
Supplementary information

Nanoparticle single-cell multiomic readouts reveal that cell heterogeneity influences lipid nanoparticle-mediated messenger RNA delivery

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Nanoparticle single-cell multiomic readouts reveal that cell heterogeneity influences lipid nanoparticle-mediated mRNA delivery

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a

G*A*T*GCTCTCATACGAACCTCGTCNNHNCCTGCTAGTCCACGTCCATGTCCACCNWNHTGATATTGNWHTGGTGTAGTCGAGCAGAGAC*T*A*G

Red = phosphorothioate linkages (*) act to increase resistance to exonucleases.
 Green = Universal primer binding sites allow for amplification from cells/tissues and linkage to next generation sequence adapters
 Blue = Probe binding site for ddPCR.
 Orange = 8nt Barcode. This 8nt sequence is referenced to identify nanoparticle composition and track nanoparticle distribution.
 Light Blue = 4nt placer used to prevent steric blocking.
 Black = Random nucleotide region used to minimize PCR bias.
 N= A, T, G, or C; W= A or T; H= A, T, or C.

b

mRNA binding oligos on beads:

End /5AmMC6/TTTAGGGATAACAGGGTAATAAGCAGTGGTATCAACGCAGAGTACGTBBBBBPCGACTCACTACAGGGBBBBBTGGTGACACGATCGBBBBBNNNNNNT(20)
 Adapter BC1 L1 BC2 L2 BC3 L3 UMI

1st /5AmMC6/TTTAGGGATAACAGGGTAATAAGCAGTGGTATCAACGCAGAGTAC
 CCATAGTTGCTCTCATGCAAAATCCGCTGAGTGATGTCCC (Round 1 barcodes)

2nd /5AmMC6/TTTAGGGATAACAGGGTAATAAGCAGTGGTATCAACGCAGAGTACGTTTATGGCGACTCACTACAGGG
 GCTGAGTGATGTCCBBBBBAGCCACTGTGCTAGC (Round 2 barcodes)

3rd /5AmMC6/TTTAGGGATAACAGGGTAATAAGCAGTGGTATCAACGCAGAGTACGTTTATGGCGACTCACTACAGGGBBBBBTGGTGACACGATCG
 AGCCACTGTGCTAGCBBBBBNNNNNNA(20) (Round 3 barcodes)

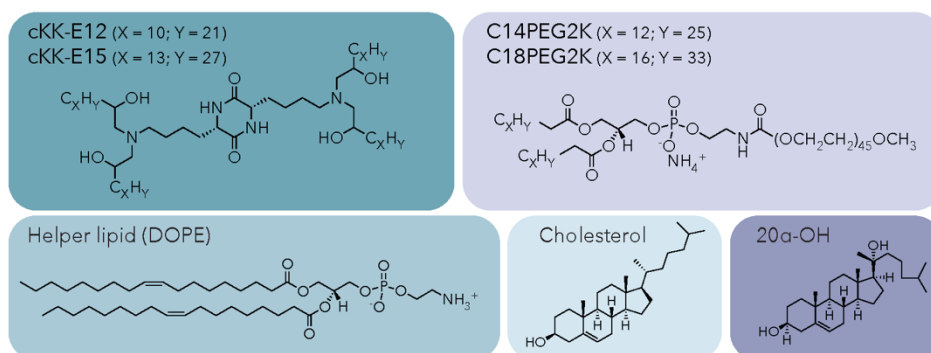
Barcode binding oligos on beads:

End /5AmMC6/TTTAGGGATAACAGGGTAATAAGCAGTGGTATCAACGCAGAGTACGTTTATGGCGACTCACTACAGGGBBBBBTGGTGACACGATCGBBBBBNNNNNNT(20)CTACGAGAGTATGCTTGAGCAGG-3'
 LNP barcode capture site

1st /5AmMC6/TTTAGGGATAACAGGGTAATAAGCAGTGGTATCAACGCAGAGTACGTBBBBBPCGACTCACTACAGGGBBBBBTGGTGACACGATCGBBBBBNNNNNNT(20)
 A(15)GATGCTCTCATACGAACCTCGTCC

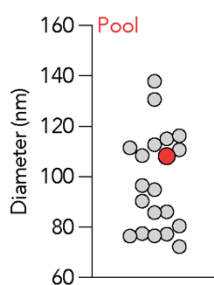
Supplementary Fig. 1. SENT-seq utilizes orthogonal capture sequences to generate tunable multiomic readouts. (a) Barcode structure for screening of LNPs; barcodes contain different regions highlighted above. (b) Beads used for microwell-based single-cell RNA sequencing were modified to include both an mRNA binding site and a barcode binding site.

a

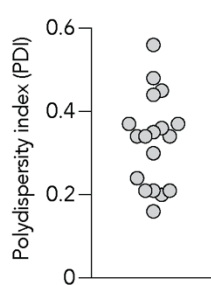


LNP #	Lipomer	Cholesterol	PEG	Helper lipid	Lipomer mole %	Cholesterol mole %	PEG mole %	Helper Lipid mole %	Included in screen?
LNP-1	cKK-E12	Cholesterol	C18PEG2K	DOPE	35	46.5	2.5	16	Yes
LNP-2	cKK-E12	Cholesterol	C18PEG2K	DOPE	45	39.5	2.5	13	Yes
LNP-3	cKK-E12	Cholesterol	C18PEG2K	DOPE	50	35	2.5	12.5	Yes
LNP-4	cKK-E12	20 α -OH	C18PEG2K	DOPE	35	46.5	2.5	16	Yes
LNP-5	cKK-E12	20 α -OH	C18PEG2K	DOPE	45	39.5	2.5	13	Yes
LNP-6	cKK-E12	20 α -OH	C18PEG2K	DOPE	50	35	2.5	12.5	Yes
LNP-7	cKK-E12	Cholesterol	C14PEG2K	DOPE	35	46.5	2.5	16	Yes
LNP-8	cKK-E12	Cholesterol	C14PEG2K	DOPE	45	39.5	2.5	13	Yes
LNP-9	cKK-E12	Cholesterol	C14PEG2K	DOPE	50	35	2.5	12.5	No
LNP-10	cKK-E12	20 α -OH	C14PEG2K	DOPE	35	46.5	2.5	16	Yes
LNP-11	cKK-E12	20 α -OH	C14PEG2K	DOPE	45	39.5	2.5	13	No
LNP-12	cKK-E12	20 α -OH	C14PEG2K	DOPE	50	35	2.5	12.5	Yes
LNP-13	cKK-E15	Cholesterol	C14PEG2K	DOPE	35	46.5	2.5	16	Yes
LNP-14	cKK-E15	Cholesterol	C14PEG2K	DOPE	45	39.5	2.5	13	Yes
LNP-15	cKK-E15	Cholesterol	C14PEG2K	DOPE	50	35	2.5	12.5	Yes
LNP-16	cKK-E15	20 α -OH	C14PEG2K	DOPE	35	46.5	2.5	16	No
LNP-17	cKK-E15	20 α -OH	C14PEG2K	DOPE	45	39.5	2.5	13	Yes
LNP-18	cKK-E15	20 α -OH	C14PEG2K	DOPE	50	35	2.5	12.5	Yes
LNP-19	cKK-E15	Cholesterol	C18PEG2K	DOPE	35	46.5	2.5	16	Yes
LNP-20	cKK-E15	Cholesterol	C18PEG2K	DOPE	45	39.5	2.5	13	Yes
LNP-21	cKK-E15	Cholesterol	C18PEG2K	DOPE	50	35	2.5	12.5	Yes
LNP-22	cKK-E15	20 α -OH	C18PEG2K	DOPE	35	46.5	2.5	16	No
LNP-23	cKK-E15	20 α -OH	C18PEG2K	DOPE	45	39.5	2.5	13	No
LNP-24	cKK-E15	20 α -OH	C18PEG2K	DOPE	50	35	2.5	12.5	Yes

