Supporting Information

1. Instrumentation, Materials, and Animal Protocols

Reactions were performed in round bottom flasks. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded with a Varian inverse probe INOVA-500 spectrometer (with a Magnex Scientific superconducting actively-shielded magnet), are reported in parts per million on the δ scale, and are referenced from the residual protium in the NMR solvent ($CDCl_3$: δ 7.24).[1] All commercial reagents and solvents were used as received.

Anti-firefly luciferase siRNA with the following sequence was used:

sense: AAcGcuGGGcGuuAAucAAdTsdT
antisense: UUGAUuAACGCCcAGCGUUdTsdT

Firefly luciferase (FLuc) mRNA was generously provided by Translate Bio (Lexington, MA). FLuc mRNA contained a 5' cap (Cap1), a 5' UTR consisting of a partial sequence of the cytomegalovirus (CMV) immediate early 1 (IE1) gene, a coding region as described below, a 3' UTR consisting of a partial sequence of the human growth hormone (hGH) gene, and a 3' polyA tail.

AUGGAAGAUGCCAAAAACAUUAAGAAGGGCCCAGCGCCAUUCUACCCACU
CGAAGACGGGACCGCCGGCGAGCAGCUGCACAAAGCCAUGAAGCGCUACGC
CCUGGUGCCCGGCACCAUCGCCUUUACCGACGCACAUAUCGAGGUGGACAU
UACCUACGCCGAGUACUUCGAGAUGAGCGUUCGGCUGGCAGAAGCUAUGA
AGCGCUAUGGGCUGAAUACAAACCAUCGGAUCGUGGUGUGCAGCGAGAAU
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AGAUCCUCAACGUGCAAAAGAAGCUACCGAUCAUACAAAAGAUCAUCAUC
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GACUUCCCAUUUGCCACCCGGCUUCAACGAGUACGACUUCGUGCCCGAGAG
CUUCGACCGGGACAAAACCAUCGCCCUGAUCAUGAACAGUAGUGGCAGUA
CCGGAUUGCCCAAGGGCGUAGCCCUACCGCACCGCACCGCUUGUGUCCGAU
UCAGUCAUGCCCGCGACCCCAUCUUCGGCAACCAGAUCAUCCCCGACACCG
CUAUCCUCAGCGUGGUGCCAUUUCACCACGGCUUCGGCAUGUUCACCACGC
UGGGCUACUUGAUCUGCGGCUUUCGGGUCGUGCUCAUGUACCGCUUCGAG
GAGGAGCUAUUCUUGCGCAGCUUGCAAGACUAUAAGAUUCAAUCUGCCCU
GCUGGUGCCCACACUAUUUAGCUUCUUCGCUAAGAGCACUCUCAUCGACAA
GUACGACCUAAGCAACUUGCACGAGAUCGCCAGCGGCGGGGCGCCGCUCAG
CAAGGAGGUAGGUGAGGCCGUGGCCAAACGCUUCCACCUACCAGGCAUCCG
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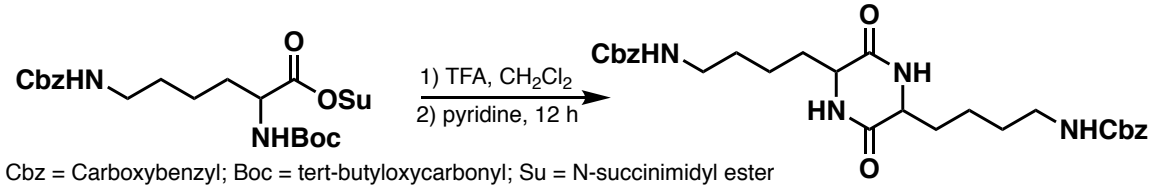
AGGGGACGACAAGCCUGGCGCAGUAGGCAAGGUGGUGCCCUUCUUCGAGG
CUAAGGUGGUGGACUUGGACACCGGUAAGACACUGGGUGUGAACCAGCGC
GGCGAGCUGUGCGUCCGUGGCCCCAUGAUCAUGAGCGGCUACGUUAACAA
CCCCGAGGCUACAAACGCUCUCAUCGACAAGGACGGCUGGCUGCACAGCGG

