACCCTGAAGTTTATTTGCACAACAGGCAAATTACCCGTTCCGTGGCC
CACCTTAGTGACCACCCTGACCTATGGCGTTCAGTGCTTCAGTCGTTACCCAGATCATATG
AAACAACACGATTTTTTCAAATCAGCCATGCCTGAAGGATATGTTCAAGAGCGTACAATC
AGCTTCAAGGACGATGGCACCTATAAAACGCGTGCGGAAGTGAAATTTGAAGGCGACAC
ATTAGTAAACCGTATCGAACTGAAAGGTATCGACTTCAAAGAAGACGGCAACATTTTAGG
CCATAAGCTGGAATATAACTTTAATTCTCATAACGTGTATATTACGGCCGATAAACAGAA
AAACGGTATCAAGGCAAATTTCAAAATTCGCCATAACGTGGAAGACGGCAGCGTTCAATT
AGCGGATCATTATCAACAAAACACGCCGATTGGTGACGGGCCTGTACTGTTACCTGACAA
CCACTACCTGAGCACCCAGTCAGCACTGAGCAAAGATCCGAACGAAAAACGCGATCACA
TGGTTCTGTTAGAATTCGTGACCGCTGCAGGCATTACTCACGGAATGGACGAACTCTACA
AGACCGGTGGTAGCGGTGGTTCTGGTGGTTCTGGTGGTAGCGGCGGTAGCGGTGGTAGCG
GTGGTAGCGGTGGCAGCGGCGGTACCGCGAGCAATTTACTGACCGTACACCAAAATTTGC
CTGCATTGCCGGTCGATGCAACGAGTGATGAGGTTCGCAAGAACCTGATGGACATGTTCA
GGGATCGCCAGGCGTTTTCTGAGCATACCTGGAAAATGCTTCTGTCCGTTTGCCGGTCGTG
GGCGGCATGGTGCAAGTTGAATAACCGGAAATGGTTTCCCGCAGAACCTGAAGATGTTCG
CGATTATCTTCTATATCTTCAGGCGCGCGGTCTGGCAGTAAAAACTATCCAGCAACATTTG
GGCCAGCTAAACATGCTTCATCGTCGGTCCGGGCTGCCACGACCAAGTGACAGCAATGCT
GTTTCACTGGTTATGCGGCGTATCCGAAAAGAAAACGTTGATGCCGGTGAACGTGCAAAA
CAGGCTCTAGCGTTCGAACGCACTGATTTCGACCAGGTTCGTTCACTCATGGAAAATAGC
GATCGCTGCCAGGATATACGTAATCTGGCATTTCTGGGGATTGCTTATAACACCCTGTTAC
GTATAGCCGAAATTGCCAGGATCAGGGTTAAAGATATCTCACGTACTGACGGTGGGAGAA
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TTAGCCTGGGGGTAACTAAACTGGTCGAGCGATGGATTTCCGTCTCTGGTGTAGCTGATG
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TCGGAGCCGCGCGAGATATGGCCCGCGCTGGAGTTTCAATACCGGAGATCATGCAAGCTG
GTGGCTGGACCAATGTAAATATTGTCATGAACTATATCCGTAACCTGGATAGTGAAACAG
GGGCAATGGTGCGCCTGCTGGAAGATGGCGACGGCGGATCCCATCACCACCACCATCAC

(–20)GFP-Cre-6xHis:

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GACGGCGACGTGAACGGTCACAAATTTAGCGTGCGCGGCGAAGGCGAAGGTGACGCTAC
CAATGGTAAATTGACCCTGAAGTTTATTTGCACAACAGGCAAATTACCCGTTCCGTGGCC
CACCTTAGTGACCACCCTGACCTATGGCGTTCAGTGCTTCAGTCGTTACCCAGATCATATG
GATCAACACGATTTTTTCAAATCAGCCATGCCTGAAGGATATGTTCAAGAGCGTACAATC
AGCTTCAAGGACGATGGCACCTATAAAACGCGTGCGGAAGTGAAATTTGAAGGCGACAC
ATTAGTAAACCGTATCGAACTGAAAGGTATCGACTTCAAAGAAGACGGCAACATTTTAGG
CCATAAGCTGGAATATAACTTTAATTCTCATGACGTGTATATTACGGCCGATAAACAGGA
AAACGGTATCAAGGCAGAATTTGAAATTCGCCATAACGTGGAGGACGGCAGCGTTCAATT
AGCGGATCATTATCAACAAAACACGCCGATTGGTGATGGGCCTGTACTGTTACCTGACGA

TCACTACCTGAGCACGGAGTCAGCCCTGAGCAAAGATCCGAACGAAGACCGCGATCACA
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CCAGCCAGCTATCAACTCGCGCCCTGGAAGGGATTTTTGAAGCAACTCATCGATTGATTT
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TCGGAGCCGCGCGAGATATGGCCCGCGCTGGAGTTTCAATACCGGAGATCATGCAAGCTG
GTGGCTGGACCAATGTAAATATTGTCATGAACTATATCCGTAACCTGGATAGTGAAACAG
GGGCAATGGTGCGCCTGCTGGAAGATGGCGACGGCGGATCCCATCACCACCACCATCAC

(–30)GFP-Cre-6xHis:

ATGGGTGCTAGCAAAGGTGAAGAGCTGTTTGACGGTGTAGTACCGATCTTAGTGGAATTA
GACGGCGACGTGAACGGTCACGAATTTAGCGTGCGCGGCGAGGGCGAAGGTGACGCTAC
CGAGGGTGAATTGACCCTGAAGTTTATTTGCACAACAGGCGAATTACCCGTTCCGTGGCC
CACCTTAGTGACCACCCTGACCTATGGCGTTCAGTGCTTCAGTGATTACCCAGATCATATG
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AGACCGGTGGTAGCGGTGGTTCTGGTGGTTCTGGTGGTAGCGGCGGTAGCGGTGGTAGCG
GTGGTAGCGGTGGCAGCGGCGGTACCGCGAGCAATTTACTGACCGTACACCAAAATTTGC
CTGCATTGCCGGTCGATGCAACGAGTGATGAGGTTCGCAAGAACCTGATGGACATGTTCA
GGGATCGCCAGGCGTTTTCTGAGCATACCTGGAAAATGCTTCTGTCCGTTTGCCGGTCGTG
GGCGGCATGGTGCAAGTTGAATAACCGGAAATGGTTTCCCGCAGAACCTGAAGATGTTCG
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CAGGCTCTAGCGTTCGAACGCACTGATTTCGACCAGGTTCGTTCACTCATGGAAAATAGC
GATCGCTGCCAGGATATACGTAATCTGGCATTTCTGGGGATTGCTTATAACACCCTGTTAC
GTATAGCCGAAATTGCCAGGATCAGGGTTAAAGATATCTCACGTACTGACGGTGGGAGAA
TGTTAATCCATATTGGCAGAACGAAAACGCTGGTTAGCACCGCAGGTGTAGAGAAGGCAC

TTAGCCTGGGGGTAACTAAACTGGTCGAGCGATGGATTTCCGTCTCTGGTGTAGCTGATG
ATCCGAATAACTACCTGTTTTGCCGGGTCAGAAAAAATGGTGTTGCCGCGCCATCTGCCA
CCAGCCAGCTATCAACTCGCGCCCTGGAAGGGATTTTTGAAGCAACTCATCGATTGATTT
ACGGCGCTAAGGATGACTCTGGTCAGAGATACCTGGCCTGGTCTGGACACAGTGCCCGTG
TCGGAGCCGCGCGAGATATGGCCCGCGCTGGAGTTTCAATACCGGAGATCATGCAAGCTG
GTGGCTGGACCAATGTAAATATTGTCATGAACTATATCCGTAACCTGGATAGTGAAACAG
GGGCAATGGTGCGCCTGCTGGAAGATGGCGACGGCGGATCCCATCACCACCACCATCAC

Cre-6xHis:

ATGGCGAGCAATTTACTGACCGTACACCAAAATTTGCCTGCATTGCCGGTCGATGCAACG
AGTGATGAGGTTCGCAAGAACCTGATGGACATGTTCAGGGATCGCCAGGCGTTTTCTGAG
CATACCTGGAAAATGCTTCTGTCCGTTTGCCGGTCGTGGGCGGCATGGTGCAAGTTGAAT
AACCGGAAATGGTTTCCCGCAGAACCTGAAGATGTTCGCGATTATCTTCTATATCTTCAGG
CGCGCGGTCTGGCAGTAAAAACTATCCAGCAACATTTGGGCCAGCTAAACATGCTTCATC
GTCGGTCCGGGCTGCCACGACCAAGTGACAGCAATGCTGTTTCACTGGTTATGCGGCGTA
TCCGAAAAGAAAACGTTGATGCCGGTGAACGTGCAAAACAGGCTCTAGCGTTCGAACGC
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CCCTGGAAGGGATTTTTGAAGCAACTCATCGATTGATTTACGGCGCTAAGGATGACTCTG
GTCAGAGATACCTGGCCTGGTCTGGACACAGTGCCCGTGTCGGAGCCGCGCGAGATATGG
CCCGCGCTGGAGTTTCAATACCGGAGATCATGCAAGCTGGTGGCTGGACCAATGTAAATA
TTGTCATGAACTATATCCGTAACCTGGATAGTGAAACAGGGGCAATGGTGCGCCTGCTGG
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(–30)dGFP-NLS-Cas9-6xHis:

ATGGGTGCTAGCAAAGGTGAAGAGCTGTTTGACGGTGTAGTACCGATCTTAGTGGAATTA
GACGGCGACGTGAACGGTCACGAATTTAGCGTGCGCGGCGAGGGCGAAGGTGACGCTAC
CGAGGGTGAATTGACCCTGAAGTTTATTTGCACAACAGGCGAATTACCCGTTCCGTGGCC
CACCTTAGTGACCACCCTGACCTCCGGCGTTCAGTGCTTCAGTGATTACCCAGATCATATG
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CCATAAGCTGGAATATAACTTTAATTCTCATGACGTGTATATTACGGCCGATAAACAGGA
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TGCCACTCTTATCCATCAATCCATCACTGGTCTTTATGAAACACGCATTGATTTGAGTCAG
CTAGGAGGTGACCATCACCACCACCATCAC

(+36)dGFP-NLS-Cas9-6xHis:

ATGGGTGCTAGCAAAGGTGAACGTCTGTTTCGTGGTAAAGTACCGATCTTAGTGGAATTA
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Cas9-NLS-6xHis:

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