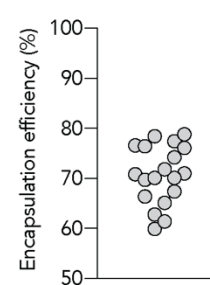
b



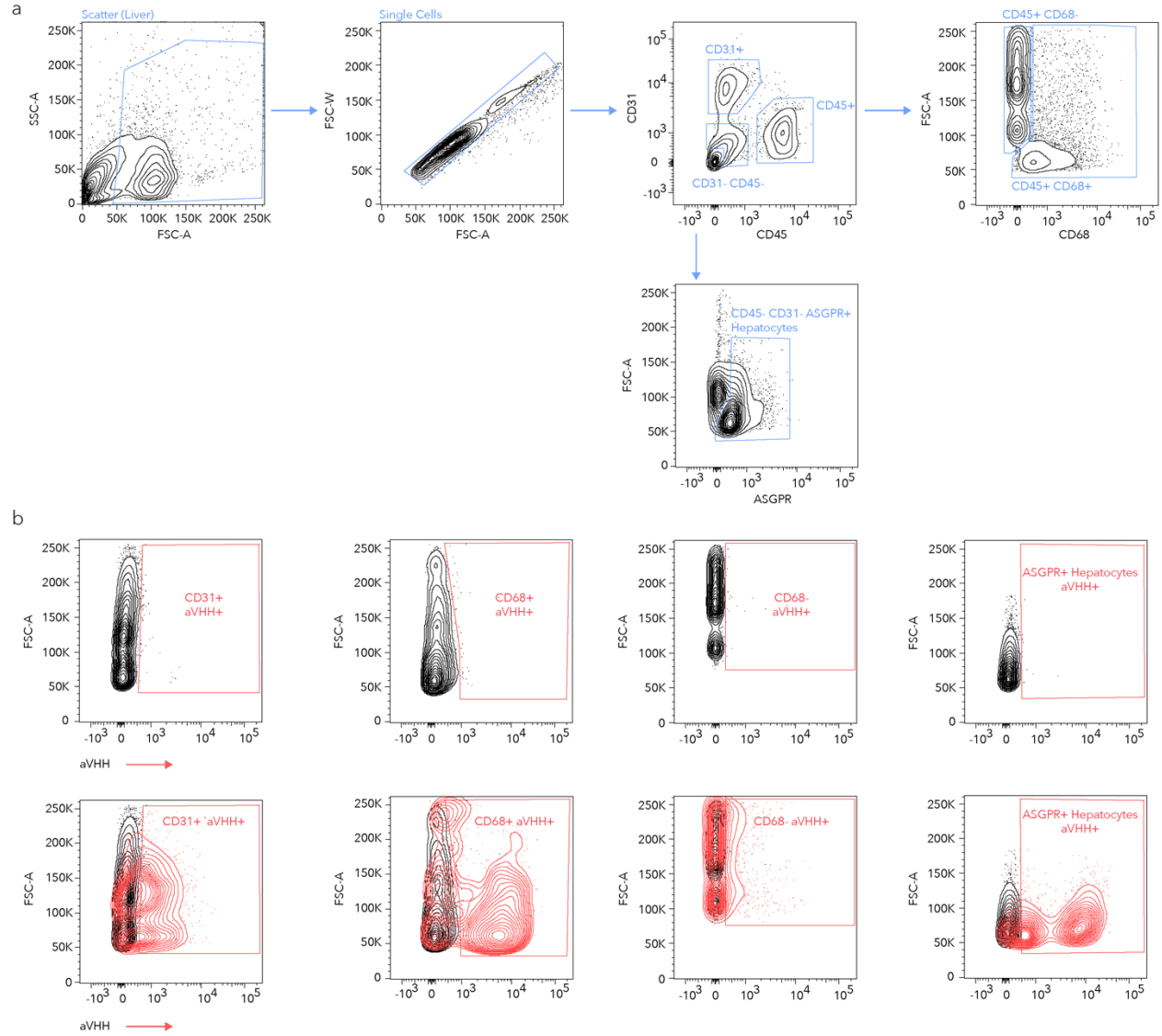
c



d



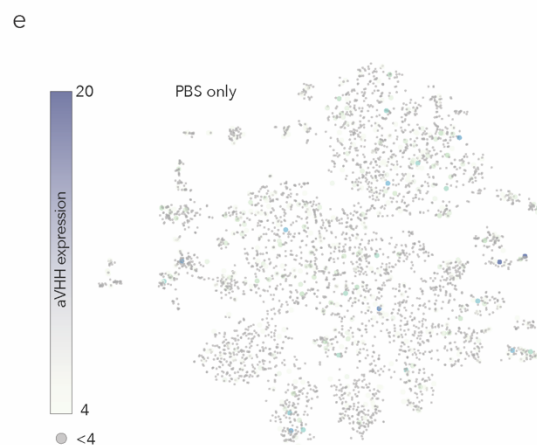
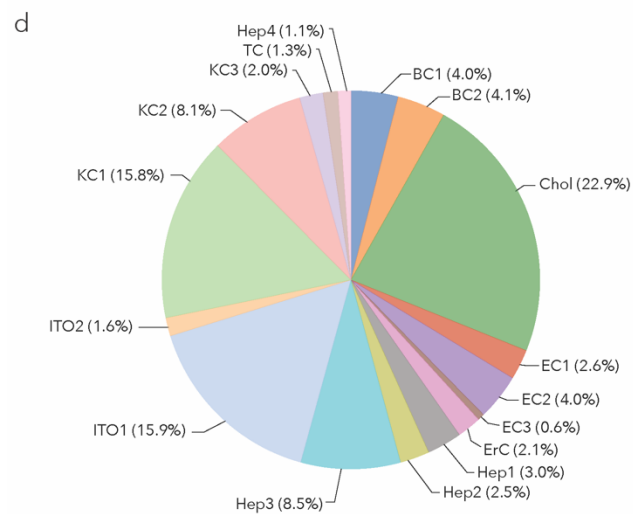
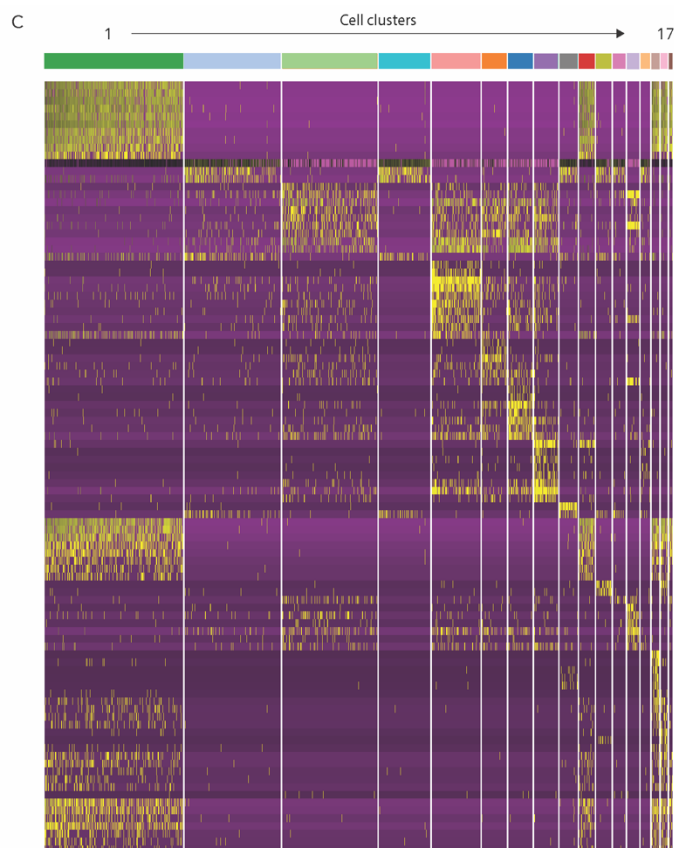
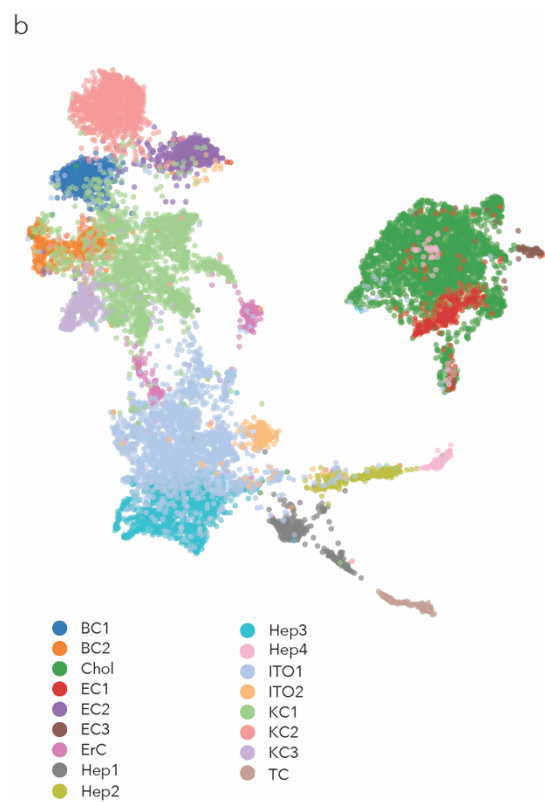
Supplementary Fig. 2. SENT-seq utilizes orthogonal capture sequences to generate tunable multiomic readouts. (a) Compounds included in LNP formulation as well as molar ratios screened. LNPs were formulated so that they contained an ionizable lipid, PEG-lipid, phospholipid, and cholesterol. Molar ratios of LNP constituents are shown for each LNP. LNP characteristics such as (b) LNP diameter, (c) polydispersity index, and (d) encapsulation efficiency are shown for all individual pooled LNPs.