CGACAUCGCCUACUGGGACGAGGACGAGCACUUCUUCAUCGUGGACCGGCU
GAAGAGCCUGAUCAAAUACAAGGGCUACCAGGUAGCCCCAGCCGAACUGG
AGAGCAUCCUGCUGCAACACCCCAACAUCUUCGACGCCGGGGUCGCCGGCC
UGCCCGACGACGAUGCCGGCGAGCUGCCCGCCGCAGUCGUCGUGCUGGAAC
ACGGUAAAACCAUGACCGAGAAGGAGAUCGUGGACUAUGUGGCCAGCCAG
GUUACAACCGCCAAGAAGCUGCGCGGUGGUGUUGUGUUCGUGGACGAGGU
GCCUAAAGGACUGACCGGCAAGUUGGACGCCCGCAAGAUCCGCGAGAUUC
UCAUUAAGGCCAAGAAGGGCGGCAAGAUCGCCGUGUAA

2. Synthetic Procedures for Ionizable Lipids and all Synthetic Intermediates

2.1. Carboxybenzyl Protected Diketopiperazine Synthesis

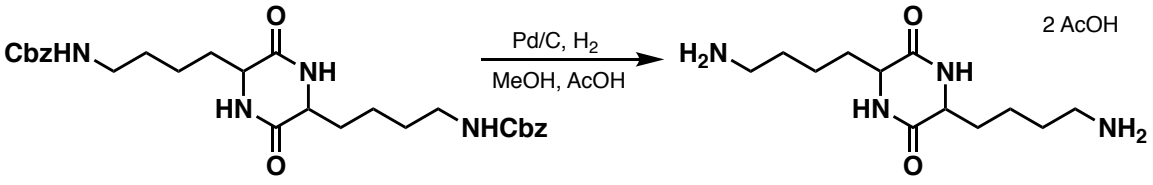


Cbz = Carboxybenzyl; Boc = tert-butyloxycarbonyl; Su = N-succinimidyl ester

The triply-protected lysine derivative (10.47 mmol, 1 equiv) was suspended in CH_2Cl_2 (15 mL*)*. To this suspension was added trifluoroacetic acid (15 mL). The reaction was stirred for 30 minutes after which point it was concentrated to afford a yellow oil. The oil was then resuspended in pyridine (200 mL) and was stirred for 24 hours. The reaction was then concentrated to dryness, was resuspended in a minimal amount of CH_2Cl_2, and then EtOAc was added to the flask to precipitate a white solid. The white solid was collected using vacuum filtration and was concentrated to dryness under reduced pressure to afford the desired caboxybenzyl proteted bis-lysine diketopiperazine product (55% yield).

Carboxybenzyl protected bis-lysine diketopiperazine: ^{1}H NMR (500 MHz, DMSO, ppm): 8.10 (s, 2H, N*H*), 7.33-7.37 (m, 8H, aromatic), 7.22 (t, 2H, aromatic), 3.79 (br, 2H, COC*H*), 2.97 (dd, 4H, NCH_2), 1.61-1.68 (m, 4H, CH_2), 1.29-1.40 (m, 8H, CH_2CH_2)

2.2. Bis-lysine Diketopiperazine Synthesis

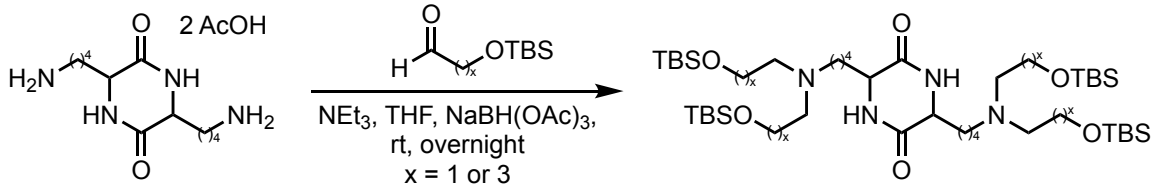


The carboxybenzyl protected bislysine diketopiperazine (5.55 mmol, 1 equiv) was suspended in a 1:1 mixture of degassed methanol and acetic acid (0.03 *M*). Palladium on carbon (25 mol% of 10% weight loaded Pd/C) was then added to the suspension. The flask was fitted with a H_2 balloon and the needle was suspended directly above the solution after briefly bubbling H_2 gas through the solution. The reaction was stirred

