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Efficient Delivery of Genome-Editing Proteins *In Vitro* and *In Vivo*

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Abstract

Efficient intracellular delivery of proteins is needed to fully realize the potential of protein therapeutics. Current methods of protein delivery commonly suffer from low tolerance for serum,

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AUTHOR CONTRIBUTIONS

J.A.Z., D.B.T., Y.S., Z.-Y.C., and D.R.L. designed the research and analyzed the data. J.A.Z., D.B.T., Y.S., J.P.G., and J.L.B. generated research materials and performed the experiments. M.L.M. designed and constructed TALEs and dCas9 activator sgRNAs. J.P.G., J.A.Z., and J.H.H. analyzed DNA sequencing data. J.K.J., Z.-Y.C., and D.R.L. supervised the research. All authors wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: the co-authors have filed a provisional patent application related to this work. J.K.J. and D.R.L. are consultants for Editas Medicine, a company that applies genome-editing technologies. J.K.J. is a consultant for Horizon Discovery. J.K.J. has financial interests in Editas Medicine and Transposagen Biopharmaceuticals. J.K.J.'s interests were reviewed and are managed by Massachusetts General Hospital and Partners HealthCare in accordance with their conflict of interest policies. M.L.M. is currently an employee of Editas Medicine.

poor endosomal escape, and limited *in vivo* efficacy. Here we report that common cationic lipid nucleic acid transfection reagents can potently deliver proteins that are fused to negatively supercharged proteins, that contain natural anionic domains, or that natively bind to anionic nucleic acids. This approach mediates the potent delivery of nM concentrations of Cre recombinase, TALE- and Cas9-based transcriptional activators, and Cas9:sgRNA nuclease complexes into cultured human cells in media containing 10% serum. Delivery of Cas9:sgRNA complexes resulted in up to 80% genome modification with substantially higher specificity compared to DNA transfection. This approach also mediated efficient delivery of Cre recombinase and Cas9:sgRNA complexes into the mouse inner ear *in vivo*, achieving 90% Cre-mediated recombination and 20% Cas9-mediated genome modification in hair cells.

Introduction

Therapeutic proteins including peptide hormones¹, cytokines², and monoclonal antibodies³ have achieved widespread success as research tools and are among the fastest growing classes of drugs.⁴ Many powerful and potentially therapeutic proteins have been discovered or engineered over the past two decades, including enzymes capable of metabolic complementation,⁵ neutralizing antibodies against intracellular targets,⁶ engineered transcription factors,⁷ and programmable genome-editing enzymes.⁸ While protein biologics have proven effective for extracellular targets, their use to address intracellular targets is comparatively undeveloped due to the inability of most proteins to spontaneously enter mammalian cells. Enabling exogenous proteins to access intracellular targets is most commonly achieved by delivery of their encoding DNA sequences through chemical transfection,⁹ electroporation,¹⁰ or viral delivery.¹¹ The introduction of exogenous DNA into cells, however, raises the possibility of permanent recombination into the genome, potential disruption of endogenous genes, and long-term exposure to the encoded agent. The recent development of methods to deliver *in vitro* transcribed mRNAs or mRNA analogs has offered an alternative to DNA delivery without requiring nuclear transport of an encoding gene, and with greatly reduced potential for genomic insertion of the foreign nucleic acid. While promising, mRNA delivery continues to face challenges including immunogenicity and RNA stability.¹² For genome editing applications that seek to effect a one-time, permanent modification of genomic DNA, the functional delivery of non-replicable protein agents may offer improved specificity, increased safety, and broader applicability.

We and others have previously developed protein delivery technologies based on fusion or conjugation to cationic molecules that facilitate endocytosis, such as unstructured peptides^{13,14} or engineered superpositively charged proteins¹⁵⁻¹⁷. While such methods can be effective in cell culture,^{15,17} and has even shown some success *in vivo*, they have not seen widespread adoption. Unprotected proteins can be rapidly degraded by extracellular and endosomal proteases, or neutralized by binding to serum proteins, blood cells, and the extracellular matrix.¹⁸ In addition, the low efficiency of endosomal escape and avoidance of lysosomal degradation are major challenges to all endocytic protein delivery strategies, as evidenced by ongoing interest in endosome altering¹⁷ and destabilizing strategies.¹⁹ These challenges have proven especially difficult *in vivo*.²⁰

Nucleic acid delivery has benefited greatly from the development of liposomal reagents over the past two decades. Cationic lipid formulations have enabled DNA and RNA transfection to become a routine technique in basic research and have even been used in clinical trials.²¹ The lipid bilayer of the vehicle protects complexed nucleic acids from degradation and can prevent neutralization by antibodies.²² Notably, fusion of liposomes with the endosomal membrane during endosome maturation can enable the efficient endosomal escape of cationic lipid-delivered cargo.²³

Because proteins, in contrast to nucleic acids, are chemically diverse with no dominant electrostatic property, no lipid formulation is likely to drive the efficient delivery of all proteins into mammalian cells. While proteins can be complexed non-specifically and delivered by rehydrated lipids *in vitro*,¹⁸ protein complexation is dependent on high protein concentrations, is generally inefficient,²⁴ and has not been widely adopted. Specialty commercial reagents developed for protein delivery^{25,26} have shown modest and variable efficiency with different protein cargoes.²⁷

We hypothesized that proteins that are highly anionic could be delivered by the same electrostatics-driven complexation used by cationic liposomal reagents for nucleic acid delivery (Fig. 1a). Although few proteins natively possess the highly anionic character of nucleic acids, we speculated that translational fusion or non-covalent complexation with a polyanionic molecule may render the resulting protein or protein complex sufficiently anionic to be efficiently delivered by common cationic lipid reagents. We demonstrate that fusion of proteins with an engineered supernegatively charged GFP²⁸ enables efficient complexation and delivery of proteins into cultured mammalian cells by cationic lipids. Our approach is effective even at low nanomolar protein concentrations and in the presence of serum, requiring 1,000-fold less protein to achieve similar functional protein delivery levels than methods that use fusion to cationic peptides or proteins.¹⁵ We further show that Cas9 nuclease protein complexed with polyanionic single guide RNA (sgRNA) can be efficiently delivered in functional form into mammalian cells using cationic lipid formulations. Delivery of Cas9:sgRNA complexes is highly efficient (up to 80% modification of cultured human cells from a single treatment) and also induces higher genome modification specificity (typically ~10-fold) compared with plasmid transfection. Finally, we demonstrate that this protein delivery approach can be effective *in vivo* by delivering functional Cre recombinase and functional Cas9:sgRNA complexes to hair cells in the inner ear of live mice. These findings suggest that the intracellular delivery of polyanionic proteins and protein:nucleic acid complexes by cationic lipids may significantly expand the scope of research and therapeutic applications of proteins.

RESULTS

Delivery of Cre recombinase fused to anionic proteins

First, we tested whether the engineered supernegatively charged GFP variant,²⁸ (–30)GFP, could mediate complexation and delivery of fused protein cargo (Fig. 1b). We translationally fused (–30)GFP to Cre recombinase to generate (–30)GFP-Cre; note that (–30) refers to the net theoretical charge of the GFP moiety, not the net charge of the fusion. We assayed a variety of commercially available cationic lipids for their ability to

functionally deliver (–30)GFP-Cre into HeLa cells that only express DsRed upon Cre-mediated recombination (Fig. 2a). Lipofectamine RNAiMAX (Life Technologies), hereafter referred to as “RNAiMAX”, is a commercial reagent designed for delivery of siRNAs. Delivery of 10 nM (–30)GFP-Cre complexed with 1.5 μ L RNAiMAX in DMEM (Dulbecco’s Modified Eagle’s Media plus GlutaMAX, Life Technologies) containing 10% fetal bovine serum (FBS) led to strong DsRed fluorescence signal among treated cells. Flow cytometry revealed that 48 hours after treatment, 52% of cells expressed DsRed consistent with Cre recombination (Fig. 2b).

Optimization resulted in recombination efficiencies of 65% using 25 nM (–30)GFP-Cre complexed with 1.5 μ L RNAiMAX in 275 μ L of DMEM containing 10% FBS (Fig. 2c). The potency of lipid-mediated (–30)GFP-Cre delivery is remarkable when compared to that of cationic protein-mediated delivery. Only 1 nM (–30)GFP-Cre with cationic lipid was needed to result in 15–20% recombined cells, whereas 1 μ M (+36)GFP-Cre was required to achieve this extent of recombination, corresponding to a 1,000-fold difference in the required protein dose (Fig. 2c). Nearly identical results were observed in a second Cre reporter cell line (BSR TdTomato) (Supplementary Fig. 1a). Under the same conditions that deliver (–30)GFP-Cre most efficiently, cationic lipids did not increase the delivery potency of neutral or cationic Cre recombinase fusions (Fig. 2c), indicating that the highly negative charge of (–30)GFP-Cre is required to participate in cationic lipid-mediated protein delivery. We also observed that increasing the amount of cationic lipid increased the concentration of protein required for maximal recombination, consistent with a model in which deliverable proteins are complexed with specific stoichiometries of cationic lipids (Fig. 2d).

To compare this method to standard plasmid DNA transfection, we optimized plasmid transfection on HeLa reporter cells. Optimized DNA transfection resulted in a maximum of 33% DsRed fluorescent cells (Supplementary Fig. 1b). We also observed that lipid-mediated delivery of (–30)GFP-Cre protein was much less toxic than plasmid transfection (Supplementary Figs. 1c–d). In addition, flow cytometry revealed that the high potency of lipid-mediated delivery of (–30)GFP-Cre does not arise from unusually high protein uptake in each cell, but rather from post-endocytosis processes that likely include endosomal escape and/or the avoidance of lysosomal protein degradation (Supplementary Fig. 2 and Supplementary Results). These observations collectively indicate that cationic lipids can mediate the potent delivery of polyanionic proteins into mammalian cells, even in the presence of serum, and with low toxicity.