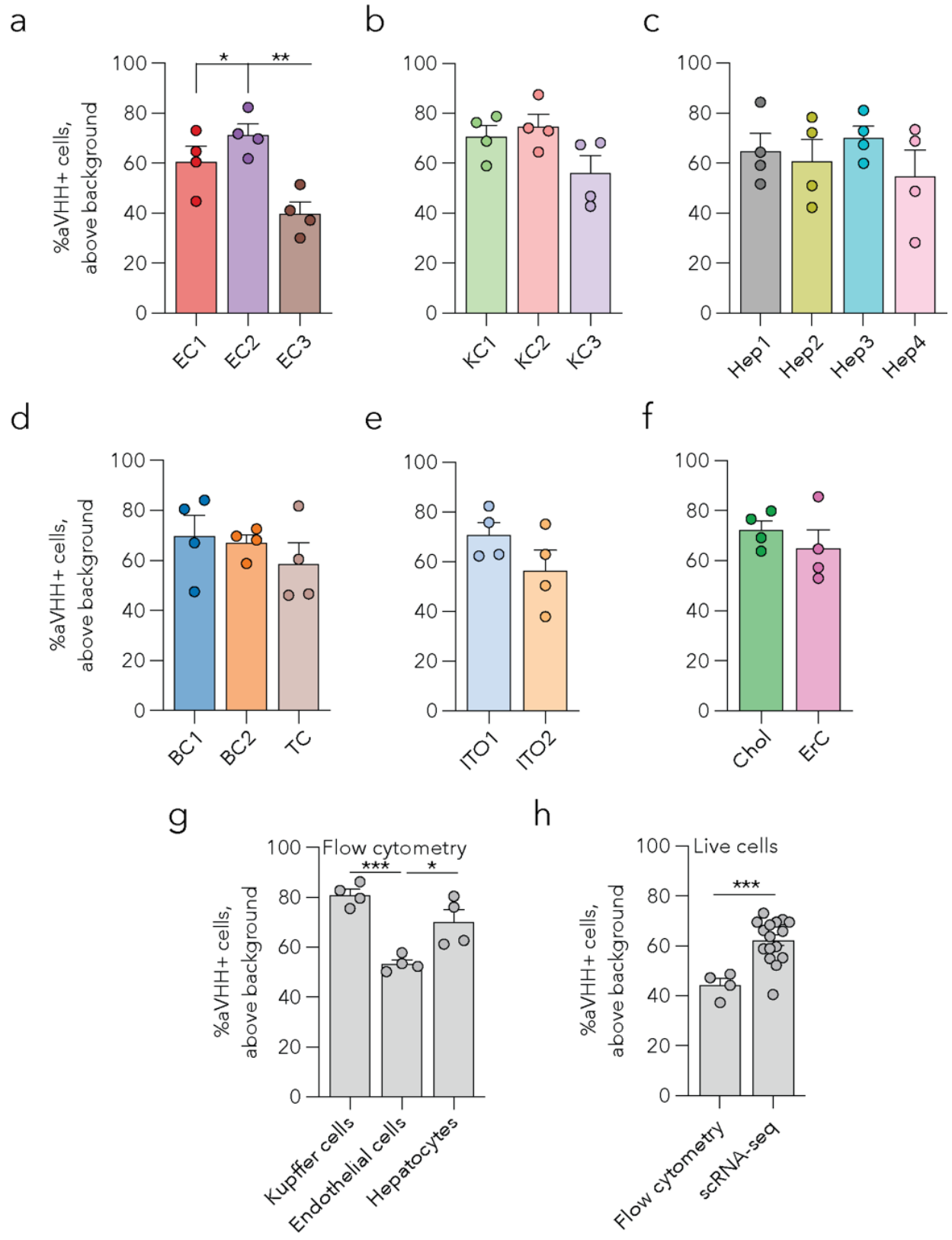
Supplementary Fig. 3. *In vivo* multiomic single-cell readouts of transcriptome, functional LNP-mediated mRNA delivery, and LNP-mediated DNA barcode delivery. Representative *in vivo* gating strategies for (a) liver cell populations, and (b) aVHH⁺ cells within those cell populations.

a

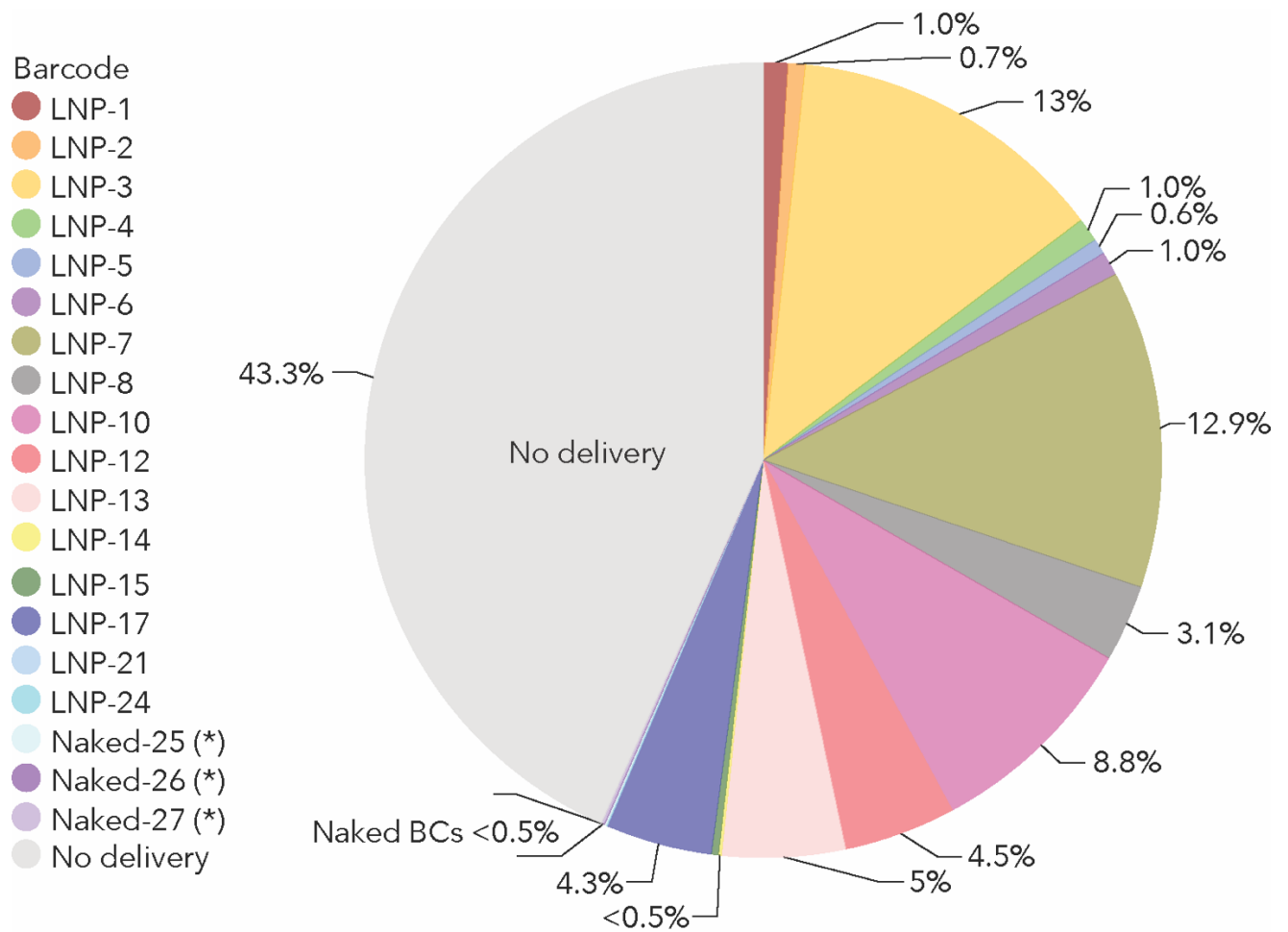
	Mean reads per cell	Median reads per cell	Mean gene count	Median gene count	Mean UMI count	Median UMI count	Number of Cells	Total Counts	Percent mapped to Exon	Percent mapped to Intron
1_PBS	11751	2823	2175	1920	2320	2078	1499	5272051	66.20%	33.80%
2_PBS	11216	2765	2277	2005	2422	2150	540	2020180	75.05%	24.95%
3_PBS	10044	2640	2215	1870	2359	2030	622	1831709	63.70%	36.30%
4_PBS	9694	2695	2414	2168	2558	2335	1532	4527792	75.06%	24.94%
1_Pool	10191	2700	2272	2010	2425	2160	3330	10091943	69.97%	30.03%
2_Pool	13006	2930	2254	1980	2408	2125	1796	7614914	67.85%	32.15%
3_Pool	9929	2713	2199	1955	2366	2113	1706	4960815	66.23%	33.77%
4_Pool	9371	2790	2379	2035	2701	2180	1803	5055979	67.15%	32.85%
Mean	10650	2757	2273	1993	2445	2146	1604	5171923	68.90%	31.10%
Standard Deviation	1158	85	79	83	117	84	805	2543331	3.92%	3.92%