overnight, was filtered through celite, and filtrate was concentrated under reduced pressure. The resultant goo was resuspended in a minimal amount of CH_2Cl_2 and was washed with EtOAc to crash out a white solid. The white solid was isolated using vacuum filtration to afford the bis-lysine diketopiperazine (48% yield).

Bis-lysine diketopiperazine: ^{1}H NMR (500 MHz, D_2O, ppm): 4.14 (t, 2H, COCH), 2.99 (t, 4H, NCH_2), 1.84-1.88 (m, 4H, CH_2), 1.67-1.71 (m, 4H, CH_2), 1.39-1.52 (m, 4H, CH_2).

2.3. Reductive Amination General Protocols and Characterization

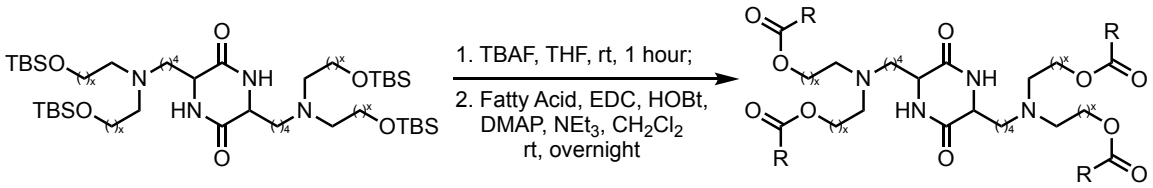


The bis-lysine diketopiperazine (2.66 mmol, 1 equiv) was dissolved in THF at 0.03 *M* with NEt_3 (5.85 mmol, *2*.2 equiv). This reaction mixture was stirred for 1.5 h until the solid was nearly fully dissolved. To this solution was then added the protected aldehyde (18.3 mmol, 6.9 equiv; x = 1 or 3) and $NaBH(OAc)_3$ (13.3 mmol, 5 equiv). The reaction was then stirred overnight at room temperature. The reaction was then diluted in EtOAc, washed with brine, filtered over Na_2SO_4, and concentrated under reduced pressure. The crude product mixture was purified using silica gel chromatography eluting with a 0→100% gradient of dichlormethane/methanol/ammonium hydroxide (75%/22%/3% v/v/v):dichloromethane. Fractions were concentrated under reduced pressure to afford the desired products (for x = 1, 58% yield; for x = 3, 41% yield).

For x = 1: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): 6.15 (br, 2H, N*H*), 3.99 (br, 2H, COC*H*N), 3.8 (t, 8H, NCH_2CH_2OSi), 2.65 (t, 8H, NCH_2CH_2OSi), 2.55 (t, 4 H, NCH_2), 1.99 (m, 2H, $COCHCH_2$), 1.8 (m, 2H, $COCHCH_2$), 1.45 (m, 4H, $NCH_2CH_2CH_2CH_2$), 1.40 (m, 4H, $NCH_2CH_2CH_2CH_2$), 0.89 (s, 36H, $OSi(CH_3)_2C(CH_3)_3$), 0.04 (s, 24H, $OSi(CH_3)_2C(CH_3)_3$)

For x = 3: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): 6.4 (br, 2H, N*H*), 3.95 (br, 2H, COC*H*N), 3.62 (t, 8H, CH_2OSi), 2.40 (m, 12H, NCH_2), 1.98 (m, 4H, $COCHCH_2$), 1.33-1.84 (m, 24H, CH_2), 0.89 (s, 36H, $OSi(CH_3)_2C(CH_3)_3$), 0.04 (s, 24H, $OSi(CH_3)_2C(CH_3)_3$)

