ADVANCED MATERIALS

Supporting Information

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All-In-One Dendrimer-Based Lipid Nanoparticles Enable Precise HDR-Mediated Gene Editing In Vivo

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Materials

A) Nucleic acids and other reagents for biological assays. Cas9 messenger RNA (CleanCap Cas9 mRNA – (L-7607)), and Luciferase mRNA were purchased from TriLink BioTechnologies. End modified sgRNA was purchased from Synthego (2'-O-methyl at 3 first and last bases, and 3' phosphorothioate bonds between first 3 and last 2 bases.). DNA oligonucleotides (ssDNA HDR template (Alt-R), sequencing and reference primers, sgRNA primer) were purchased from Integrated DNA Technologies. The Ribogreen reagent was purchased from Life Technologies. One-Glo + Tox was purchased from Promega. CellMask Orange Plasma membrane Stain was purchased from Thermo Fisher Scientific. BFP dest clone (plasmid #71825) was obtained from Addgene and BFP/GFP HEK293 cells were obtained from the laboratory of Professor Jacob Corn (ETH Zurich). Collagenase I, DNAse I, and Hyaluronidase were purchased from Sigma-Aldrich. 10X RBC lysis buffer and cell staining buffer were purchased from Biolegend. QIAquick Gel Extraction Kit and QIAquick PCR Purification Kit were purchased from Qiagen. PureLink Genomic DNA Mini Kit was purchased from Thermo Fisher Scientific. PCR reagents DreamTaq Green PCR Master Mix (2X) and Phusion High-Fidelity PCR Master Mix were purchased from Thermo Fisher Scientific. T7 Endonuclease I was puchased from New England Biolabs.

Lipofectamine 2000 and RNAiMAX reagents were purchased from Thermo Fisher Scientific. d-Luciferin Firefly, sodium salt monohydrate was ordered from Goldbio. Ghost Dye Red 780 was purchased from Tonbo Biosciences.

B) Lipids for dLNPs. Cholesterol was purchased from Sigma Aldrich, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was purchased from Avanti Polar Lipids. DMG-PEG2000 was purchased from NOF America Corporation (Sunbright GM-020). All dendrimer lipids were synthesized according to our previously reported protocols.[1]

C) Cell culture. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Thermo Fisher Scientific containing high glucose, sodium pyruvate, L-glutamine, and phenol red. RPMI-1640 (ATCC modified) was purchased from Thermo Fisher Scientific and contained L-glutamine, HEPES, phenol red, sodium pyruvate, high glucose, and low sodium bicarbonate. Penicillin-Streptomycin (10,000 U/mL) was purchased from Fisher Scientific. Dulbecco's modified phosphate buffered saline (PBS), Trypsin-EDTA (0.25%) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich. Mutated HEK293 and WT HEK293 cells were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin. IGROV1 cells were cultured in RPMI-1640 with 10% FBS and 1% penicillin-streptomycin.

D) Animal studies. All experiments were approved by the Institutional Animal Care & Use Committee (IACUC) of The University of Texas Southwestern Medical Center and were consistent with local, state and federal regulations as applicable. Corning Matrigel Membrane Matrix was purchased from Fisher Scientific. Athymic nude Foxn1^{nu} mice were purchased from Envigo.

Methods

Instrumentation

a) Flow cytometry. Flow cytometry for analysis of BFP/GFP signal was performed using BD LSRFortessa machine and HEK293 cell sorting was performed on BD FACSAria^{T} Fusion machine (BD Biosciences). Flow cytometry data was analyzed using FlowJo v10.6.1.

b) Confocal laser scanning microscopy. A Zeiss LSM-700 confocal laser scanning microscope was used to imaged treated cells as well as tumor tissue sections. Images were processed using ImageJ (NIH) and Zen 2.6 Blue Edition (Zeiss).

c) Nanoparticle size and polydispersity. Dynamic light scattering (DLS) was performed to assess nanoparticle size and polydispersity using a Malvern Zetasizer Nano ZS (He-Ne laser, $\lambda = 632$ nm).

d) Tissue sectioning. Tumors were sectioned using a Leica CM1900 Cryostat.

e) *Ex vivo* animal imaging. All *ex vivo* imaging was performed using Perkin Elmer IVIS Lumina system and images were processed using Living Image analysis software (Perkin Elmer).

f) *In vitro* luminescence and fluorescence assays. Luminescence assays and fluorescence assays were performed using a Tecan Infinite M200 Pro plate reader.

g) Sanger DNA sequencing. All DNA sequencing was done through the Eugene McDermott Center for Human Growth and Development.

h) PCR. PCR (Polymerase Chain Reaction) was performed using SimpliAmp Thermocycler from AppliedBiosystems (Thermo Fisher Scientific).

Nucleic Acid Sequences

Modified sgRNA

5’ – GCTGAAGCACTGCACGCCAT – 3’

Scrambled sgRNA (SCsgRNA)

5‘ – CAGCATCTTATCTGAGTGGA - 3 ‘

DNA sgRNA Recognition Sequence

5’ – ATGGCGTGCAGTGCTTCAGC – 3’

sgRNA Primer

5’ – TAATACGACTCACTATAGGGGCTGAAGCACTGCACGCCATGTTTTAGAGCTAGAAATAGC – 3’

Cas9 mRNA

5‘ -

AUGGCCCCCAAGAAGAAGCGGAAGGUGGGCAUCCACGGCGUGCCCGCCGCCGACAAGAAGU
ACAGCAUCGGCCUGGACAUCGGCACCAACAGCGUGGGCUGGGCCGUGAUCACCGACGAGUA
CAAGGUGCCCAGCAAGAAGUUCAAGGUGCUGGGCAACACCGACCGGCACAGCAUCAAGAAG
AACCUGAUCGGCGCCCUGCUGUUCGACAGCGGCGAGACCGCCGAGGCCACCCGGCUGAAGCG
GACCGCCCGGCGGCGGUACACCCGGCGGAAGAACCGGAUCUGCUACCUGCAGGAGAUCUUCA
GCAACGAGAUGGCCAAGGUGGACGACAGCUUCUUCCACCGGCUGGAGGAGAGCUUCCUGGU
GGAGGAGGACAAGAAGCACGAGCGGCACCCCAUCUUCGGCAACAUCGUGGACGAGGUGGCC
UACCACGAGAAGUACCCCACCAUCUACCACCUGCGGAAGAAGCUGGUGGACAGCACCGACAA
GGCCGACCUGCGGCUGAUCUACCUGGCCCUGGCCCACAUGAUCAAGUUCCGGGGCCACUUCC
UGAUCGAGGGCGACCUGAACCCCGACAACAGCGACGUGGACAAGCUGUUCAUCCAGCUGGU
GCAGACCUACAACCAGCUGUUCGAGGAGAACCCCAUCAACGCCAGCGGCGUGGACGCCAAGG
CCAUCCUGAGCGCCCGGCUGAGCAAGAGCCGGCGGCUGGAGAACCUGAUCGCCCAGCUGCCC
GGCGAGAAGAAGAACGGCCUGUUCGGCAACCUGAUCGCCCUGAGCCUGGGCCUGACCCCCAA
CUUCAAGAGCAACUUCGACCUGGCCGAGGACGCCAAGCUGCAGCUGAGCAAGGACACCUAC
GACGACGACCUGGACAACCUGCUGGCCCAGAUCGGCGACCAGUACGCCGACCUGUUCCUGGC
CGCCAAGAACCUGAGCGACGCCAUCCUGCUGAGCGACAUCCUGCGGGUGAACACCGAGAUCA
CCAAGGCCCCCCUGAGCGCCAGCAUGAUCAAGCGGUACGACGAGCACCACCAGGACCUGACC
CUGCUGAAGGCCCUGGUGCGGCAGCAGCUGCCCGAGAAGUACAAGGAGAUCUUCUUCGACC
AGAGCAAGAACGGCUACGCCGGCUACAUCGACGGCGGCGCCAGCCAGGAGGAGUUCUACAA
GUUCAUCAAGCCCAUCCUGGAGAAGAUGGACGGCACCGAGGAGCUGCUGGUGAAGCUGAAC
CGGGAGGACCUGCUGCGGAAGCAGCGGACCUUCGACAACGGCAGCAUCCCCCACCAGAUCCA
CCUGGGCGAGCUGCACGCCAUCCUGCGGCGGCAGGAGGACUUCUACCCCUUCCUGAAGGACA
ACCGGGAGAAGAUCGAGAAGAUCCUGACCUUCCGGAUCCCCUACUACGUGGGCCCCCUGGCC

CGGGGCAACAGCCGGUUCGCCUGGAUGACCCGGAAGAGCGAGGAGACCAUCACCCCCUGGA
ACUUCGAGGAGGUGGUGGACAAGGGCGCCAGCGCCCAGAGCUUCAUCGAGCGGAUGACCAA
CUUCGACAAGAACCUGCCCAACGAGAAGGUGCUGCCCAAGCACAGCCUGCUGUACGAGUAC
UUCACCGUGUACAACGAGCUGACCAAGGUGAAGUACGUGACCGAGGGCAUGCGGAAGCCCG
CCUUCCUGAGCGGCGAGCAGAAGAAGGCCAUCGUGGACCUGCUGUUCAAGACCAACCGGAA
GGUGACCGUGAAGCAGCUGAAGGAGGACUACUUCAAGAAGAUCGAGUGCUUCGACAGCGUG
GAGAUCAGCGGCGUGGAGGACCGGUUCAACGCCAGCCUGGGCACCUACCACGACCUGCUGA
AGAUCAUCAAGGACAAGGACUUCCUGGACAACGAGGAGAACGAGGACAUCCUGGAGGACAU
CGUGCUGACCCUGACCCUGUUCGAGGACCGGGAGAUGAUCGAGGAGCGGCUGAAGACCUAC
GCCCACCUGUUCGACGACAAGGUGAUGAAGCAGCUGAAGCGGCGGCGGUACACCGGCUGGG
GCCGGCUGAGCCGGAAGCUGAUCAACGGCAUCCGGGACAAGCAGAGCGGCAAGACCAUCCU
GGACUUCCUGAAGAGCGACGGCUUCGCCAACCGGAACUUCAUGCAGCUGAUCCACGACGAC
AGCCUGACCUUCAAGGAGGACAUCCAGAAGGCCCAGGUGAGCGGCCAGGGCGACAGCCUGC
ACGAGCACAUCGCCAACCUGGCCGGCAGCCCCGCCAUCAAGAAGGGCAUCCUGCAGACCGUG
AAGGUGGUGGACGAGCUGGUGAAGGUGAUGGGCCGGCACAAGCCCGAGAACAUCGUGAUCG
AGAUGGCCCGGGAGAACCAGACCACCCAGAAGGGCCAGAAGAACAGCCGGGAGCGGAUGAA
GCGGAUCGAGGAGGGCAUCAAGGAGCUGGGCAGCCAGAUCCUGAAGGAGCACCCCGUGGAG
AACACCCAGCUGCAGAACGAGAAGCUGUACCUGUACUACCUGCAGAACGGCCGGGACAUGU
ACGUGGACCAGGAGCUGGACAUCAACCGGCUGAGCGACUACGACGUGGACCACAUCGUGCC
CCAGAGCUUCCUGAAGGACGACAGCAUCGACAACAAGGUGCUGACCCGGAGCGACAAGAAC
CGGGGCAAGAGCGACAACGUGCCCAGCGAGGAGGUGGUGAAGAAGAUGAAGAACUACUGGC
GGCAGCUGCUGAACGCCAAGCUGAUCACCCAGCGGAAGUUCGACAACCUGACCAAGGCCGA
GCGGGGCGGCCUGAGCGAGCUGGACAAGGCCGGCUUCAUCAAGCGGCAGCUGGUGGAGACC
CGGCAGAUCACCAAGCACGUGGCCCAGAUCCUGGACAGCCGGAUGAACACCAAGUACGACG
AGAACGACAAGCUGAUCCGGGAGGUGAAGGUGAUCACCCUGAAGAGCAAGCUGGUGAGCGA
CUUCCGGAAGGACUUCCAGUUCUACAAGGUGCGGGAGAUCAACAACUACCACCACGCCCACG
ACGCCUACCUGAACGCCGUGGUGGGCACCGCCCUGAUCAAGAAGUACCCCAAGCUGGAGAGC
GAGUUCGUGUACGGCGACUACAAGGUGUACGACGUGCGGAAGAUGAUCGCCAAGAGCGAGC
AGGAGAUCGGCAAGGCCACCGCCAAGUACUUCUUCUACAGCAACAUCAUGAACUUCUUCAA
GACCGAGAUCACCCUGGCCAACGGCGAGAUCCGGAAGCGGCCCCUGAUCGAGACCAACGGCG
AGACCGGCGAGAUCGUGUGGGACAAGGGCCGGGACUUCGCCACCGUGCGGAAGGUGCUGAG
CAUGCCCCAGGUGAACAUCGUGAAGAAGACCGAGGUGCAGACCGGCGGCUUCAGCAAGGAG
AGCAUCCUGCCCAAGCGGAACAGCGACAAGCUGAUCGCCCGGAAGAAGGACUGGGACCCCA
AGAAGUACGGCGGCUUCGACAGCCCCACCGUGGCCUACAGCGUGCUGGUGGUGGCCAAGGU
GGAGAAGGGCAAGAGCAAGAAGCUGAAGAGCGUGAAGGAGCUGCUGGGCAUCACCAUCAUG
GAGCGGAGCAGCUUCGAGAAGAACCCCAUCGACUUCCUGGAGGCCAAGGGCUACAAGGAGG
UGAAGAAGGACCUGAUCAUCAAGCUGCCCAAGUACAGCCUGUUCGAGCUGGAGAACGGCCG

GAAGCGGAUGCUGGCCAGCGCCGGCGAGCUGCAGAAGGGCAACGAGCUGGCCCUGCCCAGC
AAGUACGUGAACUUCCUGUACCUGGCCAGCCACUACGAGAAGCUGAAGGGCAGCCCCGAGG
ACAACGAGCAGAAGCAGCUGUUCGUGGAGCAGCACAAGCACUACCUGGACGAGAUCAUCGA
GCAGAUCAGCGAGUUCAGCAAGCGGGUGAUCCUGGCCGACGCCAACCUGGACAAGGUGCUG
AGCGCCUACAACAAGCACCGGGACAAGCCCAUCCGGGAGCAGGCCGAGAACAUCAUCCACCU
GUUCACCCUGACCAACCUGGGCGCCCCCGCCGCCUUCAAGUACUUCGACACCACCAUCGACC
GGAAGCGGUACACCAGCACCAAGGAGGUGCUGGACGCCACCCUGAUCCACCAGAGCAUCACC
GGCCUGUACGAGACCCGGAUCGACCUGAGCCAGCUGGGCGGCGACAGCGGCGGCAAGCGGCC
CGCCGCCACCAAGAAGGCCGGCCAGGCCAAGAAGAAGAAGGGCAGCUACCCCUACGACGUGC
CCGACUACGCCUGA - 3‘

Scrambled ssDNA

5‘ –
CCAGGCCTCTGATTCCTCACTGATTGCTCTTAGGTCTGGCCCCTCCTCAGCATCTTATCCGCGTT
GAAGGAAATTTGCGTGTGGAGTATTTGGATGACAGAAACACTTTTCGGCATAGTG – 3‘

ssDNA HDR Template

5’–
GCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGG
CCCACCCTCGTGACCACCCTGACGTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGA
– 3’

Sequencing Primers

B/GFP Fwd 1

5’ – AGCTGGCTAGGTAAGCTTGG – 3’

B/GFP Fwd 2

5’ – TGGGTGGAGACTGAAGTTAGGC – 3’

B/GFP Rev 1

5’ – CTTGTACAGCTCGTCCATGC – 3’

B/GFP Rev 2

5’ – GGGTGCTCAGGTAGTGGTT – 3’

Reference Sequence Primers (TIDER)

B/GFP Ref Fwd

5’ – CCCTGACGTACGGCGTG – 3’

B/GFP Ref Rev

5’ – CACGCCGTACGTCAGGG – 3’

Experimental Details

dLNP component mole ratio screen. dLNPs were prepared via the ethanol dilution method using a Box-Behnken experimental design with five different variables for analysis: Mole ratio of dendrimer:nucleic acid (2000, 4000, 6000); intra-particle dendrimer (30, 50, 70); cholesterol (20, 40, 60); DOPE (20, 40, 60); DMG-PEG2K (0.5, 1.5, 2.5). IGROV1 cells were transfected with the dLNPs and luminescence values were determined as described in the luciferase mRNA delivery assay protocol listed below.