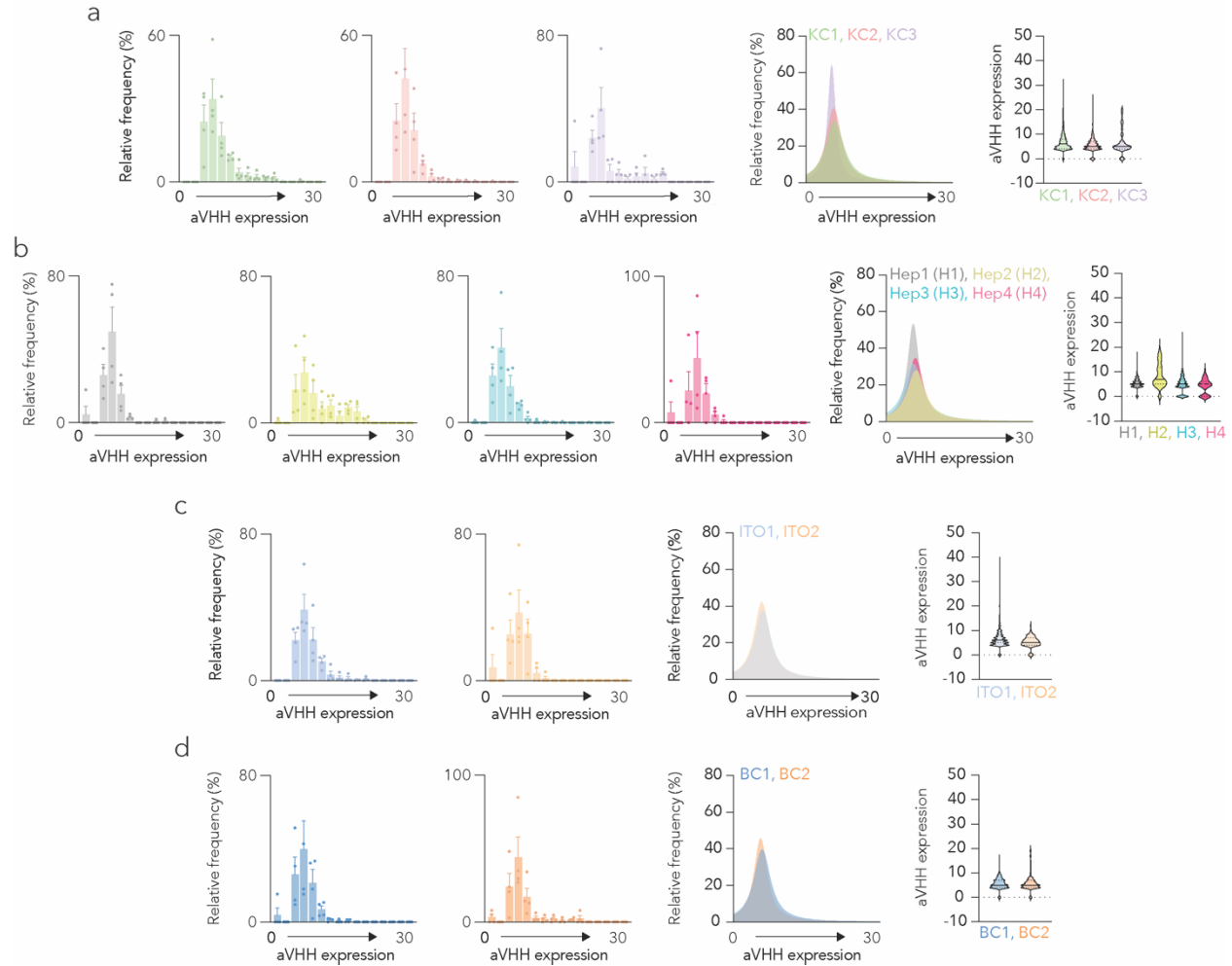
Supplementary Fig. 4. *In vivo* multiomic single-cell readouts of transcriptome, functional LNP-mediated mRNA delivery, and LNP-mediated DNA barcode delivery. (a) Single-cell RNA sequencing metrics. (b) UMAP projection showing the distribution of hepatic clusters from mouse livers treated with LNP pool and PBS. (c) Gene expression analysis shows 17 distinct cell clusters that contain different transcriptomic profiles. (d) The percentage of each hepatic cell cluster. (e) Background levels of aVHH expression in control mice treated with 1X PBS represent a stringent aVHH expression cutoff.