2.4. Desilylation-Esterification General Protocols and Characterization



The appropriate reductive amination product (0.09 mmol, 1 equiv; x = 1 for **OF-Deg-C9**, **OF-Deg-C12**, **OF-Deg-C15**, **OF-Deg-C18**, **OF-Deg-Ole**, **OF-Deg-Ela**, and **OF-Deg-Lin**; x = 3 for **OF-C4-Deg-Lin**) was dissolved in THF at 0.015 *M*. To this solution was added tetrabutylammonium fluoride solution in THF (1.08 mmol, 12 equiv). After stirring at room temperature for 1 hour, the reaction was quenched with 1 drop of distilled H_2O. The reaction was then concentrated to dryness under reduced pressure. The remaining material in the flask was then dissolved in CH_2Cl_2 at 0.015 *M*. To this solution was added HOBt (0.9 mmol, 10 equiv), EDC (0.9 mmol, 10 equiv), the requisite carboxylic acid (0.9 mmol, 10 equiv), DMAP (0.09 mmol, 1 equiv), and NEt_3 (2.25 mmol, 25 equiv). The reaction was then stirred overnight and was then diluted with ethyl acetate. The organic phase was washed with 1 X 1 N HCl, 1 X saturated $NaHCO_3$, and then 1 X brine. The organic phase was dried over sodium sulfate, was filtered through cotton, and was then concentrated under reduced pressure. The crude product was purified using silica gel chromatography eluting with a 0→100% gradient of dichlormethane/methanol/ammonium hydroxide (75%/22%/3% v/v/v):dichloromethane. Fractions were concentrated under reduced pressure to afford the desired products in moderate yields (see **Figure 2** for percent yields).

OF-Deg-C9: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): 6.15 (br, 2H, N*H*), 4.1 (m, 8H, NCH_2CH_2OCO), 3.99 (br, 2H, $COCHN$), 2.73 (t, 8H, $OCOCH_2$), 2.53 (t, 4 H, NCH_2), 2.29 (t, 8H, NCH_2CH_2OCO), 1.96 (m, 2H, $COCHCH_2$), 1.78 (m, 2H, $COCHCH_2$), 1.61 (m, 4H, $NCH_2CH_2CH_2CH_2$), 1.47 (m, 4H, $NCH_2CH_2CH_2CH_2$), 1.1-1.35 (m, 48H, CH_2), 0.88 (t, 12H, CH_3)

OF-Deg-C12: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): 6.12 (br, 2H, N*H*), 4.11 (m, 8H, NCH_2CH_2OCO), 3.97 (br, 2H, $COCHN$), 2.75 (t, 8H, $OCOCH_2$), 2.54 (t, 4 H, NCH_2), 2.29 (t, 8H, NCH_2CH_2OCO), 1.97 (m, 2H, $COCHCH_2$), 1.77 (m, 2H, $COCHCH_2$), 1.61 (m, 4H, $NCH_2CH_2CH_2CH_2$), 1.45 (m, 4H, $NCH_2CH_2CH_2CH_2$), 1.1-1.17 (m, 72H, CH_2), 0.9 (t, 12H, CH_3)

OF-Deg-C15: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): 6.17 (br, 2H, N*H*), 4.11 (m, 8H, NCH_2CH_2OCO), 3.97 (br, 2H, $COCHN$), 2.74 (t, 8H, $OCOCH_2$), 2.53 (t, 4 H, NCH_2), 2.29 (t, 8H, NCH_2CH_2OCO), 1.97 (m, 2H, $COCHCH_2$), 1.76 (m, 2H, $COCHCH_2$), 1.59 (m, 4H, $NCH_2CH_2CH_2CH_2$), 1.45 (m, 4H, $NCH_2CH_2CH_2CH_2$), 1.17-1.34 (m, 96H, CH_2), 0.9 (t, 12H, CH_3)

OF-Deg-C18: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): 6.14 (br, 2H, N*H*), 4.11 (m, 8H, NCH_2CH_2OCO), 3.97 (br, 2H, $COCHN$), 2.73 (t, 8H, $OCOCH_2$), 2.53 (t, 4 H, NCH_2),

