Supplementary Information

Selective ORgan Targeting (SORT) nanoparticles for tissue specific mRNA delivery and CRISPR/Cas gene editing

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Additional Supplementary Methods

Cell lines

Huh7 and A549 cells were originally obtained from ATTC and used without further authentication. Both cell lines were cultured in DMEM medium supplemented with 10% FBS and 50U/mL penicillin/streptomycin. All cells were maintained at 37 °C and 5% CO_2. These cell lines were not tested for mycoplasma contamination.

In vitro luciferase expression and cell viability tests

Huh-7 or A549 cells were seeded into white 96-well plates at a density of $1x10^4$ cells per well the day before transfection. The media was replaced by 150 µL fresh DMEM medium (5% FBS), then 50 µL Luc mRNA formulations were added with fixed 25 ng mRNA per well. After incubation for another 24h, ONE-Glo + Tox kits were used to detect mRNA expression and cytotoxicity based on Promega's standard protocol.

Animal experiments

All animal experiments were approved by the Institution Animal Care and Use Committees of The University of Texas Southwestern Medical Center and were consistent with local, state and federal regulations as applicable. C57BL/6 mice were obtained from the UTSW Mouse Breeding Core Facility. B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J mice (also known as Ai9 or Ai9(RCL-tdT) mice) were obtained from The Jackson Laboratory (007909) and bred to maintain homozygous expression of the Cre reporter allele that has a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent tdTomato protein. Following Cre-mediated recombination, Ai9 mice will express tdTomato fluorescence. Ai9 mice are congenic on the C57BL/6J genetic background.

Statistical analyses

Data for all bar charts were plotted via mean ± standard error of the mean (s.e.m.). Statistical analyses were performed using Prism 7 (GraphPad Software). A two-tailed unpaired t-test was used to determine the significance of the indicated comparisons. (*P < 0.05; **P<0.01; ***P < 0.001; ****P<0.0001).

Coding sequences for mouse IL-10, human EPO, mouse Klotho ECD, Cre recombinase, and Cas9 mRNAs:

Mouse IL-10-6Xhis

ATGCCTGGCTCAGCACTGCTATGCTGCCTGCTCTTACTGACTGGCATGAGGATCAGCAGG
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CAGGTGAAGAGTGATTTTAATAAGCTCCAAGACCAAGGTGTCTACAAGGCCATGAATGAA

TTTGACATCTTCATCAACTGCATAGAAGCATACATGATGATCAAAATGAAAAGCGGCGGC
TCTCACCACCACCATCACCACTAA

Human EPO

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Mouse Klotho ExtraCellular Domain (ECD)-6Xhis

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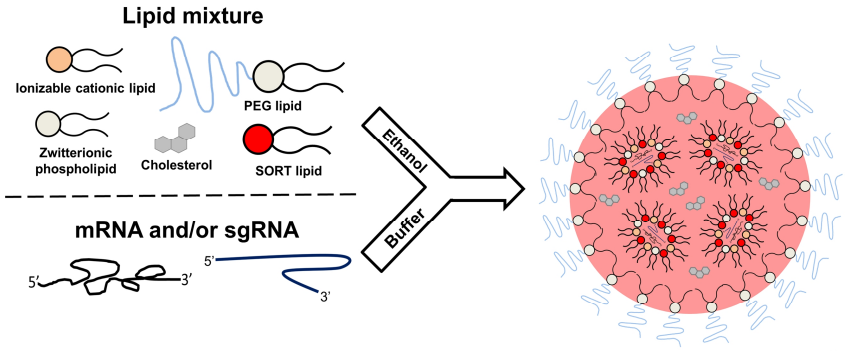
Supplementary Figures

a

Name	Molar Ratios					Molar Percentage (%)					Total Lipids/ mRNA (wt/wt)	Size (nm)	PDI	Zeta-Potential (mV)
	5A2-SC8	DOPE	Chol	DMG-PEG	DOTAP	5A2-SC8	DOPE	Chol	DMG-PEG	DOTAP				
0% (mDLNP)	15	15	30	3	0	23.81	23.81	47.62	4.76	0	40	93.2	0.14	-3.58
5%	15	15	30	3	3.315	22.62	22.62	45.24	4.52	5	40	156.3	0.16	-3.86
10%	15	15	30	3	7	21.43	21.43	42.86	4.29	10	40	152.8	0.10	-2.28
15%	15	15	30	3	11.12	20.24	20.24	40.47	4.05	15	40	123.0	0.11	-2.28
20%	15	15	30	3	15.75	19.05	19.05	38.10	3.81	20	40	113.0	0.18	-2.44
25%	15	15	30	3	21	17.86	17.86	35.71	3.57	25	40	108.8	0.21	-2.07
30%	15	15	30	3	27	16.67	16.67	33.33	3.33	30	40	101.6	0.18	-2.07
35%	15	15	30	3	33.93	15.48	15.48	30.95	3.10	35	40	104.0	0.23	-2.03
40%	15	15	30	3	42	14.29	14.29	28.57	2.86	40	40	108.0	0.27	-1.45
45%	15	15	30	3	51.54	13.10	13.10	26.19	2.62	45	40	115.1	0.19	-0.74
50%	15	15	30	3	63	11.90	11.90	23.81	2.38	50	40	113.1	0.22	-0.52
55%	15	15	30	3	77	10.71	10.71	21.43	2.14	55	40	108.3	0.19	0.13
60%	15	15	30	3	94.5	9.52	9.52	19.05	1.90	60	40	113.5	0.19	-0.94
65%	15	15	30	3	117	8.33	8.33	16.67	1.67	65	40	125.3	0.16	5.13
70%	15	15	30	3	147	7.14	7.14	14.29	1.43	70	40	115.8	0.17	3.11
75%	15	15	30	3	188.95	5.95	5.95	11.91	1.19	75	40	117.7	0.20	6.50
80%	15	15	30	3	252	4.76	4.76	9.52	0.95	80	40	129.7	0.15	6.86
85%	15	15	30	3	357	3.57	3.57	7.14	0.71	85	40	119.1	0.19	14.87
90%	15	15	30	3	567	2.38	2.38	4.76	0.48	90	40	137.6	0.21	15.97
95%	15	15	30	3	1198	1.19	1.19	2.38	0.24	95	40	124.5	0.22	14.50
100%	0	0	0	0	100	0	0	0	0	100	40	118.2	0.20	25.50

Name	5A2-SC8	DOPE	Chol	DMG-PEG	18PA	5A2-SC8	DOPE	Chol	DMG-PEG	18PA	Total Lipids/ mRNA (wt/wt)	Size (nm)	PDI	Zeta-Potential (mV)
5%	15	15	30	3	3.315	22.62	22.62	45.24	4.52	5	40	95.2	0.13	-1.88
10%	15	15	30	3	7	21.43	21.43	42.86	4.29	10	40	102.7	0.13	-1.55
20%	15	15	30	3	15.75	19.05	19.05	38.10	3.81	20	40	116.6	0.07	-1.74
30%	15	15	30	3	27	16.67	16.67	33.33	3.33	30	40	142.1	0.13	-2.11
40%	15	15	30	3	42	14.29	14.29	28.57	2.86	40	40	165.2	0.14	-5.57
50%	15	15	30	3	63	11.90	11.90	23.81	2.38	50	40	142.4	0.16	-3.07
60%	15	15	30	3	94.5	9.52	9.52	19.05	1.90	60	40	141.0	0.11	-4.60
100%	0	0	0	0	100	0	0	0	0	100	40	73.3	0.53	-38.87

b



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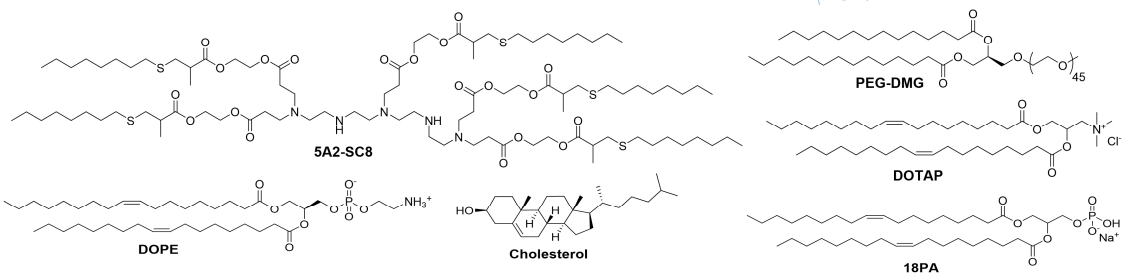
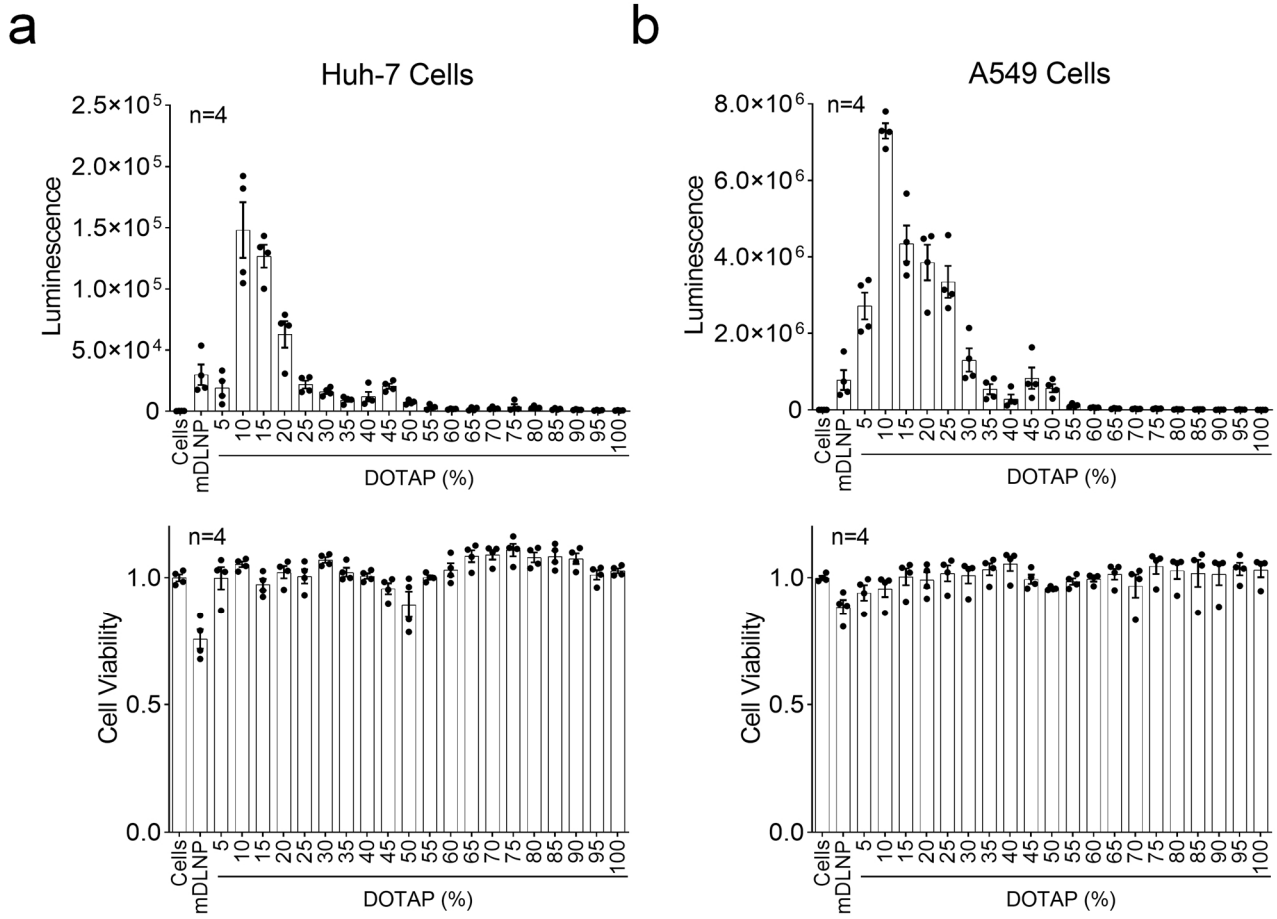


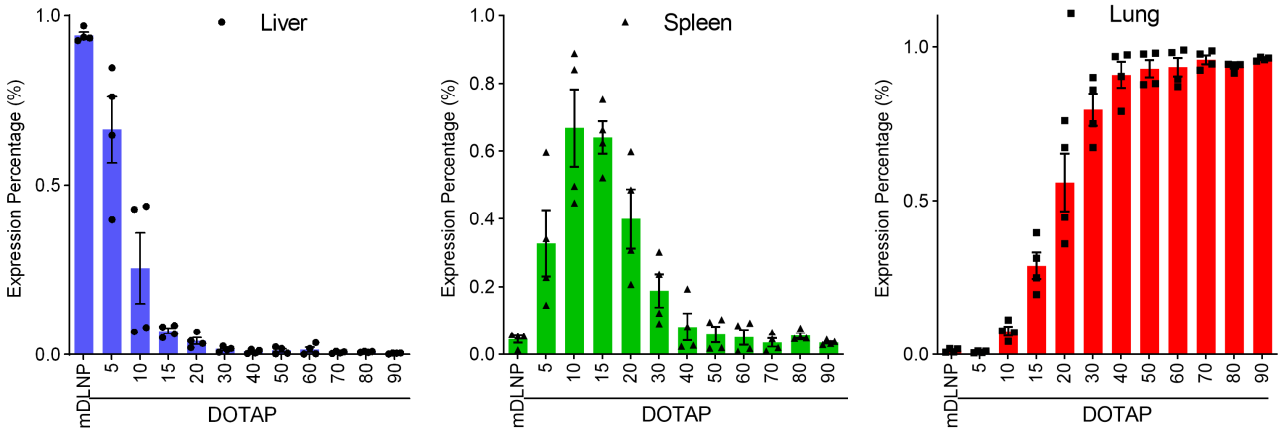
Supplementary Fig. 1 | RNA-loaded DOTAP and 18PA SORT LNPs were formed using the ethanol dilution. a, Details of DOTAP and 18PA SORT LNPs, including molar ratios, molar percentages, weight ratios of total lipid to mRNA, sizes, PDI and zeta potentials. **b**, LNPs were formulated using a modified

ethanol dilution method. SORT lipids are included in the ethanol phase and sgRNA/mRNA are encapsulated during LNP formation. **c**, The chemical structures of lipids used in standard mDLNP and DOTAP / 18PA SORT formulations are shown. For the development of SORT, we focused on a degradable dendrimer-based ionizable cationic lipid named 5A2-SC8 used in LNPs that can deliver siRNAs/miRNAs to extend survival in a genetically engineered mouse model of *MYC*-driven liver cancer [1-3] and toggle polyploidy in the liver. We further focused on an LNP molar composition that was optimized for mRNA delivery to the liver named mDLNPs [4]. This liver-targeted base mRNA formulation of 5A2-SC8 / DOPE / Cholesterol / DMG-PEG2000 = 15/15/30/3 (mol) were prepared and supplemented with SORT lipids to prepare SORT LNPs (details in **a**). For the sake of further clarity, we note that traditional 4-component LNPs are composed of ionizable cationic lipids (herein defined as containing an amino group with pKa <8), zwitterionic phospholipids (defined as a lipid bearing equal number of positive and negative charges), cholesterol, and poly(ethylene glycol) (PEG) lipids (most commonly, PEG2000-DMG). SORT LNPs include a 5th lipid, such as a permanently cationic lipid (defined as positively charged without pKa or pKa >8) or a permanently anionic lipid (defined as negatively charged).