In vitro dLNP formulation. dLNPs were prepared via the ethanol dilution method. Luciferase mRNA was diluted in acidic aqueous buffer (0.01M citric acid/sodium citrate buffer, pH 3). Stock solutions of each lipid component at specific molar concentrations were created via dilution in ethanol. These stock solutions were then combined together at the molar ratio of 38.5:30:30:1.5 (dendrimer:cholesterol:DOPE:DMG-PEG). This lipid mixture was added to the mRNA solution at a volumetric ratio of 1:3 (lipid mixture:mRNA solution) and mixed rapidly using a micropipette. Following mixing, the dLNPs were allowed to incubate at room temperature (RT) for 15-20 minutes and then either diluted in (by volume, 3X) or dialyzed against 1X Dulbecco’s Modified PBS without calcium and magnesium (Sigma-Aldrich). If dialyzed, a 2 L solution of 1X PBS was created via dilution of 10X PBS (Sigma-Aldrich) in deionized and autoclaved H_2O and dLNPs were loaded into Pur-A-Lyzer Midi dialysis chambers (Sigma-Aldrich). The loaded dLNPs were then dialyzed in the 1X PBS for a duration of 1 hour per 200 μL of sample in the chamber.

Nucleic acid binding experiments. Nucleic acid binding was determined using the Quant-iT Ribogreen assay (Fisher Scientific). dLNPs were first prepared using the *in vitro* or *in vivo* dLNP preparation method. These dLNPs were added to a 96-well black opaque polystyrene microplate (Corning – Fisher Scientific) at a volume corresponding to the treatment dosage. A standard curve of the appropriate nucleic acid was prepared in the same medium as the dLNPs for consistency.

The Ribogreen reagent was diluted 1000-fold in 1X PBS and added to each well at a volume of 50 μL using a multichannel micropipette. Using an orbital shaker, the dLNPs and Ribogreen reagent mixture were shaken for 5 minutes at RT. Each well was then measured for fluorescence (λ_{Ex} 485 nm, λ_{Em} 535 nm) and the amount of free mRNA was assessed once fitted to the standard curve. Free mRNA was used to determine encapsulated mRNA percentage via the following formula: Fraction of bound nucleic acid = [(total nucleic acid added – free nucleic acid)/(total nucleic acid added)] (N = 4 +/- standard deviation in all cases).

dLNP physical property characterization. dLNP properties were determined using a Zetasizer Nano ZS (Malvern) with a helium-neon laser (λ = 632 nm). Size and polydispersity of the dLNPs were measured using dynamic light scattering (DLS) by 173º back scattering with the following settings: 5 measurements, 3 runs at 10 seconds per run, and attenuation set to automatic. The refraction index was adjusted appropriately for each sample to account for any possible viscosity differences between dLNP formulations as indicated via Malvern guidance.

Luciferase mRNA delivery assay. dLNPs were made with Luciferase mRNA (Tri-Link Biotechnologies) using the ethanol dilution method described above. IGROV1 cells (for molar ratio optimization, other cell lines were used for the dendrimer library screen as described above) were seeded in a white opaque 96 well microplate (Corning – Fisher Scientific) at a density of 4 x 10^3 cells per well in 100 μL of RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin and allowed to adhere to the well overnight at 37 ºC. Following overnight incubation, the dLNPs were prepared containing Luciferase mRNA and added to each well using a multichannel micropipette at a dose of 50 ng of mRNA/well. 24 hours after the addition of dLNPs, 100 μL of additional RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin was then added to each well via a multichannel micropipette. The cells were then incubated at 37 ºC for 24 additional hours resulting in a total transfection time of 48 hours. After the 48-hour incubation period, cell viability and luciferase expression were assessed using the ONE-Glo + Tox Assay (Promega) according to the manufacturer protocol and normalized to viability (N = 4 +/- standard deviation).

Dendrimer library screen. The dendrimer library analyzed consisted of the amine cores 3A3, 3A5, 4A3, and 4A1, as well as the alkyl periphery groups SC5, SC6, SC7, SC8, SC9, SC10, SC11, SC12, and SC14; totaling 36 distinct dendrimer compounds. Each of these compounds were used to transfect three cell lines (IGROV1, HEK293T, and HeLa). All cells were seeded in white opaque

96 well microplates at different densities in 100 μL of their respective media (IGROV1: 4 x 10^3 cells/well – RPMI1640 10% FBS 1% penicillin/streptomycin, HEK293T 1 x 10^4 cells/well – DMEM 10% FBS 1% penicillin/streptomycin, HeLa: 4 x 10^3 cells/well – DMEM 10% FBS 1% penicillin/streptomycin) and incubated at 37 ºC overnight so that they could adhere to the wells. Each of the dendrimer lipids were combined with ethanol to form a 10 mM working stock solution. These stock solutions were then combined with cholesterol, DOPE, and DMG-PEG at a molar ratio of 38.5:30:30:1.5 (dendrimer:cholesterol:DOPE:DMG-PEG), wherein each dendrimer was fixed at a molar ratio of 10,000:1 (dendrimer:mRNA). Each dLNP mixture was then combined with luciferase mRNA in acidic aqueous buffer (as previously described) at a ratio of 1:3 (lipid mixture in ethanol:mRNA in acidic aqueous buffer) by volume and rapidly mixed using a micropipette. Once mixed, the dLNPs were allowed to incubate at RT for 15 minutes and then diluted threefold by volume in 1X PBS. After overnight incubation, all cells were transfected with the following dLNPs containing 12.5 ng luciferase mRNA and then 100 μL of additional media was added to each well using a multichannel micropipette. Cells were incubated for 48 hours at 37 ºC following transfection and then analyzed using a microplate reader for luciferase expression and toxicity. Luciferase expression and toxicity were evaluated using the ONE-Glo + Tox Assay (Promega) according to the manufacturer protocol and normalized to background and viability (N = 4 +/- standard deviation).

Dose response. Selected dendrimer compounds with the 4A3 amine core and alkyl peripheries SC5, SC6, SC7, SC8, SC9, SC10, SC11, SC12, and SC14 were examined in a dose-response fashion for efficacy and toxicity. dLNPs containing luciferase mRNA were created via the ethanol dilution method (molar ratio of 38.5:30:30:1.5, dendrimer:cholesterol:DOPE:DMG-PEG) and administered to three different cell lines (IGROV1, HEK293T, HeLa) in the same fashion as the dendrimer library screen, but at 4 different doses (6.25 ng, 12.5 ng, 25 ng, 50 ng). Luciferase expression and toxicity were determined via the ONE-Glo + Tox Assay (Promega) (N = 3 +/- standard deviation).

sgRNA sequence validation. The sgRNA sequence was validated using an *in vitro* cutting assay. Briefly, 300 ng of linearized pDNA (BFP dest clone, addgene) was mixed with 160 ng of Cas9 protein and 33 ng of IVT sgRNA RNPs in 1X NE buffer 3.1 (final volume: 20 μL). Then, the mixture was incubated at 37 ºC for 2 hours. The cleavage was detected by agarose electrophoresis (2% agarose gel).

dLNP multi-encapsulation of HDR components. HEK293 B/GFP cells were plated in a 12 well tissue culture dish (CytoOne) at a density of 1.5 x 10^5 cells per well in 500 μL of DMEM containing 10% FBS and 1% penicillin/streptomycin and allowed to attach overnight at 37 º C. After overnight incubation, 4A3-SC8 dLNPs (38.5:30:30:1.5; 4A3-SC8:cholesterol:DOPE:DMG-PEG) were made using the ethanol dilution method, however multiple components were added into the nucleic acid mixture for encapsulation. In the first round of evaluation, a two particle, sequential delivery system was implemented with the first dLNPs containing only Cas9 mRNA (500 ng) and the other dLNPs containing one of three different cargoes: modified sgRNA only (250 ng), both ssDNA HDR template and modified sgRNA (fixed at a 1:1 ratio by weight, 250 ng each), or both ssDNA HDR template and modified sgRNA (fixed at a 1:1 ratio by moles, 500 ng total nucleic acid). The dLNPs containing Cas9 mRNA were administered to the cells at a dose of 500 ng per well following overnight incubation. 24 hours after transfection with dLNPs containing Cas9 mRNA, the second dLNPs were administered to the cells containing one of three different cargoes and cells were incubated at 37 ºC for 48 hours. 24 hours after transfection with the second round of dLNPs, 1 mL of DMEM was added to each well. Following the 48-hour incubation, cells were prepared for analysis via flow cytometry. Briefly, all media in each well was aspirated and each well was then rinsed with 1 mL of 1X PBS. 1X PBS was aspirated and 500 μL of Trypsin-EDTA was added to each well and the cells were incubated at 37 ºC for 2 minutes. After incubation, 1 mL of DMEM was added to each well and the contents of all wells were collected in 1.5 mL tubes (Eppendorf) and spun at 300 G for 4 minutes to generate a cell pellet. The cell pellet was then resuspended in 1 mL of 1X PBS and placed on ice until analyzed via flow cytometry. In the second round of evaluation, a simultaneous delivery system consisting of a three, two, or a single particle was evaluated. HEK293 B/GFP cells were seeded in a 12 well plate using the same method as described above. In the three-particle system, 4A3-SC8 dLNPs were created using the ethanol dilution method and contained a single nucleic cargo each (500 ng Cas9 mRNA, 250 ng modified sgRNA, 250 ng ssDNA HDR template). In the two-particle system, one set of 4A3-SC8 dLNPs were created containing 500ng of Cas9 mRNA, and the other set of 4A3-SC8 dLNPs contained a 1:1 ratio of ssDNA HDR Template and modified sgRNA (500 ng total nucleic acid). Finally, in the single particle system, 4A3-SC8 dLNPs were created that contained all three nucleic acid cargoes fixed by weight at a ratio of 2:1:1 (Cas9 mRNA:modified sgRNA:ssDNA HDR template, 1000 ng total nucleic acid). Each of the respective particle mixtures were added to the cells simultaneously and the same protocol listed above was followed for media addition and incubation time (48 hours). Following incubation, cells were prepared for analysis via flow cytometry as described above.

In vitro HDR titer. HEK293 B/GFP cells were plated in a 12 well tissue culture dish (CytoOne) at a density of 1.5 x 10^5 cells per well in 500 μL of DMEM containing 10% FBS and 1% penicillin/streptomycin and allowed to attach overnight at 37 ºC. After overnight incubation, 4A3-SC8 dLNPs were generated using the ethanol dilution method and contained a mixture of three nucleic acids (Cas9 mRNA, modified sgRNA, ssDNA HDR template) and an internal molar ratio of components fixed at 38.5:30:30:1.5 of 4A3-SC8:cholesterol:DOPE:DMG-PEG. The molar ratio of 4A3-SC8 to nucleic acid was fixed at 2500:1. In all formulations, Cas9 mRNA and modified sgRNA were fixed at one of the following ratios by weight: 1:1, 1:2, or 2:1, wherein 1 signifies 250 ng of that particular nucleic acid. Once the ratio between those two components was fixed, the amount of ssDNA HDR template was added into each individual RNA mixture at one of the following ratios: 0.5, 1, 2, 3, 4, 6, 8, 10. The dLNPs were then administered to the cells using a micropipette. 24 hours after transfection, 1 mL of DMEM was added to each well. Following the 48-hour incubation, cells were prepared for analysis via flow cytometry as described above (N = 3 +/- standard deviation).

In vitro genomic DNA sequencing. For all cells, gDNA was extracted 48h post transfection with HDR dLNPs as follows. Briefly, cells were trypsinized at 37 °C for 3 min wherein 500 mL of DMEM was then added to neutralize the trypsin. Cells were then collected in 1.5 mL DNA LoBinid tubes (Eppendorf) and centrifuged at 300 G for 5 min to form a cell pellet. This pellet was then resuspended in cell digestion media. The digestion media consists of 2 uL proteinase K (Ambion, 20 mg/mL), 10 uL of passive cell lysis buffer (Promega), and 40 uL of DEPC treated H_2O (Ambion) per sample. 50 uL of this cell lysis buffer was then added to each cell pellet and the pellet was resuspended using a micropipette. The mixtures were then transferred to 0.5 mL PCR tubes (Fisherbrand) and run on the following PCR cycle: 1X(65 °C - 20 min; 95 °C – 10 min; 4 °C – hold). The extracted gDNA was then amplified via PCR using the following protocol: 40 uM working solutions of primers B/GFP Fwd 1 and B/GFP Rev 2 were prepared and combined at equal volumes to create a 20 uM mix of both primers. The PCR mixtures for each reaction consisted of 2 uL extracted gDNA from lysed cells, 25 uL DreamTaq Green PCR Master Mix (2X), 1 uL of 20 uM B/GFP Fwd + B/GFP Rev primer mix, and 22 uL DEPC treated H_2O. Each mixture was run at the following cycle conditions: (1X (95 °C - 5 min) 35X (95 °C - 30 sec; 61 °C - 30 sec; 72 °C - 1 min) 1X (72 °C - 7 min) 1X (4 °C - hold). Following this, PCR purification was performed according to the manufacturer protocol (Qiagen). All samples were then run in a 2% agarose gel at 130 V for 30 min – 1h, verified for correct size, and extracted using Qiagen Gel Purification kit according to the manufacturer's protocol. Purified samples were then submitted for sequencing via

Sanger DNA sequencing utilizing a mixture of 6 uL of PCR-amplified DNA at 8.333 ng/uL and 6 uL of 1 uM stock of primer B/GFP Rev 2.

Xenograft HEK293 B/GFP tumor formation. A suspension HEK293 B/GFP cells were resuspended in 1X PBS at a concentration of 1 x 10^6 cells per 50 μL and combined with Corning Matrigel Membrane Matrix at a 1:1 volumetric ratio. 100 μL of the mixture was then injected subcutaneously into the right hind leg of athymic nude Foxn1^{nu} mice using a 29G1/2 insulin syringe (Excelint). Tumors were allowed to grow in size for ~2-3 weeks until measuring 125 mm^3.

In vivo dLNP delivery. 4A3-SC8 dLNPs were created for *in vivo* experiments using the ethanol dilution method and dialyzed against 1X PBS for 2 hours. All dLNPs used for in vivo work were dialyzed against 1X PBS for 2 hours before injection. Molar ratio of internal components was fixed at 38.5:30:30:1.5 of 4A3-SC8:cholesterol:DOPE:DMG-PEG and the mole ratio of 4A3-SC8 to nucleic acid was fixed at 2500:1. The nucleic acid mixture consisted of 1:1:8, 1:1:3, or 2:1:3 fixed ratio by weight of internal components of Cas9 mRNA:modified sgRNA:ssDNA HDR template. 4A3-SC8 dLNPs were injected intratumorally at a dose of 0.5 mg/kg.