2.29 (t, 8H, NC*H*$_2$CH$_2$OCO), 1.97 (m, 2H, COCHC*H*$_2$), 1.76 (m, 2H, COCHC*H*$_2$), 1.58 (m, 4H, NCH$_2$C*H*$_2$CH$_2$CH$_2$), 1.45 (m, 4H, NCH$_2$CH$_2$C*H*$_2$CH$_2$), 1.17-1.34 (m, 120H, C*H*$_2$), 0.9 (t, 12H, C*H*$_3$)

OF-Deg-Ole: ^{1}H NMR (500 MHz, CDCl$_3$, ppm): 6.13 (br, 2H, N*H*), 5.34 (m, 8H, CH$_2$C*H*C*H*CH$_2$), 4.11 (m, 8H, NCH$_2$C*H*$_2$OCO), 3.98 (br, 2H, COC*H*N), 2.74 (t, 8H, OCOC*H*$_2$), 2.53 (t, 4H, NCH$_2$), 2.29 (t, 8H, NC*H*$_2$CH$_2$OCO), 2.00 (m, 16H, C*H*$_2$CHCHC*H*$_2$), 1.94 (m, 2H, COCHC*H*$_2$), 1.77 (m, 2H, COCHC*H*$_2$), 1.61 (m, 4H, NCH$_2$C*H*$_2$CH$_2$CH$_2$), 1.45 (m, 4H, NCH$_2$CH$_2$C*H*$_2$CH$_2$), 1.21-1.36 (m, 88H, C*H*$_2$), 0.9 (t, 12H, C*H*$_3$)

OF-Deg-Ela: ^{1}H NMR (500 MHz, CDCl$_3$, ppm): 6.13 (br, 2H, N*H*), 5.39 (m, 8H, CH$_2$C*H*C*H*CH$_2$), 4.10 (m, 8H, NCH$_2$C*H*$_2$OCO), 3.97 (br, 2H, COC*H*N), 2.74 (t, 8H, OCOC*H*$_2$), 2.52 (t, 4H, NCH$_2$), 2.27 (t, 8H, NC*H*$_2$CH$_2$OCO), 1.95 (m, 16H, C*H*$_2$CHCHC*H*$_2$), 1.78 (m, 2H, COCHC*H*$_2$), 1.69 (m, 2H, COCHC*H*$_2$), 1.60 (m, 4H, NCH$_2$C*H*$_2$CH$_2$CH$_2$), 1.45 (m, 4H, NCH$_2$CH$_2$C*H*$_2$CH$_2$), 1.20-1.37 (m, 88H, C*H*$_2$), 0.87 (t, 12H, C*H*$_3$)

OF-Deg-Lin: ^{1}H NMR (500 MHz, CDCl3, ppm): 6.13 (br, 2H, N*H*), 5.39 (m, 16H, CH$_2$C*H*C*H*CH$_2$), 4.10 (m, 8H, NCH$_2$C*H*$_2$OCO), 3.97 (br, 2H, COC*H*N), 2.74 (m, 16H, NC*H*$_2$CH$_2$OCO and CH$_2$CHCHC*H*$_2$CHCHCH$_2$), 2.53 (t, 4H, NC*H*$_2$), 2.29 (t, 8H, OCOC*H*$_2$), 2.00 (m, 16H, C*H*$_2$CHCHC*H*$_2$CHCHC*H*$_2$), 1.98 (m, 2H, COCHCH$_2$), 1.77 (m, 2H, COCHC*H*$_2$), 1.61 (m, 8H, OCOCH$_2$C*H*$_2$), 1.45 (m, 8H, NCH$_2$C*H*$_2$CH$_2$CH$_2$ and NCH$_2$CH$_2$C*H*$_2$CH$_2$), 1.21- 1.36 (m, 56H, C*H*$_2$), 0.9 (t, 12H, C*H*$_3$).