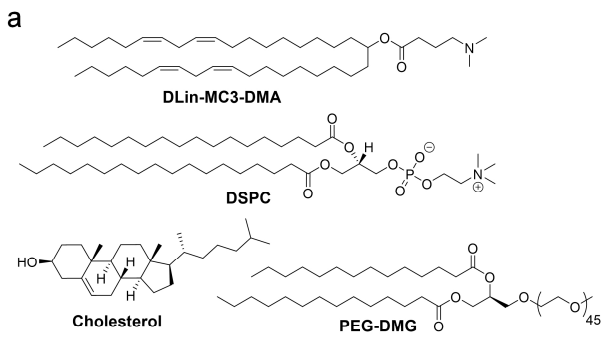


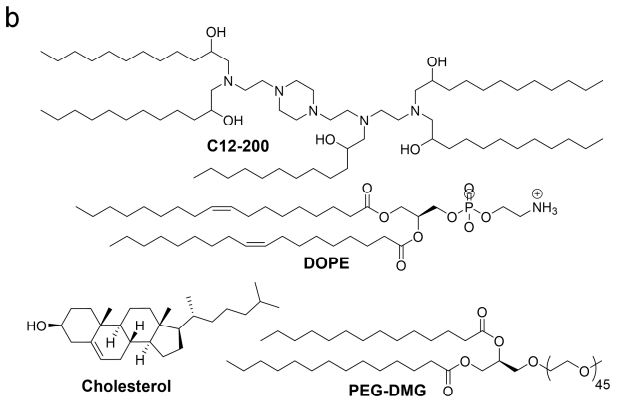
Supplementary Fig. 2 | DOTAP SORT LNPs effectively delivered Luc mRNA into cells. *In vitro* Luciferase (Luc) mRNA delivery results for DOTAP-modified SORT mDLNPs in (**a**) Huh-7 liver cells and (**b**) A549 lung cells as a function of the incorporated DOTAP percentage. Luc mRNA delivery results showed that LNPs with DOTAP percentages of 5%-50% delivered the most mRNA in both Huh-7 liver cells and A549 lung cells. SORT LNPs with 10% DOTAP were much more efficacious in vitro

than the previously reported base mDLNP. No appreciable cytotoxicity was observed for any formulation and all were uniform (low PDI) with diameters ranging from 90 nm to 150 nm (Supplementary Fig. 1). Measurements of surface charge revealed that DOTAP was encapsulated inside together with mRNA and not on the LNP surface as the zeta potentials were close to 0 when DOTAP was less than 60%. The surface charge became positive only at percentages above 65% (Supplementary Fig. 1), revealing that PEG lipid-coated SORT LNPs with selective tissue tropism could be discovered that possess a near neutral surface charge, which is an important attribute for clinical translation. Cells were seeded into 96-well plates at a density of $1X10^4$ cells per well the day before transfection. Luc mRNA expression and cell viability were measured at 24h post treatment with the dose of 50 ng/well Luc mRNA. Data was presented as mean±s.e.m. (n=4 biologically independent samples).



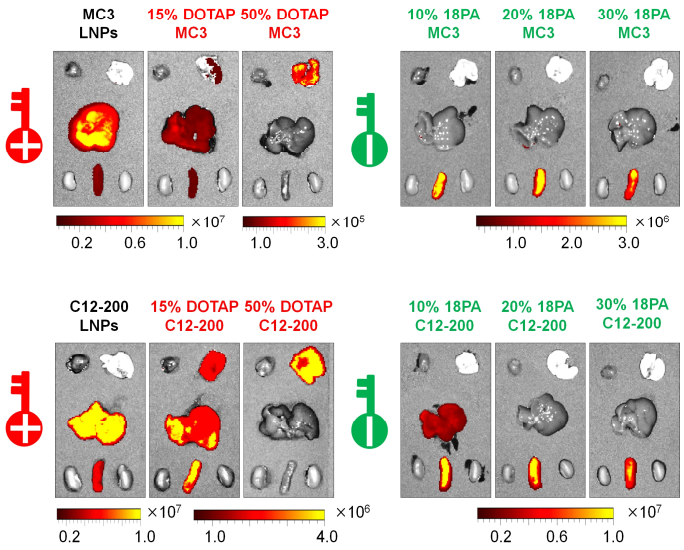
Supplementary Fig. 3 | Results showing relative luciferase expression in each organ demonstrated that fractional expression could be predictably tuned. 0.1 mg/kg Luc mRNA, IV, 6h. Data are presented as mean±s.e.m. (n=4 biologically independent animals).



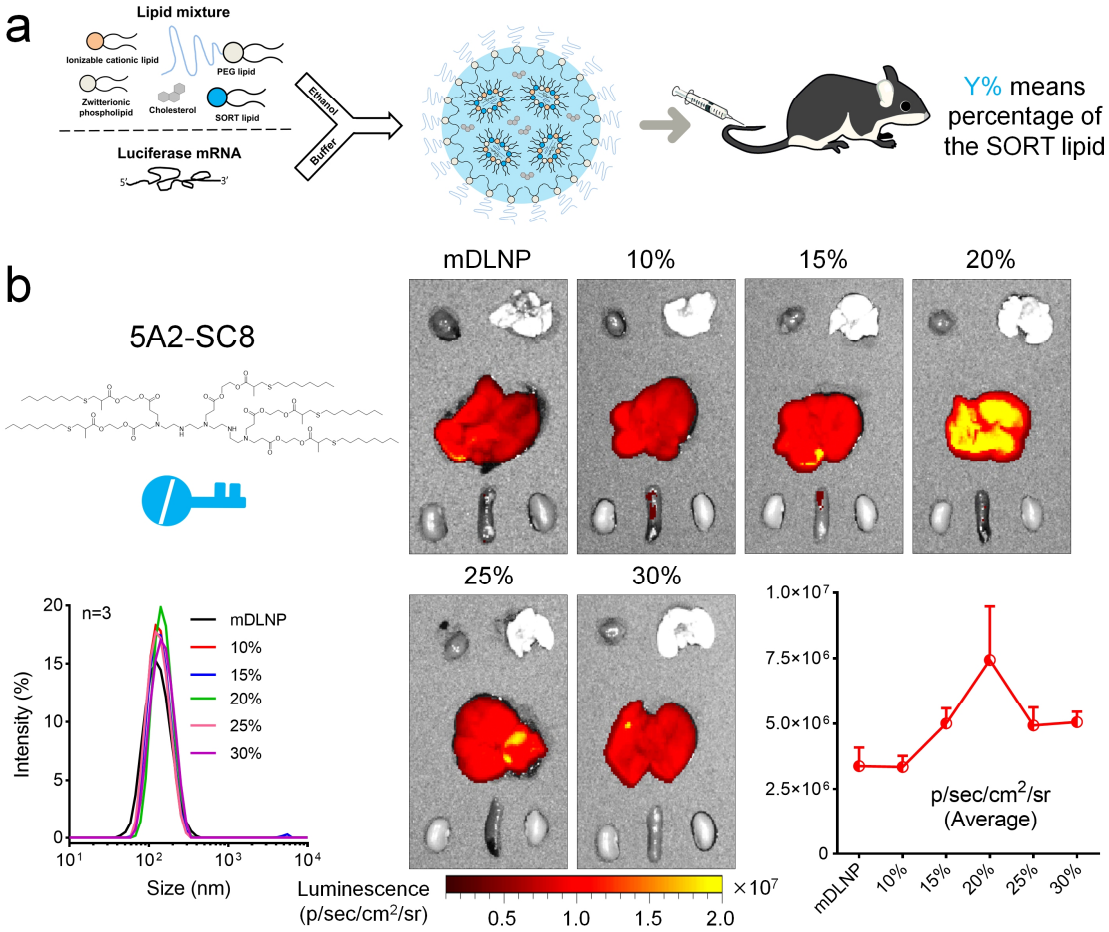


c

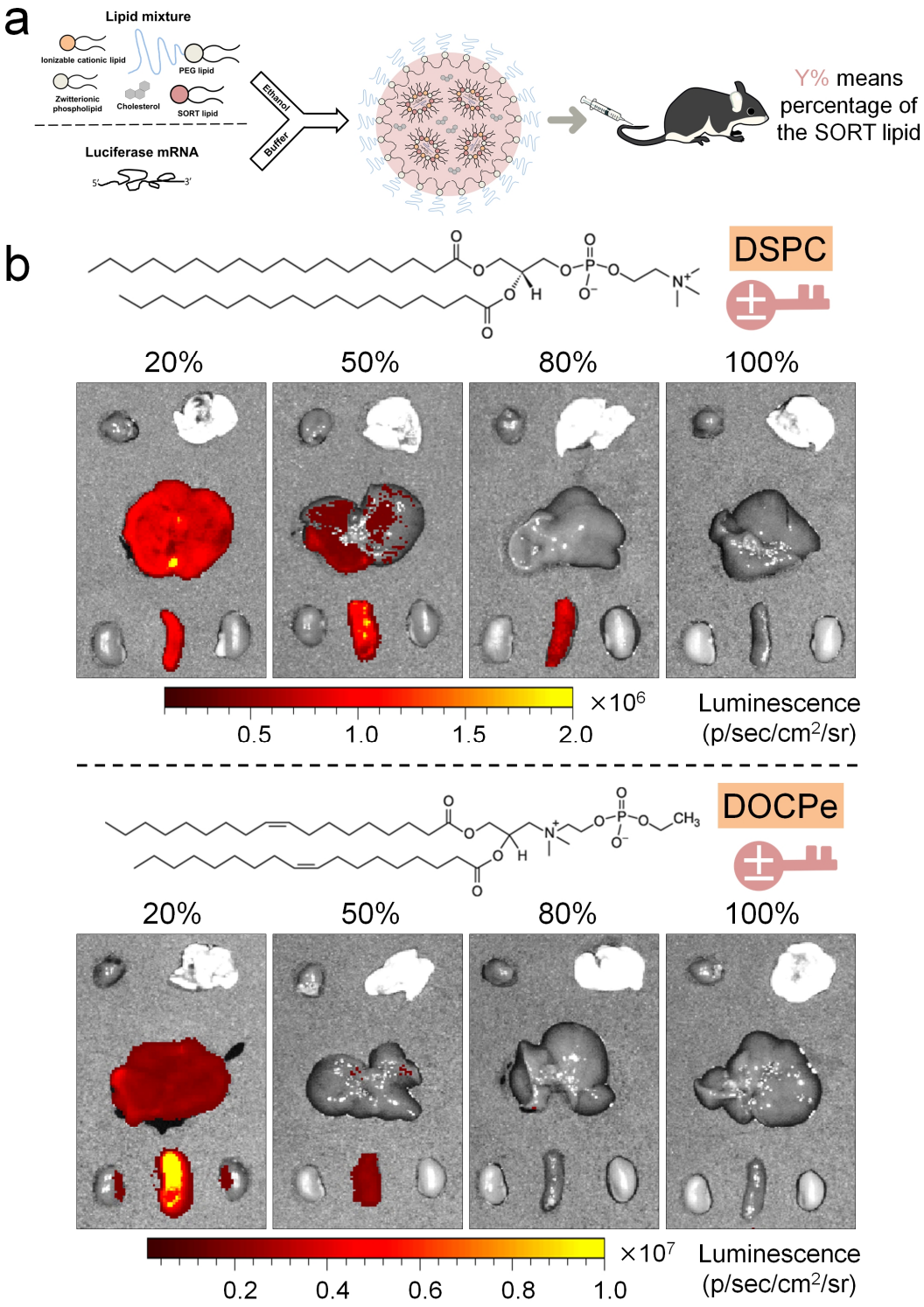
Molar % (Molar Ratios)					
DLin-MC3-DMA SORT LNPs					
Name	**DLin-MC3-DMA**	**DSPC**	**Chol**	**DMG-PEG**	**DOTAP (18PA)**
MC3 LNPs	50 (50)	10 (10)	38.5 (38.5)	1.5 (1.5)	**0 (0)**
15% DOTAP	42.5 (50)	8.5 (10)	32.7 (38.5)	1.3 (1.5)	**15 (17.65)**
10% 18PA	45 (50)	9 (10)	34.7 (38.5)	1.4 (1.5)	**10 (11.11)**
20% 18PA	40 (50)	8 (10)	30.8 (38.5)	1.2 (1.5)	**20 (25)**
C12-200 SORT LNPs					
Name	**C12-200**	**DOPE**	**Chol**	**DMG-PEG**	**DOTAP (18PA)**
C12-200 LNPs	35 (35)	16 (16)	46.5 (46.5)	2.5 (2.5)	**0 (0)**
15% DOTAP	29.8 (35)	13.6 (16)	39.5 (46.5)	2.1 (2.5)	**15 (17.65)**
10% 18PA	31.5 (35)	14.4 (16)	41.9 (46.5)	2.3 (2.5)	**10 (11.11)**
20% 18PA	28 (35)	12.8 (16)	37.2 (46.5)	2 (2.5)	**20 (25)**



Supplementary Fig. 4 | SORT was applied into MC3 LNPs and C12-200 LNPs to achieve tissue specific mRNA delivery *in vivo*. The chemical structures of lipids used in (**a**) DLin-MC3-DMA SNALPS [5] and (**b**) C12-200 LLNPs [6] are shown. Liver-targeted base mRNA formulations of DLin-MC3-DMA / DSPC / Cholesterol / DMG-PEG2000 = 50/10/38.5/1.5 (mol) and C12-200 / DOPE / Cholesterol / DMG-PEG2000 = 35/16/46.5/2.5 (mol) were prepared and later supplemented with SORT lipids to prepare SORT LNPs. (**c**) Table and results of additional SORT formulations using DLin-MC3-DMA and C12-200. The weight ratio of total lipids / mRNA was 20/1 (wt/wt) for all DLin-MC3-DMA and C12-200 LNPs. Note that some data from Fig. 1f have been reproduced here to show the Luciferase expression trend as a percentage of included SORT lipid (n=3 biologically independent animals).