To test whether the ability to deliver polyanionic proteins is dependent on proprietary components in RNAiMAX or whether other cationic lipids are capable of mediating similarly potent delivery, we tested several other transfection reagents designed to deliver nucleic acids. Several, but not all, cationic lipid formulations tested are able to potently deliver negatively charged proteins into human cells (Fig. 2e and Supplementary Results).

Several other polyanionic protein, including the VP64 transcriptional activation domain (–22 net theoretical charge), (–20)GFP, (–7)GFP, and the 3x FLAG epitope tag (–7 net theoretical charge), all enhanced cationic lipid-mediated delivery of Cre in a charge-dependent manner (Fig. 2f and Supplementary Results). Collectively these results suggest

protein delivery efficacy by cationic lipids is predominantly a function of total negative charge, and does not require a particular distribution of anionic residues.

Functional delivery of TALE activator proteins

Next we tested lipid-mediated delivery of TALE-VP64 transcriptional activators into HEK293T cells. While modestly effective cleavage of endogenous genes by delivered TALEN proteins has been demonstrated in mammalian cells in the absence of serum using cationic peptides,²⁹ the delivery of TALE-based transcription factor proteins has not yet been reported, and no effective delivery of TALE proteins in serum-containing media has been previously described to our knowledge. We targeted the gene for neurotrophin-3 (NTF3), a neural growth factor that when mutated has been associated with neurodegenerative diseases and hearing loss.³⁰ We fused a previously described NTF3-targeting TALE-VP64³¹ to (–30)GFP (Fig. 3a) and treated HEK293T cells with 25 nM (–30)GFP-NTF3 TALE1-VP64 and 1.5 μ L RNAiMAX under the conditions optimized for Cre delivery. Gene expression levels of NTF3 4 hours after treatment were 3.5-fold higher in cells treated with 25 nM (–30)GFP-NTF3 TALE1-VP64 and RNAiMAX than in untreated cells, cells treated with RNAiMAX only, or cells treated with a VEGF-targeting TALE transcriptional activator (Fig. 3b). Similar levels of NTF3 expression were observed 48 hours after transfection of plasmids encoding the same NTF3-targeting TALE-VP64 (Fig. 3b).

Since the synergistic expression of multiple TALE activators targeting different sites on the same gene has been shown to augment gene activation,³¹ we simultaneously delivered five distinct NTF3-targeting TALE activators fused to (–30) GFP using RNAiMAX. Protein-lipid complexes were prepared as above by adding the five (–30)GFP-NTF3-TALE-VP64 proteins at 5 nM each, for a total of 25 nM protein. We observed an optimized 7-fold increase in NTF3 expression after a 4-hour incubation (Fig. 3b and Supplementary Fig. 3a), while plasmid co-transfection of all five NTF3 TALE activators, followed by a 48-hour incubation, resulted in a similar 10-fold increase in NTF3 expression levels (Fig. 3b). To characterize TALE activity over time using these two methods, we measured NTF3 mRNA levels over a 48-hour period following protein or DNA delivery. TALE activator activity following protein delivery peaks ~4 hours post-treatment and falls over the next 44 hours (Fig. 3c), whereas plasmid DNA transfection required ~24 hours to show above-background levels of NTF3 activation, which plateaued at ~36–48 hours (Fig. 3c). These findings collectively demonstrate that TALE activator proteins can be delivered using cationic lipids to rapidly and transiently activate gene expression in human cells. This capability may prove especially valuable for proteins that induce a permanent change in cell state or cell fate when transiently expressed.

Functional delivery of Cas9:sgRNA protein:RNA complexes

Given the potent lipid-mediated delivery of polyanionic Cre and TALE activator protein variants in full-serum media, we speculated that CRISPR-Cas9:sgRNA complexes, either as fusions with (–30)GFP or as native polyanionic Cas9:guide RNA complexes, might also be delivered into human cells using this approach. Using a well-established Cas9-induced gene disruption assay,³² we targeted specific sites within a genomic EGFP reporter gene in

human U2OS cells (Supplementary Fig. 4a). On-target Cas9 cleavage induces non-homologous end joining (NHEJ) in *EGFP* and the loss of cell fluorescence. To avoid interference from the fluorescence of (–30)GFP, we introduced a Y67S mutation into (–30)GFP to eliminate its fluorescence, and designated this non-fluorescent variant as (–30)dGFP. Treatment of U2OS reporter cells with 25 nM (–30)dGFP-NLS-Cas9 and 25 nM EGFP-targeting sgRNA with RNAiMAX in DMEM containing 10% FBS showed loss of EGFP expression in 48% of cells (Fig. 4a). No significant *EGFP* disruption was observed upon transfection of plasmids encoding EGFP sgRNA alone, Cas9 alone, or cotransfection of plasmids encoding Cas9 and an sgRNA designed to target a *VEGF* locus (Fig. 4a and Supplementary Fig. 4b).

We confirmed that the robust disruption of EGFP was not a result of cellular toxicity (Supplementary Figs. 4c and d). We also observed that treatment of cells with (+36)dGFP-NLS-Cas9 and sgRNA in the presence of 10% FBS did not lead to efficient gene disruption (Fig. 4a), suggesting that cationic-protein based methods of delivery for Cas9 and sgRNA may not be effective, perhaps due to interference of Cas9:sgRNA complex formation or nuclease function by cationic proteins,³³ consistent with a recent study describing the delivery of Cas9 protein with an oligoarginine peptide tag which achieved only moderate levels of gene disruption.³⁴ Optimization of plasmid transfection conditions did not yield higher than 40% EGFP disruption (Fig. 4a and Supplementary Fig. 5a), and the transfection conditions required to achieve this level of gene disruption resulted in high levels of cellular toxicity (Supplementary Fig. 5b). Together, these results establish that cationic lipid-mediated delivery of (–30)dGFP-NLS-Cas9:sgRNA complexes can result in efficient sgRNA-dependent target gene disruption in human cells with minimal toxicity, unlike cationic peptide-based protein delivery or plasmid DNA transfection methods.

Anionic sgRNA is necessary and sufficient for Cas9 delivery

Since the complex of native Cas9 protein (+22 net theoretical charge) and an sgRNA (~103 anionic phosphate groups) should be overall highly anionic, next we tested whether native Cas9:sgRNA complexes without fusion to polyanionic proteins can be delivered into human cells using cationic lipids. Treatment of U2OS EGFP reporter cells with 100 nM Cas9, 50 nM EGFP sgRNA, and 0.8 μ L RNAiMAX resulted in 65% disruption of the *EGFP* reporter gene (Fig. 4a). These observations suggest that sgRNA alone, even in the absence of a supernegatively charged fusion protein, can provide the highly anionic character needed to mediate cationic lipid-based delivery of Cas9. We evaluated several different Cas9 constructs over a broad range of conditions (Supplementary Figs. 6a–g and Supplementary Results) and lipid formulations (Supplementary Fig. 7a and Supplementary Results) for their effect on *EGFP* disruption and observed that up to 80% targeted gene disruption resulted from Cas9:sgRNA complexed with Lipofectamine 2000 (Fig. 4a). Due to the modestly higher toxicity of Lipofectamine 2000 compared to RNAiMAX across a range of doses (Supplementary Figs. 7b–d and Supplementary Results), we continued using RNAiMAX for cell culture studies unless otherwise noted.

To verify that *EGFP* disruption arose from genome modification and not only from Cas9 binding,³⁵ we used the T7 endonuclease I (T7EI) assay³⁶ to detect and quantify the

frequency of Cas9-mediated genomic insertion/deletion mutations (indels) at the target *EGFP* locus (Fig. 4b). The T7EI assay results showed that only those cells treated with both Cas9 and EGFP sgRNA plasmids, or Cas9 protein and purified EGFP sgRNA, contained indels at the target site 48 hours after treatment. We also treated U2OS EGFP reporter cells with a single lipid-mediated delivery treatment of Cas9 complexed with a mixture of four sgRNAs targeting *EGFP*, *CLTA*, *EMX*, and *VEGF*, resulting in cleavage efficiencies of 58%, 28%, 16%, and 40%, respectively, as measured by T7EI cleavage assay (Fig. 4c). These high gene disruption efficiencies from a single delivery of 50 nM Cas9 and 12.5 nM of each sgRNA (50 nM total sgRNA) demonstrate that lipid-mediated Cas9:sgRNA delivery can support efficient multiplexed genome editing.

We also tested whether delivered Cas9 nuclease:sgRNA complexes are capable of effecting homology-directed repair (HDR) using an EGFP-repair reporter cell line.³⁷ We combined Cas9 and *EGFP*-targeting sgRNA, mixed the resulting protein:RNA complexes with varying concentrations of single-stranded DNA oligonucleotide (ssODN) donor template (Supplementary Notes), and delivered the Cas9:sgRNA + ssODN mixture using Lipofectamine 2000 (Supplementary Fig. 8a). Cas9:sgRNA delivery achieved EGFP HDR frequencies of ~8–11%, similar to that of optimized plasmid transfection-based HDR (Supplementary Figs. 8b and c), and consistent with previous reports using the same reporter cell line³⁷, suggesting that cationic lipid-based delivery of Cas9:sgRNA is a viable approach to efficient HDR.