Xenograft tumor and organ resection for IVIS imaging. 5 days after injection with HDR dLNPs, the mice were euthanized and the tumor was resected by peeling back the skin. Additionally, the heart, lungs, spleen, and kidneys were resected as well for imaging with IVIS Lumina.

Xenograft tumor preparation for confocal imaging. Resected tumors were placed in a well containing O.C.T. Compound (Tissue-Tek) and then transferred to -80 ºC until ready to section (minimum overnight). Tumors frozen in O.C.T. Compound were then placed in the Cryostat (Leica Biosystems) and sectioned at a thickness varying from 7 μm to 15 μm where they were then mounted to a slide. After mounting, the tissue slices were fixed via incubation at RT for 2 hours with 4% paraformaldehyde solution, washed 3X with 1X PBS, and covered.

10X digestion media. Digestion media for tumors consisted of the following mixture: Collagenase I (450 units/μL, Sigma Aldrich), DNAse I (250 units/μL, Sigma Aldrich) and Hyaluronidase (300 units/μL, Sigma Aldrich) in 1X PBS. The mixture was stored at -20º C.

In vivo tumor genomic DNA sequencing. For all tumors, gDNA was extracted from tumors using PureLink Genomic DNA Mini Kit according to manufacturer protocol. The GFP sequence in tumor

gDNA was then amplified via PCR. Briefly, 40 uM working solutions of the F'1 and R'2 sequencing primers were prepared and added to a mix consisting of 40 ng gDNA, 25 uL DreamTaq Green Master Mix (2X), 1 uL of F'1 and R'2 primers at a 20 uM concentration, and 22 uL of milliQ H_2O. This mix was run at the following PCR cycle: 1X (95 °C - 5 min) 30X (95 °C - 30 sec; 62.5 °C - 30 sec; 72 °C - 1 min) 1X (72 °C - 7 min) 1X (4 °C - hold). Samples were then run in a 2% agarose gel at 130 V for 30 min – 1h, verified for correct size, and extracted using Qiagen Gel Purification kit according to the manufacturer's protocol. Purified samples were then submitted for sequencing via Sanger DNA sequencing utilizing a mixture of 6 uL of PCR-amplified DNA at 8.333 ng/uL and 6 uL of 1 uM stock of primer B/GFP Rev 2.

Reference sequence template generation. A reference sequence was generated using the following protocol: gDNA was extracted from PBS treated tumors and amplified using the above PCR cycle. Following amplification and purification, each end of the sequence was amplified using a combination of the F'1 primer + Rev. reference primer, or Fwd. reference primer + R'2 primer for the first half, and second half of the template, respectively. Each of the two reaction mixtures consisted of 1 uL of 20 uM primer mixture, 25 uL of Phusion High-Fidelity PCR Master Mix (2X), 1 ng of PBS PCR product, and H_2O up to 50 uL total reaction volume. The PCR cycle used to generate each half of the template sequence was as follows: 1X (95 °C - 5 min) 30X (95 °C - 30 sec; 62 °C - 30 sec; 72 °C - 1 min) 1X (72 °C - 7 min) 1X (4 °C – hold). After this amplification, the two sequences were then annealed using the following PCR mixture and cycle: 5 uL of each end of the two reference templates, 25 uL of DreamTaq Green PCR Master Mix (2X), and H_2O up to 50 uL. The PCR cycle was: 1X (95 °C - 5 min) 10X (95 °C - 30 sec; 62 °C - 30 sec; 72 °C - 1 min) 1X (72 °C - 7 min) 1X (4 °C - hold). Finally, with both ends amplified and annealed together, the resulting PCR product was diluted 200X and then amplified using 1 uL of the 200X diluted PCR product, 1 uL of 20 uM F'1+R'2 primer set, 25 uL of DreamTaq Green PCR Master Mix (2X), and H_2O up to 50 uL under the following conditions: 1X (95 °C - 5 min) 30X (95 °C - 30 sec; 66 °C - 30 sec; 72 °C - 1 min) 1X (72 °C - 7 min) 1X (4 °C - hold). The resulting product was run in a 2% agarose gel at 130 V for 1h, purified using the Qiagen Gel Extraction kit, and then sequenced using Sanger DNA Sequencing.

TIDER analysis for HDR via DNA sequencing. The TIDER webtool (https://tide.nki.nl/#about-tider) was used to calculate HDR from Sanger DNA sequencing data. From TIDER website: "TIDER is a modified version of TIDE that estimates the frequency of targeted small nucleotide changes introduced by CRISPR in combination with homology-directed repair using a donor

template. In addition, it determines the spectrum and frequency of non-templated indels. Compared to TIDE, TIDER requires one additional sequencing trace (i.e., three instead of two). Preparation of this third "reference" DNA can be done with a simple two-step PCR protocol. The web tool reports the estimated frequencies of the templated mutation and of all non-templated indels."

Flow cytometry. For detection of unedited cells (BFP+), NHEJ (no fluorescence), and HDR (GFP+), cells were analyzed with BD LSRFortessa machine (BD Biosciences). GFP+, BFP+, and non-fluorescent cells were quantified using FlowJo.
Statistical snalysis. Statistical analysis was performed using a Student's t-test or one-way ANOVA with Tukey's multiple comparisons test or Dunnett's multiple comparisons test in GraphPad Prism.

In vivo Luciferase assay. HEK293 B/GFP cells were used to form tumors in the right hind leg of athymic nude Foxn1^{nu} mice. Once the tumors reached ~125 mm^3 in size, the tumors were injected with 4A3-SC8 dLNPs containing luciferase mRNA at a dose of 0.25 mg/kg. 6 h post intratumoral (IT) injection, the mice were injected intraperitoneally (IP) with d-Luciferin and imaged for luminescence using IVIS. After imaging, the mice were then returned to their housing. 24 h post initial IT injection with dLNPs, the mice were again injected IP with d-Luciferin and imaged using IVIS for luminescence. Immediately following that, whole mouse IVIS imaging, the tumor mass, lung, liver, heart, kidney, and spleen were resected from each mouse for luminescence imaging using IVIS (N = 3).

In vivo PTEN off-target assay. HEK293 B/GFP cells were used to form tumors in the right hind leg of athymic nude Foxn1^{nu} mice. Once the tumors reached ~125 mm^3 in size, the tumors were injected with 4A3-SC8 dLNPs containing a 2:1 weight ratio of Cas9 mRNA:PTEN sgRNA at a dose of 0.25 mg/kg. 5 days after IT injection of 4A3-SC8 dLNPs containing Cas9 mRNA and PTEN sgRNA, the liver, lung, and spleen were resected from the mice for downstream off-target analysis (N = 3).

In vivo T7E1 and DNA sequencing for PTEN off-target editing. The Cas-OFFinder webtool was used to predict likely off-target editing sites for the PTEN sgRNA. gDNA was extracted from the resected lung, liver, and spleen using the PureLink Genomic DNA mini kit according to the manufacturer protocol. Each of the off-target sites in the gDNA were then amplified via PCR. All samples were then run in a 2% agarose gel at 130 V for 30 min – 1h, verified for correct size, and extracted using Qiagen Gel Purification kit according to the manufacturer's protocol. The gel

purified off-target amplicons were then used for the T7E1 assay according to the manufacturer's protocol. Additionally, the gel purified off-target amplicons were submitted for Sanger DNA sequencing (N = 3).

In vitro T7E1 and sequencing for off-target HEK293 B/GFP editing. The Cas-OFFinder webtool was used to predict likely off-target editing sites for the B/GFP sgRNA. For all cells, gDNA was extracted 48h post transfection with HDR dLNPs as follows. Briefly, cells were trypsinized at 37 °C for 3 min wherein 500 mL of DMEM was then added to neutralize the trypsin. Cells were then collected in 1.5 mL DNA LoBinid tubes (Eppendorf) and centrifuged at 300 G for 5 min to form a cell pellet. This pellet was then resuspended in cell digestion media. The digestion media consists of 2 uL proteinase K (Ambion, 20 mg/mL), 10 uL of passive cell lysis buffer (Promega), and 40 uL of DEPC treated H_2O (Ambion) per sample. 50 uL of this cell lysis buffer was then added to each cell pellet and the pellet was resuspended using a micropipette. The mixtures were then transferred to 0.5 mL PCR tubes (Fisherbrand) and run on the following PCR cycle: 1X(65 °C - 20 min; 95 °C – 10 min; 4 °C – hold). The extracted gDNA was then amplified via PCR at each of the predicted off-target sites and gel purified using the Qiagen Gel Purification Kit according to the manufacturer's protocol. Following amplification, the T7E1 assay was performed using the manufacterer's protocol to check of off-target editing. Additionally, the purified samples were submitted for Sanger DNA sequencing (N = 3).

Scramble and commericaly available reagent in vitro HDR assessment. HEK293 B/GFP cells were plated in a 12 well tissue culture dish (CytoOne) at a density of 1.5 x 10^5 cells per well in 500 µL of DMEM containing 10% FBS and 1% penicillin/streptomycin and allowed to attach overnight at 37 ºC. After overnight incubation, Lipofectamine 2000 and RNAiMAX lipoplexes were prepared with a 1:1:3 ratio of sgRNA:Cas9 mRNA:ssDNA according to the manufacturer's protocols. Additionally, 4A3-SC8 dLNPs were created containing the following: a 1:1:3 ratio of Cas9 mRNA:sgRNA:ssDNA; a 1:1:3 ratio of Cas9 mRNA:SCsgRNA:ssDNA; a 1:1:3 ratio of Cas9 mRNA:sgRNA:SCssDNA; a 1:3 ratio of Cas9 mRNA:ssDNA (no sgRNA); and a 1:1 ratio of Cas9 mRNA:sgRNA (no ssDNA). All formulations were then administered to the cells. 24 h after transfection, an additional 1 mL of DMEM was added to the cells. Following the 48 h incubation, the cells were prepared for analysis via flow cytometry as previously described (N = 3 +/- standard deviation).

HDR cytotoxicity analysis. HEK293 B/GFP cells were plated in a 12 well tissue culture dish (CytoOne) at a density of 1.5 x 10^5 cells per well in 500 μL of DMEMcontaining 10% FBS and 1% penicillin/streptomycin and allowed to attach overnight at 37 ºC. After overnight incubation, Lipofectamine 2000 and RNAiMAX lipoplexes were prepared with a 1:1:3 ratio of sgRNA:Cas9 mRNA:ssDNA according to the manufacturer's protocol. All formulations were then administered to the cells. 24 h after transfection, an additional 1 mL of DMEM was added to the cells. Following the 48 h incubation, the cells were prepared for analysis via flow cytometry as previously described. During preparation for flow cytometry, cells were stained with Ghost Dye Red 780 according to the manufacturer's protocol and washed 3X with PBS thereafter. Cytotoxicity was assessed using flow cytometry (N = 3 +/- standard deviation; statistical analysis performed using one-way ANOVA with Dunnett's multiple comparisons test against PBS).

Supporting Information Figures

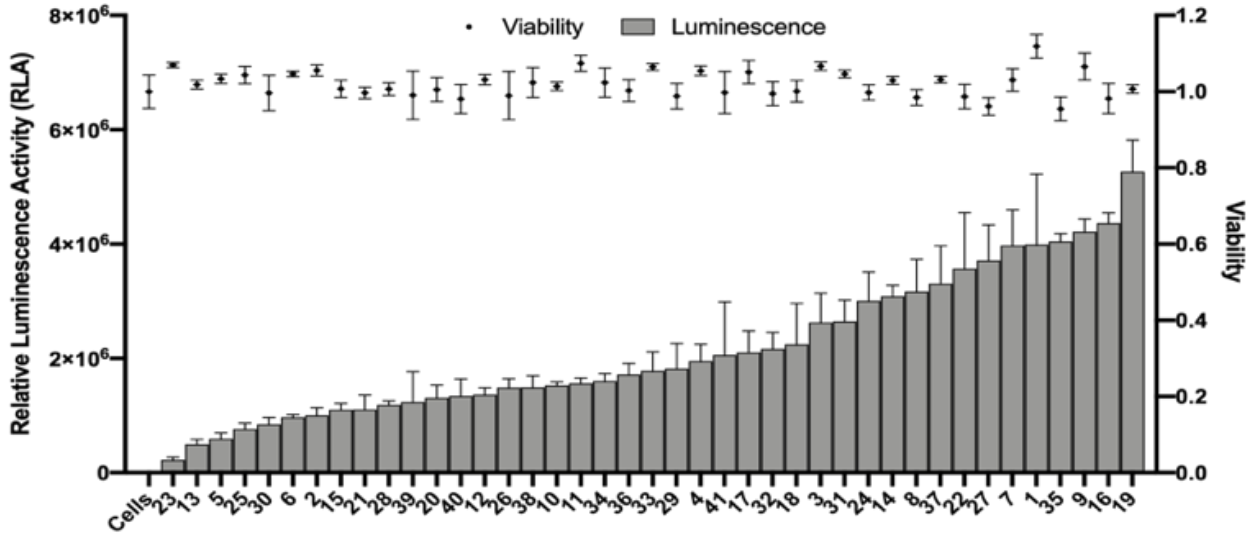

Figure S1. The internal molar ratios of 4A3-SC8, Cholesterol, DOPE, and PEG-DMG were varied in a systematic fashion to create particles with different lipid properties. Various formulations were screened where 50 ng of luciferase mRNA was delivered per well. Luciferase activity and cytotoxicity were measured (N = 4 +/- standard deviation). See **Table S1** for formulation details.