OF-C4-Deg-Lin: ^{1}H NMR (500 MHz, CDCl$_3$, ppm): 6.29 (br, 2H, N*H*), 5.35 (m, 16H, CH$_2$C*H*C*H*CH$_2$), 4.07 (m, 8H, CH$_2$C*H*$_2$OCO), 3.96 (br, 2H, COC*H*N), 2.77 (t, 8H, OCOC*H*$_2$), 2.44 (br, 12H, NC*H*$_2$), 2.29 (t, 8H, CHC*H*$_2$CH), 2.04 (m, 16H, C*H*$_2$CHCHCH$_2$CHCHC*H*$_2$), 1.96 (m, 2H, COCHC*H*$_2$), 1.77 (m, 2H, COCHC*H*$_2$), 1.22-1.68 (m, 88H, C*H*$_2$), 0.88 (t, 12H, C*H*$_3$)

3. General Lipid Nanoparticle Synthesis

An organic phase was prepared by solubilizing with ethanol a mixture of the ionizable lipid, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, Avanti), cholesterol (Sigma), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy-(polyethyleneglycol)-2000] (ammonium salt) (C14-PEG 2000, Avanti) at a molar ratio of 35:16:46.5:2.5. The ionizable lipid:nucleic acid weight ratio was 10:1 for all studied formulations. All ethanolic stock solutions were prepared at a concentration of 10 mg/mL. The aqueous phase was prepared in 10 mM citrate buffer (pH 3) with either Firefly luciferase mRNA (Shire) or anti-firefly siRNA. All nucleic acids were stored at -80 °C and were allowed to thaw on ice prior to use. The ethanol and aqueous phases were mixed at a 3:1 ratio in a microfluidic chip device using syringe pumps at a final mRNA concentration of 0.1 mg/mL. Resultant LNPs were dialyzed against 1X PBS in a 20,000 MWCO cassette at 4°C for 2 hours and were stored at 4°C prior to injection.

4. General Lipid Nanoparticle Characterization

To calculate the siRNA and mRNA encapsulation efficiencies, modified Quant-iT RiboGreen RNA assays (Invitrogen) were used. Briefly, RiboGreen fluorescence was compared in the presence and absence of 2% Triton X-100 in TE buffer. The fluorescence was quantified using a Tecan infinite M200 Pro. The diameter and polydispersity (PDI) of the LNPs were measured using dynamic light scattering (ZetaPALS, Brookhaven Instruments). LNP diameters are reported as the largest intensity mean peak average, which constituted >95% of the nanoparticles present in the sample.

5. In Vitro Screen and Dose Response With Anti-Luciferase siRNA

HeLa cells (American Type Culture Collection, Manasas, VA) expressing both Firefly and Renilla Luciferase were maintained at 37 ºC in high glucose Dulbecco's Modified Eagles Medium with phenol red (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). 12–24 h before transfection, cells were seeded in white 96-well plates at a density of 20,000 cells per well. Cells were transfected with a 50 ng dose of anti-Luc siRNA that had been formulated with each OF-Deg-XX lipid into LNPs as described above. After 24 hours, both the firefly and renilla luciferase levels were quantified using a Bright-Glo Luciferase Assay kit (Promega, Madison, WI) using a Tecan multiplate reader. Firefly luciferase levels were normalized to their renilla counterparts. Cell viability was assessed by measuring levels of Renilla Luciferase expression in the cells (**Figure S1**).

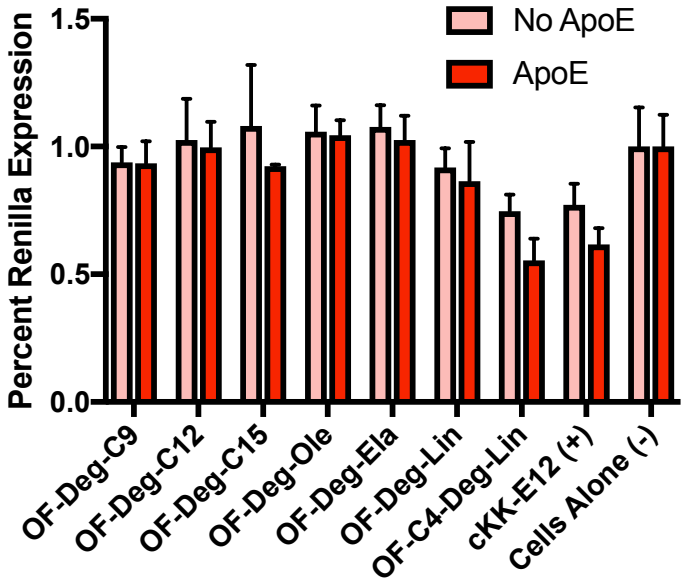


Figure S1: Percent renilla expression in cells treated with 50 ng doses of siRNA LNPs