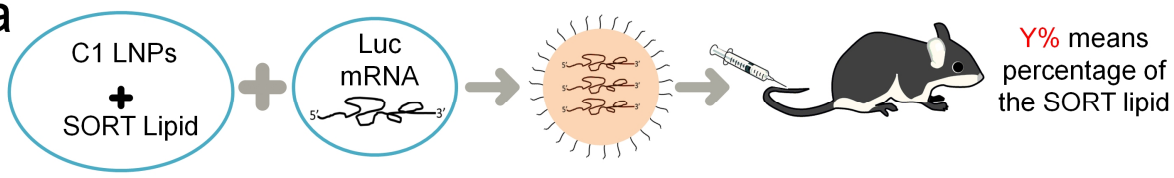
Supplementary Fig. 5 | SORT was applied to utilize ionizable cationic lipids as SORT lipids to further enhance mDLNP liver delivery. a, Schematic illustration of SORT. **b**, 5A2-SC8 was used as a SORT lipid, essentially supplementing base the mRNA mDLNP formulation (5A2-SC8 / DOPE / Cholesterol / DMG-PEG2000 = 15/15/30/3 (mol)) with additional 5A2-SC8 using the SORT method. *Ex vivo* luciferase images and quantified data showed that mRNA delivery potency was dramatically improved when an extra 15% - 25% SORT lipid was added. Maximal expression was produced with 20% incorporation (0.05 mg/kg, 6h). SORT thus allowed development of a 2nd generation mDLNP with increased efficacy. Data are presented as mean±s.e.m. (n=4 biologically independent animals).



Supplementary Fig. 6 | Zwitterionic SORT lipids improved mRNA spleen targeting delivery. Inclusion of zwitterionic SORT lipids into liver-targeted mDLNPs altered expression from the liver to the spleen with increasing incorporation of the SORT lipid. 80% DSPC and 50% DOCPe SORT LNPs delivered mRNA exclusively to the spleen after IV injection. **a**, Schematic illustration of SORT method. **b**, *Ex vivo* images of luminescence in major organs at 6h post IV injection. DSPC and DOCPe,

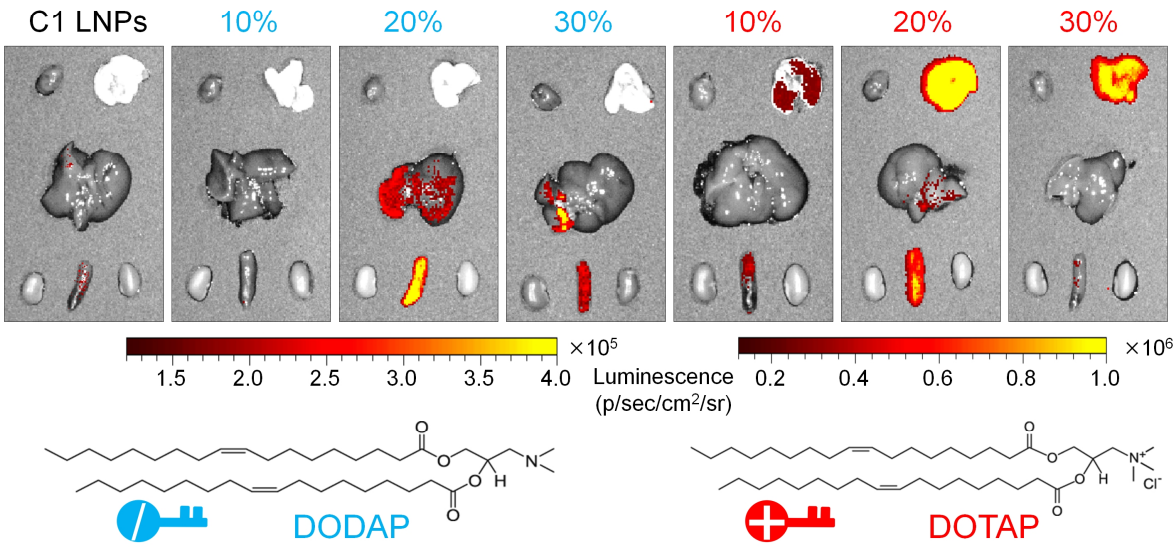
zwitterionic lipids with different structures, improved Luc mRNA delivery into spleen with increased percentages (0.1 mg/kg, 6h, n=4 biologically independent animals).

a



b

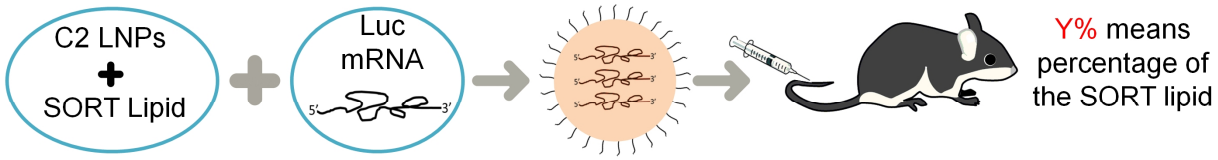
Name	Molar Ratios					Molar Percentage (%)					Lipids/ mRNA (wt/wt)	Size (nm)	PDI
	5A2-SC8	DSPC	Chol	DMG-PEG	DODAP (DOTAP)	5A2-SC8	DSPC	Chol	DMG-PEG	DODAP (DOTAP)			
C1 LNPs	10	60	26	4	0	10.0	60.0	26.0	4.0	0.0	40	181.4	0.31
10% DODAP C1	10	60	26	4	11.11	9.0	54.0	23.4	3.6	10.0	40	169.3	0.25
20% DODAP C1	10	60	26	4	25	8.0	48.0	20.8	3.2	20.0	40	155.1	0.23
30% DODAP C1	10	60	26	4	42.85	7.0	42.0	18.2	2.8	30.0	40	148.7	0.25
10% DOTAP C1	10	60	26	4	11.11	9.0	54.0	23.4	3.6	10.0	40	239.0	0.40
20% DOTAP C1	10	60	26	4	25	8.0	48.0	20.8	3.2	20.0	40	149.2	0.31
30% DOTAP C1	10	60	26	4	42.85	7.0	42.0	18.2	2.8	30.0	40	132.9	0.33



Supplementary Fig. 7 | SORT presented a potential strategy to "activate" inactive LNP formulations. a, Schematic illustration of supplementing an inactive C1 formulation with a SORT lipid to test if SORT can endow activity. **b**, The detailed information of C1 LNPs (inactive LNPs) and DOTAP

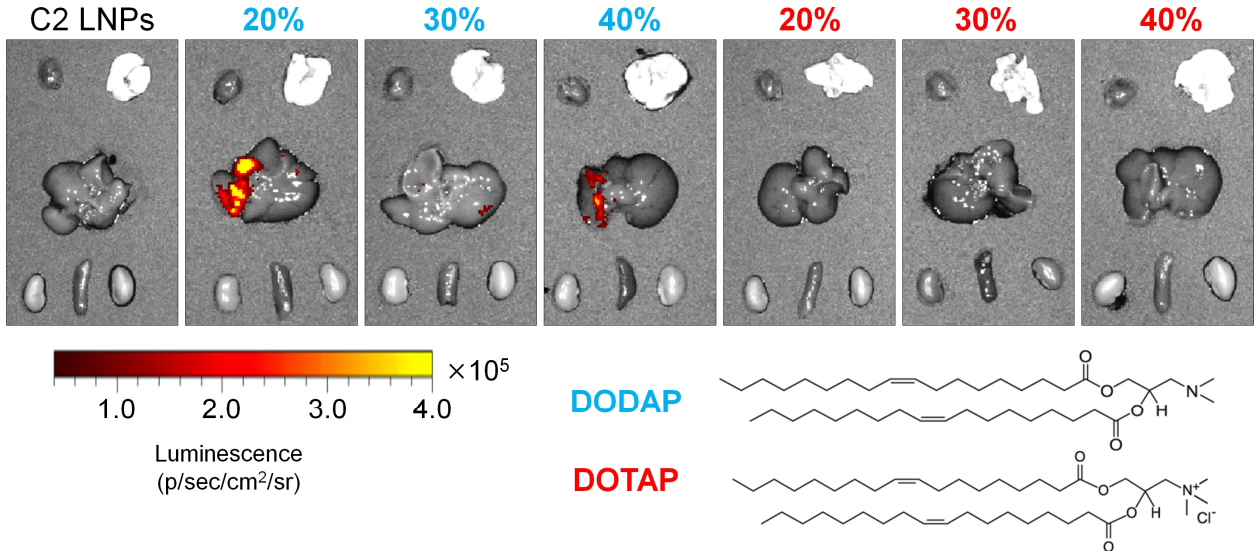
(or DODAP) C1 SORT LNPs, including lipid molar ratios, molar percentages, weight ratios of total lipids to mRNA, sizes, and PDI. C1 LNPs were prepared in a way that allowed for mRNA encapsulation and favorable biophysical properties (uniform <200 nm size). However, no protein expression at all resulted following IV injection of C1 LNPs. Thus, we asked if SORT could "activate" dead LNPs. DODAP and DOTAP SORT lipids were evaluated. DODAP@C1 LNPs delivered mRNA into spleen and liver, and DOTAP@C1 LNPs delivered mRNA into lung and spleen (0.1 mg/kg, 6h, n=4 biologically independent animals). Therefore, we show that SORT can activate dead LNPs and provide tissue selectivity.

a



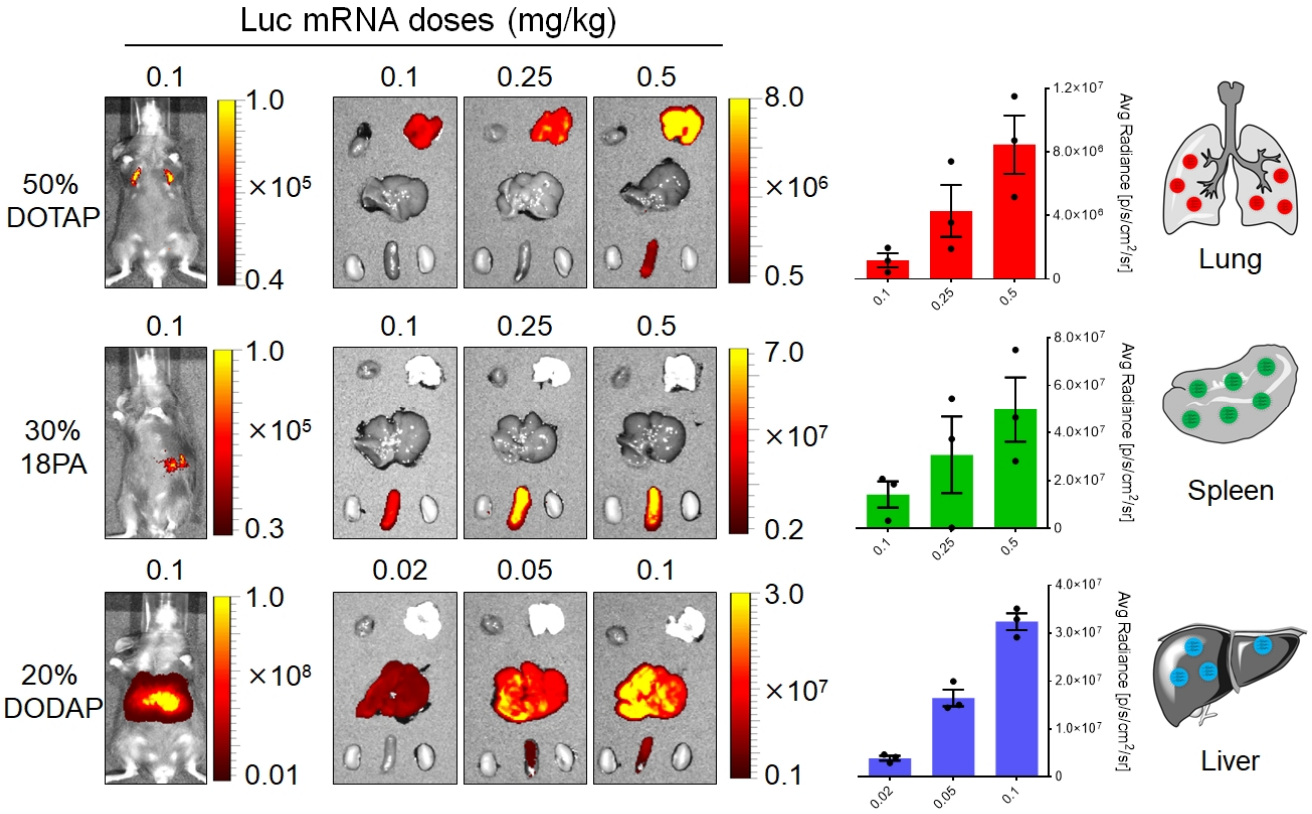
b

Name	Molar Ratios					Molar Percentage (%)					Total Lipids/ mRNA (wt/wt)	Size (nm)	PDI
	5A2 -SC8	DOPE	Chol	DMG-PEG	DODAP (DOTAP)	5A2 -SC8	DOPE	Chol	DMG-PEG	DODAP (DOTAP)			
C2 LNPs	0	15	30	3	0	0.00	31.25	62.50	6.25	0.00	40	85.5	0.14
20% DODAP C2	0	15	30	3	12	0.00	25.00	50.00	5.00	20.00	40	112.1	0.14
30% DODAP C2	0	15	30	3	20.57	0.00	21.88	43.75	4.38	30.00	40	122.9	0.14
40% DODAP C2	0	15	30	3	32	0.00	18.75	37.50	3.75	40.00	40	124.8	0.13
20% DOTAP C2	0	15	30	3	12	0.00	25.00	50.00	5.00	20.00	40	93.9	0.19
30% DOTAP C2	0	15	30	3	20.57	0.00	21.88	43.75	4.38	30.00	40	101.3	0.19
40% DOTAP C2	0	15	30	3	32	0.00	18.75	37.50	3.75	40.00	40	91.2	0.20