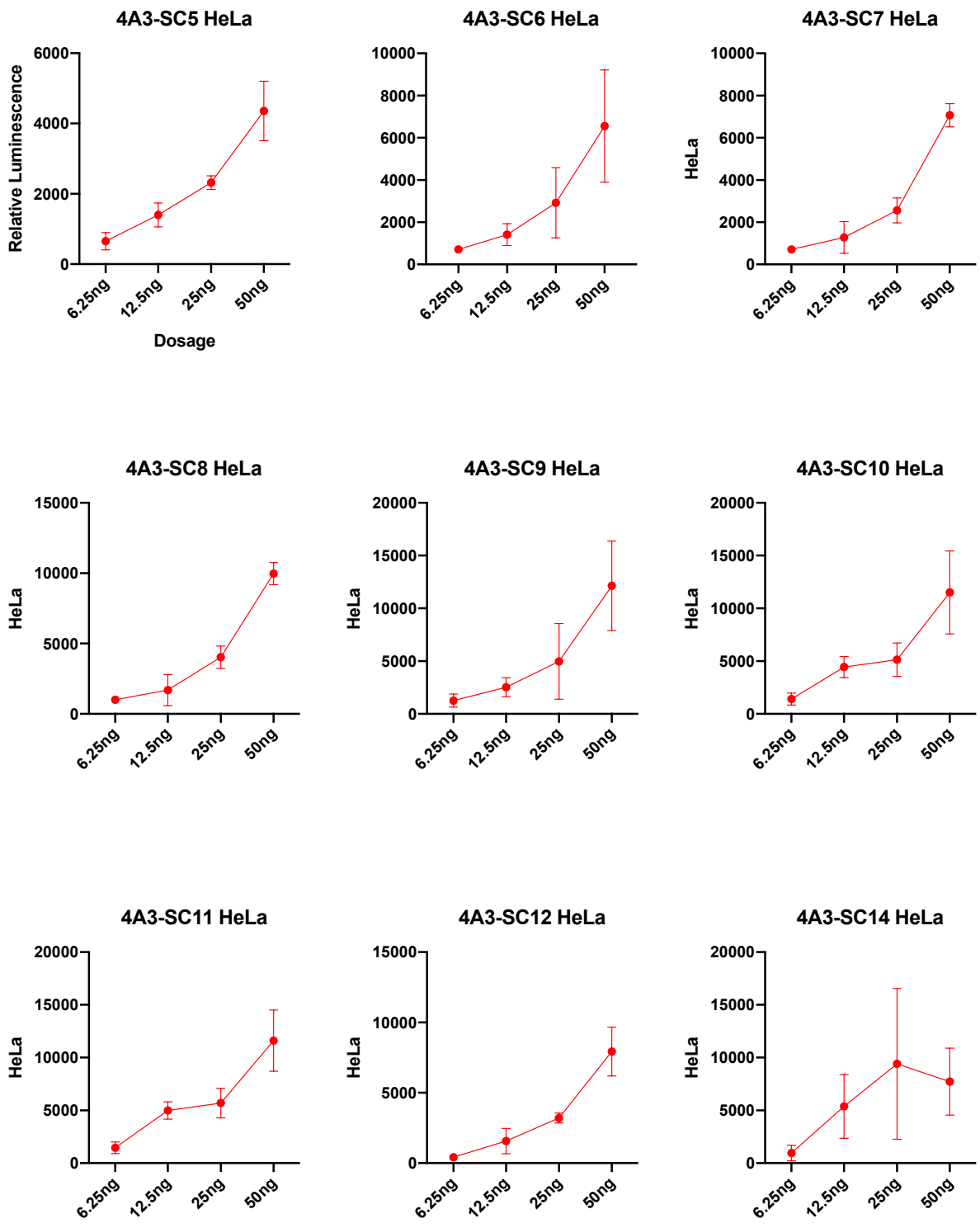

Figure S2. A dose response experiment was performed with dLNPs containing luciferase mRNA. The dLNPs were comprised of the 4A3 amine core and 9 different alkyl peripheries (SC5, SC6, SC7, SC8, SC9, SC10, SC11, SC12, SC14) in HeLa Cells. Notably, HeLa cells exhibited dramatic increases in luminescence in accordance with increasing dose of luciferase mRNA (N = 4).

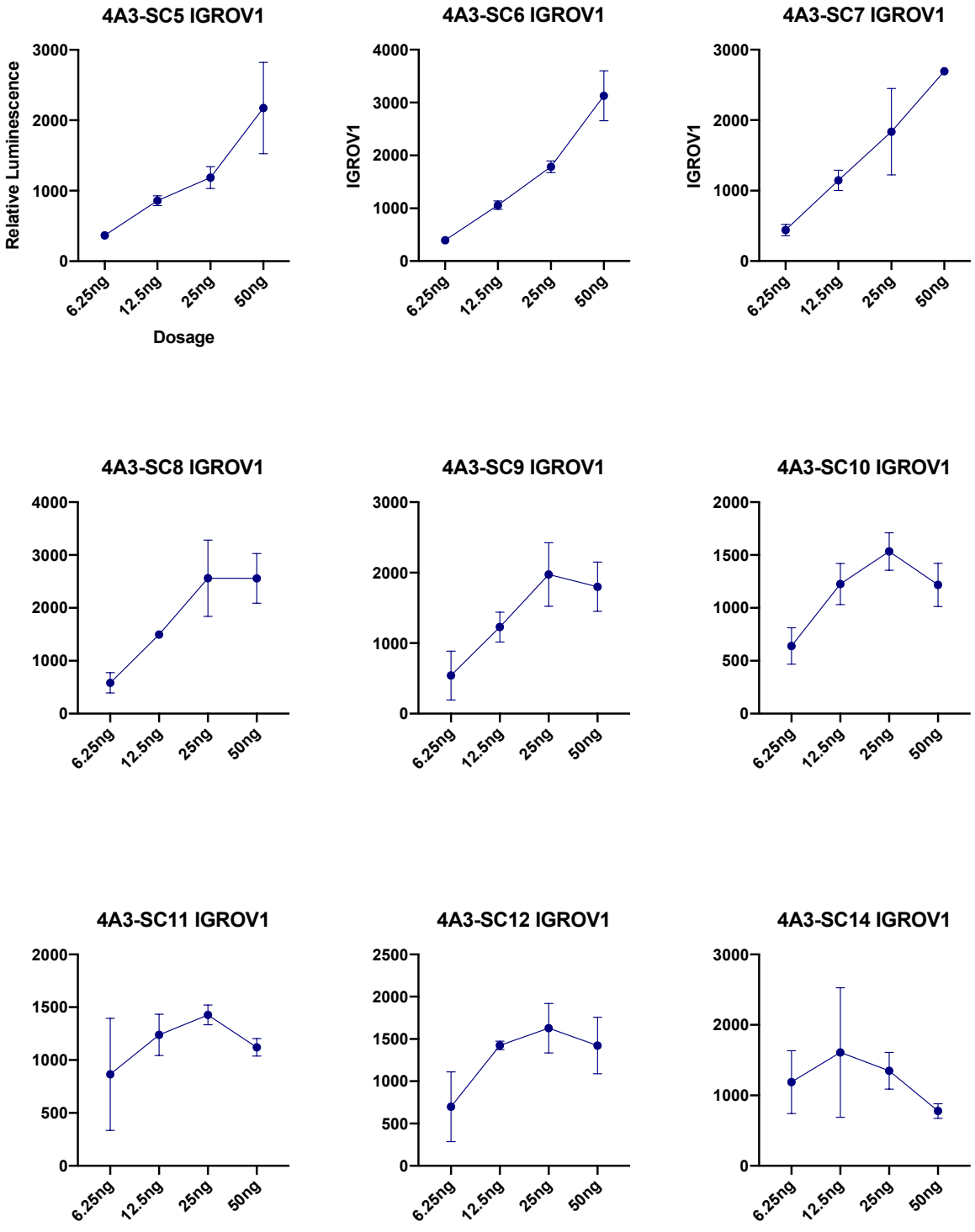

Figure S3. A dose response experiment was performed with dLNPs containing luciferase mRNA. The dLNPs were comprised of the 4A3 amine core and 9 different alkyl peripheries (SC5, SC6, SC7, SC8, SC9, SC10, SC11, SC12, SC14) in IGROV1 cells (N = 4).

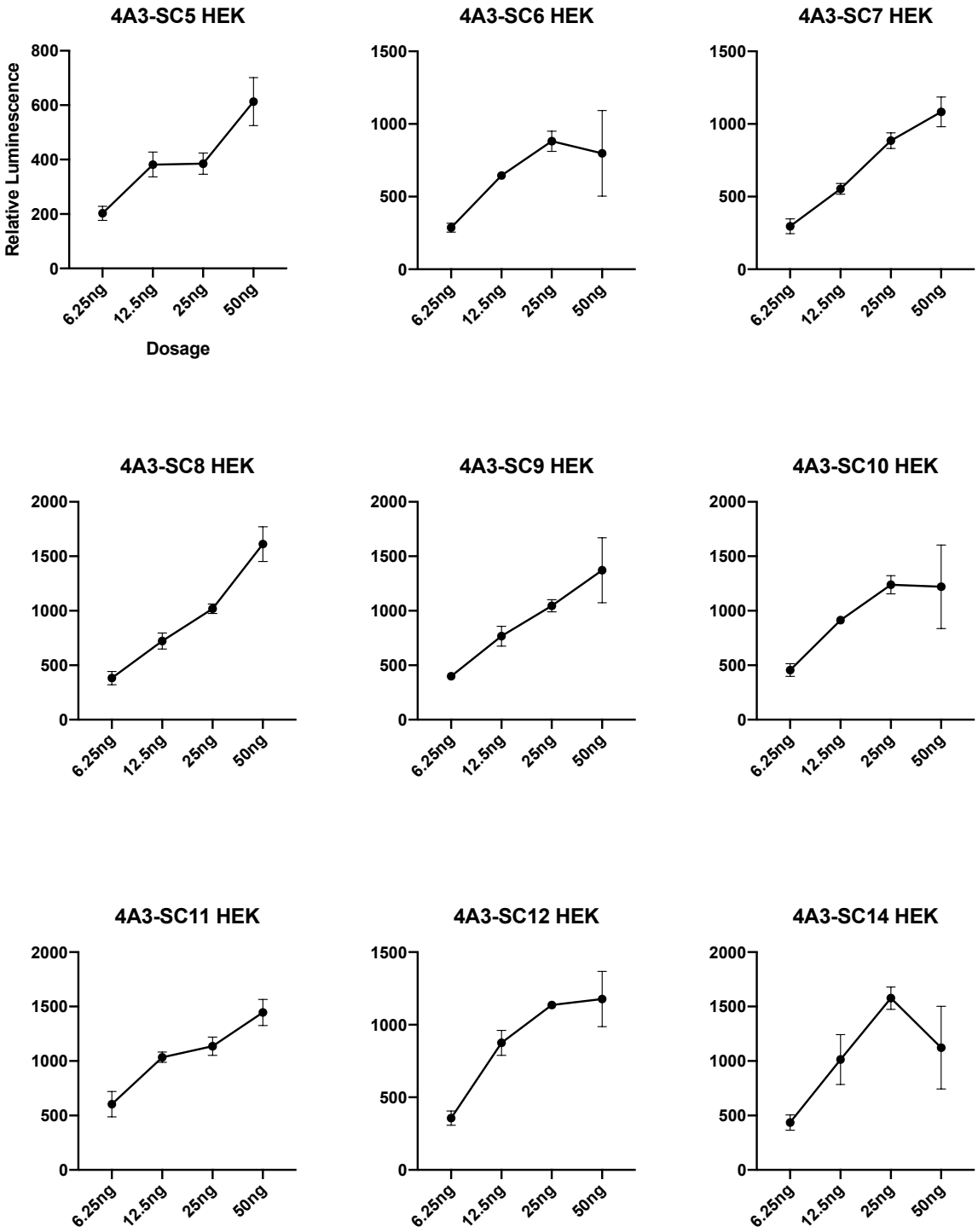

Figure S4. A dose response experiment was performed with dLNPs containing luciferase mRNA. The dLNPs were comprised of the 4A3 amine core and 9 different alkyl peripheries (SC5, SC6, SC7, SC8, SC9, SC10, SC11, SC12, SC14) in HEK293T cells (N = 4).

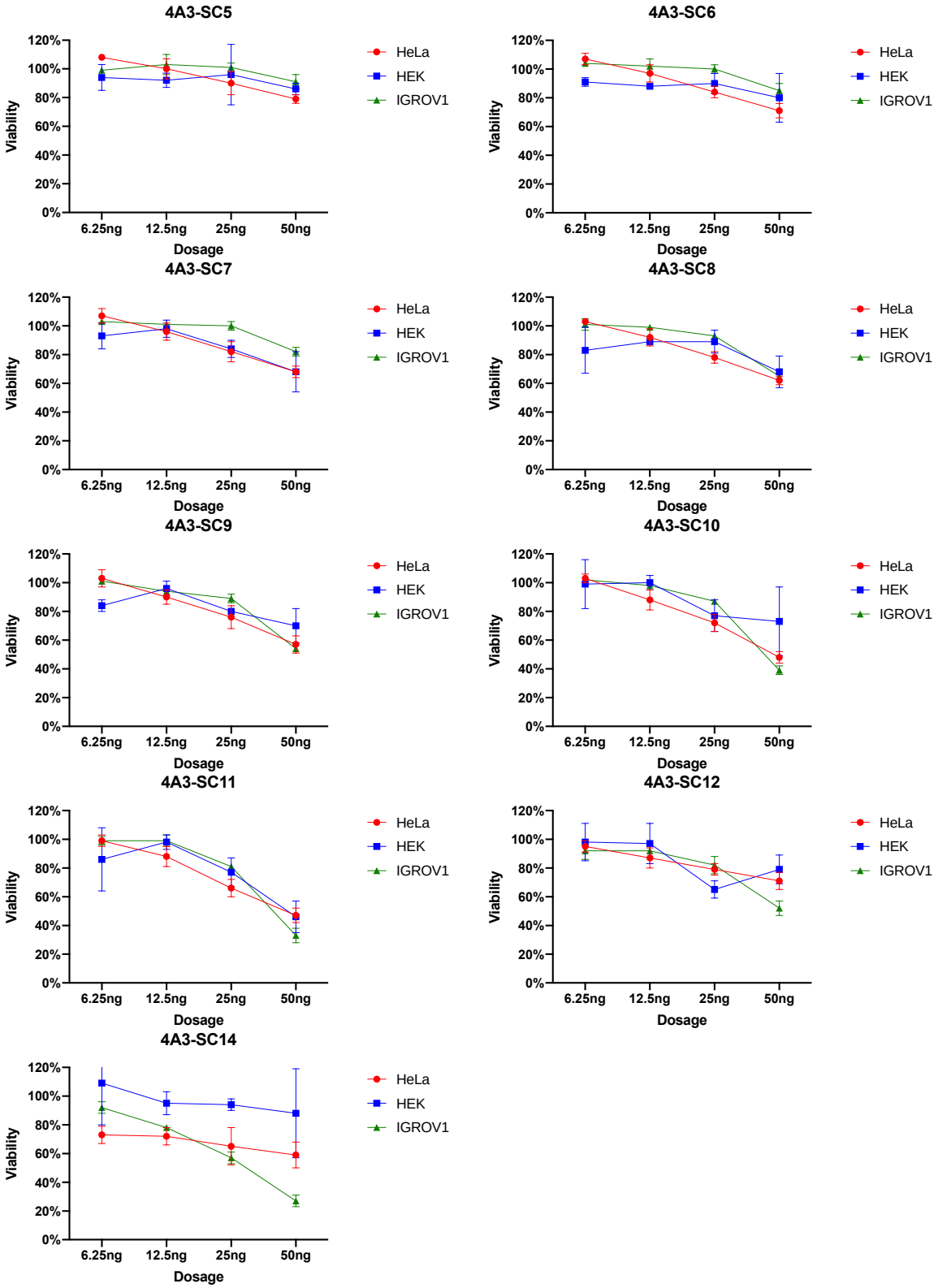

Figure S5. The ONE-Glo + Tox assay was used to assess cell viability in the dose response experiment performed with dLNPs containing luciferase mRNA. The dLNPs were comprised of the 4A3 amine core and 9 different alkyl peripheries (SC5, SC6, SC7, SC8, SC9, SC10, SC11, SC12, SC14). (N = 4).