Next we determined whether cationic lipid-based protein delivery could be applied to deliver other Cas9-derived genome engineering tools such as Cas9 nickases³⁸ and Cas9-based transcriptional activators.³⁹ We measured gene disruption efficiency in U2OS EGFP reporter cells resulting from delivery of Cas9 D10A nickase (Fig. 4d and Supplementary Results) and achieved results similar to previous reports using plasmid transfection.⁴⁰ 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). As observed above with TALE activators (Fig. 3c), dCas9-VP64 protein delivery resulted in fast-acting and transient transcriptional activation compared to DNA delivery (Supplementary Fig. 9b and Supplementary Results). 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 improves genome modification specificity

Transient delivery of functional Cas9:sgRNA protein:RNA complexes circumvents risks associated with viral or other gene delivery methods and has the potential to improve the specificity of genome editing by minimizing the opportunity of agents to modify off-target substrates after the target locus is modified, or to reverse on-target modification. To test whether our approach can disrupt endogenous genes in human cells, we targeted genomic loci in the *EMX1*, *CLTA2*, and *VEGF* genes due to their potential biomedical relevance and their use in previous studies^{32,40,41} of Cas9 off-target cleavage activity. Cationic lipid-mediated delivery of Cas9:sgRNA complexes into HEK293T cells resulted in robust

cleavage of all three human genes with efficiencies similar to or greater than those of plasmid transfection methods as revealed by the T7EI assay (Fig. 5a).

To compare the endogenous gene modification specificity of plasmid versus protein:RNA delivery methods for Cas9, we amplified the on-target locus as well as several known off-target sites (Supplementary Table 1) from genomic DNA isolated from HEK293T cells treated either by transfection of Cas9 and sgRNA expression plasmids, or by RNAiMAX-mediated Cas9:sgRNA complex delivery under conditions that resulted in similar on-target modification efficiencies. The indel frequencies at the three on-target and 11 off-target sites were assayed by high-throughput DNA sequencing (Supplementary Table 2). For all three target genes, the frequency of on-target DNA modification resulting from either plasmid or protein:sgRNA delivery was $10\pm 2\%$ (Supplementary Fig. 10), enabling the comparison of off-target modification under conditions that result in very similar on-target modification efficiencies. Importantly, the frequency of off-target genome modification for all 11 off-target sites was lower from protein:sgRNA delivery compared with plasmid delivery (Supplementary Figs. 10a–c), and as a result the ratio of on-target to off-target modification ratio for all sites tested was up to 19-fold higher for protein:sgRNA delivery than for plasmid delivery (Figs. 5b–d).

We also observed that the increase in specificity for Cas9 protein delivery relative to DNA transfection persists across a wide range of on-target cleavage efficiencies (~1%, ~10%, and ~40%) (Supplementary Fig. 11 and Supplementary Results). This increase in specificity using protein delivery is consistent with the transient nature of the delivered protein:sgRNA complexes compared to plasmid transfection (Supplementary Fig. 12 and Supplementary Results). We also measured the amount of protein internalized by cells using our cationic lipid-based protein delivery approach and determined that ~4% of the total protein used in the treatment was internalized by cells (Supplementary Fig. 13 and Supplementary Results). We note that the majority of this protein likely exists within endosomes and may not be available to effect genome modification.^{17,42} Taken together, these results show that the delivery of Cas9:sgRNA complexes using cationic lipids can effect target gene modification at high efficiency and with substantially greater specificity than the delivery of DNA expressing Cas9 and sgRNA.

***In vivo* delivery of Cre recombinase and Cas9:sgRNA**

Efficient delivery of functional genome-editing proteins *in vivo* could enable a wide range of applications including non-viral therapeutic genome editing to correct genetic diseases. To evaluate this protein delivery method in a live animal, we chose delivery to the mouse inner ear due to its confined space, well-characterized inner ear cell types, and the existence of genetic deafness mouse models that may enable future hearing recovery studies. We attempted the *in vivo* delivery of two types of proteins into the mouse inner ear. First, we tested the delivery of (–30)GFP-Cre protein to assess the targeting of inner ear cell types and the efficiency of functional protein delivery. Second, we evaluated the delivery of Cas9:sgRNA complexes to the inner ear to determine whether cationic lipid-mediated protein:sgRNA complex delivery can support CRISPR-based gene editing *in vivo*.

We previously showed that (+36)GFP-Cre could be delivered to mouse retina,¹⁵ although the protein resulted in only modest levels of recombinant conversion suggestive of inefficient *in vivo* delivery. For our initial inner ear delivery trials, we complexed (–30)GFP-Cre with RNAiMAX and injected the complex into the cochlea of postnatal day 1 (P1) reporter mice with a genomically integrated floxed-STOP tdTomato reporter. As with our *in vitro* Cre reporter cell line, functional delivery of Cre to the inner ear cells, followed by endosomal escape, nuclear localization, and Cre-mediated recombination results in expression of tdTomato. After injection, the cochleas were harvested for immunolabeling with inner ear cell markers for co-localization with tdTomato. RNAiMAX injection alone was used as control. Five days following injection of (–30)GFP-Cre and RNAiMAX, cochlear outer hair cells, a type of auditory sensory cells that detect sound, showed strong tdTomato signal that co-localized with the hair cell marker myosin VIIa (*Myo7a*), demonstrating functional Cre delivery to hair cells (Figs. 6a–b). No tdTomato expression was detected in control cochleas (Fig. 6a). The tdTomato signal was concentrated in the region of the injection site at the basal turn of the cochlea. On average 33±3% of outer hair cells were tdTomato positive at the base of the cochlea ($P < 0.001$; mean ± SEM, $n = 4$) and intact stereocilia were observed indicative of healthy hair cells (Fig. 6b). We also tested delivery using Lipofectamine 2000 due to its higher potency *in vitro* (Supplementary Fig. 7a) and observed dramatically higher recombination efficiency: 91 ± 5% outer hair cells in cochleas treated with (–30)GFP-Cre + Lipofectamine 2000 were tdTomato positive (Fig. 6c). In comparison to control samples, some outer hair cell loss was observed (Fig. 6c), consistent with our previous observation of higher cell toxicity of Lipofectamine 2000, although overall cochlear architecture was preserved.

After validating Cas9:sgRNA delivery in reporter cells (Figs. 4a–e), and in neuron-derived mouse embryonic stem cells (Supplementary Fig. 14 and Supplementary Results), we tested Cas9:sgRNA delivery *in vivo*. Cas9 and sgRNA targeting *EGFP* were combined with RNAiMAX and the resulting complexes were injected into postnatal day 2 (P2) transgenic *Atoh1-GFP* mouse cochlea in which all hair cells express GFP under the control of a hair cell-specific enhancer for transcription factor *Atoh1*.⁴³ Using this model, Cas9:sgRNA-mediated disruption of *Atoh1-GFP* results in loss of GFP fluorescence in outer hair cells. Ten days after injection of Cas9:sgRNA with cationic lipid, we observed the absence of GFP in 13% of outer hair cells near the injection site. In contrast, control cochlea injected with Cas9 protein and RNAiMAX without any sgRNA showed no loss of EGFP signal (Fig. 6d). The outer hair cells of cochlea injected with Cas9:sgRNA RNAiMAX complexes appeared to be otherwise unaffected, with stereotypical expression of *Myo7a* and healthy nuclei, consistent with minimal hair cell toxicity (Fig. 6d). High-throughput DNA sequencing of genomic DNA isolated from cochlea tissue samples revealed indels consistent with *GFP* target gene disruption in the treated samples, but not in the control samples that lacked sgRNA (Supplementary Fig. 15a). In addition, we repeated inner ear *in vivo* delivery of Cas9:sgRNA using an sgRNA that targets the *EMX* gene and similarly observed indels in the *EMX* gene in treated animals, but not control animals (Supplementary Fig. 15b).

As (–30)GFP-Cre complexed with Lipofectamine 2000 resulted in more efficient modification of the target hair cell population than (–30)GFP-Cre complexed with

RNAiMAX (Figs. 6a and c), we tested its use on Cas9:sgRNA delivery to Atoh1-GFP cochlea as above. We observed loss of GFP expression in $20 \pm 3\%$ of outer hair cells near the injection site after 10 days, whereas all outer hair cells maintained strong GFP expression in control cochlea injected with Cas9 and Lipofectamine 2000 but no sgRNA (Fig. 6d). In contrast to modest hair cell loss observed following Lipofectamine 2000 delivery of (-30)GFP-Cre (Fig. 6c), outer hair cells targeted by Cas9:sgRNA exhibited no obvious toxicity or structural alteration (Fig. 6d).

As with (-30)GFP-Cre, virus-free, cationic lipid-mediated delivery of Cas9:sgRNA into the mouse inner ear successfully modified a specific genomic locus in the outer hair cell population, leading to loss of target gene expression. As nearly half of all types of genetic deafness arise from hair cell loss or dysfunction,⁴⁴ our results suggest a potential strategy based on the delivery of Cas9:sgRNA complexes to genetically modify these cells to effect hearing recovery. Taken together, these findings suggest that cationic lipid-mediated delivery of genome-editing proteins can serve as a powerful tool and a potential *in vivo* strategy for the treatment of genetic disease.