6. In Vitro Screen and Dose Response With Luciferase mRNA

HeLa cells (American Type Culture Collection, Manassas, VA) were maintained at 37 ºC in high glucose Dulbecco's Modified Eagles Medium with phenol red (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). 12–24 h before transfection, cells were seeded in white 96-well plates at a density of 20,000 cells per well. Cells were transfected with a 50 ng dose of Luc mRNA that had been formulated into lipid nanoparticles with each OF-Deg-XX lipid as described above. After 24 hours, luciferase expression was quantified using a Bright-Glo Luciferase Assay kit (Promega, Madison, WI) using a Tecan multiplate reader. Expression levels were normalized to live cell signal. Cell viability was assessed using a MultiTox-Fluor multiplex cytotoxicity assay (Promega, Madison, WI; **Figure S2**).

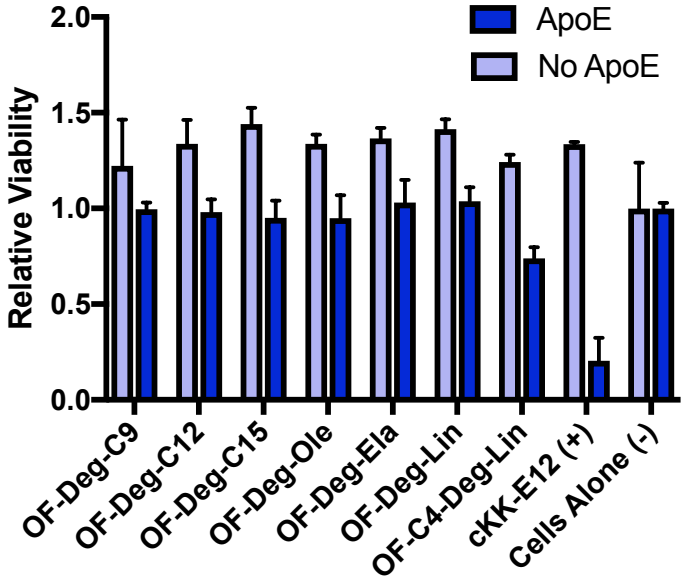


Figure S2: Cell viability for populations treated with 50 ng doses of mRNA LNPs

7. In Vivo Luciferase Expression/IVIS Imaging/Fluorescence Localization

All animal studies were approved by the M.I.T. Institutional Animal Care and use Committee and were consistent with local, state, and federal regulations as applicable. **OF-C4-Deg-Lin** Luc mRNA LNPs were injected via the tail vein of female C57BL/6 mice (Charles Riber Labs, 18-22 g) at a dose of 0.75 mg/kg. After 6 hours, the mice were injected intraperitoneally with 130 μL of D-Luciferin (30 mg/mL in PBS). After 15 minutes, mice were sacrificed and the organs were isolated (pancreas, spleen, liver, kidneys, lungs, heart, uterus, and ovaries) and imaged with an IVIS imaging system (Perkin Elmer, Waltham, MA). Luminescence was quantified using LivingImage software (Perkin Elmer). An identical process was used for positive control **cKK-E12** Luc mRNA LNPs, and Invivofectamine Luc LNPs were prepared according to manufacturer's instructions (ThermoFisher).

For fluorescence localization studies, **OF-C4-Deg-Lin** was formulated into LNPs in the same ratio and process as described above except the mRNA was a non-translating

Cy5 modified sequence. The resultant OF-C4-Deg-Lin Cy5 mRNA LNPs were injected at a 075 mg/kg dose via the tail vein of female C57Bl/6 mice. After 1 hour, the mice were sacrificed and organs were isolated (pancreas, spleen, liver, kidneys, lungs, heart) and imaged using an excitation wavelength of 640 nm and an emission wavelength of 680 nm. A PBS control was performed alongside and the resected organs from the PBS mice were also imaged.