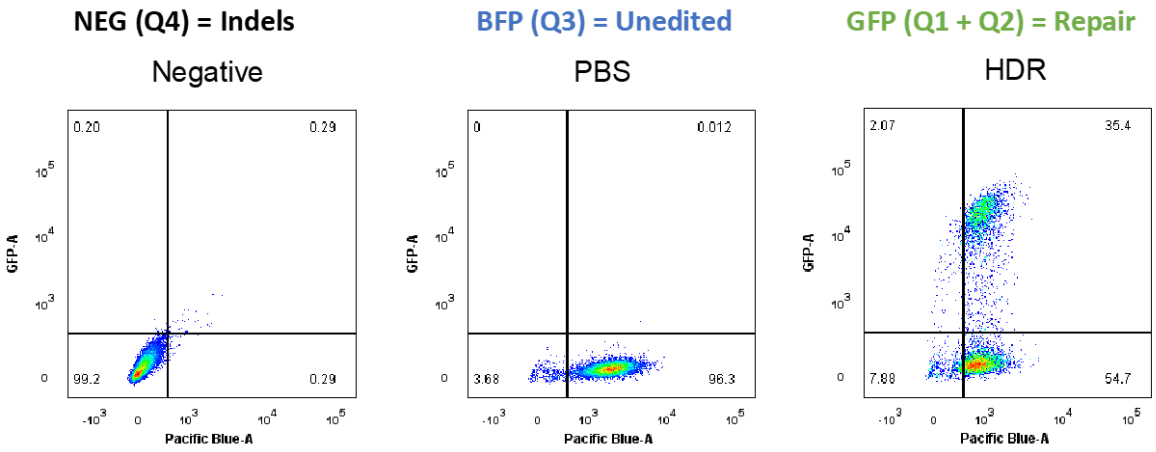

Figure S6. Quantitation of editing via NHEJ and HDR was performed via flow cytometry. In the above representative figures, HEK293 B/GFP cells with no fluorescence are shown as a negative control for gating purposes and quantification of editing via NHEJ wherein cells lose fluorescence due to the introduction of indels. Additionally, unedited cells are shown wherein blue fluorescence is observed. Finally, cells that have undergone gene correction via HDR are shown as indicated by a shift in the population of cells upwards on the GFP axis, indicating bright green fluorescence.

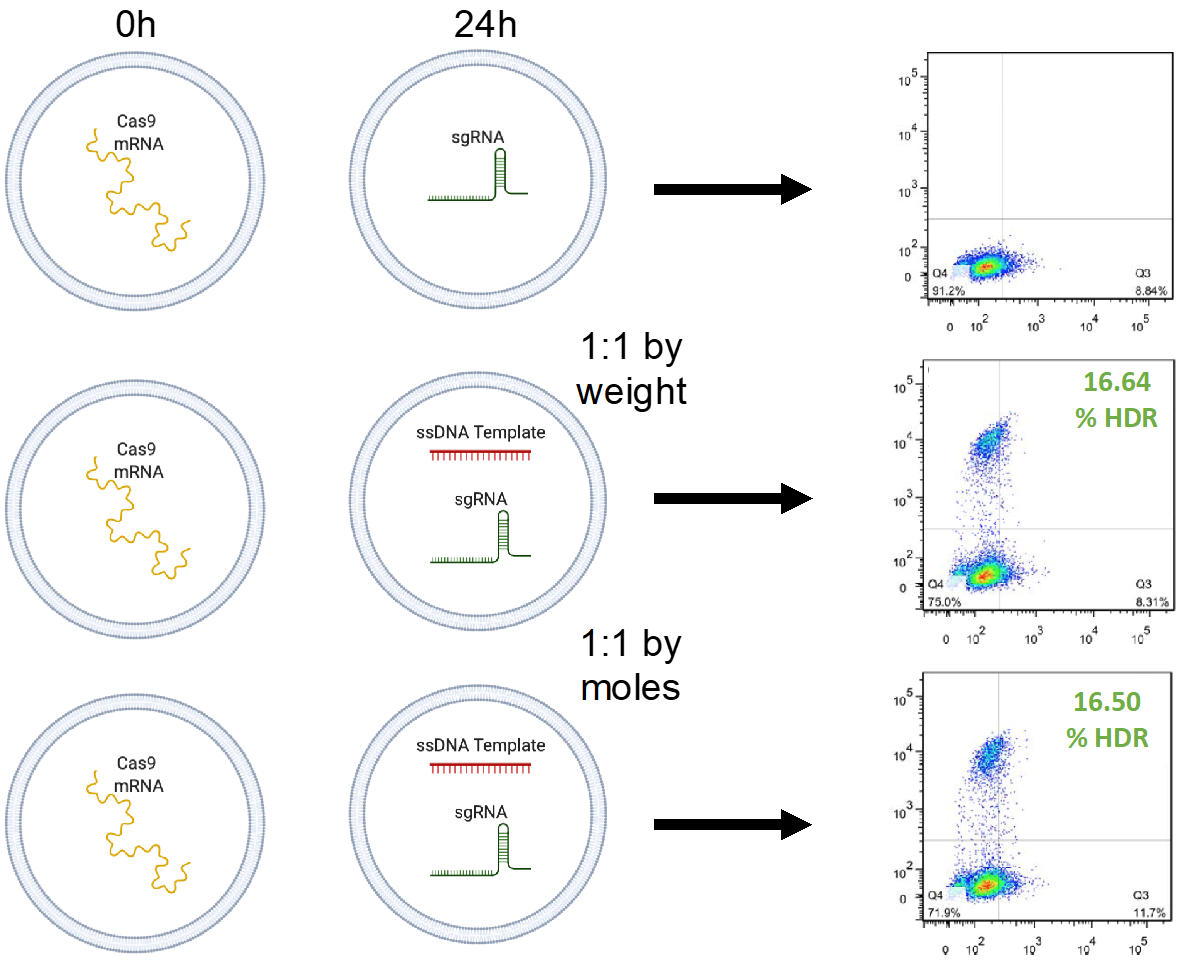

Figure S7. Sequential delivery was achieved via a two particle system wherein the first dLNPs were loaded only with Cas9 mRNA and administered to the cells at a dose of 500 ng. 24h after transfection with the Cas9 mRNA dLNPs, a second round of particles containing either sgRNA only or sgRNA and ssDNA template were administered to the cells at doses of 250 ng for sgRNA only particles and 500 ng total nucleic acid for the particles containing sgRNA and ssDNA template.

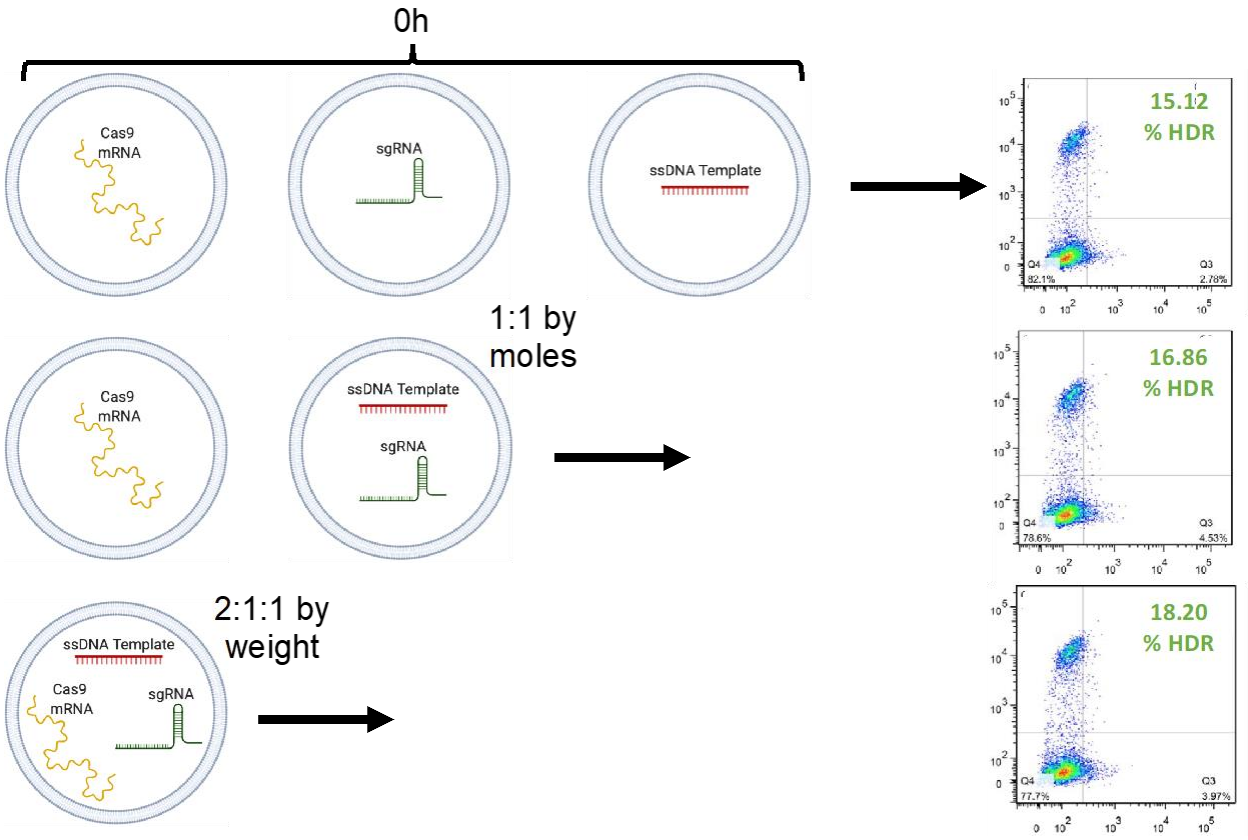

Figure S8. Simultaneous delivery was achieved via multi or single particle systems. The first dLNP system consisted of three different particles each loaded only with one nucleic acid, either Cas9 mRNA 500 ng, sgRNA 250 ng, or ssDNA template 250 ng. All particles were administered to the cells at the same time point. The second system used two particles, one loaded with Cas9 mRNA (500 ng) and the other loaded with sgRNA and ssDNA template (500 ng total nucleic acid) which were administered to the cells at the same time point. Finally, a particle containing 1000 ng total nucleic acid at a 2:1:1 ratio of Cas9 mRNA:sgRNA:ssDNA template was administered to the cells.

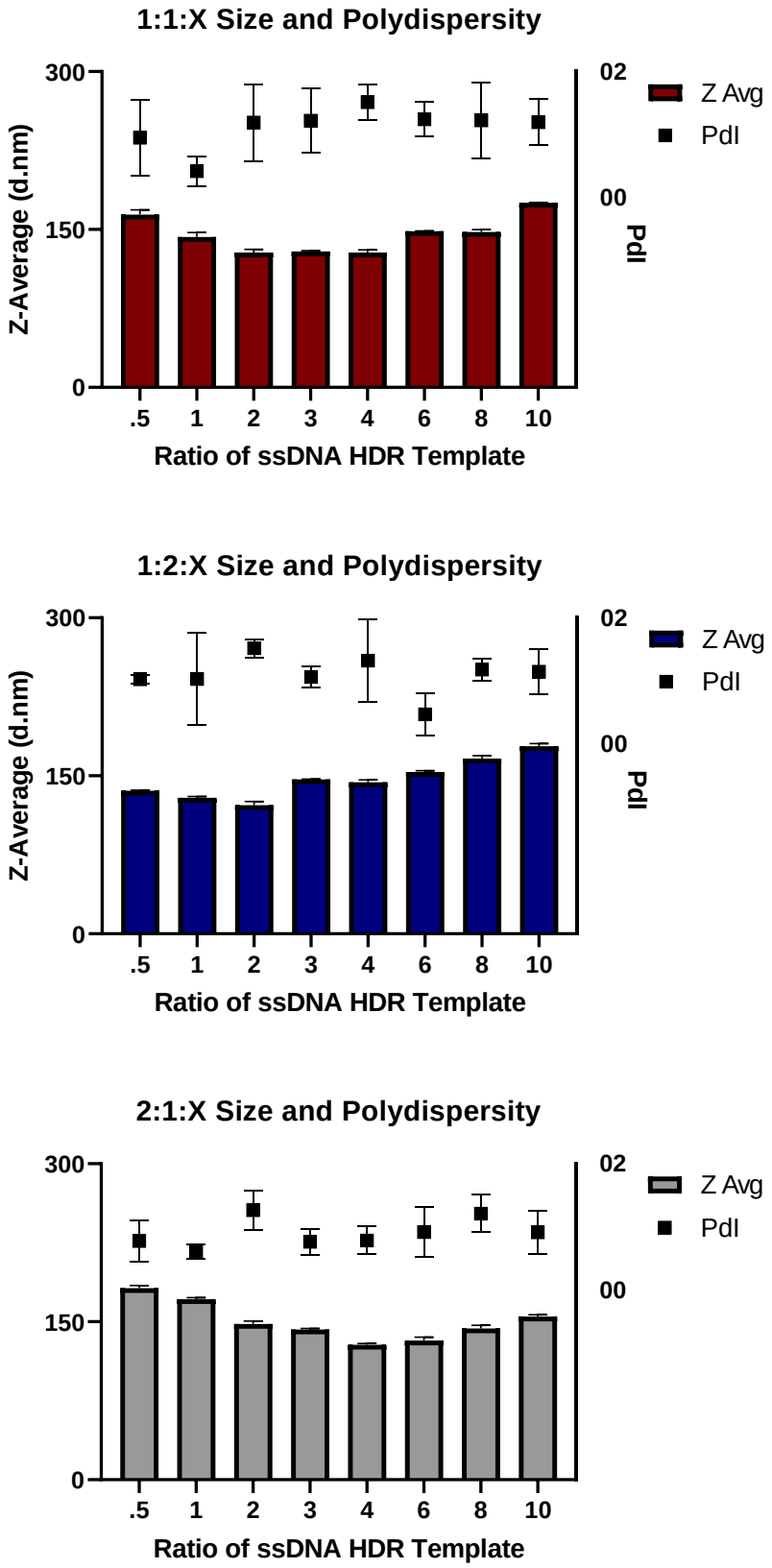

Figure S9. HDR dLNPs were characterized for size and polydispersity using dynamic light scattering.

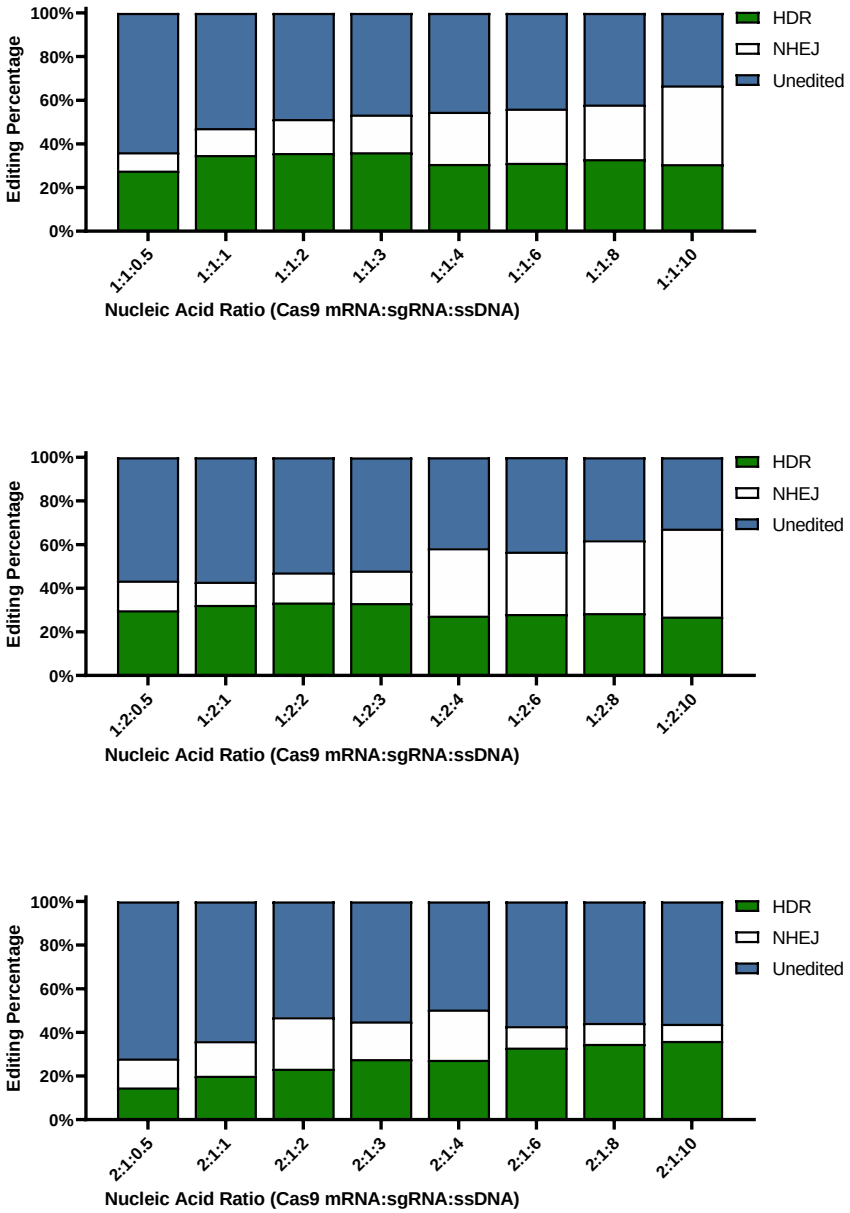

Figure S10. HDR dLNPs were created containing differing ratios of nucleic acid components wherein the ratio of Cas9 mRNA:sgRNA was fixed at one of three ratios (1:1, 1:2, or 2:1) by weight (1 = 250 ng) and the amount of ssDNA HDR template was titrated in at ratios of 0.5, 1, 2, 3, 4, 6, 8, or 10. Following treatment with HDR dLNPs, the cells were analyzed for GFP+ (HDR), BFP+ (unedited) or non-fluorescent (NHEJ) signal using flow cytometry. HDR was accomplished in all groups, however, the amount of HDR, NHEJ, and unedited cells changed with respect to ratio of nucleic acid components in each of the dLNP formulations (N = 3).