DISCUSSION

Efficient intracellular protein delivery *in vitro* and especially *in vivo* has been a persistent challenge in biomedical research and protein therapeutics. Here we report a general strategy for protein delivery that makes use of anionic protein complexation with cationic liposomes. We used this method to deliver diverse protein classes, including the Cre tyrosine recombinase, TALE transcriptional activators, and Cas9 nucleases, nickases, and transcriptional activators (Fig. 1a) to cultured cell lines, stem cell colonies, and therapeutically relevant *in vivo* sites within the mouse inner ear. Our approach is highly efficient, producing modification rates similar to or exceeding those of established nucleic acid transfection methods in cell culture, and enabling Cre recombinase- and Cas9-mediated genome modification rates of up to 90% and 20%, respectively, within the inner ear hair cell population of live mice (Figs. 6c and d). For Cas9 nuclease delivery, this approach also typically results in >10-fold more specific genome modification than traditional plasmid transfection (Figs. 5b–d), likely due to the transient window of Cas9 activity to which each genome is exposed (Supplementary Fig. 12) compared to DNA delivery methods, consistent with previous reports.⁴⁵

Others groups have reported the *in vivo* delivery of Cas9 expression constructs in DNA or mRNA form.^{46,47} This study establishes that protein delivery is a viable approach to *in vivo* genome editing. Since the commercial lipid reagents used in the current study were optimized for the delivery of DNA and RNA, it is likely that future development of specific components of the liposomal formulation will further improve the performance of this strategy, especially for *in vivo* use. These results also suggest that cationic lipids can efficiently deliver other proteins *in vitro* and *in vivo*, including natively anionic proteins or proteins that can be fused or bound to polyanionic macromolecules.

ONLINE METHODS

Construction of Cas9, Cre, and TALE fusion and sgRNA expression plasmids

Sequences of all constructs used in this paper are listed in the Supplementary Notes. All protein constructs were generated from previously reported plasmids for protein of interest cloned into a pET29a expression plasmid. All plasmid constructs generated in this work will be deposited with Addgene. sgRNA expression plasmids were obtained from J. Keith Joung and are available from Addgene.

Expression and purification of *S. pyogenes* Cas9 and other proteins

E. coli BL21 STAR (DE3) competent cells (Life Technologies) were transformed with pMJ806⁴¹ encoding the *S. pyogenes* Cas9 fused to an N-terminal 10xHis-tag/maltose binding protein. The resulting expression strain was inoculated in Luria-Bertani (LB) broth containing 100 µg/mL of ampicillin at 37 °C overnight. The cells were diluted 1:100 into the same growth medium and grown at 37 °C to OD₆₀₀ = ~0.6. The culture was incubated at 20 °C for 30 min, and isopropyl β-D-1- thiogalactopyranoside (IPTG) was added at 0.5 mM to induce Cas9 expression. After ~16 h, the cells were collected by centrifugation at 8,000 g and resuspended in lysis buffer (50 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 8.0, 1 M NaCl, 20 % glycerol, 10 mM tris(2-carboxyethyl)phosphine (TCEP; Soltec Ventures). The cells were lysed by sonication (1 sec pulse-on, 1 sec pulse-off for 15 min total at 6 W output) and the soluble lysate was obtained by centrifugation at 20,000 g for 30 min.

The cell lysate was incubated with His-Pur nickel-nitriloacetic acid (nickel-NTA) resin (Thermo Scientific) at 4 °C for 30 min to capture His-tagged Cas9. The resin was transferred to a 20-mL column and washed with 20 column volumes of lysis buffer. Cas9 was eluted in 50 mM Tris-HCl (pH 8), 0.1 M NaCl, 20 % glycerol, 10 mM TCEP, and 300 mM imidazole, and concentrated by Amicon ultra centrifugal filter (Millipore, 100-kDa molecular weight cut-off) to ~50 mg/mL. The 6xHis tag and maltose-binding protein were removed by TEV protease treatment at 4 °C for 20 hours and captured by a second Ni-affinity purification step. The eluent, containing Cas9, was injected into a HiTrap SP HP column (GE Healthcare) in purification buffer containing 50 mM Tris-HCl (pH 8), 0.1 M NaCl, 20 % glycerol, and 10 mM TCEP. Cas9 was eluted with purification buffer containing a linear NaCl gradient from 0.1 M to 1 M over five column volumes. The eluted fractions containing Cas9 were concentrated down to a concentration of 200 µM as quantified by Bicinchoninic acid (BCA) assay (Pierce Biotechnology), snap-frozen in liquid nitrogen, and stored in aliquots at -80 °C. All other proteins were purified by this method but without TEV cleavage step and proteins containing (-30)GFP were purified by anion exchange using a HiTrap Q HP anion exchange column (GE Healthcare) using the same purification protocol.

In vitro transcription of sgRNAs

Linear DNA fragments containing the T7 promoter binding site followed by the 20-bp sgRNA target sequence were transcribed *in vitro* using the T7 High Yield RNA Synthesis Kit (NEB) according to the manufacturer's instructions. *In vitro* transcribed RNA was precipitated with ethanol and purified by gel electrophoresis on a Criterion 10%

polyacrylamide TBE-Urea gel (Bio-Rad). Excised gel fragments were extracted in 420 μL of 300 mM NaCl overnight on a rocking surface at 4 $^{\circ}\text{C}$. Gel-purified sgRNA was precipitated with ethanol and redissolved in water and sgRNA concentration was finally quantified by UV absorbance and snap-frozen at -80°C .

Plasmid transfection

Plasmid DNA was transfected using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. For Cre recombinase experiments, 500 ng of DNA was transfected. For TALE activator plasmids, 300 ng of DNA was transfected, and for the activator synergy experiments 60 ng of each of five plasmids was pooled and transfected. For Cas9 nuclease plasmid delivery experiments targeting genomic sites in *CLTA*, *EMX*, or *VEGF*, linear PCR products expressing sgRNAs were used along with the normal Cas9 plasmid. Linear PCR products were generated using plasmid containing the U6 promoter as template, forward primers containing the U6 promoter upstream sequence, and reverse primers containing U6 downstream sequence followed by the sgRNA sequence (20-bp sequences unique to each target plus constant sgRNA backbone architecture sequence). sgRNAs expressed from linear DNA templates contained at least two 5' guanosines to match *in vitro*-transcribed sgRNAs that required these bases for transcription by T7 RNA polymerase. Primer sequences and PCR conditions are referred to in the Supplementary Notes. For dCas9 activator experiments, 750 ng of Cas9 or dCas9-VP64 plasmid DNA was co-transfected with 250 ng of the appropriate sgRNA expression plasmid. For Cas9 activator synergy experiments 50 ng of DNA from each of the six sgRNA were pooled and co-transfected with 750 ng of dCas9-VP64 plasmid.

Delivery of proteins complexed with cationic lipids in cell culture

Cultured cells were plated in 48-well format (250 μL volume) in Dulbecco's Modified Eagle's Media plus GlutaMAX (DMEM, Life Technologies) with 10% FBS ("full serum media") and antibiotics at a cell density necessary to reach ~70% confluence the next day. Full serum media was replaced with the same media but containing no antibiotics at least one hour before delivery. Delivery of Cre and TALE proteins was performed by combining 1 nM to 1 μM protein (based on a 275 μL final volume) with 0.2–2.5 μL commercially available cationic lipids in 25 μL OPTIMEM media (Life Technologies) according to the manufacturer's protocol for normal plasmid transfection, including incubation time. 25 μL of the OPTIMEM mixture containing cationic lipids and protein was then added to cells in 250 μL of full serum media. For Cas9 delivery *in vitro*, purified sgRNA was incubated with Cas9 protein for 5 min before complexing with the cationic lipid reagent in 25 μL OPTIMEM and treating cells in 250 μL of full serum media. All complexing steps were performed at room temperature. For all protein deliveries that lasted longer than 12–16 hours, media containing lipid complexes was replaced with fresh full serum media without antibiotics after 12–16 hours unless otherwise noted. Cells were assayed for recombination 48 hours after delivery, for optimal gene activation either 4 hours (TALE) or 12–16 hours (dCas9-VP64) after delivery, and for gene modification (Cas9) 48 hours after delivery.

T7 endonuclease I assay to detect genomic modifications

U2OS-EGFP cells or HEK293T cells were transfected with Cas9 expression and sgRNA expression plasmids or linear DNA PCR products as described above or treated with only Cas9 protein, only *in vitro* transcribed sgRNA, or only RNAiMAX. Genomic DNA was isolated from cells 2 days after transfection using the DNAdvance Kit (Agencourt) following the manufacturer's instructions. 200 ng of genomic DNA was used as template in PCR reactions to amplify the targeted genomic loci with flanking survey primer pairs specified in the Supplementary Notes. PCR products were purified with a QIAquick PCR Purification Kit (Qiagen) and quantified with Quant-iT™ PicoGreen® dsDNA Kit (Life Technologies). 250 ng of purified PCR DNA was combined with 2 µL of NEBuffer 2 (NEB) in a total volume of 19 µL and denatured then re-annealed with thermocycling at 95 °C for 5 min, 95 to 85 °C at 2 °C/s; 85 to 20 °C at 0.2 °C/s. The re-annealed DNA was incubated with 1 µL of T7 Endonuclease I (10 U/µL, NEB) at 37 °C for 15 min. 10 µL of 50 % glycerol was added to the T7 Endonuclease reaction and 12 µL was analyzed on a 5 % TBE 18-well Criterion PAGE gel (Bio-Rad) electrophoresed for 30 min at 200 V, then stained with 1x SYBR Gold (Life Technologies) for 30 min. Cas9-induced cleavage bands and the uncleaved band were visualized on an AlphaImager HP (Alpha Innotech) and quantified using ImageJ software⁴⁸. The peak intensities of the cleaved bands were divided by the total intensity of all bands (uncleaved + cleaved bands) to determine the fraction cleaved which was used to estimate gene modification levels as previously described.⁴⁰ For each sample, transfections and subsequent modification measurements were performed in triplicate on different days.