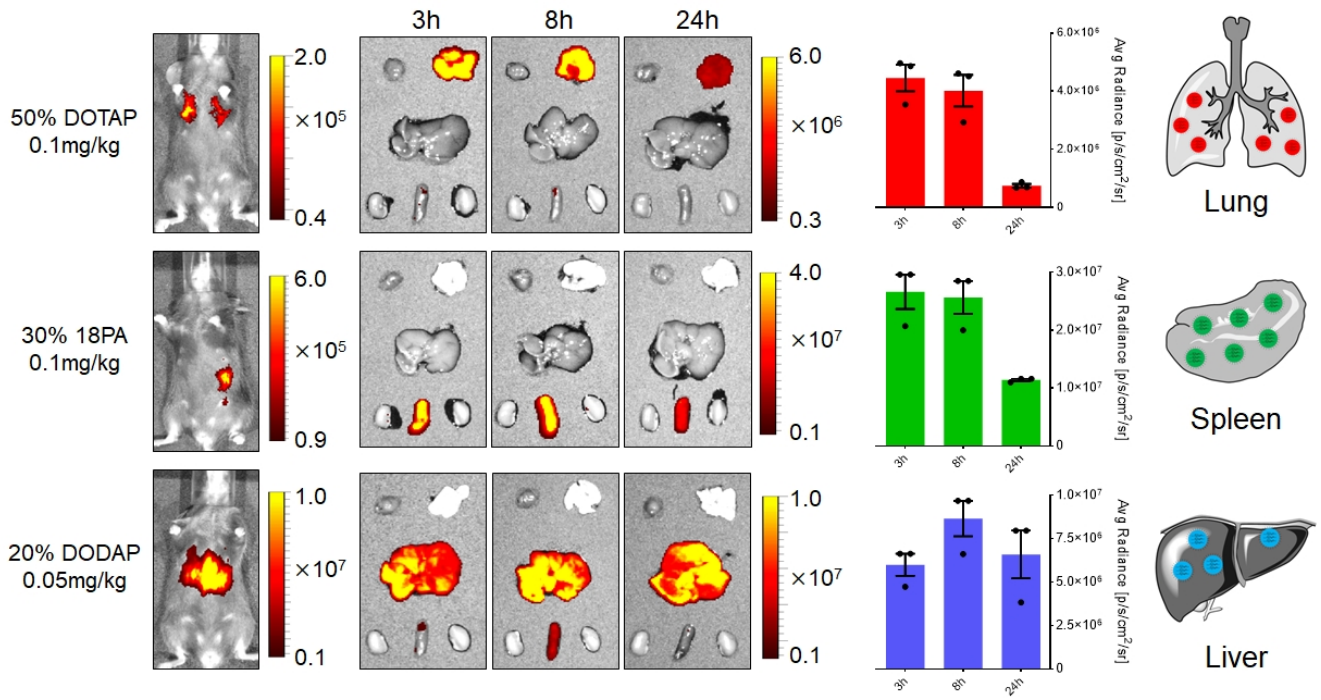


Supplementary Fig. 8 | Inclusion of an ionizable lipid (e.g. 5A2-SC8) was required for efficacy. LNPs that contained SORT lipids, but no ionizable cationic lipids were inactive. **a**, Schematic illustration

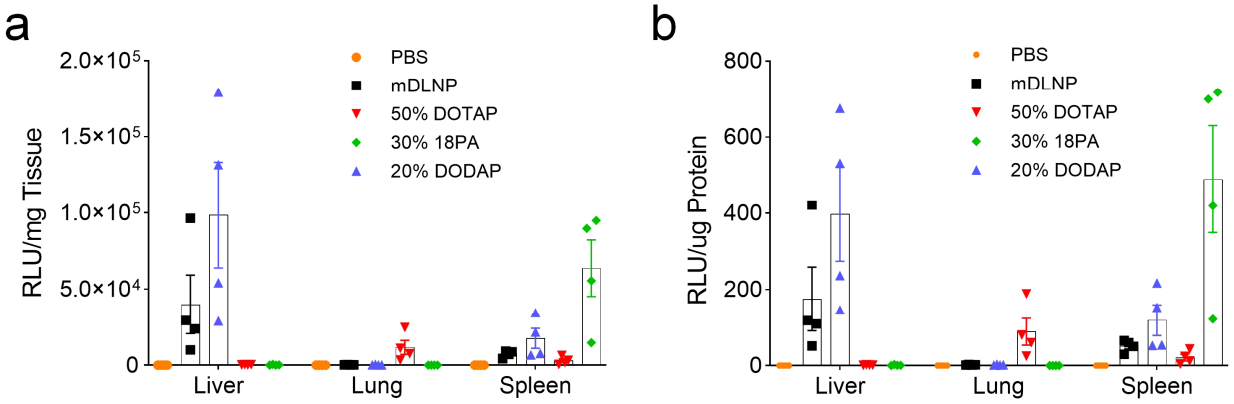
of SORT C2 LNPs. **b**, Details of C2 and SORT lipids C2 LNPs. *Ex vivo* luciferase images showed that both DODAP and DOTAP failed to enable significant mRNA delivery of C2 LNPs. These results indicate that the ionizable amino lipid is required for successful mRNA delivery (0.1 mg/kg, 6h, n=4 biologically independent animals).



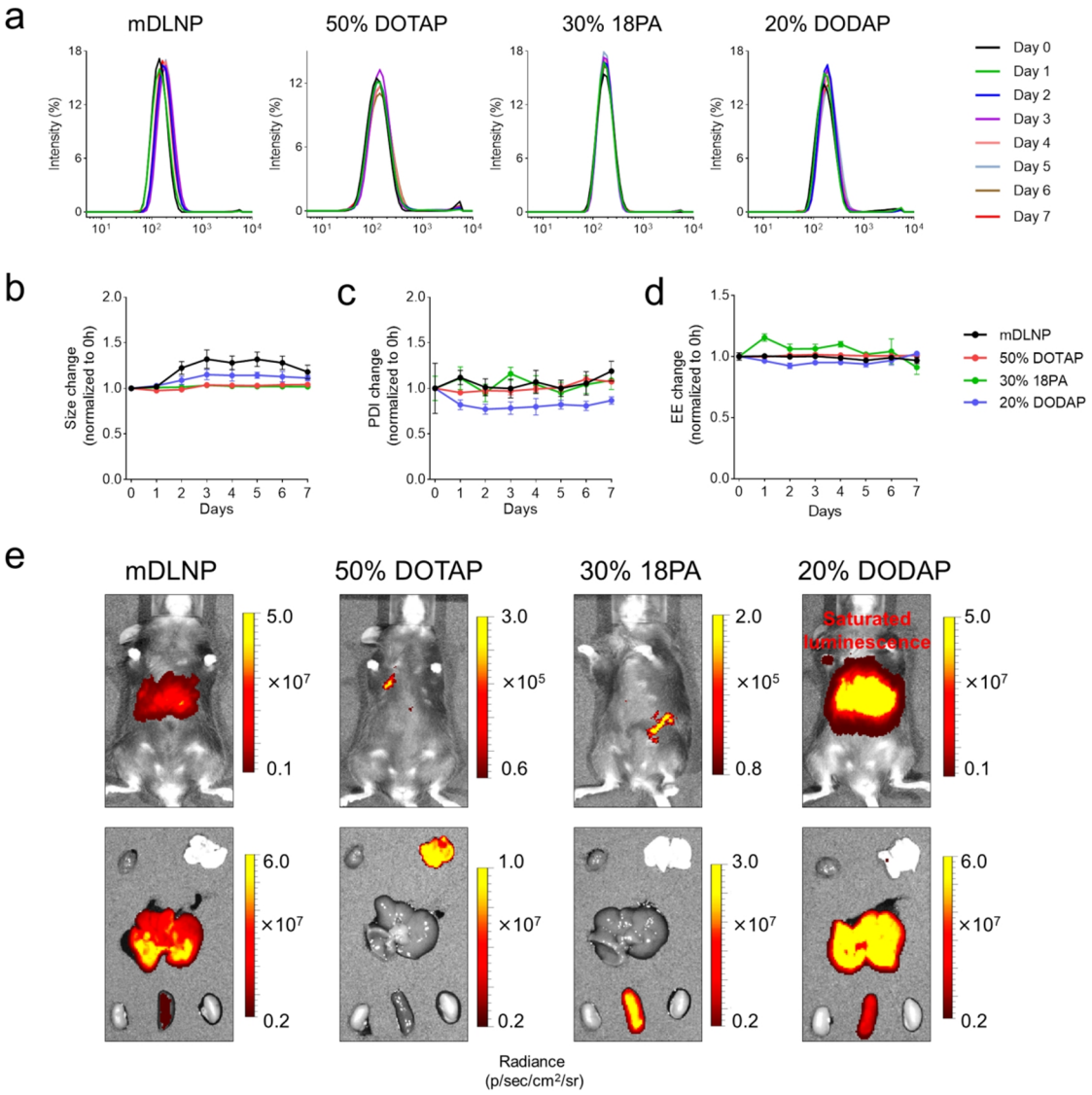
Supplementary Fig. 9 | SORT LNPs enabled dose-responsive Luc mRNA delivery efficacy in specific organs. Lung (50% DOTAP), spleen (30% 18PA) and liver (20% DODAP) SORT LNPs with various mRNA doses (0.02 to 0.5 mg/kg) were I.V. injected into mice, then whole body and organs were imaged at 6 hours. For all three formulations, Luciferase production is dose responsive. Notably, the organ specificity is precise and is the same at all doses. Data are presented as mean±s.e.m. (n=3 biologically independent animals).



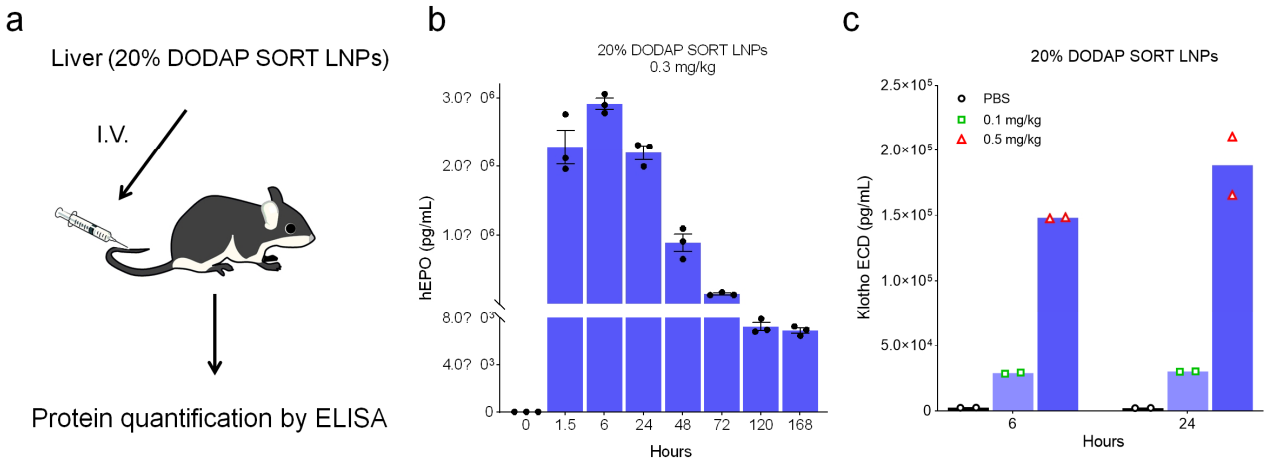
Supplementary Fig. 10 | Tissue specific mRNA delivery of SORT LNPs is not dependent on time. Lung (50% DOTAP, 0.1 mg/kg), spleen (30% 18PA, 0.1 mg/kg) and liver (20% DODAP, 0.05 mg/kg) SORT LNPs were I.V. injected into mice, then whole body and organs were imaged at 3h, 8h, and 24h. Luciferase production was occurs quickly and was maintained in specific organs over all three time-points, suggesting the tissue specific mRNA delivery by SORT LNPs is not time dependent. Data are presented as mean±s.e.m. (n=3 biologically independent animals).



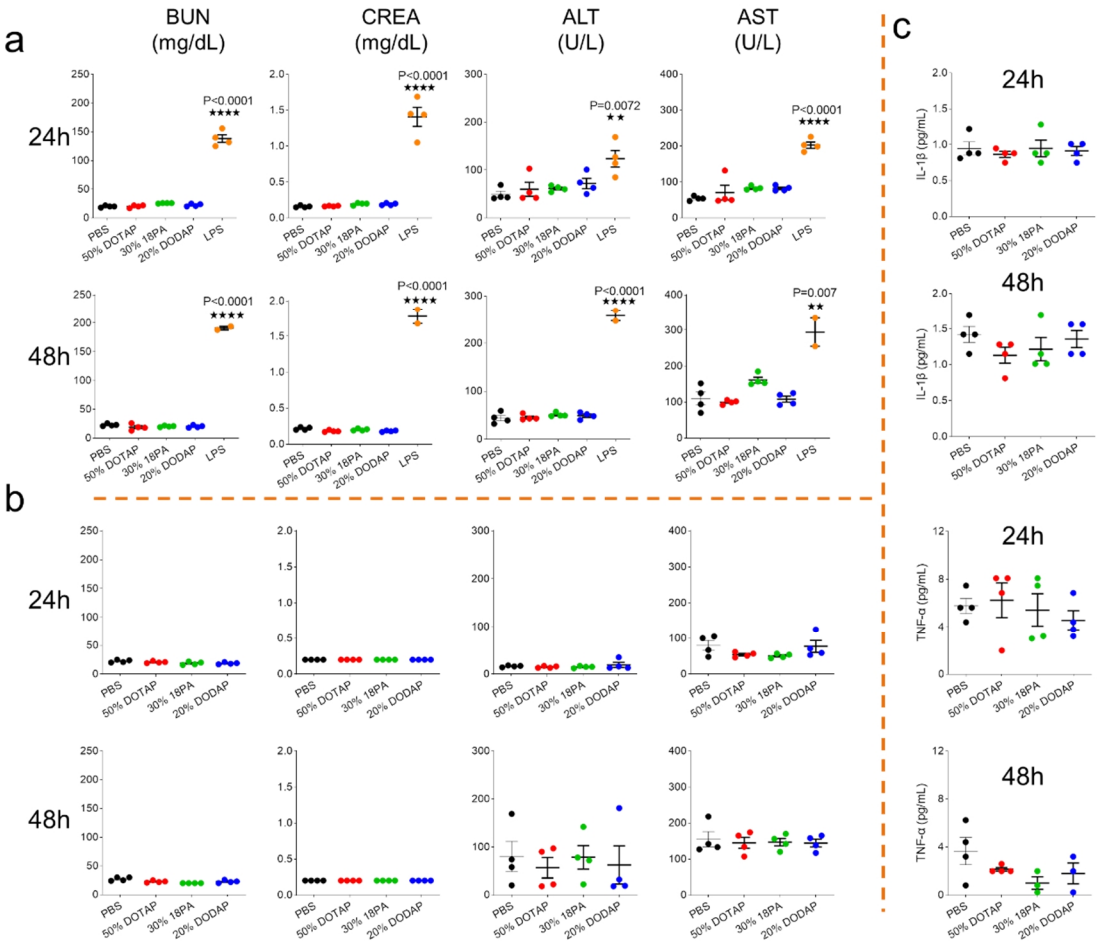
Supplementary Fig. 11 | SORT LNPs enabled Luc mRNA delivery efficacy in specific organs. Luminescence expression was normalized to (**a**) weight of tissue and (**b**) total protein. Base mDLNP, lung (50% DOTAP), spleen (30% 18PA) and liver (20% DODAP) SORT LNPs were injected IV into mice (0.1 mg/kg mRNA). After 6 hours, tissues were collected and homogenized for luminescence detection. Relative luciferase expression was calculated based on weight of tissue and total protein. Data are presented as mean±s.e.m. (n=4 biologically independent animals).