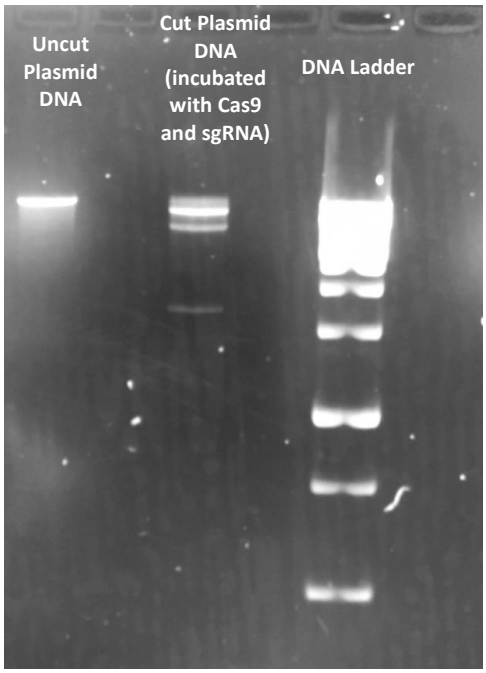

Figure S11. The sgRNA sequence was evaluated for target site cutting using an *in vitro* cutting assay. The sgRNA used in this assay was created using IVT. Cutting at the target site is indicated in the agarose gel image above by the presence of multiple bands in the group that was incubated with sgRNA and Cas9 protein.

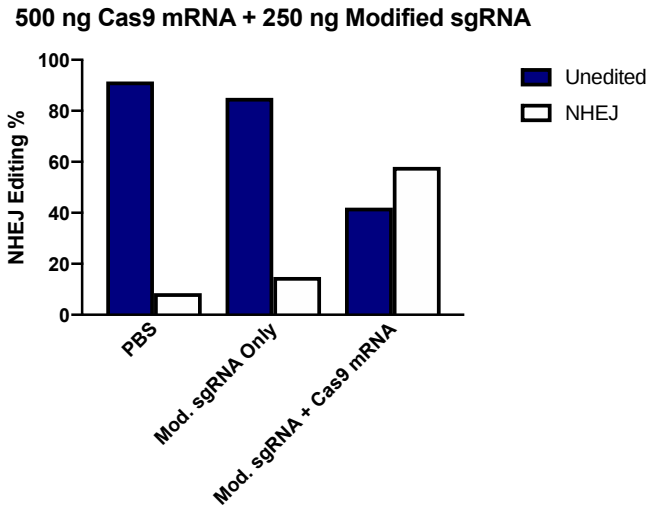

Figure S12. Modified sgRNA was encapsulated along with Cas9 mRNA into dLNPs at a ratio of 2:1 by weight (500 ng Cas9 mRNA, 250 ng modified sgRNA). Similarly, 250ng of modified sgRNA was encapsulated into dLNPs that did not contain Cas9 mRNA. The dLNPs were administered to HEK293 B/GFP cells and the cells were incubated at 37 ºC for 48 h before being collected and prepared for analysis via flow cytometry. In the group receiving dLNPs containing both Cas9 mRNA and modified sgRNA there was significant gene editing via NHEJ as indicated by the large amount of non-fluorescent cells (white bar, 58%). Editing was further confirmed by controlling for antisense activity that is known to be induced by the presence of sgRNA in cells. As indicated above, when modified sgRNA was delivered to cells alone, the amount of non-fluorescent cells present was similar to the PBS control.

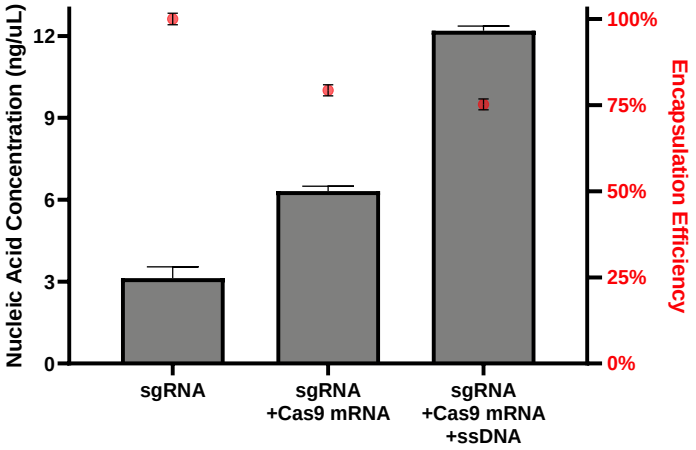

Figure S13. Three 4A3-SC8 dLNPs were created using the same lipid composition and loaded with 250 ng sgRNA only, 250 ng sgRNA and 500 ng Cas9 mRNA, or 250 ng sgRNA, 500 ng Cas9 mRNA, and 750 ng ssDNA. The Ribogreen assay was used to evaluate nucleic acid encapsulation in each of the three 4A3-SC8 dLNPs revealing that the amount of encapsulated nucleic acid increased in each formulation. Encapsulation efficiency was >75% for all three dLNPs.

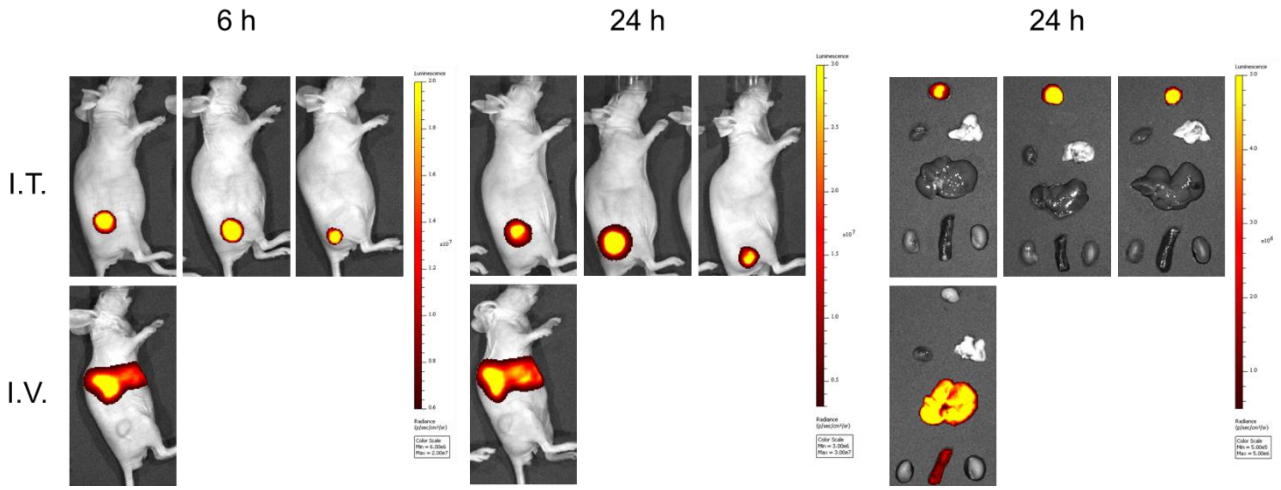

Figure S14. Nude mice bearing HEK293 B/GFP tumors were injected intravenously (IV) or intratumorally (IT) with 4A3-SC8 dLNPs containing luciferase mRNA at a dose of 0.25 mg/kg. 6 h post IT/IV injection, mice were administered d-luciferin and imaged using IVIS for luminescence. In mice injected IT with the Luc mRNA 4A3-SC8 dLNPs, imaging at 6 h showed bright luminescence in the tumor mass and no luminescence in other tissues. In mice injected IV with Luc mRNA 4A3-SC8 dLNPs, bright luminescence can be seen in the liver, but no luminescence is visible in the tumor mass. To ensure enough time passed for any potential leakage of dLNPs into systemic circulation, mice were imaged 24 h following the initial IV/IT injection of dLNPs. Again, luminescence was present only in the tumor mass of mice injected IT. In mice injected IV, luminescence was only present in the liver. Additionally, the tumor mass, heart, lungs, liver, kidneys, and spleen were resected from the mice and imaged for luminescence. Confirming previous images, bright luminescence was visible only in the tumor mass of mice injected IT, indicating no leak through of dLNPs into systemic circulation. Mice injected IV with dLNPs showed bright luminescence in the liver and minor luminescence in spleen, but no luminescence in tumor mass.

	ID	Aligned Site (20ng+PAM)	# mismatches	Strand	Locus
On-Target	sgPTEN	AGATCGTTAGCAGAAACAAAAGG	0	+	chr19:32758464
	OT-1	AGCACGTTAGCAGAAACCAAAGG	3	-	chr15:15308342
	OT-2	AGAATGTTAACAGAAACAAATGG	3	+	chr15:60681817
	OT-3	AGAGCGTTAGCAGACCCAAATGG	3	+	chr19:30941492
	OT-4	AGATGGTTAGCAGTAGCAAAGGG	3	-	chrX:152078091
	OT-5	AGATAGTTAGCACAAAGAAATGG	3	+	chr13:111927102

Primers used for PCR		
ID	Forward (5' to 3')	Reverse (5' to 3')
On-Target	ATCCGTCTTCTCCCCATTCCG	GACGAGCTCGCTAATCCAGTG
OT-1	GCTTCACTGGGTTTGAAAGTTCCC	TCCAAGAAGCATGGAGTTAATGAGACAAA
OT-2	CATATGTAATCGAGATGAATTTACACTGCCT	CCCAAGATTAGGGAGATGATTCCTCAC
OT-3	AGTTGCTCAGTGACATGCCTTACT	TGAGCAAACCTCCAAACACTCAAAGT
OT-4	CAGCACCAGCTCTAGATATAGGTAGGT	TTAGATGTTACACAGCCACTAGAATTCATTCC
OT-5	CTGACTGGCTTATGCTGGAGAG	CCACTCTGCAGCTGATATTAAATAGCT

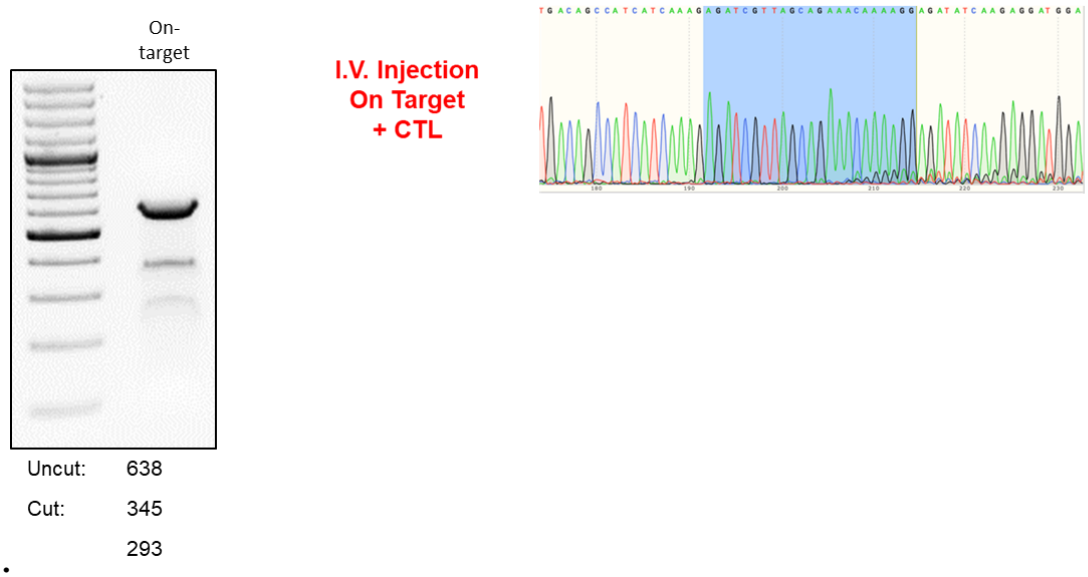

Figure S15. Cas-OFFinder was used to identify 5 top off-target sites for PTEN in the mouse genome. Nude mice bearing HEK293 B/GFP tumors were injected intratumorally with a 2:1 ratio of Cas9 mRNA:PTEN sgRNA at a dose of 0.25 mg/kg and analyzed for on-target and off-target editing via Sanger DNA Sequencing and the T7E1 assay.