Stem cell culture and delivery

Mouse embryonic stem cell (ES) line Tau-GFP (courtesy of Dr. A. Edge, Massachusetts Eye & Ear Infirmary, Boston) containing a permanent GFP gene insertion was cultured in DMEM with 10% FBS (Gibco), 100 mM MEM nonessential amino acids (Gibco), 0.55 mM 2-mercaptoethanol (Sigma), and leukemia inhibitory factor (1,000 units/ml; Chemicon). After 3 days floating spheres were formed that exhibited GFP fluorescence. Complexes of Cas9:sgRNA and Lipofectamine 2000 were added to the culture containing the floating spheres for 16 hours. After Cas9:sgRNA treatment, the cells were cultured in the above media for 3 days. The floating spheres were treated with trypsin for 5 min then passed through a 70 µm filter to collect single cells. The cells were cultured on laminin-coated slides in DMEM/F12 (1:1) supplemented with 1xN2, 1xB27, penicillin-streptomycin (100 µg/mL; Life Technologies) and 10% FBS for two days before labeling. Immunohistochemistry was performed using an anti-GFP antibody (#ab13970, Abcam) to assess GFP expression. To quantify the number of GFP-negative cells, we counted the total number of GFP-positive and GFP-negative cells from three representative visual fields at 20X magnification, and calculated the average efficiency. Three independent experiments were performed for each condition.

Microinjection of proteins to mouse inner ear

Animals were used under protocols approved by the Massachusetts Eye & Ear Infirmary IACUC committee. P0 floxP-tdTomato mice (The Jackson Laboratory) were used for

(-30)GFP-Cre injection and P2 Atoh1-GFP mice (courtesy of Dr. J. Johnson, Southwestern Medical Center, University of Texas) were used for Cas9:sgRNA injection. Mice were anesthetized by lowering their temperature on ice. Cochleostomies were performed by making an incision behind the ear to expose the cochlea. Glass micropipettes held by a micromanipulator were used to deliver the complex into the scala media, which allows access to inner ear hair cells. For delivery of (-30)GFP-Cre, 3 μ L of 45 μ M protein was mixed with 3 μ L of either RNAiMAX or Lipofectamine 2000 and incubated at room temperature for 30 minutes prior to injection. Four mice were injected per treatment group. For delivery of Cas9:sgRNA complexes, 1 μ L of 200 μ M Cas9 protein was mixed with 2 μ L of 50 μ M sgRNA and incubated for 5 minutes at room temperature before mixing with 3 μ L of either RNAiMAX or Lipofectamine 2000 and incubating for an additional 30 minutes prior to injection. Three mice were injected per treatment group. The total delivery volume for every injection was 0.3 μ L per cochlea and the release was controlled by a micromanipulator at the speed of 3 nL/sec.

Immunohistochemistry and quantification

5–10 days after injection, the mice were sacrificed and cochlea were harvested by standard protocols.⁴⁹ For immunohistochemistry, antibodies against hair-cell markers (Myo7a and Esp) and supporting cells (Sox2) were used following a previously described protocol.⁴⁹ To quantify the number of tdTomato positive cells after (-30)GFP-Cre or GFP negative cells after Cas9:sgRNA delivery, we counted the total number of outer hair cells in a region spanning 200 μ m around the site of injection in the base turn of the cochlea. The efficiency of (-30)GFP-Cre-induced recombination or Cas9:sgRNA-induced genome modification was calculated as the percentage of outer hair cells that expressed tdTomato or that lost GFP expression.

High-throughput DNA sequencing of genome modifications

HEK293T cells were either transfected with Cas9 and sgRNA expression plasmids or linear DNA PCR products or treated with 50 nM Cas9 protein, 125 nM purified sgRNA, and cationic lipids as described earlier for Cas9 protein delivery to U2OS-EGFP reporter cells. For plasmid-based transfection experiments, 700 ng of Cas9 expression plasmid plus 250 ng of sgRNA plasmid or 50 ng of a linear DNA PCR product expressing sgRNA for targeting either the EMX1, CLTA2, or VEGF locus were transfected with Lipofectamine 2000 (Life Technologies) and cells were isolated 2 days later. For protein delivery experiments *in vivo*, ~30 mg of mouse tissue was isolated as previously described⁴⁹ from anesthetized mice and genomic DNA was extracted using the Agencourt DNAdvance Genomic DNA Isolation Kit (Beckman Coulter). For cell culture experiments genomic DNA was isolated as described above. 150 ng of genomic DNA was used as template to amplify by PCR the on-target and off-target genomic sites with flanking HTS primer pairs specified in the Supplementary Notes. Relative amounts of crude PCR products were quantified by gel electrophoresis and samples treated with different sgRNA pairs or Cas9 nuclease types were separately pooled in equimolar concentrations before purification with the QIAquick PCR Purification Kit (Qiagen). ~150 ng of pooled DNA was electrophoresed using a 5% TBE 18-well Criterion PAGE gel (Bio-Rad) for 30 min at 200 V and DNAs ~125 bp to ~300 bp in length were isolated and purified by QIAquick PCR Purification Kit (Qiagen). Purified DNA was

amplified by PCR with primers containing sequencing adapters, purified, and sequenced on a MiSeq high-throughput DNA sequencer (Illumina) as previously described.⁴¹

Quantification of Cas9 protein uptake

We used Alexa Fluor 647 C2 Maleimide (Life Technologies) to fluorescently label Cas9 protein on surface cysteines. A 10 mM stock solution of Alexa 647 was prepared in anhydrous DMSO (Sigma). In a 0.4 mL reaction, 10 nmol of purified Cas9 protein and 200 nmol of Alexa 647 maleimide were combined in buffer conditions used for Cas9 protein storage. The labeling reaction was incubated at 4° C for 16 hours. At the end of the reaction, excess unconjugated Alexa 647 was removed by re-purifying the labeled Cas9 protein by cation exchange chromatography as described above. To measure the amount of protein delivered into treated cells, 20,000 cells were plated in the wells of a 48-well plate 1 day prior to treatment. On the day of treatment, 50 nM of Alexa 647-labeled Cas9 (Cas9-Alexa 647) and 50 nM of EGFP sgRNA were prepared for delivery using 0.8 µL of Lipofectamine 2000 as described above, and applied to the cells. After 4 hours, Cas9-Alexa 647:sgRNA Lipofectamine-containing media was removed, and cells were washed three times with 500 µL of PBS containing 20 U/mL heparin. The cells were trypsinized and prepared for counting and flow cytometry as described above. Cas9-Alexa 647 uptake was measured by flow cytometry, while 10,000 cells of the treated population were transferred to a black, flat-bottomed, opaque 96-well plate. Standard curves of Cas9-Alexa 647 were prepared by complexing 50 pmol of the Cas9-Alexa 647 protein with Lipofectamine 2000 exactly as described for Cas9-Alexa 647 delivery, followed by serial 2-fold dilutions in DMEM with 10% FBS containing 10,000 U2OS cells per well in the 96-well plate. The effect of U2OS cells or complexation with Lipofectamine 2000 on Alexa 647 fluorescence was determined by preparing three additional Cas9-Alexa 647 standard curves: (i) with Lipofectamine 2000 in media lacking U2OS cells, (ii) without Lipofectamine 2000 in media containing U2OS cells, and (iii) without Lipofectamine 2000 in media lacking U2OS cells.

Data Analysis

Illumina sequencing reads were filtered and parsed with scripts written in Unix Bash as outlined in Supplementary Notes. DNA sequences are deposited in NCBI's Sequencing Reads Archive (SRA). Sample sizes for sequencing experiments were maximized (within practical experimental considerations) to ensure greatest power to detect effects. Statistical analyses for Cas9-modified genomic sites (Supplementary Table 2) were performed as previously described.⁵⁰