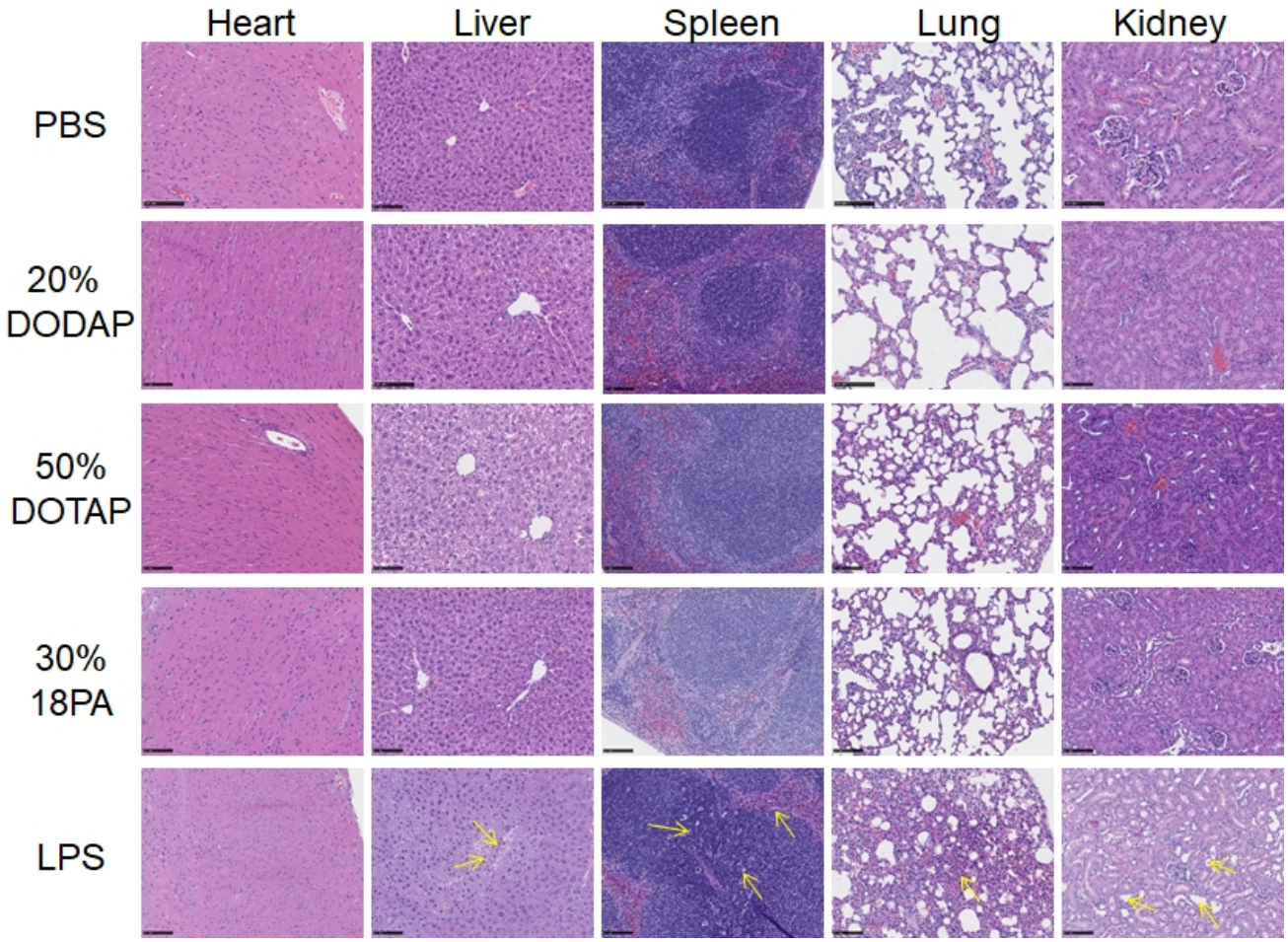
Supplementary Fig. 12| SORT LNPs maintained stability and delivery efficacy after storage. Base mDLNPs, lung SORT LNPs (50% DOTAP), spleen SORT LNPs (30% 18PA), and liver SORT LNPs (20% DODAP) were formed and stored at 4 °C. Using dynamic light scattering (DLS) and the Ribogreen assay, size (**a**, **b**), PDI (**c**), and encapsulation efficiency (EE) (**d**) were monitored every day over one week. (**e**) Following storage for 7 days at 4 °C, LNPs were administer intravenously (IV) to C57BL/6 mice to evaluate *in vivo* Luc mRNA delivery efficacy (0.1 mg/kg Luc mRNA, n=3 biologically independent animals). Whole body and organs were imaged by IVIS at the 6 hour time point. SORT LNPs maintained mRNA delivery efficacy following storage. The results of **a-d** were obtained from n=3 biologically independent samples. Data of **b-d** were presented as mean±s.e.m.



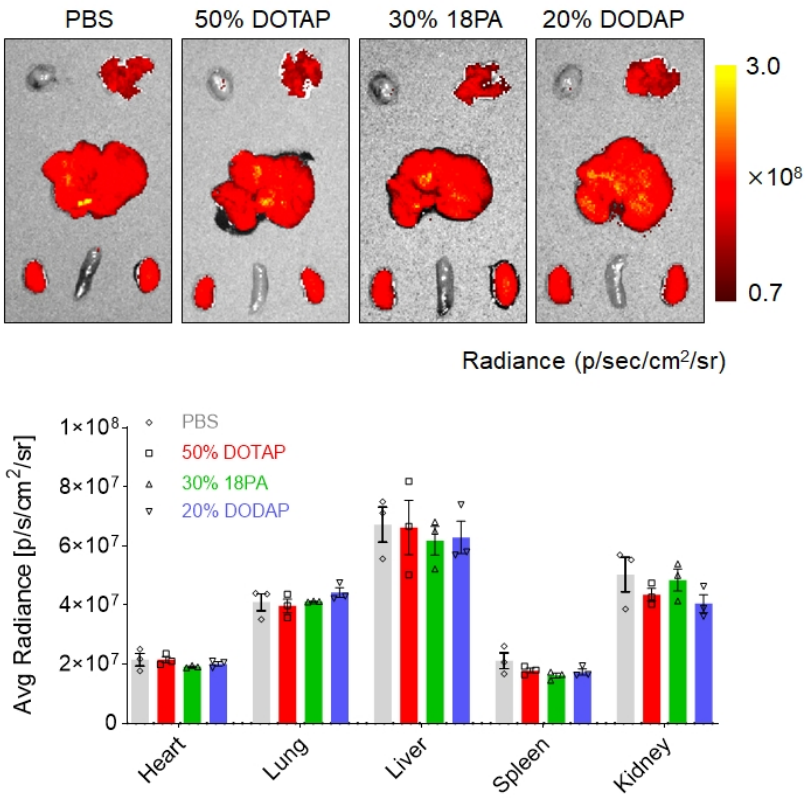
Supplementary Fig. 13 | 20% DODAP SORT LNPs effectively delivered hEPO mRNA and Klotho mRNA *in vivo*. a, Scheme for mRNA delivery of secreted proteins. **b**, Serum hEPO was quantified by ELISA following IV administration of 0.3 mg/kg hEPO mRNA in 20% DODAP SORT LNPs. High levels of hEPO expression persisted for > one week. Data were presented as mean±s.e.m. (n=3 biologically independent animals). **c**, Serum Klotho was quantified by ELISA in serum following IV administration of mouse Klotho mRNA (coding ECD) in liver-specific SORT LNPs (20% DODAP). High levels of Klotho expression were observed in 6h and 24h following administration of 0.1 and 0.5 mg/kg mRNA (n=2 biologically independent animals).



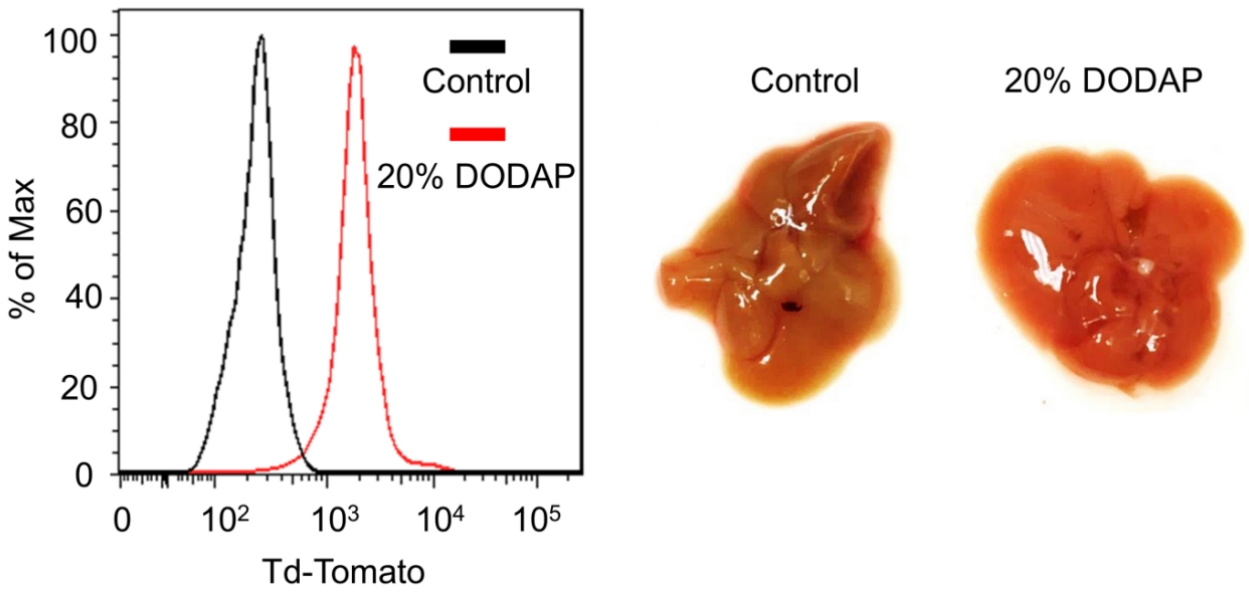
Supplementary Fig. 14 | mRNA-loaded lung-, spleen-, and liver-specific SORT LNPs were well-tolerated *in vivo*. Mice were IV administrated at an injected dose higher than needed for therapeutic benefit. Groups included SORT LNP formulations containing mCherry mRNA (1 mg/kg, IV), lipopolysaccharide (LPS, IP) as a positive control, and PBS (IV) as negative control. Mice were injected (**a**) once and (**b, c**) twice (day 0 and day 3). At 24 and 48 hours after the last injection, serum was separated, kidney function (BUN and CREA), liver function (AST and ALT), and serum cytokines (IL-1βand TNF-α) were evaluated. LPS injected animals exhibited severe kidney and liver injury at detected time-points. There were no significant differences between the SORT LNP and PBS groups. Data are presented as mean±s.e.m. (n=4 biologically independent animals). A two-tailed unpaired t-test was used to determine the significance of the indicated comparisons (*P < 0.05; **P<0.01; ***P < 0.001; ****P<0.0001).



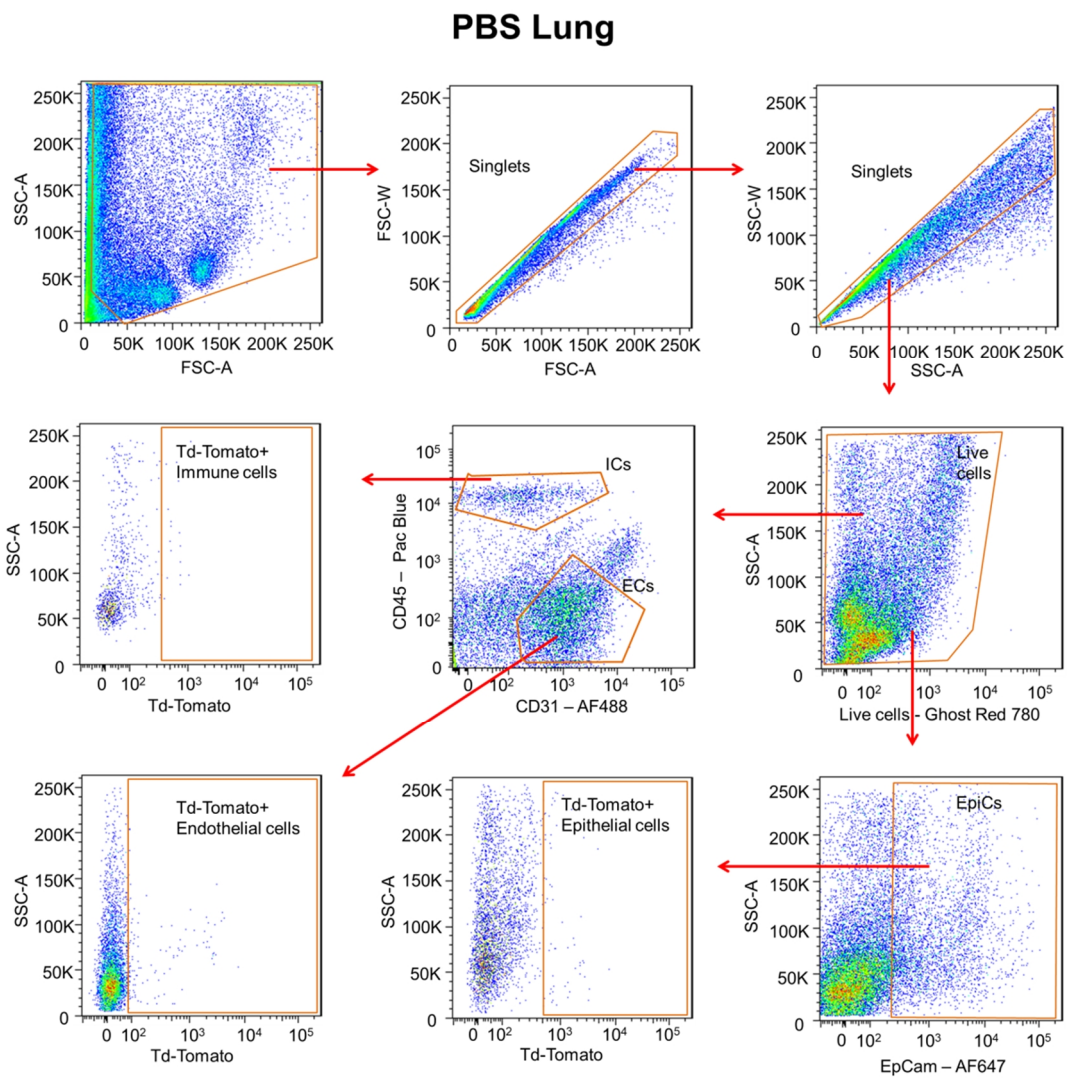
Supplementary Fig. 15 | mRNA-loaded lung-, spleen-, and liver-specific SORT LNPs were well-tolerated *in vivo*. Mice were IV administrated at an injected dose higher than needed for therapeutic benefit. Groups included SORT LNP formulations containing mCherry mRNA (1 mg/kg, IV, single dosing), lipopolysaccharide (LPS, 5 mg/kg, IP) as a positive control, and PBS (IV) as negative control (n=4 biologically independent animals). After 48 hours, tissue sections of heart, liver, spleen, lung and kidney were prepared for H&E staining. LPS injected animals exhibited significant injury in multiple organs (arrows indicated), but no obvious injury was observed in SORT LNP groups. Scale bar = 100 μm.