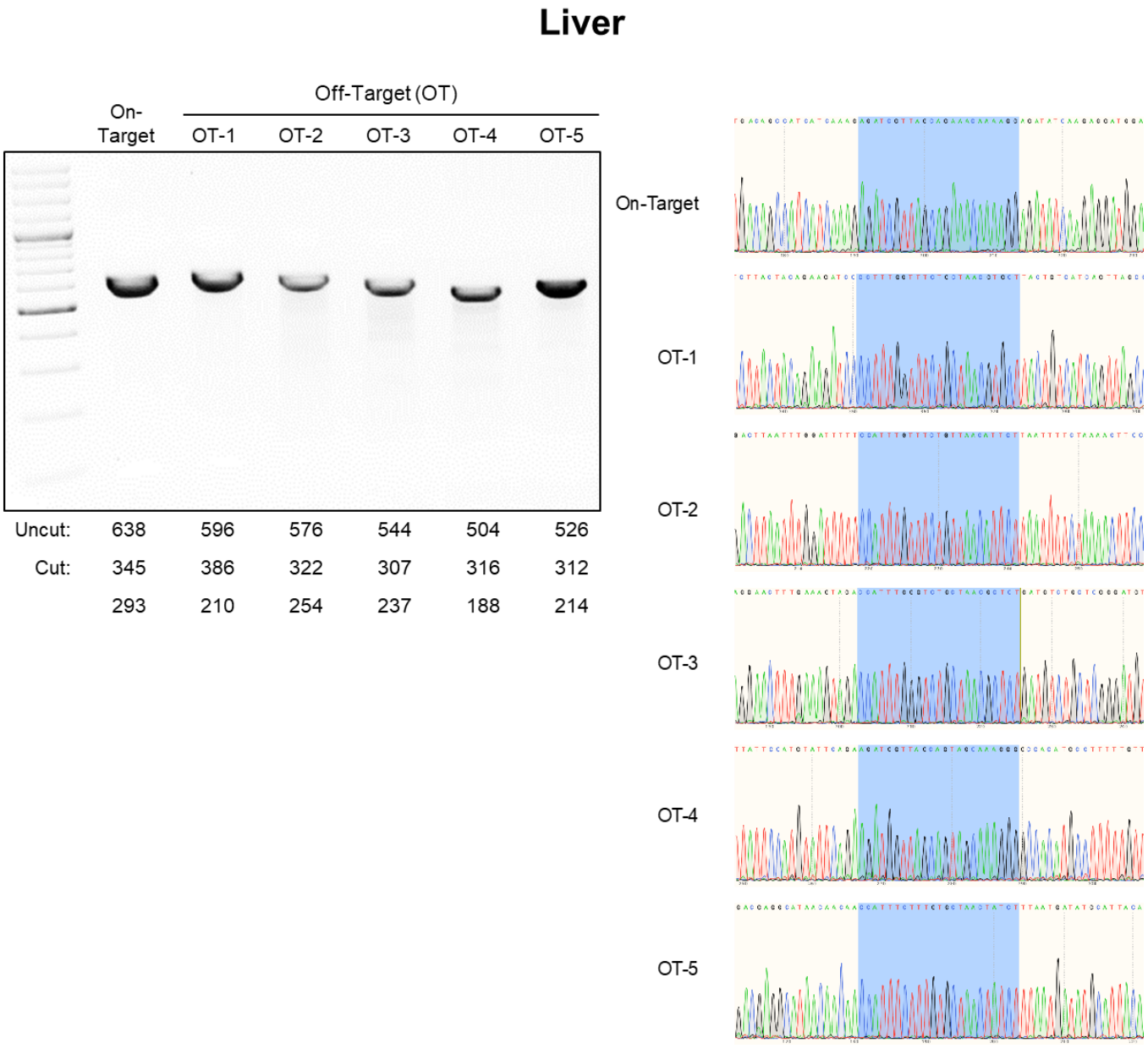

Figure S16. Sanger DNA sequencing and the T7E1 assay revealed no on-target or off-target editing at any of the 5 predicted off-target sites in liver following IT injection with 4A3-SC8 dLNPs containing a 2:1 ratio of Cas9 mRNA:PTEN sgRNA at a dose of 0.25 mg/kg.

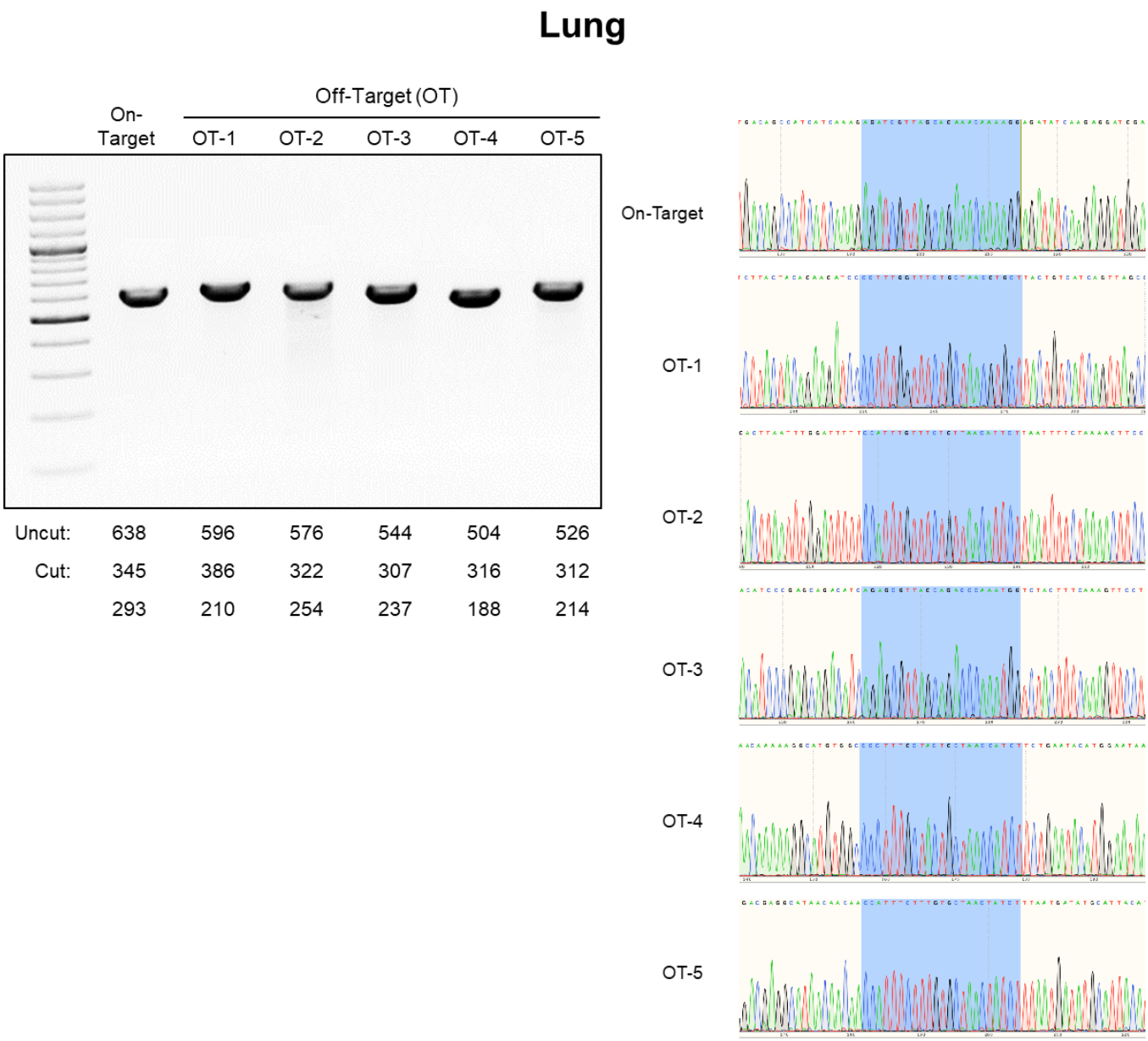

Figure S17. Sanger DNA sequencing and the T7E1 assay revealed no on-target or off-target editing at any of the 5 predicted off-target sites in lung following IT injection with 4A3-SC8 dLNPs containing a 2:1 ratio of Cas9 mRNA:PTEN sgRNA at a dose of 0.25 mg/kg.

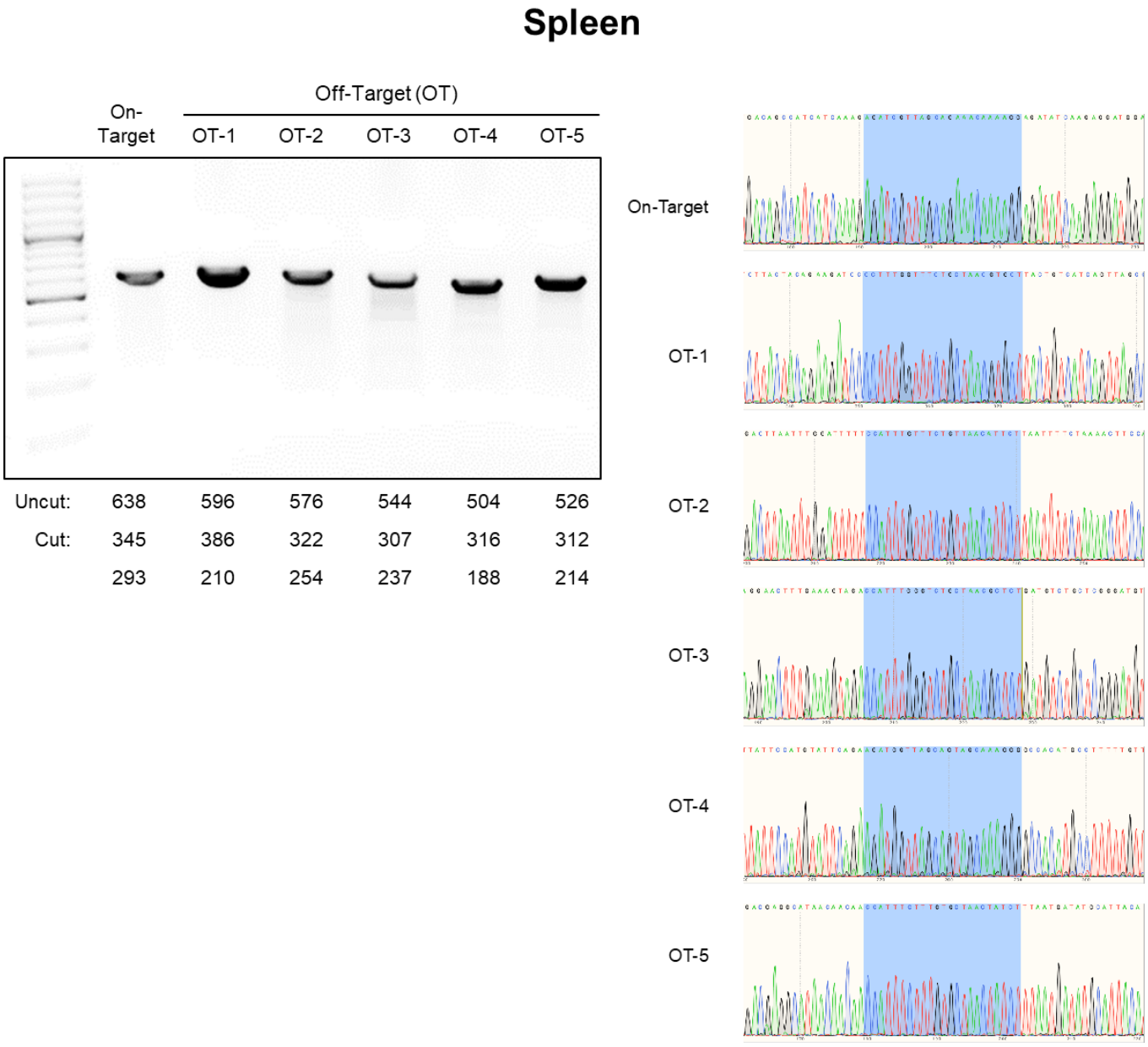

Figure S18. Sanger DNA sequencing and the T7E1 assay revealed no on-target or off-target editing at any of the 5 predicted off-target sites in spleen following IT injection with 4A3-SC8 dLNPs containing a 2:1 ratio of Cas9 mRNA:PTEN sgRNA at a dose of 0.25 mg/kg.

	ID	Aligned Site (20ng+PAM)	# mismatches	Strand	Locus
On-Target	sgBGFP	GCTGAAGCACTGCACGCCATGGG	0	-	N/A
Off-Target (OT)	OT-1	GCTGAAGCACTGCCAGACATAGG	4	-	chr8:7050317
	OT-2	GTGGAAGCACTGCAAGCCATTGG	4	-	chr7:4486342
	OT-3	GCTGAATCACAGCAGGCCATGGG	2	+	chr17:5639617
	OT-4	GCTGTAGCACTCCACGCCGTTGG	4	+	chr19:1614429
	OT-5	GCTCAAGCACTGCACCCCGTGGG	3	+	chr9:3927273

Primers used for PCR		
ID	Forward (5' to 3')	Reverse (5' to 3')
On-Target	AGCTGGCTAGGTAAGCTTGG	GGGTGCTCAGGTAGTGGTT
OT-1	ATCCACATCATATGCCAGGGTGATC	CATAGGGGGTCCATCTCCCTGAC
OT-2	CTCTCGTCCGTTAAACTAGCTATTGCT	ACATTTGATTCACCAGCTGGCAG
OT-3	CCAGCTGGATCAGAGGCACC	CCTGACCCCAAAGTGGGGTG
OT-4	CCATGATGCCCTACGATACAAGCA	ATGCGCTCAGGATCGCTG
OT-5	GTCCTCTGTACCTTGGCTGCC	GCAGGACTGGGGATGAGGG

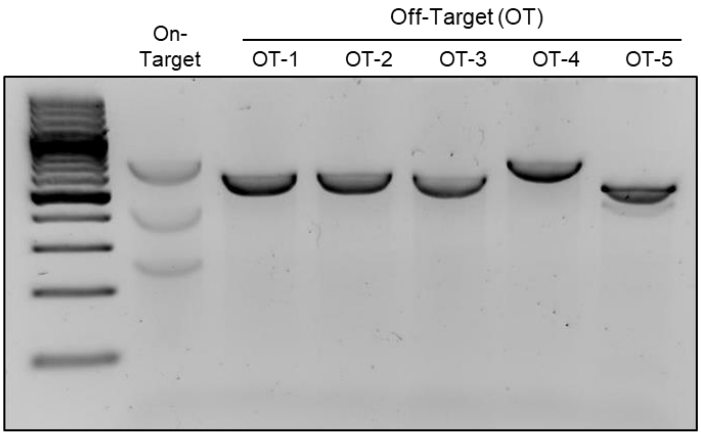

Figure S19. The Cas-OFFinder webtool was used to predict likely off-target editing sites for the B/GFP sgRNA. Five top potential off-target sites were amplified by PCR following transfection of HEK293 B/GFP cells with 4A3-SC8 dLNPs containing a 2:1 ratio of Cas9mRNA:sgRNA and then analyzed using the T7E1 assay. On-target amplicons showed clear cleavage bands of the correct predicted size but no editing at any of the five off-target sites.