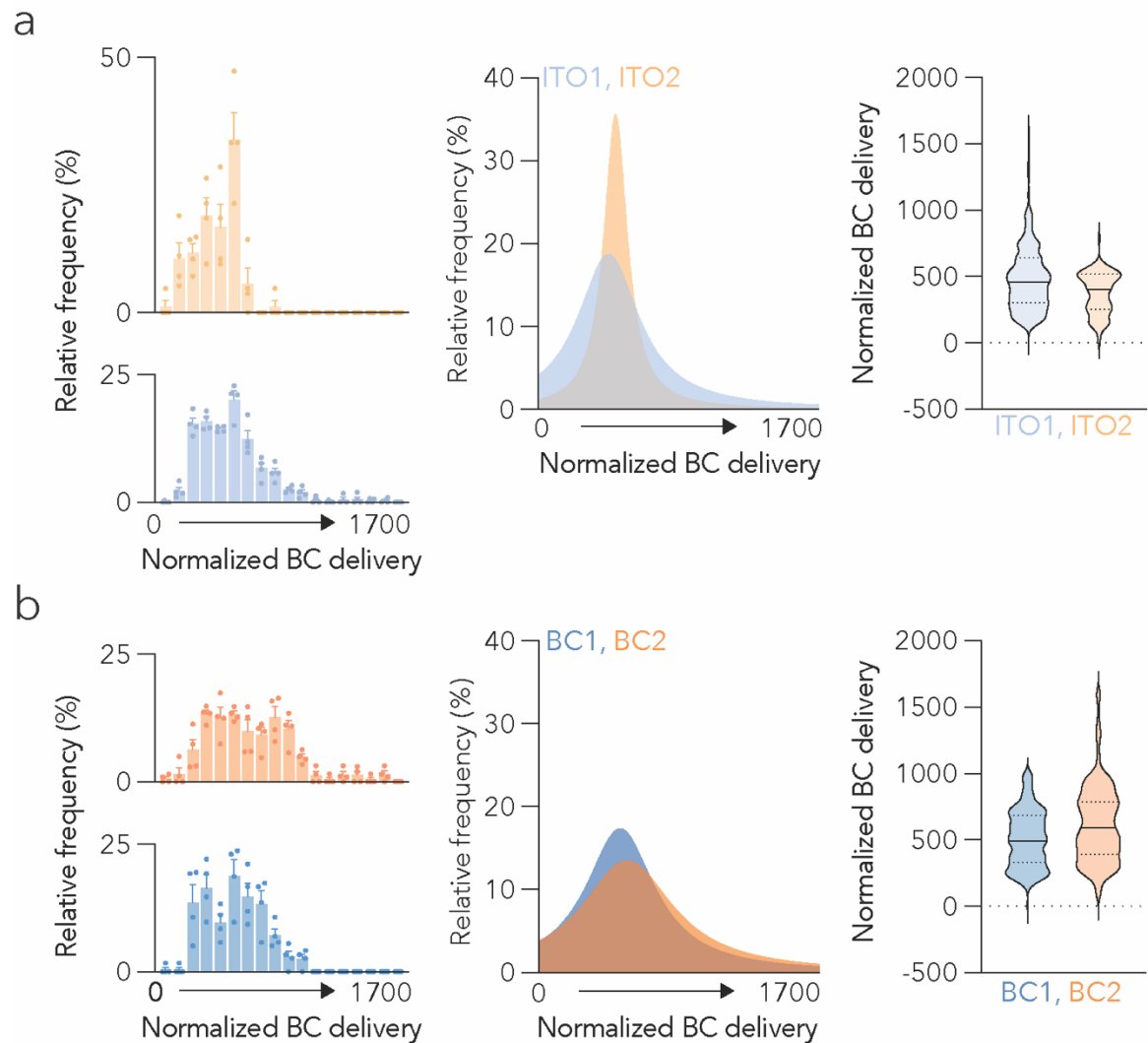
Supplementary Fig. 5. *In vivo* multiomic single-cell readouts of transcriptome, functional LNP-mediated mRNA delivery, and LNP-mediated DNA barcode delivery. Percentage of aVHH⁺ cells determined using single-cell RNA sequencing (scRNA-seq), with background levels found in control mice subtracted, in (a) endothelial cell subsets, (b) Kupffer cell subsets, (c) hepatocyte subsets, (d) B and T cells, (e) Ito cell subsets, and (f) cholangiocytes and erythroid cells. (g) The percentage of aVHH⁺ cells in populations analyzed using flow cytometry. (h) The comparison of the percentage of aVHH⁺ cells in the whole hepatic population determined using flow cytometry and scRNA-seq. In (a) – (g), n = 4 mice per group were used. In (h), n = 4 mice per group in the flow cytometry experiment were compared to an average value for each scRNA-seq determined cell population, plotted together. In all cases, data is plotted as mean ± standard error mean. Statistical analyses were conducted using a one-way factor ANOVA with Šidák's multiple comparison test for every cell population that had multiple subtypes within that population as well as for the flow cytometry and flow cytometry versus scRNA-seq comparisons. ns (p > 0.05, not shown), * (p < 0.05), ** (p < 0.01), *** (p < 0.001).



Supplementary Fig. 6. *In vivo* multiomic single-cell readouts of transcriptome, functional LNP-mediated mRNA delivery, and LNP-mediated DNA barcode delivery. Distribution of LNP barcodes in mice treated with LNP pool. As noted, naked barcodes, our negative control, each made up less than 0.5% of barcodes delivered to hepatic cells.

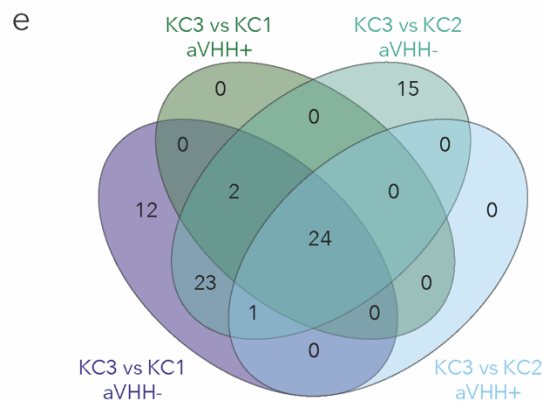
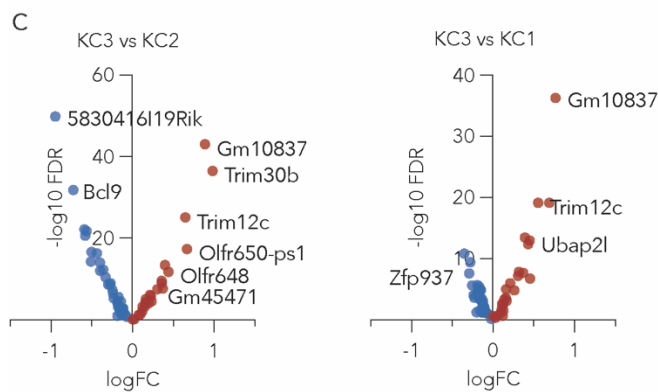
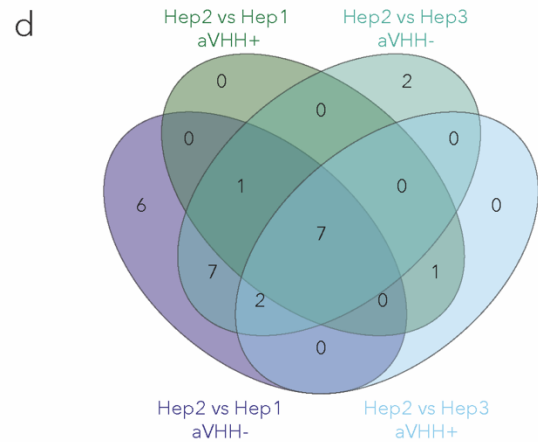
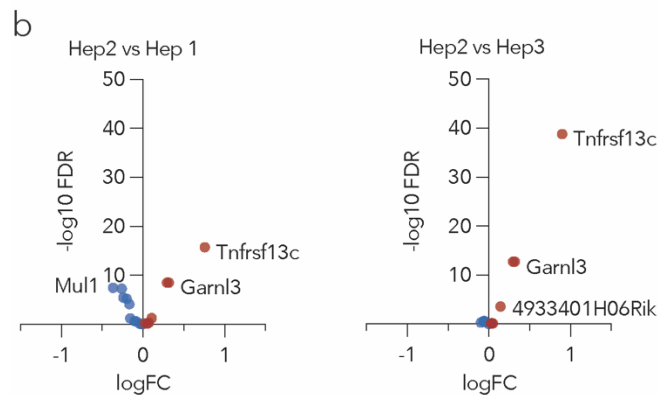


Supplementary Fig. 7. Cell subsets differentially uptake LNPs. aVHH expression profiles and violin plots representing the spread of aVHH expression shown for (a) Kupffer cells, (b) hepatocytes, (c) Ito cells, and (d) B cells. In all cases, $n = 4$ mice per group were used and data is plotted as mean $\pm$ standard error mean.