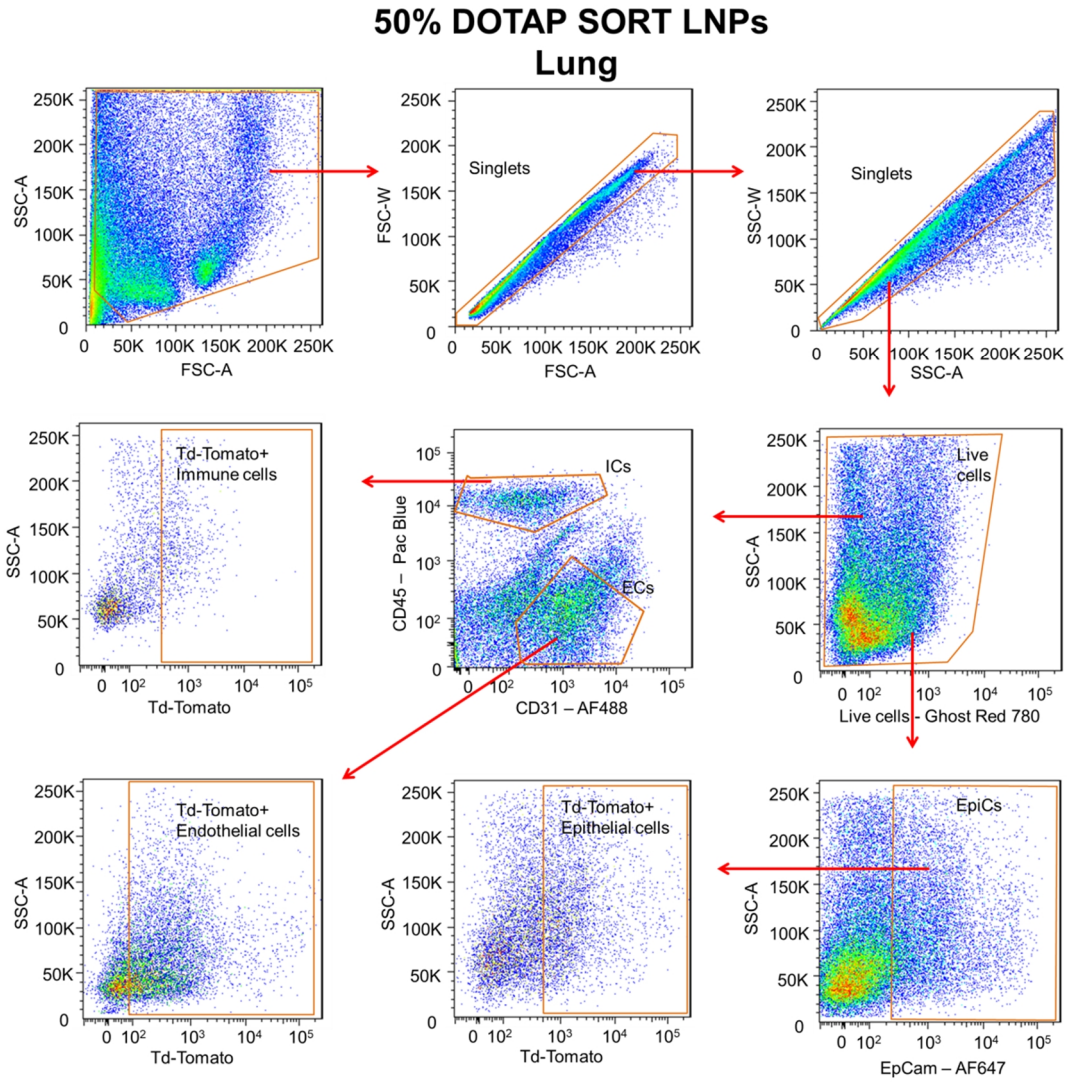


Supplementary Fig. 16 | B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J (Ai9) mice exhibit some autofluorescence in the absorption region for TdTom. Moreover, there is a large difference for TdTom autofluorescence among different organs. Liver and kidney show the highest signal and spleen shows the lowest. Although this does not interfere with detection of editing in most organs (when the excitation settings are properly adjusted to eliminate background), it does complicate detection of spleen TdTom expression because background spleen is so much lower than other organs. Therefore, inaccurate conclusions can be reached for low levels of spleen editing when the experiment is not properly controlled. Notably, no significant difference was observed between PBS group and SORT LNPs (Luc mRNA) treated groups in all tested organs. Data are presented as mean±s.e.m. (n=3 biologically independent animals).

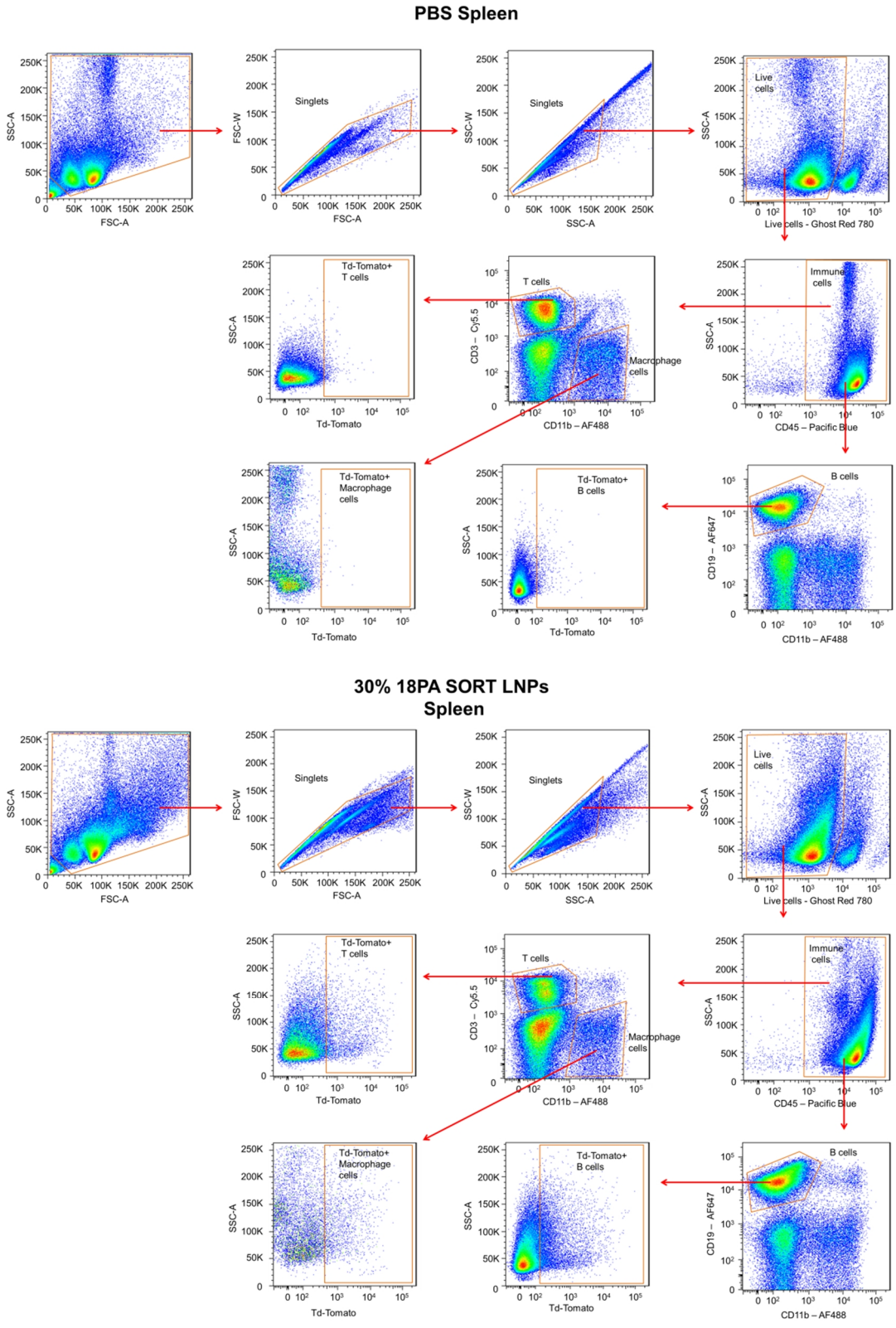


Supplementary Fig. 17 | 20% DODAP SORT LNPs achieved nearly 100% TdTom editing in hepatocytes administration of a single 0.3 mg/kg Cre mRNA dose. As shown in the flow cytometry histogram, there is full separation between TdTom- control mice and TdTom+ 20% DODAP treated mice. After liver perfusion, the resected livers of mice treated with 20% DODAP SORT LNPs were surprisingly bright red compared to control livers. Even without fluorescence excitation, the livers glowed red due to complete activation of TdTom expression. TdTom mice were injected with 0.3 mg/kg Cre mRNA, then sacrificed after two days (n=3 biologically independent animals). Hepatocytes were isolated by two-step collagenase perfusion and TdTom fluorescence was analyzed by flow cytometry.


PBS Lung
SSC-A
FSC-A
Singlets
FSC-W
SSC-W
Td-Tomato+
Immune cells
Td-Tomato
ICs
ECs
CD45 – Pac Blue
CD31 – AF488
Live
cells
Live cells - Ghost Red 780
Td-Tomato+
Endothelial cells
Td-Tomato+
Epithelial cells
EpiCs
EpCam – AF647

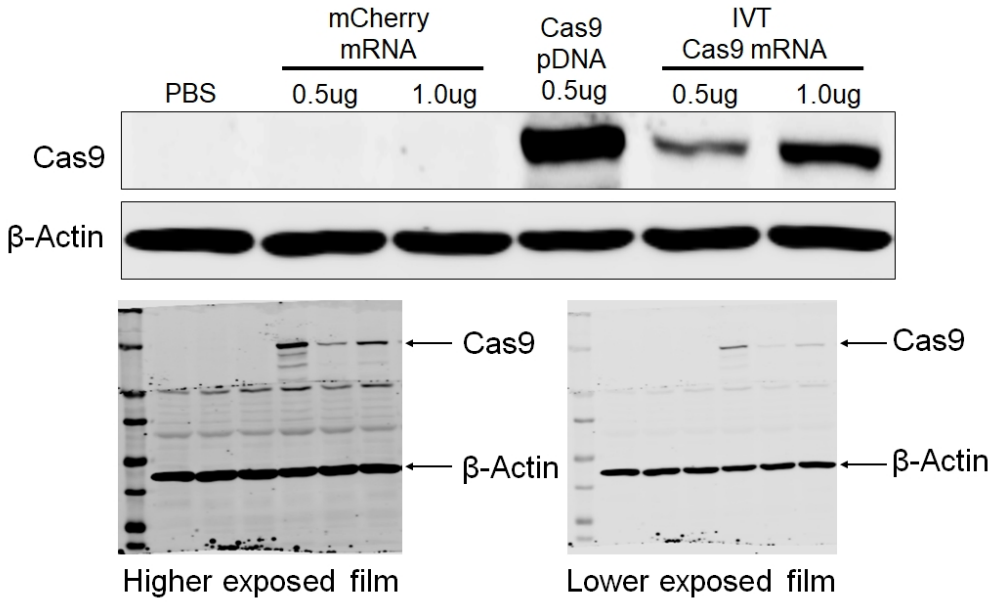


Supplementary Fig. 18 | The FACS gating strategy for analysis of TdTom+ expression in lung cells is described. See Experimental Section for a description of the antibodies used. Ghost Red 780 was used to distinguish live and dead cells. EpCam+ was used to define epithelial cells, CD45+ and CD31- were used to define immune cells, and CD45- and CD31+ were used to define endothelial cells. Gates for Td-Tom+ in cell types were drawn based on PBS injected control mice. Td-Tom mice were injected with Cre mRNA formulations and Td-Tom+ in given cell types was detected by flow after two days (n=3).