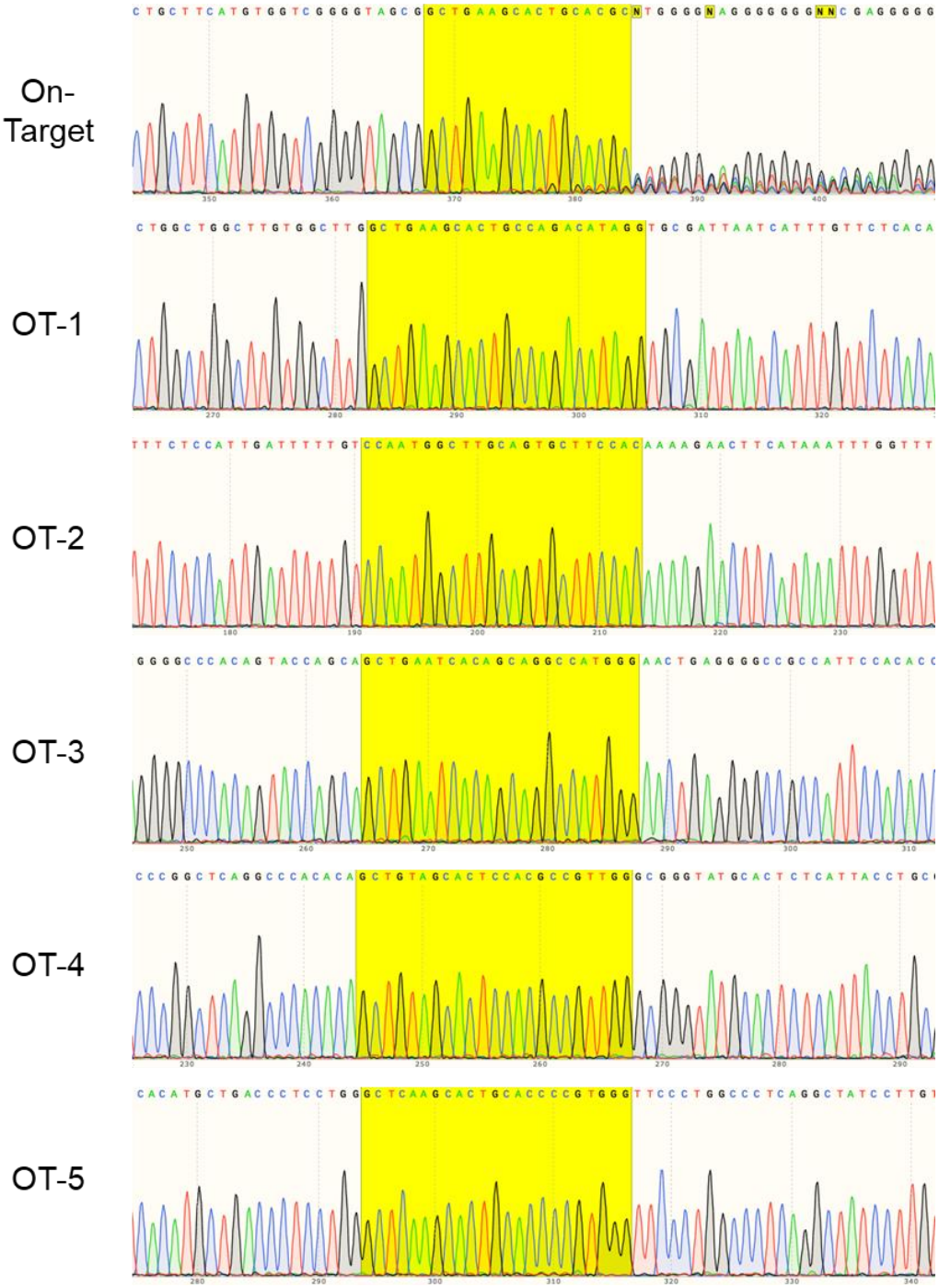

Figure S20. The Cas-OFFinder webtool was used to predict likely off-target editing sites for the B/GFP sgRNA. Five top potential off-target sites were amplified by PCR following transfection of HEK293 B/GFP cells with 4A3-SC8 dLNPs containing a 2:1 ratio of Cas9mRNA:sgRNA and then analyzed using the T7E1 assay and Sanger sequencing. Sequencing data revealed clear on-target editing with small, undefined peaks around the target cleavage site. In the off-target samples, sharp, clear peaks were present around the potential cleavage sites, indicating no Indel formation.

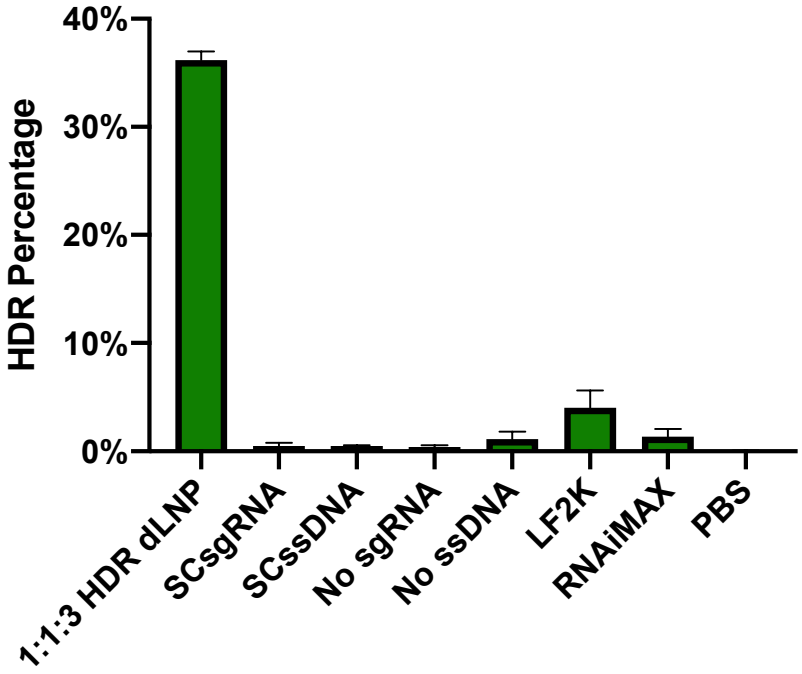

Figure S21. Flow cytometry was used to asses HDR efficiency of 1:1:3 4A3-SC8 HDR dLNPs in comparison to the commercially available reagents Lipofectamine 2000 and RNAiMAX which were both loaded with a 1:1:3 ratio of Cas9mRNA:sgRNA:ssDNA and administered to HEK293 B/GFP cells. Analysis for GFP+ cells revealed little HDR induction for the Lipofectamine 2000 (<5%) and RNAiMAX (<2%) transfection reagents in comparison to the 4A3-SC8 HDR dLNPs (>35%). Additionally, HEK293 B/GFP cells were transfected with 4A3-SC8 dLNPs containing scrambled sgRNA in place of B/GFP sgRNA (SCsgRNA), scrambled DNA template in place of ssDNA B/GFP template (SCssDNA), Cas9 mRNA and ssDNA but no sgRNA, and sgRNA and Cas9 mRNA but no ssDNA. In all cases other than the dLNP containing no ssDNA template HDR induction was <1%.

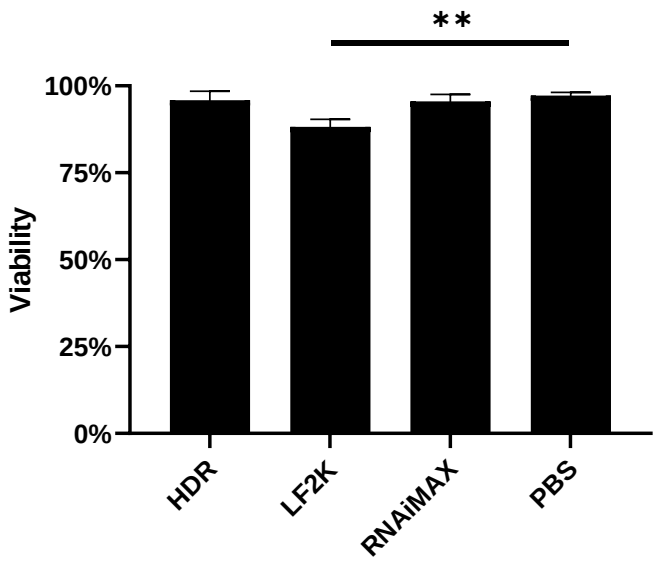

Figure S22. Flow cytometry was used to asses cytotoxicity after treatment with 4A3-SC8 dLNPs containing a 1:1:3 ratio of Cas9 mRNA:sgRNA:ssDNA (1000 ng of total nucleic acids) as well as Lipofectamine 2000 and RNAiMAX containing the same dose and ratio of nucleic acids. HEK293 B/GFP cells were plated in 500 uL of DMEM (10% FBS, 1% penicillin/streptomycin) in a 12 well plate at a density of 1.5 x 10^5 cells/well and allowed to attach overnight at 37 ºC prior to transfection. 24 h following transfection, an additional 1 mL of DMEM was added to each well. The cells were prepared for analysis via flow cytometry 48 h after initial transfection. No significant toxicity was observed as compared with PBS in cells treated with the 1:1:3 4A3-SC8 HDR dLNPs or RNAiMAX. However, ~12% toxicity was present in cells treated with Lipofectamine 2000 (N = 3 +/- standard deviation; statistical analysis performed using one-way ANOVA with Dunnett's multiple comparisons test against PBS).

Supporting Information Tables

Table S1. The ratios of nucleic acids within each formulation of HDR dLNPs.

Ratio	Cas9 mRNA (ng)	sgRNA (ng)	ssDNA HDR Temp. (ng)	Total Nucleic Acid (ng)
1:1:0.5	250	250	125	625
1:1:1	250	250	250	750
1:1:2	250	250	500	1000
1:1:3	250	250	750	1250
1:1:4	250	250	1000	1500
1:1:6	250	250	1500	2000
1:1:8	250	250	2000	2500
1:1:10	250	250	2500	3000
1:2:0.5	250	500	125	875
1:2:1	250	500	250	1000
1:2:2	250	500	500	1250
1:2:3	250	500	750	1500
1:2:4	250	500	1000	1750
1:2:6	250	500	1500	2250
1:2:8	250	500	2000	2750
1:2:10	250	500	2500	3250
2:1:0.5	500	250	125	875
2:1:1	500	250	250	1000
2:1:2	500	250	500	1250
2:1:3	500	250	750	1500
2:1:4	500	250	1000	1750
2:1:6	500	250	1500	2250
2:1:8	500	250	2000	2750
2:1:10	500	250	2500	3250

Table S2. The internal molar ratios and particle percentage breakdown of the individual components as well as the fixed molar ratios of dendrimer lipid component to nucleic acid.

		Molar Ratio				Lipid Percentage			
	Dendrimer:NA MR	4A3-SC	Cholesterol	DOPE	DMG-PEG	4A3-SC	Cholesterol	DOPE	DMG-PEG
1	2000	30	40	40	1.5	26.91%	35.87%	35.87%	1.35%
2	2000	70	40	40	1.5	46.20%	26.40%	26.40%	0.99%
3	6000	30	40	40	1.5	26.91%	35.87%	35.87%	1.35%
4	6000	70	40	40	1.5	46.20%	26.40%	26.40%	0.99%
5	4000	50	20	20	1.5	54.64%	21.86%	21.86%	1.64%
6	4000	50	20	60	1.5	38.02%	15.21%	45.63%	1.14%
7	4000	50	60	20	1.5	38.02%	45.63%	15.21%	1.14%
8	4000	50	60	60	1.5	29.15%	34.99%	34.99%	0.87%
9	4000	30	40	40	0.5	27.15%	36.20%	36.20%	0.45%
10	4000	30	40	40	2.5	26.67%	35.56%	35.56%	2.22%
11	4000	70	40	40	0.5	46.51%	26.58%	26.58%	0.33%
12	4000	70	40	40	2.5	45.90%	26.23%	26.23%	1.64%
13	2000	50	20	40	1.5	44.84%	17.94%	35.87%	1.35%
14	2000	50	60	40	1.5	33.00%	39.60%	26.40%	0.99%
15	6000	50	20	40	1.5	44.84%	17.94%	35.87%	1.35%
16	6000	50	60	40	1.5	33.00%	39.60%	26.40%	0.99%
17	4000	50	40	20	0.5	45.25%	36.20%	18.10%	0.45%
18	4000	50	40	20	2.5	44.44%	35.56%	17.78%	2.22%
19	4000	50	40	60	0.5	33.22%	26.58%	39.87%	0.33%
20	4000	50	40	60	2.5	32.79%	26.23%	39.34%	1.64%
21	4000	30	20	40	1.5	32.79%	21.86%	43.72%	1.64%
22	4000	30	60	40	1.5	22.81%	45.63%	30.42%	1.14%
23	4000	70	20	40	1.5	53.23%	15.21%	30.42%	1.14%
24	4000	70	60	40	1.5	40.82%	34.99%	23.32%	0.87%
25	2000	50	40	20	1.5	44.84%	35.87%	17.94%	1.35%
26	2000	50	40	60	1.5	33.00%	26.40%	39.60%	0.99%
27	6000	50	40	20	1.5	44.84%	35.87%	17.94%	1.35%
28	6000	50	40	60	1.5	33.00%	26.40%	39.60%	0.99%
29	4000	50	20	40	0.5	45.25%	18.10%	36.20%	0.45%
30	4000	50	20	40	2.5	44.44%	17.78%	35.56%	2.22%
31	4000	50	60	40	0.5	33.22%	39.87%	26.58%	0.33%
32	4000	50	60	40	2.5	32.79%	39.34%	26.23%	1.64%
33	2000	50	40	40	0.5	38.31%	30.65%	30.65%	0.38%
34	2000	50	40	40	2.5	37.74%	30.19%	30.19%	1.89%
35	6000	50	40	40	0.5	38.31%	30.65%	30.65%	0.38%
36	6000	50	40	40	2.5	37.74%	30.19%	30.19%	1.89%
37	4000	30	40	20	1.5	32.79%	43.72%	21.86%	1.64%
38	4000	30	40	60	1.5	22.81%	30.42%	45.63%	1.14%
39	4000	70	40	20	1.5	53.23%	30.42%	15.21%	1.14%
40	4000	70	40	60	1.5	40.82%	23.32%	34.99%	0.87%
41	4000	50	40	40	1.5	38.02%	30.42%	30.42%	1.14%

Table S3. The p values associated with in vitro HDR percentage as determined by TIDER analysis of DNA sanger sequencing are reported above for each of the replicates (N = 3).

Cas9 mRNA:sgRNA:ssDNA	N = 1	N = 2	N = 3
1:1:0.5	2.00E-120	7.60E-54	1.60E-191
1:1:1	1.90E-290	1.60E-70	6.20E-216
1:1:2	8.00E-220	0.00E+00	8.70E-295
1:1:3	0.00E+00	0.00E+00	0.00E+00
1:1:4	7.30E-246	1.30E-281	6.00E-222
1:1:6	2.20E-102	1.40E-41	7.30E-130
1:1:8	1.40E-59	6.50E-141	9.60E-18
1:1:10	3.70E-02	3.60E-29	0.00E+00
1:2:0.5	2.70E-98	6.10E-80	5.90E-166
1:2:1	1.40E-119	1.40E-137	1.40E-118
1:2:2	0.00E+00	0.00E+00	4.10E-171
1:2:3	1.50E-144	3.30E-117	1.80E-143
1:2:4	0.00E+00	0.00E+00	0.00E+00
1:2:6	2.00E-261	2.00E-88	5.70E-176
1:2:8	4.10E-16	1.30E-18	7.20E-120
1:2:10	6.00E-37	8.60E-91	1.50E-08
2:1:0.5	1.00E-122	2.70E-173	4.00E-32
2:1:1	2.30E-124	8.80E-142	4.10E-41
2:1:2	2.50E-198	2.70E-40	4.60E-235
2:1:3	2.00E-209	2.90E-246	5.80E-203
2:1:4	4.30E-123	0.00E+00	0.00E+00
2:1:6	0.00E+00	2.10E-80	3.80E-137
2:1:8	0.00E+00	0.00E+00	0.00E+00
2:1:10	7.50E-19	0.00E+00	7.20E-35

Table S4. The p values associated with in vivo HDR percentage as determined by TIDER analysis of DNA sanger sequencing are reported above for each of the replicates (N = 4 in all cases except for 1:1:3 where N = 3).

Cas9 mRNA:sgRNA:ssDNA	**N = 1**	**N = 2**	**N = 3**	**N = 4**
1:1:8	0.012	0.009	0.043	0.01
1:1:3	5.70E-15	4.40E-09	1.50E-08	
2:1:3	3.30E-12	9.60E-14	3.20E-25	3.80E-19

References

[1] K. Zhou, L. H. Nguyen, J. B. Miller, Y. Yan, P. Kos, H. Xiong, L. Li, J. Hao, J. T. Minnig, H. Zhu, D. J. Siegwart, *Proc. Natl. Acad. Sci. U.S.A.* **2016**, 113, 520.