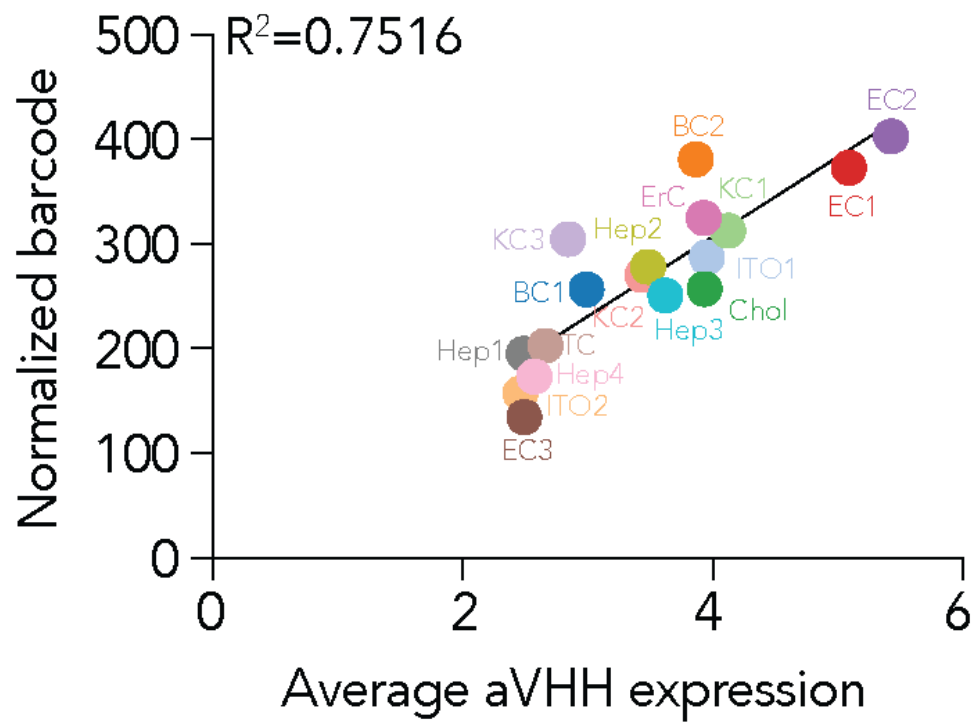


Supplementary Fig. 8. Cell subsets differentially uptake LNPs. Normalized barcode distribution profiles and violin plots representing the spread of normalized barcode distribution profiles shown for (a) Ito cells and (b) B cells. In all cases, $n = 4$ mice per group were used and data is plotted as mean $\pm$ standard error mean.

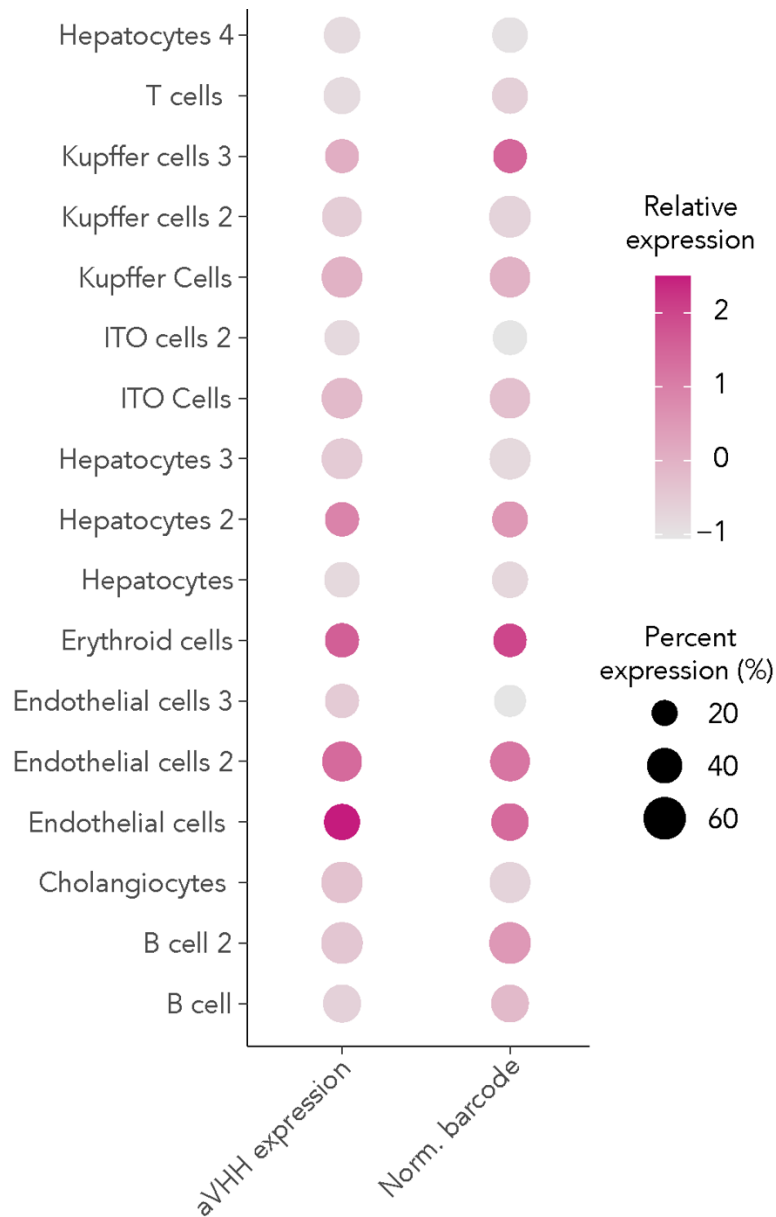
a	Gene	Putative role
	Col27a1	Preproprotein linked to playing a crucial roles in tissue growth and repair
	Fbln1	May play a role in cell adhesion and migration along protein fibers within the extracellular matrix
	Ebp4.1l4a	Thought to play an important role in regulating interactions between the cytoskeleton and plasma membrane, while also being involved in the beta-catenin signaling pathway
	Agap1	Directly and specifically regulates the adapter protein 3-dependent trafficking of proteins in the endosomal-lysosomal system
	Foxo6	Transcriptional activator
	Tcea2	Necessary for efficient RNA polymerase II transcription elongation
	Cdk13	Cyclin-dependent kinase which displays CTD kinase activity and is required for RNA splicing
	Cdk14	Kinase involved in the control of the eukaryotic cell cycle, including regulating the Wnt signaling pathway, cell cycle progression and cell proliferation.
	Taf5	A component of the transcription factor IID complex, which is essential for mediating regulation of RNA polymerase transcription
	Klhdc8a	Putatively linked to tumors potentially maintaining aggressiveness in the absence of epidermal growth factor receptor dependence
	Cldn5	Plays a major role in tight junction-specific obliteration of the intercellular space, through calcium-independent cell-adhesion activity