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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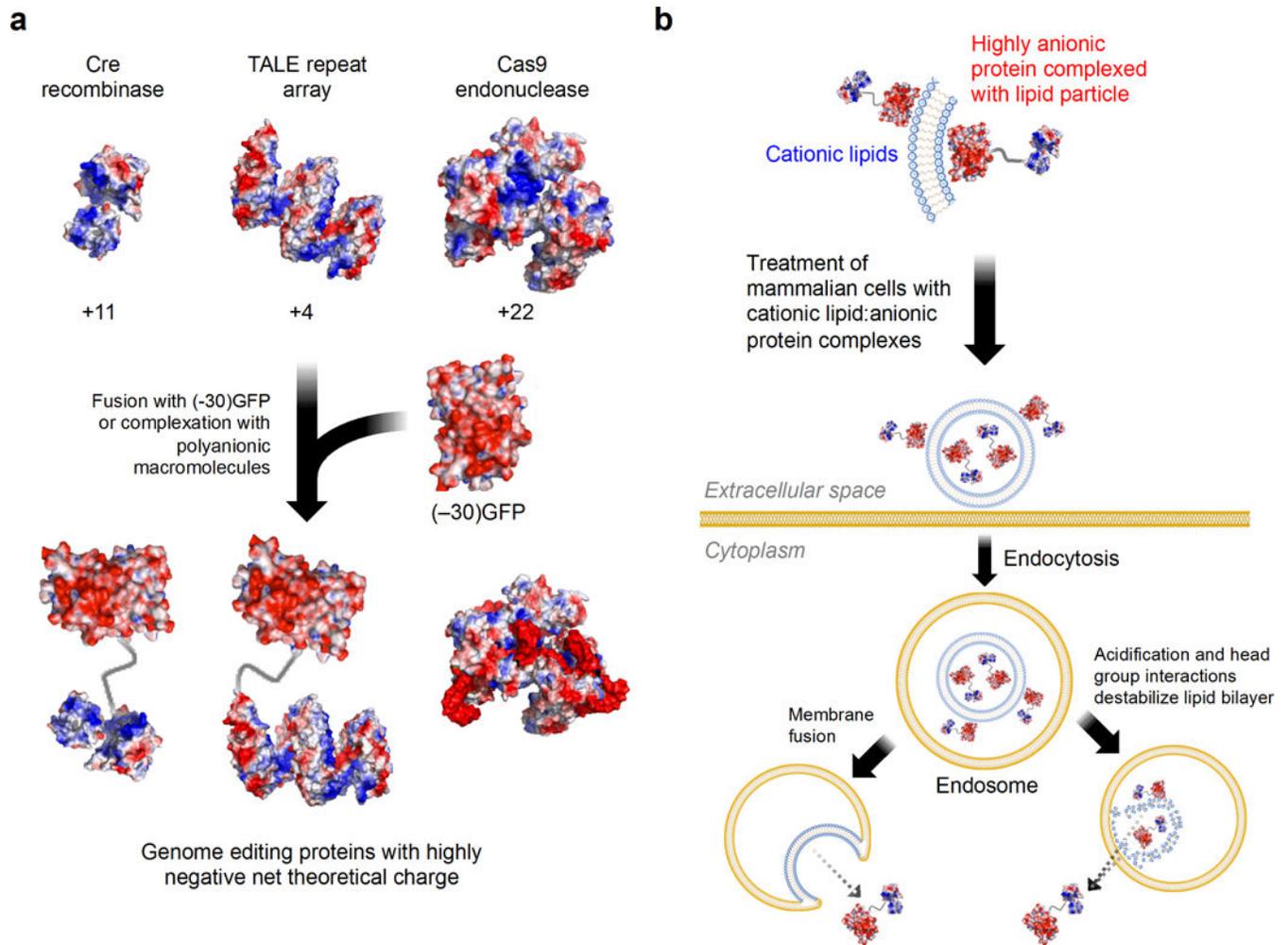
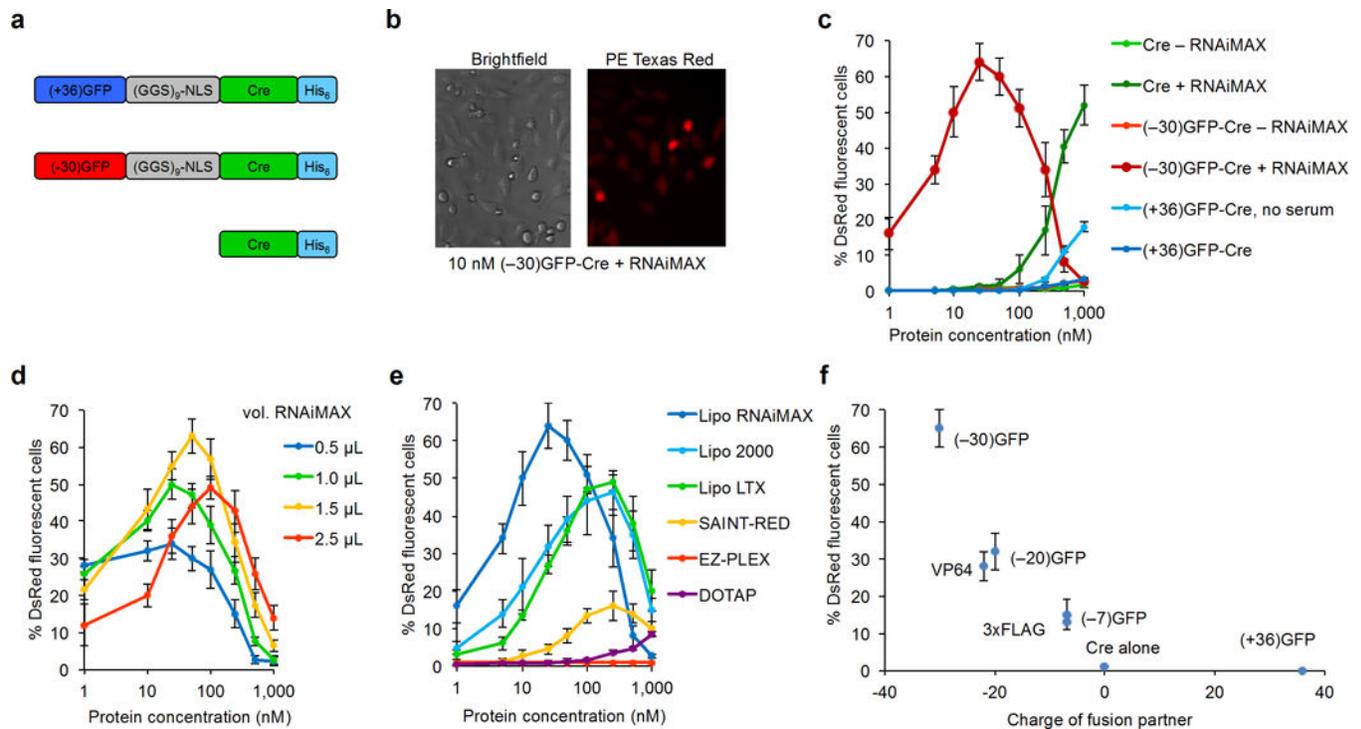


Figure 1. Strategy for delivering proteins into mammalian cells by fusion or non-covalent complexation with polyanionic macromolecules and complexation with cationic lipids. **(a)** Recombinases, transcriptional-activator-like effector (TALE) proteins, and Cas9 endonucleases bind nucleic acids and are natively cationic (net theoretical charges are shown in black) and are not efficiently complexed with cationic lipids. These proteins can be rendered highly anionic, however, by fusion to either a supernegatively charged protein such as (-30)GFP, or by complexation with polyanionic nucleic acids. **(b)** We envisioned that cationic lipids commonly used to transfect DNA and RNA would complex with the resulting highly anionic proteins or protein:nucleic acid complexes, mediating their delivery into mammalian cells.

**Figure 2.**

Delivery of Cre recombinase to cultured human cells. **(a)** Fusion of either highly cationic (+36)GFP or highly anionic (-30)GFP to Cre recombinase. We used a HeLa reporter cell line that expresses DsRed upon Cre-mediated recombination to evaluate Cre delivery efficiency. **(b)** HeLa dsRed cells treated with 10 nM (-30)GFP-Cre and 1.5 μL of the cationic lipid formulation RNAiMAX. Cells were visualized after incubation for 48 hours in media containing 10% fetal bovine serum (FBS). **(c)** Delivery of (+36)GFP-Cre in 10% FBS media or in serum-free media, and (-30)GFP-Cre with or without the cationic lipid RNAiMAX (0.8 μL) in full-serum media. **(d)** Effect of cationic lipid dose on functional (-30)GFP-Cre delivery efficacy after 48 hours. **(e)** Comparison of several commercially available cationic lipids and polymers for functional delivery efficacy of (-30)dGFP-Cre. **(f)** RNAiMAX-mediated delivery of multiple anionic peptide or protein sequences fused to Cre. The net theoretical charge of the VP64 activation domain and the 3xFLAG tag is -22 and -7, respectively. All experiments were performed with 25 nM protein in 48-well plate format using 275 μL DMEM with 10% FBS and no antibiotics. Error bars reflect s.d. from three biological replicates performed on different days.