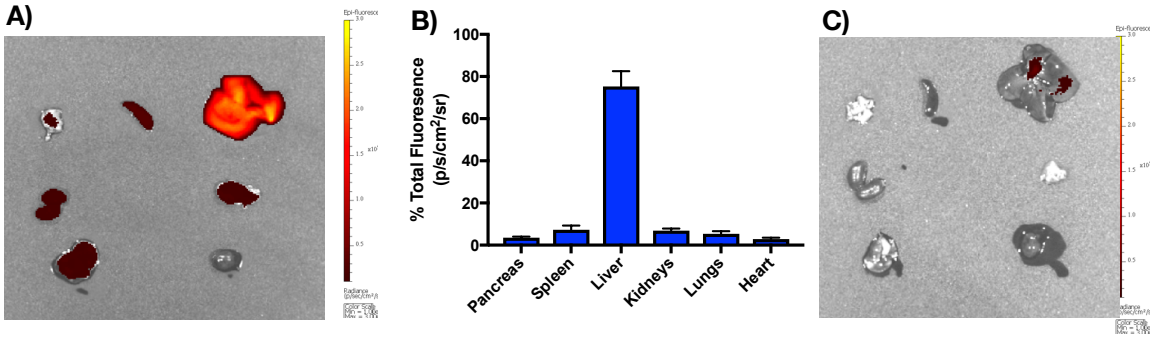


Figure S3: a) Representative fluorescence localization of a systemically administered 0.75 mg/kg dose of **OF-C4-Deg-Lin** Cy5 mRNA LNPs after 1 hour (n=3). b) Percent total fluorescence as a function of organ for data in Figure S3A. c) Representative fluorescence data for a PBS negative control.

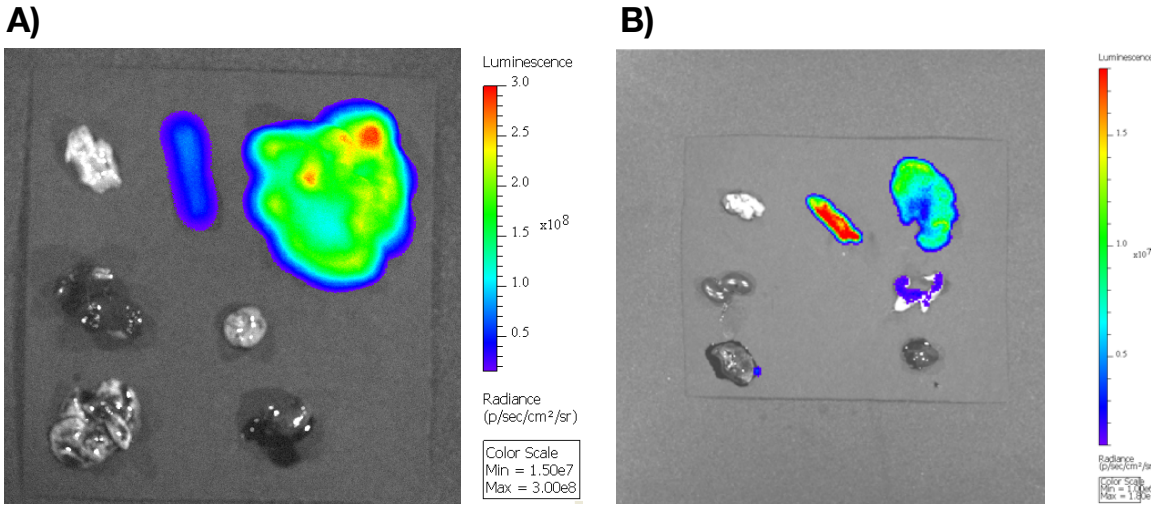


Figure S4: a) Representative *in vivo* expression profile of luciferase mRNA **ckk-E12** positive control LNPs in mice following luciferin injection and euthanasia at a 0.75 mg/kg intravenous dose (n=3) b) Representative *in vivo* expression profile of luciferase mRNA Invivofectamine positive control LNPs in mice following luciferin injection and euthanasia at a 0.75 mg/kg intravenous dose (n=3).