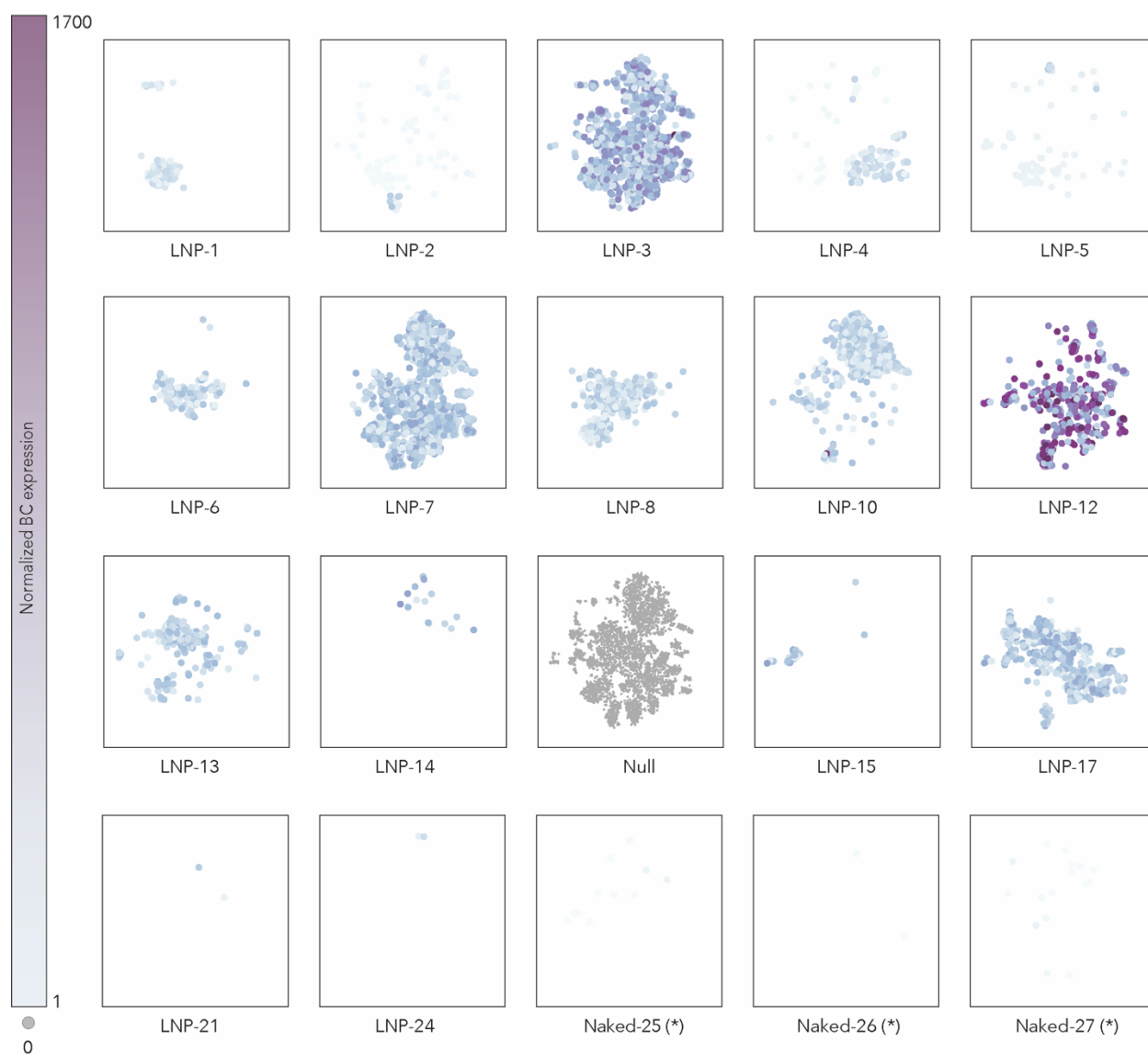
Supplementary Fig. 9. Other cell subtypes have transcriptional differences that may not affect mRNA delivery. (a) The 11 differentially regulated genes in aVHH⁺ EC1 and EC2, relative to EC3, that were not differentially expressed in aVHH⁻ cells, and the current putative roles for those genes in *mus musculus*. Differential analysis of (b) hepatocyte and (c) Kupffer cells of low-delivery clusters (Hep2 and KC3) as compared to high-delivery clusters (Hep1/3 and KC2/1). Venn diagram comparison of upregulated genes in (d) hepatocyte and (e) Kupffer cell clusters after segregation based on aVHH⁻ and aVHH⁺ expression.



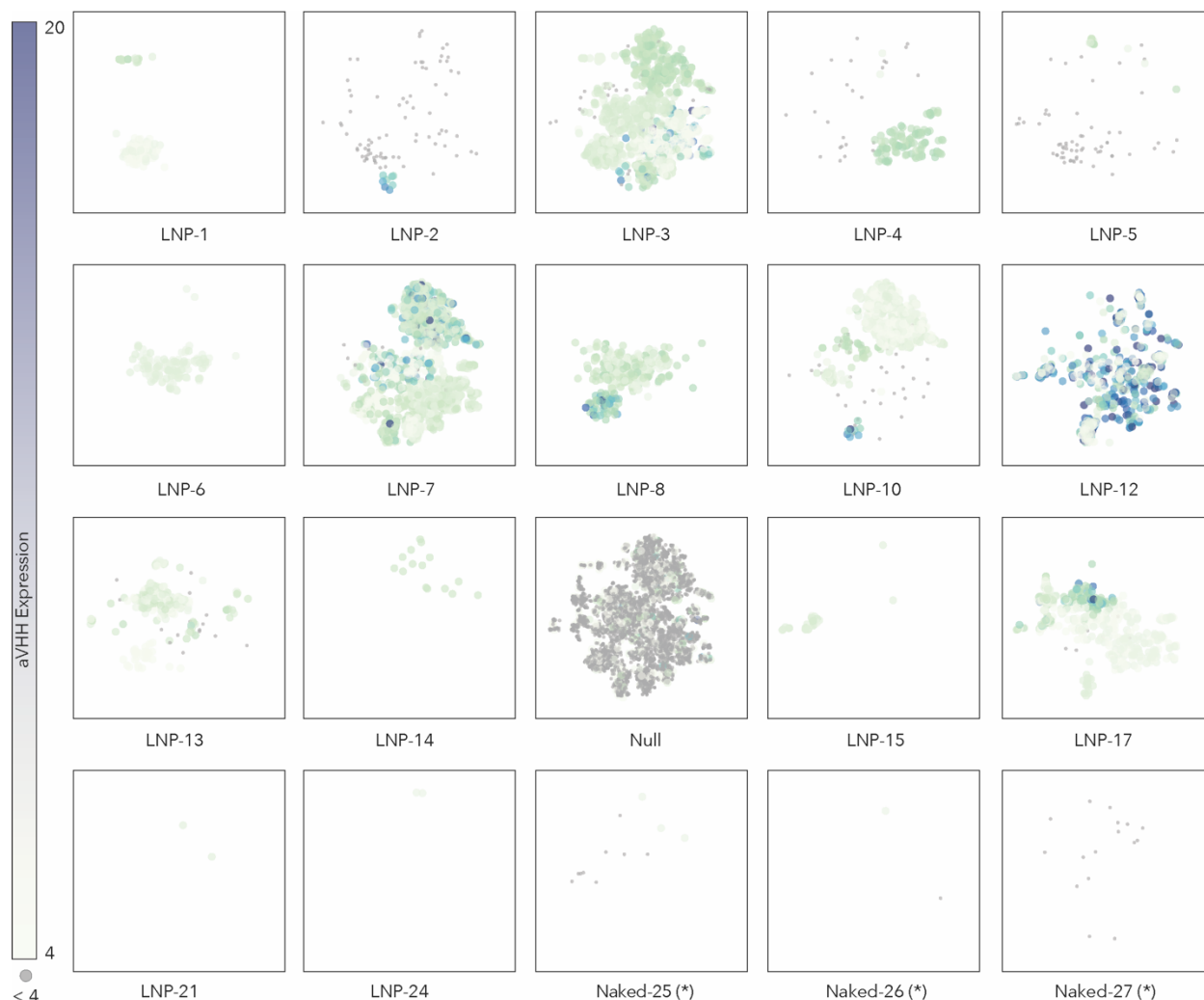
Supplementary Fig. 10. Average aVHH expression correlated to average normalized barcode values for each cell type in our dataset.



Supplementary Fig. 11. Relative and percent expression of aVHH and normalized barcode are matched for most cell populations. A comparison of relative expression of aVHH and normalized barcode, represented by the color of the dot. The percentage of single cells within each cell type that express aVHH and barcode is represented by the size of the dot in each respective column.