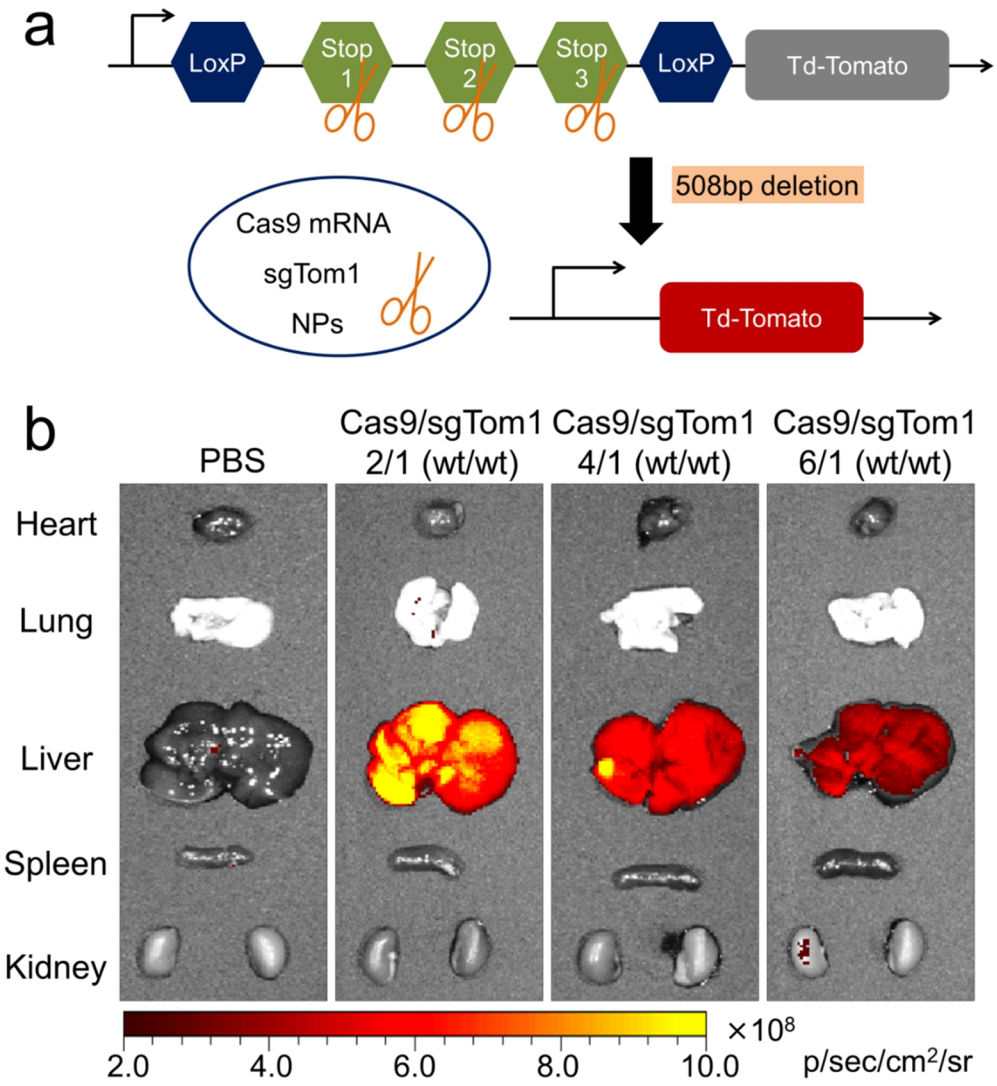


Supplementary Fig. 19 | The FACS gating strategy for analysis of TdTom+ expression in splenic cells is described. See Experimental Section for a description of the antibodies used. Ghost Red 780 was used to distinguish live and dead cells. CD44+ was used to distinguish immune cells, then CD3+ and CD11b- were used for T cells, CD3- and CD11b+ were used for macrophage cells, CD19+ and

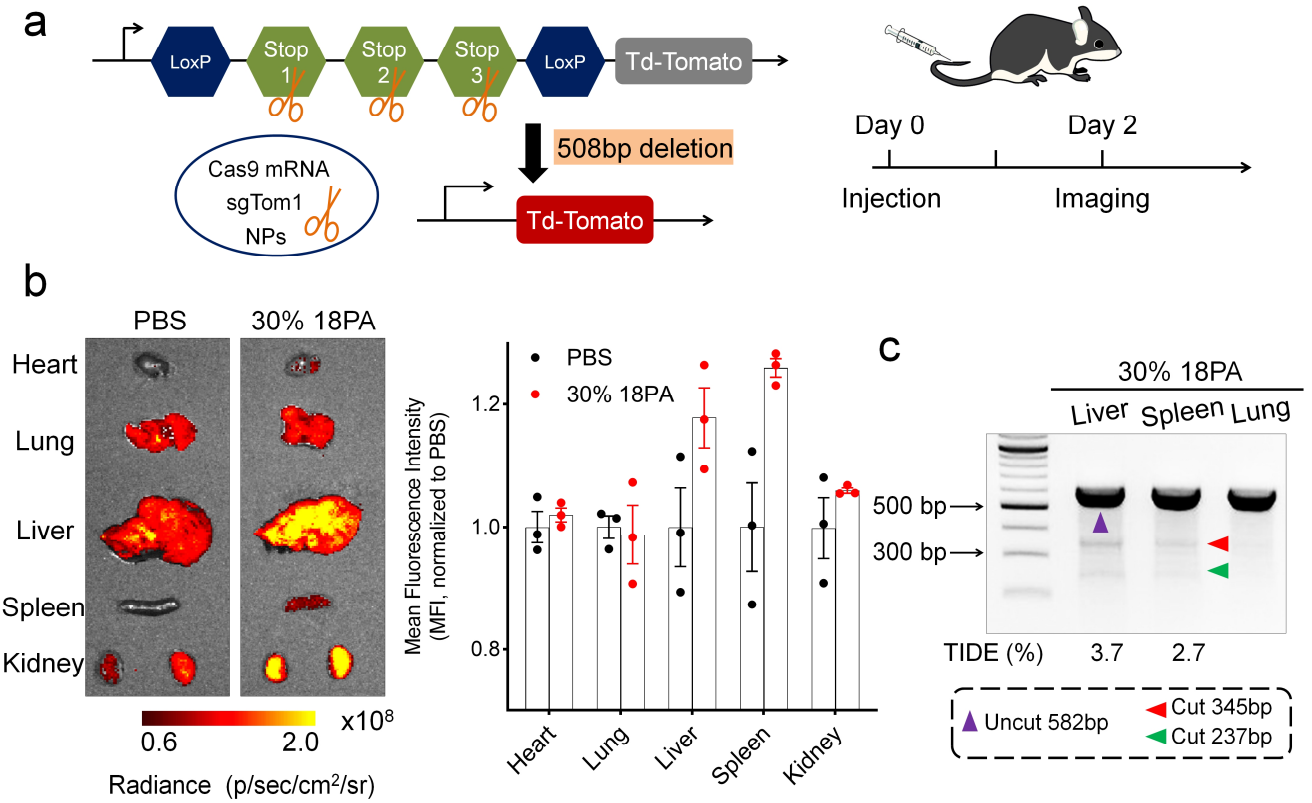
CD11b- were used for B cells. Gates for Td-Tom+ in cell types were drawn based on PBS injected control mice. Td-Tom mice were injected with Cre mRNA formulations and Td-Tomato+ in given cell types was detected by flow after two days (n=3).



Supplementary Fig. 20 | High quality of IVT Cas9 mRNA was verified by western blot. 293T cells were seeded in 12-well plate the day before transfection, cells were treated for 24h by each condition before performing western blot. Cas9 pDNA was delivered by Lipofectamine 2000 and mRNAs were delivered by mDLNPs. The result was repeated three times independently with similar results.



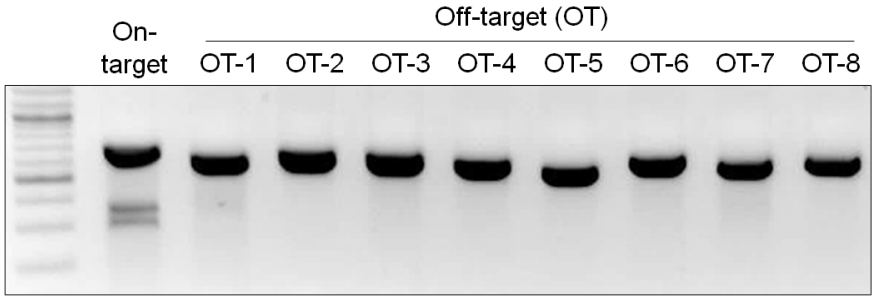
Supplementary Fig. 21 | Lower weight ratios of IVT Cas9 mRNA to sgTom1 improved gene editing efficacy. Weight ratios of Cas9 mRNA and sgRNA were optimized via co-delivery strategy. **a**, Schematic illustration shows that co-delivery of Cas9 mRNA and sgTom1 activates Td-Tom expression in transgenic mouse. **b**, Td-Tom fluorescence of major organs was imaged at day 7 after IV injection, indicating that 2/1 of Cas9/sgTom1 (wt/wt) was optimal. The total RNA dose was 1 mg/kg, IVT Cas9 mRNA and modified sgTom1 were co-encapsulated by mDLNPs (n=3 biologically independent animals).



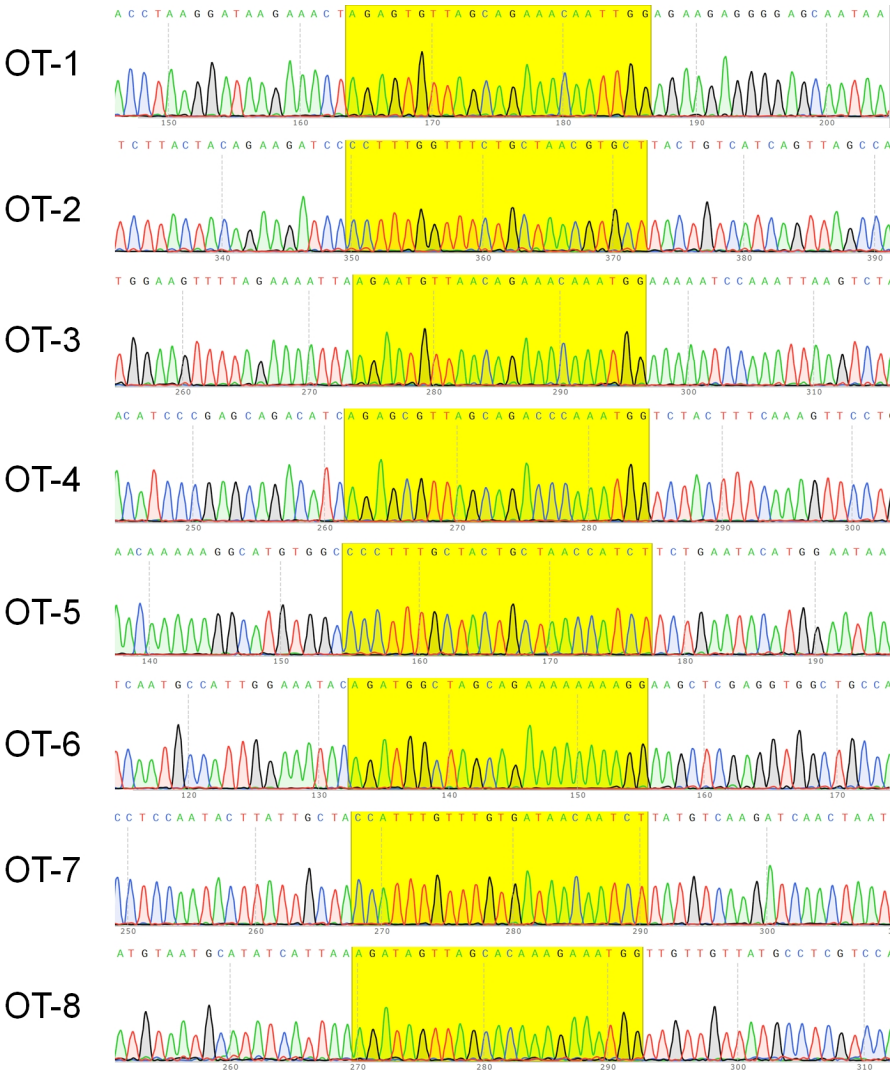
Supplementary Fig. 22 | CRISPR/Cas gene editing in the spleen was achieved in both Td-Tom transgenic mice and wild type C57/BL6 mice by co-delivering Cas9 mRNA and sgRNA. a, Schematic illustration shows that co-delivery of Cas9 mRNA and sgTom1 activates Td-Tom expression in Td-Tom mice. **b**, Td-Tom expression was induced in the spleen and liver by the spleen-targeted formulation 30% 18PA SORT LNP. Td-Tom fluorescence of main organs was detected at day 2 after IV treatment with co-delivery of Cas9 mRNA and modified sgTom1 (2/1, wt/wt) at the total doses of 4 mg/kg. Data are presented as mean±s.e.m. (n=3 biologically independent animals). **c**, T7E1 assay indicated that PTEN editing of liver and spleen was obtained by co-delivery of Cas9 mRNA (IVT) and sgPTEN. C57/BL6 mice were IV injected with 30% 18PA SORT LNPs at total dose of 4 mg/kg (Cas9 mRNA/sgPTEN, 2/1, wt/wt), and PTEN editing was detected at day 2 (n = 3 biologically independent animals).