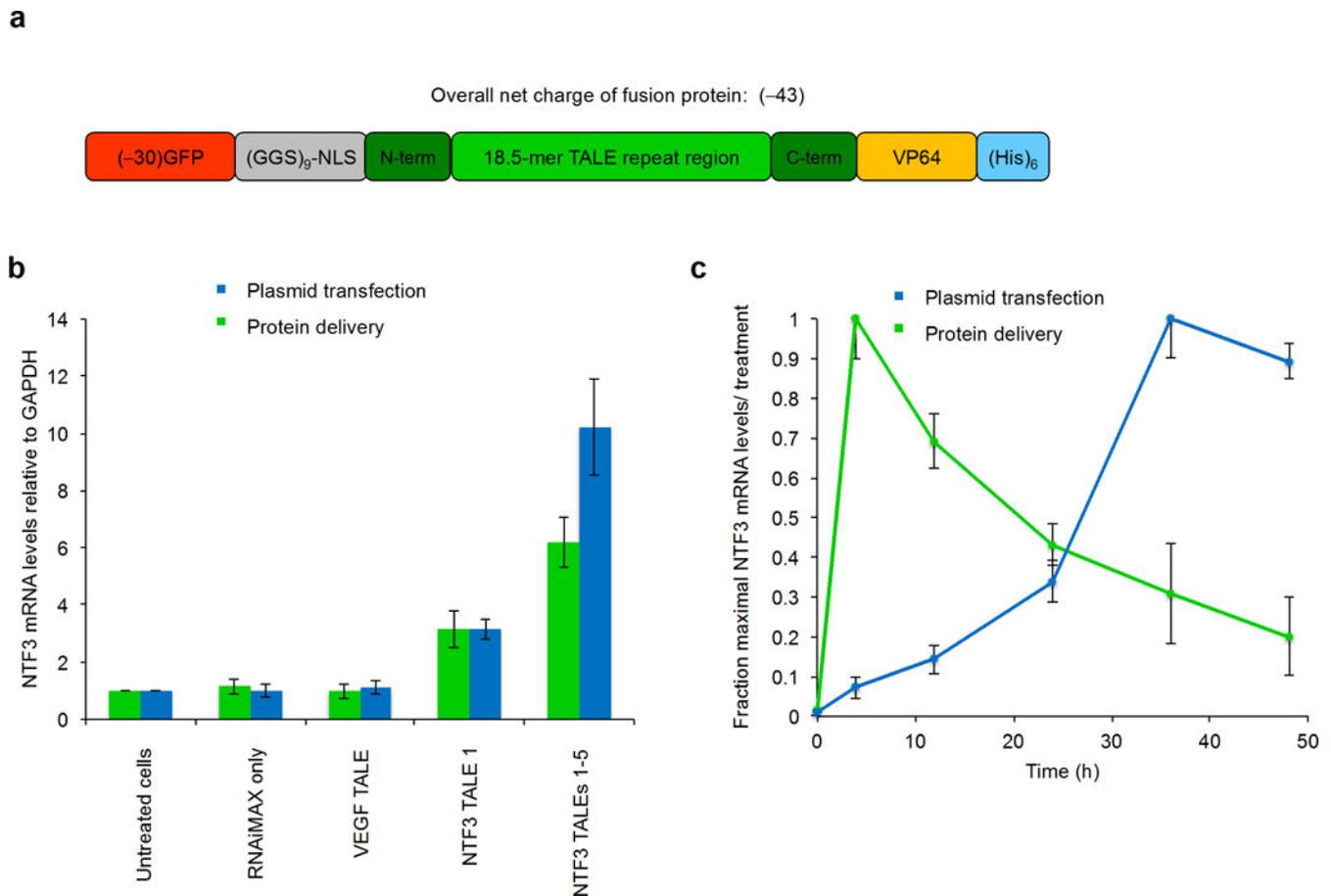
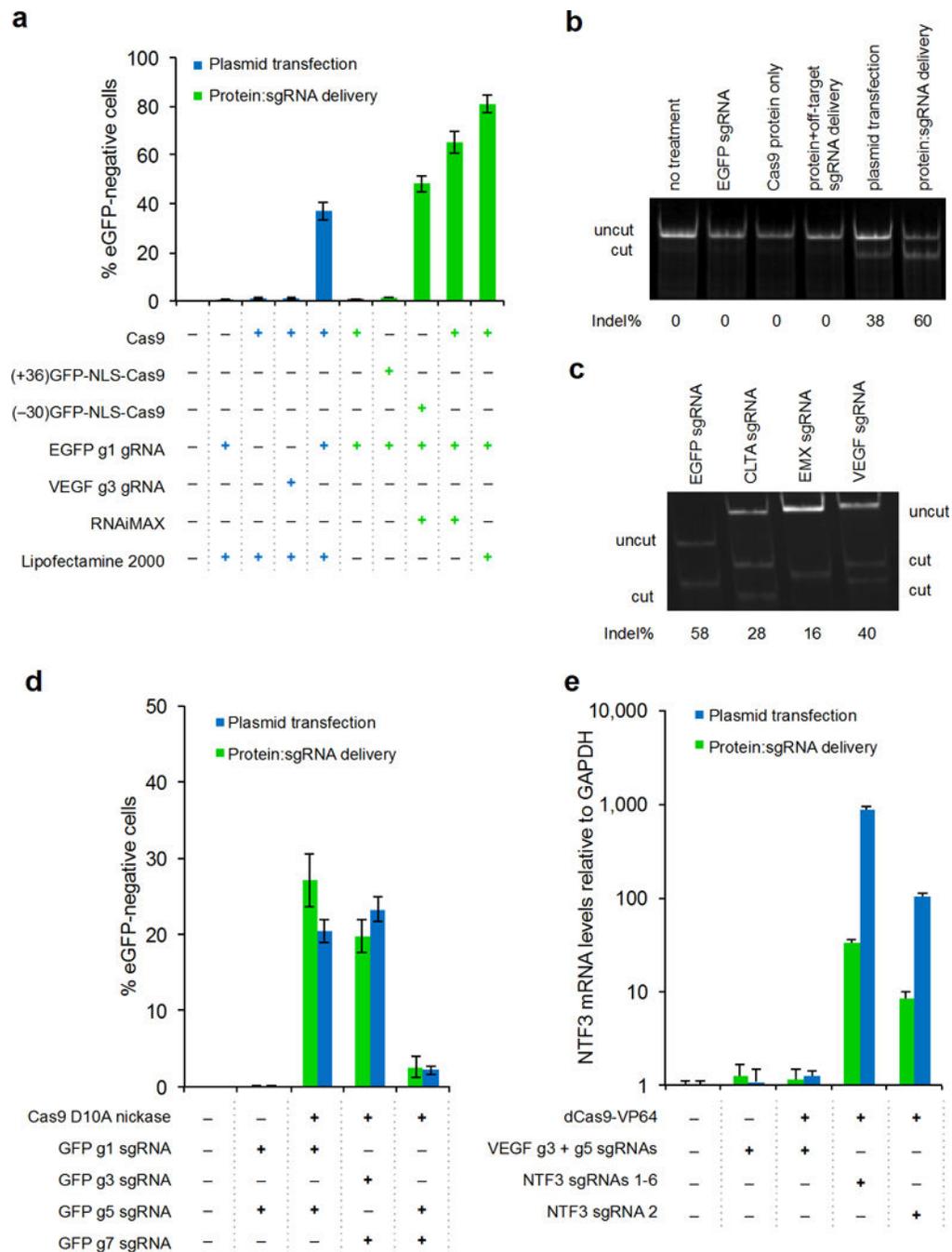


Figure 3. Delivery of TALE transcriptional activators into cultured human cells. **(a)** Design of an 18.5-repeat TALE activator fused C-terminally to a VP64 activation domain and N-terminally to (-30)GFP and an NLS. The overall net theoretical charge of the fusion is -43. **(b)** Activation of *NTF3* transcription by traditional transfection of plasmids encoding TALE-VP64 activators that target sites in the *NTF3* gene, or by RNAiMAX cationic lipid-mediated delivery of the corresponding *NTF3*-targeting (-30)GFP-TALE-VP64 proteins. For protein delivery experiments, 25 nM VEGF TALE, 25 nM NTF3 TALE 1, or 25 nM NTF3 TALEs 1–5 (5 nM each) were delivered with 1.5 μ L RNAiMAX in 275 μ L DMEM-FBS without antibiotics for 4 hours before being harvested. For plasmid transfections, a total of 300 ng of one or all five NTF3 TALE expression plasmids (60 ng each) were transfected with 0.8 μ L Lipofectamine 2000 in 275 μ L DMEM-FBS without antibiotics and harvested 48 hours later. Gene expression levels of harvested cells were measured by qRT-PCR and are normalized to GAPDH expression levels. Incubation times for TALE activators by plasmid transfection and protein delivery were those found to give maximal increases in NTF3 mRNA levels. **(c)** Time course of TALE activation for protein delivery and plasmid transfection by measuring NTF3 mRNA levels and then normalizing each method to the highest activation value achieved over any time point for that method. Optimal protein (25–50 nM) and lipid dosage (1.5 μ L RNAiMAX) was used for each delivery technique. Error bars reflect s.d. from three biological replicates performed on different days.

**Figure 4.**

Delivery of Cas9:sgRNA, Cas9 D10A nickase, and dCas9-VP64 transcriptional activators to cultured human cells. **(a)** Green entries: U2OS EGFP reporter cells were treated with 100 nM of the Cas9 protein variant shown, 0.8 μ L of the cationic lipid shown, and either 50 nM of the sgRNA shown for Cas9 protein treatment, or 125 nM of the sgRNA shown for (+36)dGFP-NLS-Cas9 and (-30)dGFP-NLS-Cas9 treatment. The fraction of cells lacking EGFP expression was measured by flow cytometry. Blue entries: plasmid DNA transfection of 750 ng Cas9 and 250 ng sgRNA expression plasmids using 0.8 μ L Lipofectamine 2000.

(b) T7 endonuclease I (T7EI) assay to measure modification of EGFP from no treatment (lane 1), treatment with EGFP-targeting sgRNA alone (lane 2), Cas9 protein alone (lane 3), Cas9 protein + VEGF-targeting sgRNA + RNAiMAX (lane 4), DNA transfection of plasmids expressing Cas9 and EGFP-targeting sgRNA (lane 5), or Cas9 protein + EGFP-targeting sgRNA + RNAiMAX (lane 6). **(c)** T7EI assay of simultaneous genome modification at *EGFP* and three endogenous genes in U2OS cells 48 hours after a single treatment of 100 nM Cas9 protein, 25 nM of each of the four sgRNAs shown (100 nM total sgRNA), and 0.8 μ L RNAiMAX. **(d)** Delivery of Cas9 D10A nickase and pairs of sgRNAs either by plasmid transfection or by RNAiMAX-mediated protein:sgRNA complex delivery under conditions described in **(a)** with 50 nM *EGFP*-disrupting sgRNAs (25 nM each) for protein delivery, and 250 ng sgRNA-expressing plasmids (125 ng each) for DNA delivery. *EGFP*-disrupting sgRNAs g1 + g5, or g3 + g7, are expected to result in gene disruption, while g5 + g7 target the same strand and are expected to be non-functional. **(e)** Delivery of dCas9-VP64 transcriptional activators that target *NTF3* either by DNA transfection or RNAiMAX-mediated protein delivery. Error bars reflect s.d. from six biological replicates performed on different days.

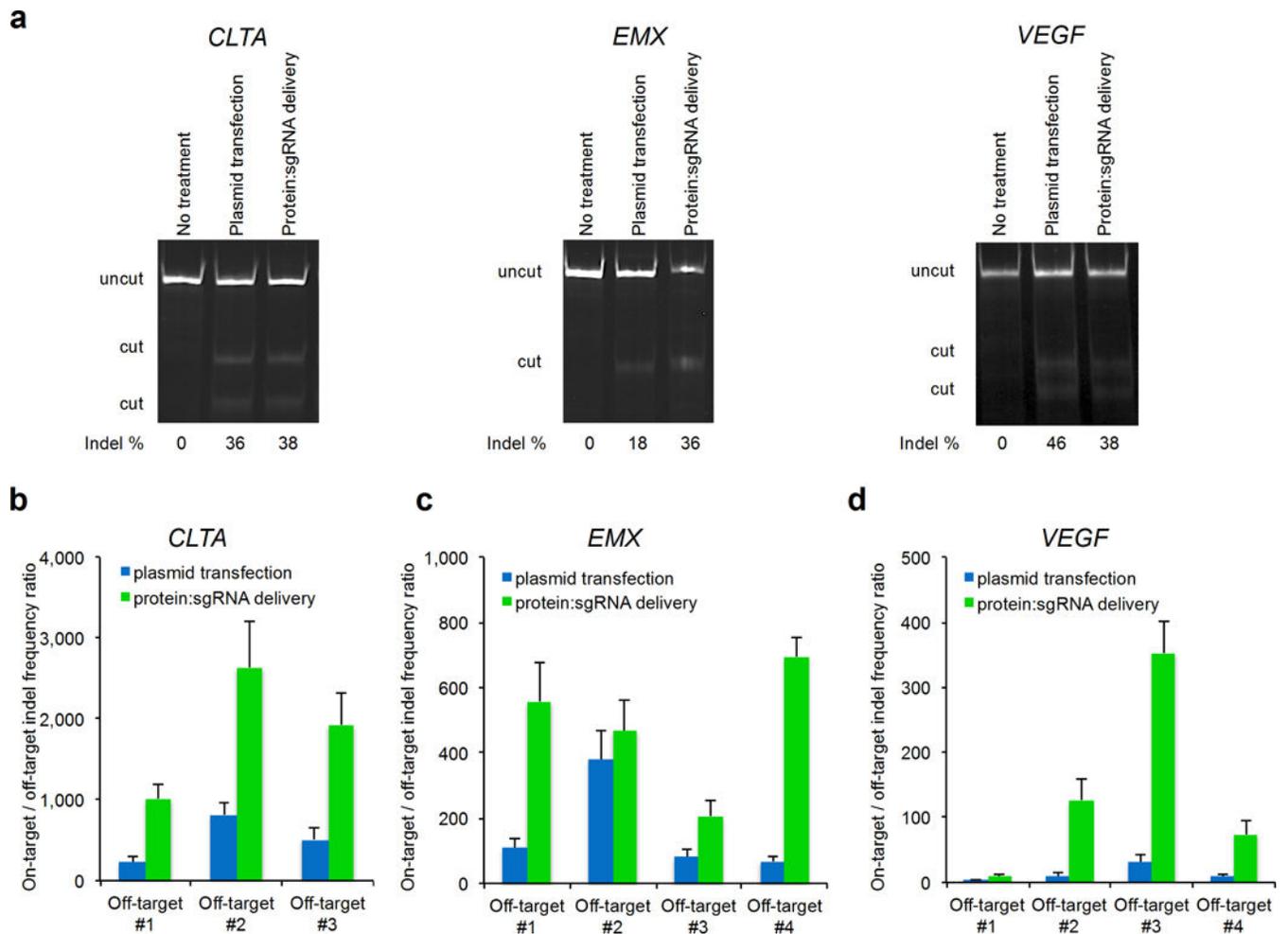


Figure 5.

DNA sequence specificity of Cas9-mediated endogenous gene cleavage in cultured human cells by plasmid transfection or by cationic lipid-mediated protein:sgRNA delivery using 1.6 μ L RNAiMAX complexed with 100 nM Cas9 and 100 nM sgRNA. **(a)** T7EI assay was performed for on-target modification of endogenous *CLTA*, *EMX*, and *VEGF* genes in HEK293T cells. **(b–d)** On-target:off-target DNA modification ratio resulting from Cas9:sgRNA for plasmid transfection or cationic lipid-mediated protein:sgRNA delivery. The conditions for each treatment were adjusted to result in \sim 10% on-target cleavage, enabling a comparison of DNA cleavage specificity between the two delivery methods under conditions in which on-target gene modification efficiencies are similar. *P* values for a single biological replicate are listed in Supplementary Table 2. 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). All protein:sgRNA deliveries and plasmid transfections were performed in 24-well format using 1.6 μ L RNAiMAX in 550 μ L DMEM-FBS without antibiotics. Error bars reflect s.d. from three biological replicates performed on different days.

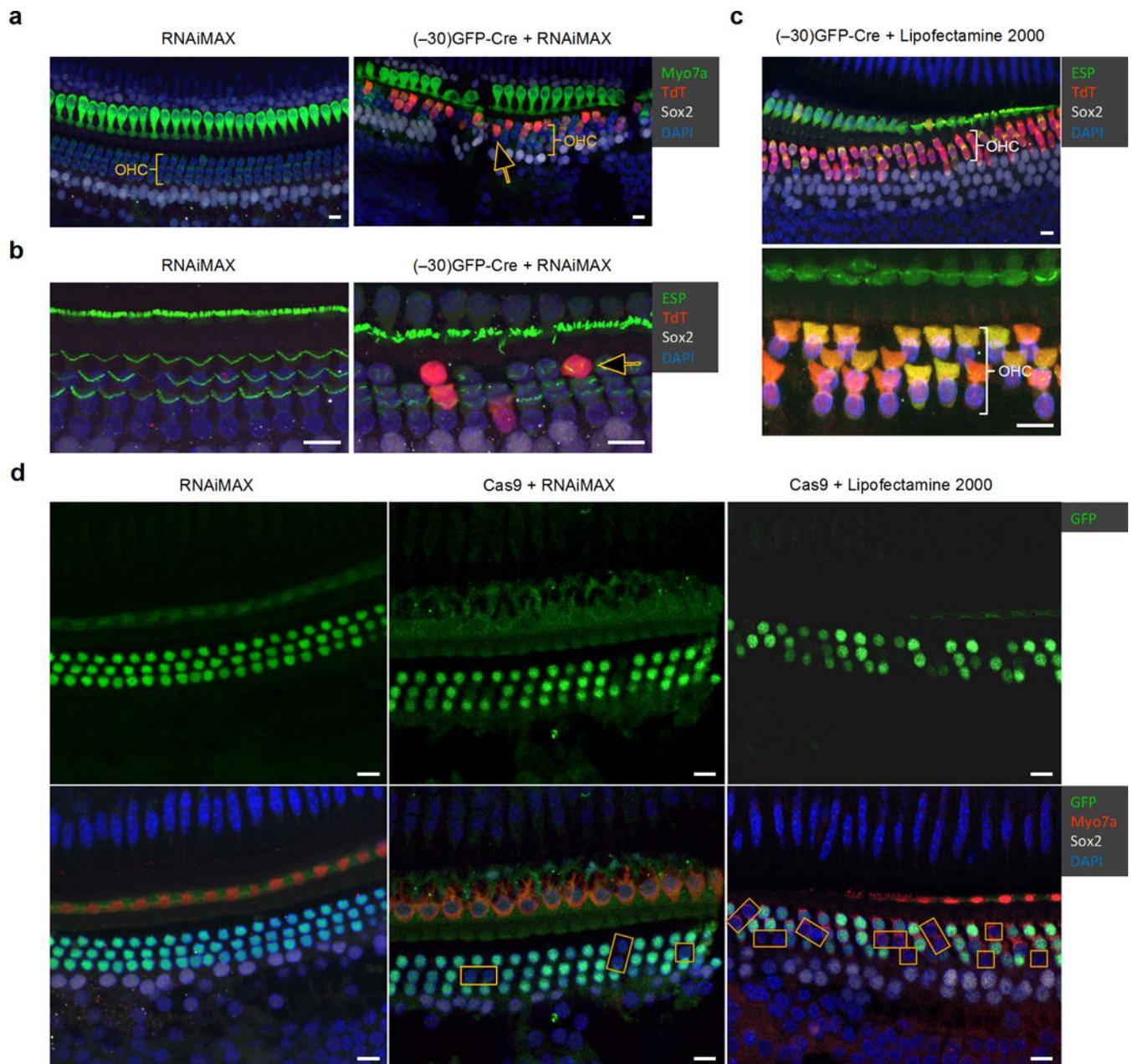


Figure 6.

In vivo delivery of Cre recombinase and Cas9:sgRNA complexes to hair cells in the mouse inner ear. (a) The scala media (cochlear duct) of P0 floxP-tdTomato mice ($n = 4$) were injected with 0.3 μ L of 23 μ M (-30)GFP-Cre in 50% RNAiMAX or with RNAiMAX alone (control). After 5 days, tdTomato expression indicative of Cre-mediated recombination was visualized using immunohistology. Red = tdTomato; green = Myo7a; white = Sox2; blue = DAPI. Yellow brackets indicate the outer hair cell (OHC) region. (b) Ten days after (-30)GFP-Cre delivery, intact espin (Esp)-expressing stereocilia of tdTomato-positive outer hair cells were present (arrow), similar to stereocilia in control cochlea. Red = tdTomato; green = Esp; white = Sox2; blue = DAPI. (c) Identical to (a) except using Lipofectamine

2000 instead of RNAiMAX. ($n = 4$). The upper and lower panels are images of mice cochlea at low and high magnification, respectively, detailing the efficiency of delivery and the effect on cochlear architecture and hair cell loss. **(d)** The scala media of P2 Atoh1-GFP mice ($n = 3$) were injected with 0.3 μL of 33 μM Cas9, 16.5 μM EGFP sgRNA in 50% RNAiMAX or Lipofectamine 2000 commercial solutions. Cas9-mediated gene disruption results in the loss of GFP expression when visualized 10 days later. The upper panels show GFP signal only, while lower panels include additional immunohistological markers. Yellow boxes in the lower panels highlight hair cells that have lost GFP expression. No obvious OHC loss was observed in the Cas9 + RNAiMAX or Cas9 + Lipofectamine 2000 groups. Red = tdTomato; green = Myo7a; white/light blue = Sox2; blue = DAPI. All scale bars (white) are 10 μm .