	ID	Aligned site (20nt+PAM)	# mismatches	Strand	Locus
On-Target	sgPTEN	AGATCGTTAGCAGAAACAAAAGG	0	+	chr19:32758464
Off-target (OT)	OT-1	AGAGTGTTAGCAGAAACAATTGG	3	+	chr11:22333524
	OT-2	AGCACGTTAGCAGAAACCAAAGG	3	-	chr15:15308342
	OT-3	AGAATGTTAACAGAAACAAATGG	3	+	chr15:60681817
	OT-4	AGAGCGTTAGCAGACCCAAATGG	3	+	chr19:30941492
	OT-5	AGATGGTTAGCAGTAGCAAAGGG	3	-	chrX:152078091
	OT-6	AGATGGCTAGCAGAAAAAAAAGG	3	+	chr10:122894687
	OT-7	AGATTGTTATCACAAACAAATGG	3	-	chr6:74056110
	OT-8	AGATAGTTAGCACAAAGAAATGG	3	+	chr13:111927102

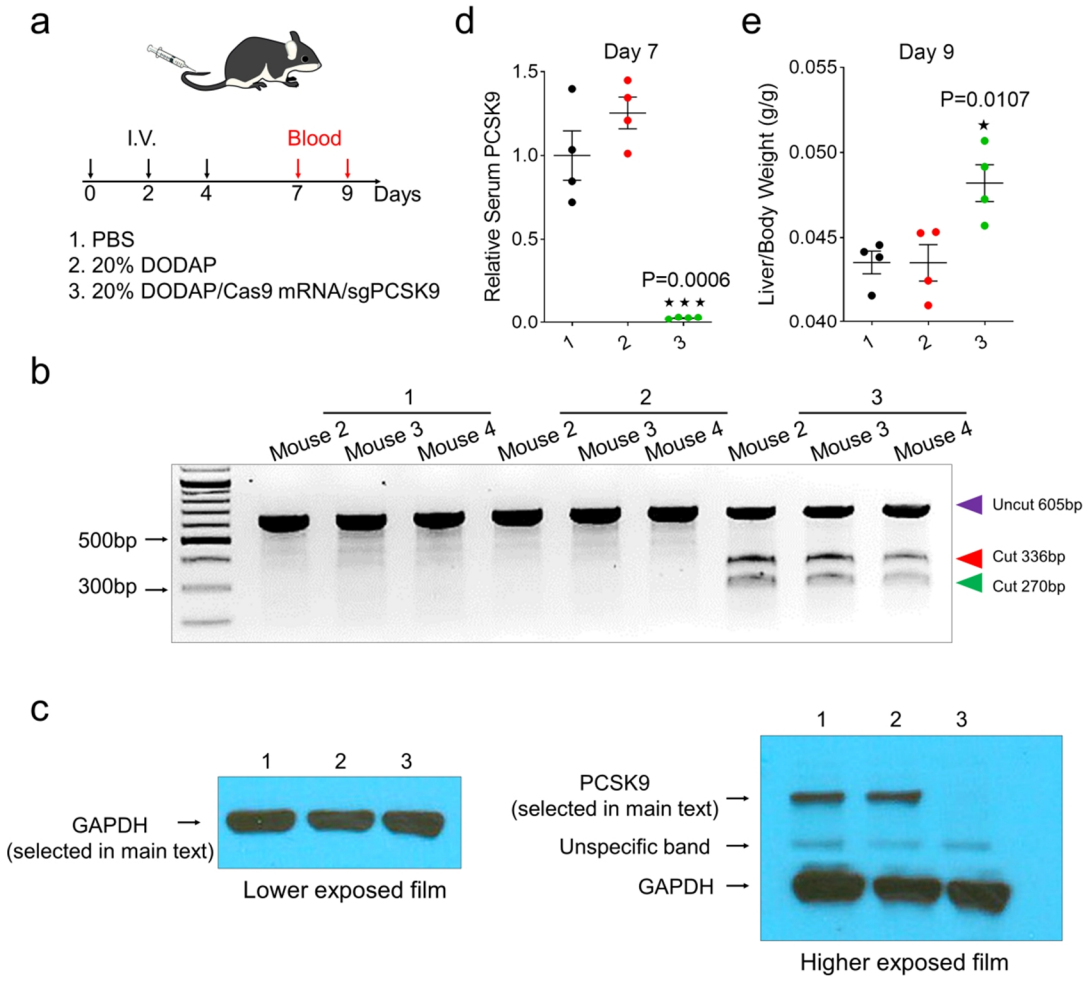
Primers used for PCR		
ID	Forward (5' to 3')	Reverse (5' to 3')
On-Target	ATCCGTCTTCTCCCCATTCCG	GACGAGCTCGCTAATCCAGTG
OT-1	TGACAAGACACACTTGGGTGGTAGTG	TCACCGTACATTTGAATTACAGGACCTG
OT-2	GCTTCACTGGGTTTGAAAGTTCCC	TCCAAGAAGCATGGAGTTAATGAGACAAA
OT-3	CATATGTAATCGAGATGAATTTACACTGCCT	CCCAAGATTAGGGAGATGATTCCTCAC
OT-4	AGTTGCTCAGTGACATGCCTTACT	TGAGCAAACCTCCAAACACTCAAAGT
OT-5	CAGCACCAGCTCTAGATATAGGTAGGT	TTAGATGTTACACAGCCACTAGAATTCATTCC
OT-6	TACCGAATCTTGTGTGAGTGTGCATTTG	AGGAGGGAGGAGAAAGCCACAG
OT-7	CTATGCATCCCCTTTTCAGACACACA	TCATGACAACTATGTAGTAACCAACCCACTT
OT-8	CTGACTGGCTTATGCTGGAGAG	CCACTCTGCAGCTGATATTAAATAGCT



Supplementary Fig. 23 | No off-target editing was detected following SORT LNP delivery of sgPTEN. The Cas-OFFinder webtool was employed to predict likely off-target editing sites for PTEN [7]. Eight potential targeted positions were amplified by PCR, then analyzed by T7E1 assay. On-target PCR amplicons showed clear cleavage bands by T7E1 enzyme digestion, but nothing for all tested off-target positions. The result was repeated three times independently with similar results.



Supplementary Fig. 24 | No off-target editing was detected following SORT LNP delivery of sgPTEN. The Cas-OFFinder webtool was employed to predict likely off-target editing sites for PTEN [7]. Eight potential targeted positions were amplified by PCR, then analyzed by T7E1 assay and Sanger sequencing. Sequencing data showed clear and uniform peaks around potential cleavage sites, suggesting no Indels formation.



Supplementary Fig. 25 | 20% DODAP SORT LNPs mediated effective PCSK9 editing in liver. a, Wild type C57BL/6 mice were IV injected three times (day 0, day 2, and day 4) by co-delivery of Cas9 mRNA and modified sgPCSK9 at a total dose of 2.5 mg/kg (1/1, mRNA/sgRNA, wt/wt) (n = 4). After 7 and 9 days, serum and tissue were collected for PCSK9 knockout evaluation. **b**, The Indel percentage at PCSK9 locus of liver tissue was verified and quantified by T7E1 assay and TIDE analysis (~60%, **Fig. 4J**). Effective PCSK9 knockout induced ~100% PCSK9 protein reduction in both (**c**) liver tissue (western blot) and (**d**) serum (ELISA), as well as increased (**e**) ratio of liver weight to body weight. Experiment **c** was repeated three times independently with similar results. Data of **d** and **e** are presented as mean±s.e.m. (n=4 biological independent animals). A two-tailed unpaired t-test was used to determine the significance of the indicated comparisons (*P < 0.05; **P<0.01; ***P < 0.001; ****P<0.0001).

Supplementary Tables

Supplementary Table 1. Details of DDAB, EPC, 14PA, 18BMP, DODAP, C12-200, 5A2-SC8, DSPC, and DOCPe modified mDLNP formulations (SORT LNPs), including molar ratio and percentage of each component, weight ratios of total lipids to mRNA, size, and PDI.

Name	Molar Ratios					Molar Percentage (%)					Lipids/ mRNA (wt/wt)	Size (nm)	PDI
	5A2-SC8	DOPE	Chol	DMG-PEG	X[a]	5A2-SC8	DOPE	Chol	DMG-PEG	X[a]			
5% DDAB	15	15	30	3	3.315	22.62	22.62	45.24	4.52	5.00	40	193.5	0.14
15% DDAB	15	15	30	3	11.12	20.24	20.24	40.47	4.05	15.00	40	167.7	0.13
40% DDAB	15	15	30	3	42	14.29	14.29	28.57	2.86	40.00	40	143.8	0.13
50% DDAB	15	15	30	3	63	11.90	11.90	23.81	2.38	50.00	40	174.7	0.21
100% DDAB	0	0	0	0	100	0.00	0.00	0.00	0.00	100.00	40	3174.3	0.23
5% EPC	15	15	30	3	3.315	22.62	22.62	45.24	4.52	5.00	40	195.3	0.14
15% EPC	15	15	30	3	11.12	20.24	20.24	40.47	4.05	15.00	40	166.3	0.14
40% EPC	15	15	30	3	42	14.29	14.29	28.57	2.86	40.00	40	116.7	0.15
50% EPC	15	15	30	3	63	11.90	11.90	23.81	2.38	50.00	40	105.7	0.17
100% EPC	0	0	0	0	100	0.00	0.00	0.00	0.00	100.00	40	455.9	0.32
5% 14PA	15	15	30	3	3.315	22.62	22.62	45.24	4.52	5.00	40	92.8	0.18
10% 14PA	15	15	30	3	7	21.43	21.43	42.86	4.29	10.00	40	98.6	0.18
20% 14PA	15	15	30	3	15.75	19.05	19.05	38.10	3.81	20.00	40	105.7	0.17
30% 14PA	15	15	30	3	27	16.67	16.67	33.33	3.33	30.00	40	107.3	0.19
100% 14PA	0	0	0	0	100	0.00	0.00	0.00	0.00	100.00	40	2607.3	0.28
5% 18BMP	15	15	30	3	3.315	22.62	22.62	45.24	4.52	5.00	40	112.0	0.13
10% 18BMP	15	15	30	3	7	21.43	21.43	42.86	4.29	10.00	40	131.4	0.10
20% 18BMP	15	15	30	3	15.75	19.05	19.05	38.10	3.81	20.00	40	172.9	0.12
30% 18BMP	15	15	30	3	27	16.67	16.67	33.33	3.33	30.00	40	195.9	0.13
100% 18BMP	0	0	0	0	100	0.00	0.00	0.00	0.00	100.00	40	87.4	0.21
10% DODAP	15	15	30	3	7	21.43	21.43	42.86	4.29	10.00	40	138.5	0.11
20% DODAP	15	15	30	3	15.75	19.05	19.05	38.10	3.81	20.00	40	122.4	0.18
50% DODAP	15	15	30	3	63	11.90	11.90	23.81	2.38	50.00	40	148.0	0.15
80% DODAP	15	15	30	3	252	4.76	4.76	9.52	0.95	80.00	40	180.4	0.13
100% DODAP	0	0	0	0	100	0.00	0.00	0.00	0.00	100.00	40	932.8	0.74
10% C12-200	15	15	30	3	7	21.43	21.43	42.86	4.29	10.00	40	179.7	0.11
20% C12-200	15	15	30	3	15.75	19.05	19.05	38.10	3.81	20.00	40	141.3	0.19
50% C12-200	15	15	30	3	63	11.90	11.90	23.81	2.38	50.00	40	156.1	0.16
80% C12-200	15	15	30	3	252	4.76	4.76	9.52	0.95	80.00	40	273.2	0.21
100% C12-200	0	0	0	0	100	0.00	0.00	0.00	0.00	100.00	40	2505.3	1.00
10% 5A2-SC8	15	15	30	3	7	21.43	21.43	42.86	4.29	10.00	40	127.1	0.14
15% 5A2-SC8	15	15	30	3	11.12	20.24	20.24	40.47	4.05	15.00	40	130.9	0.13
20% 5A2-SC8	15	15	30	3	15.75	19.05	19.05	38.10	3.81	20.00	40	137.6	0.10
25% 5A2-SC8	15	15	30	3	21	17.86	17.86	35.71	3.57	25.00	40	126.0	0.12
30% 5A2-SC8	15	15	30	3	27	16.67	16.67	33.33	3.33	30.00	40	134.7	0.10
20% DSPC	15	15	30	3	15.75	19.05	19.05	38.10	3.81	20.00	40	115.7	0.19
50% DSPC	15	15	30	3	63	11.90	11.90	23.81	2.38	50.00	40	202.0	0.29
80% DSPC	15	15	30	3	252	4.76	4.76	9.52	0.95	80.00	40	1024.7	0.67
100% DSPC	0	0	0	0	100	0.00	0.00	0.00	0.00	100.00	40	2134.8	0.99
20% DOCPe	15	15	30	3	15.75	19.05	19.05	38.10	3.81	20.00	40	147.7	0.15
50% DOCPe	15	15	30	3	63	11.90	11.90	23.81	2.38	50.00	40	173.6	0.20
80% DOCPe	15	15	30	3	252	4.76	4.76	9.52	0.95	80.00	40	127.7	0.21
100% DOCPe	0	0	0	0	100	0.00	0.00	0.00	0.00	100.00	40	93.7	0.36

[a] X represents DDAB, EPC, 14PA, 18BMP, DODAP, C12-200, 5A2-SC8, DSPC and DOCPe.

Supplementary Table 2. All sgRNA sequences used in this research work are listed.

Name	Sequences (5' to 3')	PAM (5' to 3')	Notes
sgTom1	AAGTAAAACCTCTACAAATG	TGG	Chemical modified sgTom1, sgPTEN and sgPCSK9 were selected
sgPTEN	AGATCGTTAGCAGAAACAAA	AGG	
sgPCSK9	CCCATACCTTGGAGCAACGG	CGG	

Supplementary Table 3. All primers used in this research work are listed, including the length of PCR products and their purposes.

Name	Forward Primers (5' to 3')	Reverse Primers (5' to 3')	Length	Notes
IL-10 -6xHis	ATATATGGATCCGCCACCATGCCTGGC TCAGCACTGCTATG	ATATATGAATTCTTAGTGGTGATGGT GGTGGTGAGAGCCGCCGCTTTTC ATTTTGATCATCAT	594bp	For IVT clone
hEPO	ATATATGGATCCGCCACCATGGGGGTG CACGAATGTCCTG	ATATATGAATTCTCATCTGTCCCCTG TCCTGCAGGC	612bp	For IVT clone
Klotho ECD-6xHis	ATATATGGATCCGCCACCATGCTAGCCC GCGCCCCTC	ATATATGAATTCTTAGTGGTGATGGT GGTGGTGAGAGCCGCCCTTCCGG GTTTGAAAAAATCCACATTCG	3006bp	For IVT clone
NLS-Cre	ATATATGGATCCGCCACCATGCCCAAG AAGAAGAGGAAGGTGGCCAATTTACTG ACCGTACACCAAAATTTGCCTG	ATATATGAATTCTTAATCGCCATCTT CCAGCAG	1083bp	For IVT clone
SV40 NLS-Cas9-Nucleoplasmin NLS	ATATATGGATCCGCCACCATGGCCCCA AAGAAGAAGCGGAAGGTC	ATATATGAATTCTTACTTTTTCTTTTT TGCCTGGCCGGCCTTTTTCGTGGC CGCCGGCCTTTTGTCGCCTCCCAG	4233bp	For IVT clone
Ca9 Seq-1	CTGAGCGACATCCTGAGAGTGAAC			For sequencing to confirm the whole Cas9 sequences
Ca9 Seq-2		AGCAGGTCCTCTCTGTTCAG		
Ca9 Seq-3	GACGGCTTCGCCAACAGAAACTTC			
Ca9 Seq-4		TTTGATGCCCTCTTCGATCCG		
Ca9 Seq-5	GGGAGATCGTGTGGGATAAG			
Ca9 Seq-6		ACTTCTTAGGGTCCCAGTCC		
Ca9 Seq-7	AAGAGAGTGATCCTGGCCGAC			
PTEN	AAGCAGGCCCAGTCTCTG	GACGAGCTCGCTAATCCAGTG	582bp	For T7E1 assay
PCSK9	CTGAGGCTAGAGGACTGAGCCA	CGGAGGACACGTTTTCTGCATGAC	605bp	For T7E1 assay

Additional Supplementary References

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6. Love, K. et al. Lipid-like materials for low-dose, in vivo gene silencing. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 1864-1869 (2010).
7. Cas-OFFinder. http://www.rgenome.net/cas-offinder/.