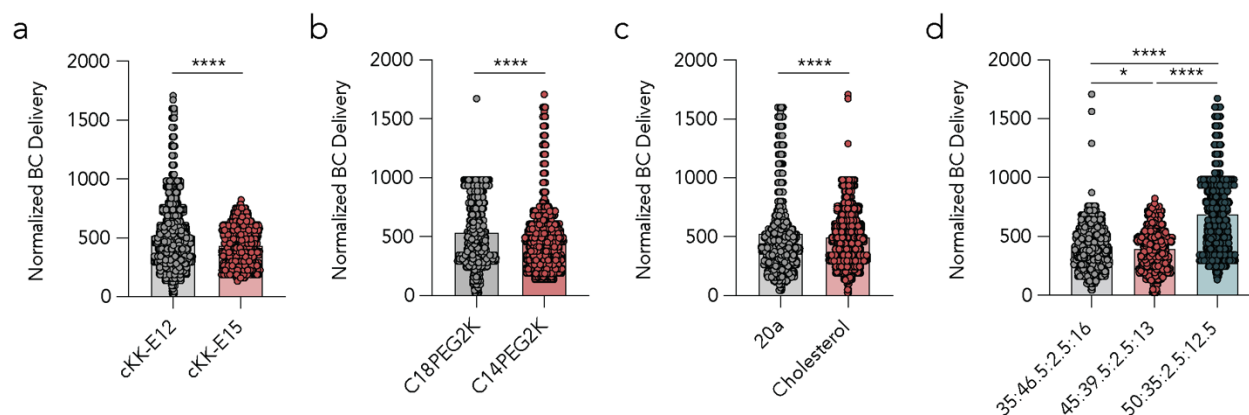
Supplementary Fig. 12. Chemically distinct LNPs exhibit different tropism within the liver microenvironment. t-SNE plots showing normalized barcode expression in each cell type for each LNP in the pool. Naked barcodes (*) had low normalized expression across all cells.



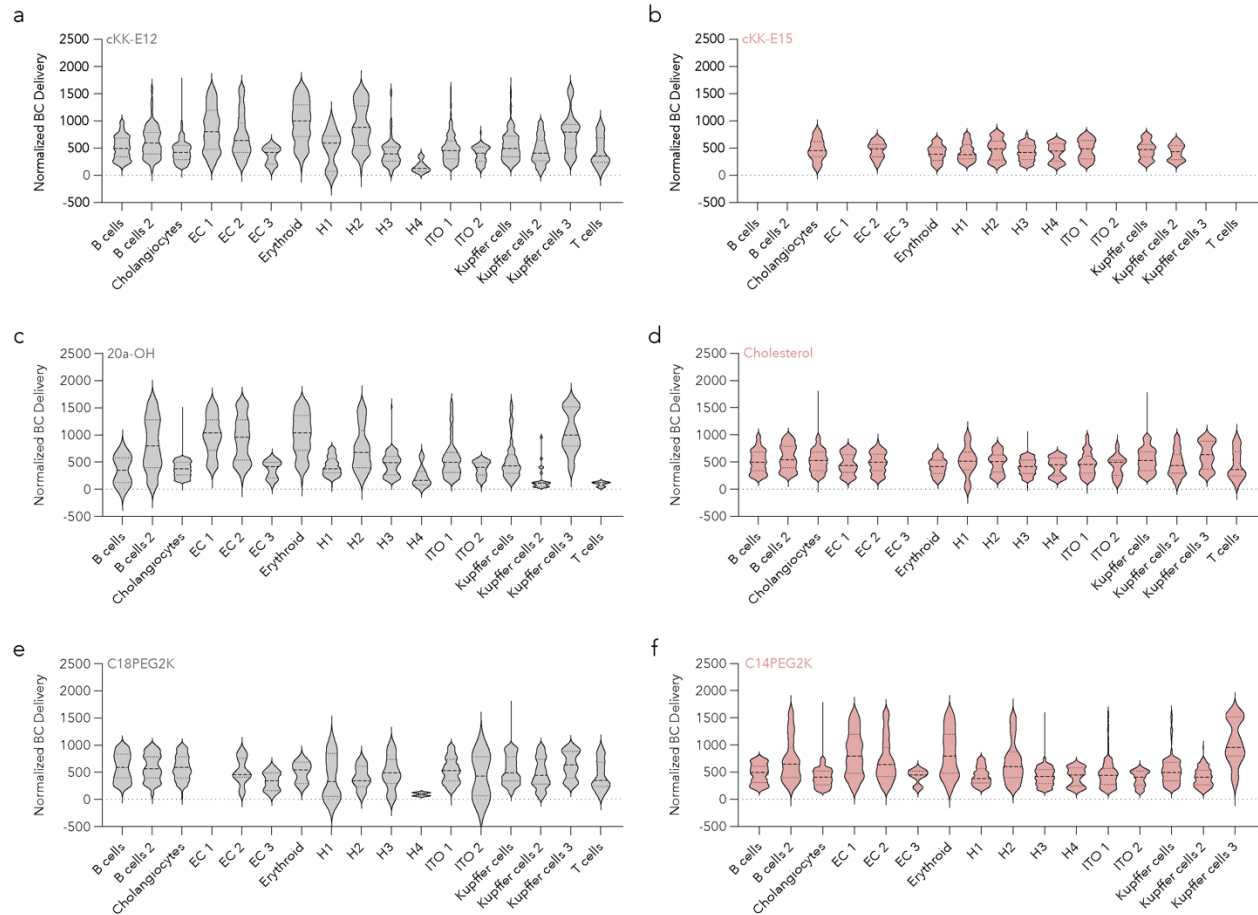
Supplementary Fig. 13. Chemically distinct LNPs exhibit different tropism within the liver microenvironment. t-SNE plots showing aVHH expression in each cell type for each LNP in the pool. Naked barcodes (*) had low aVHH expression across all cells.



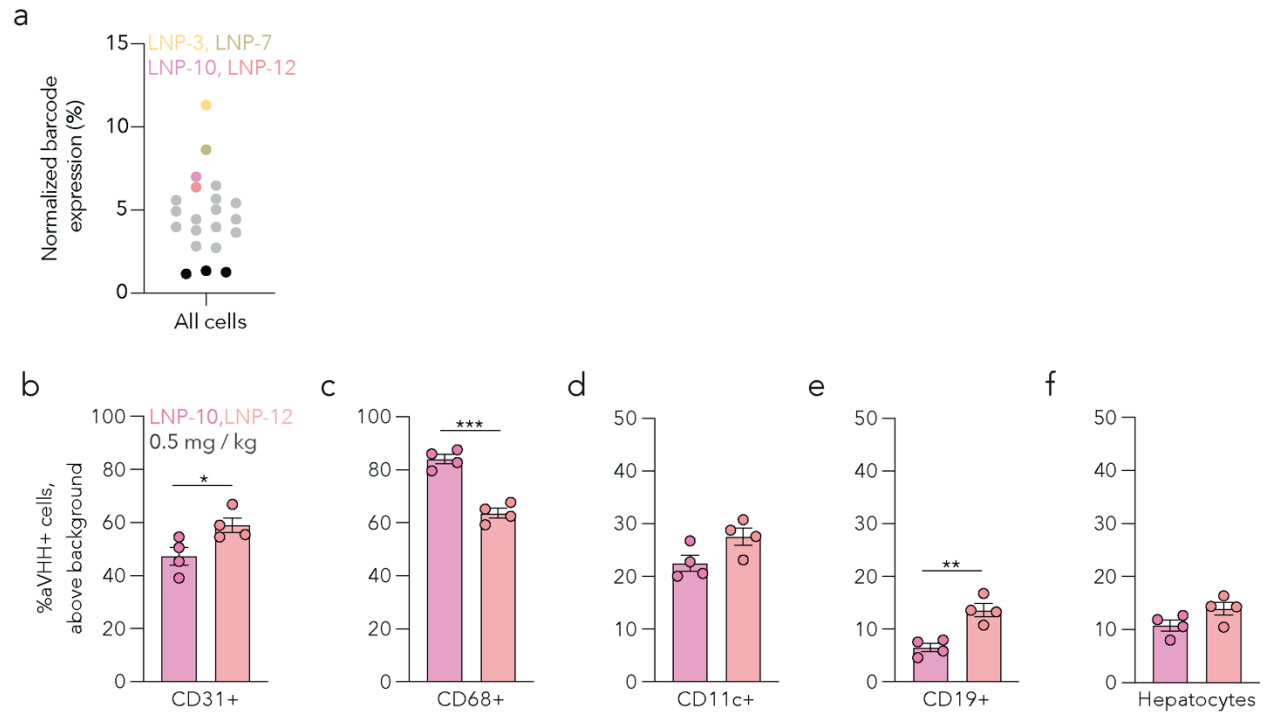
Supplementary Fig. 14. Chemically distinct LNPs exhibit different tropism within the liver microenvironment. t-SNE plots showing the ratio of aVHH expression to barcode expression in each cell type for each LNP in the pool.



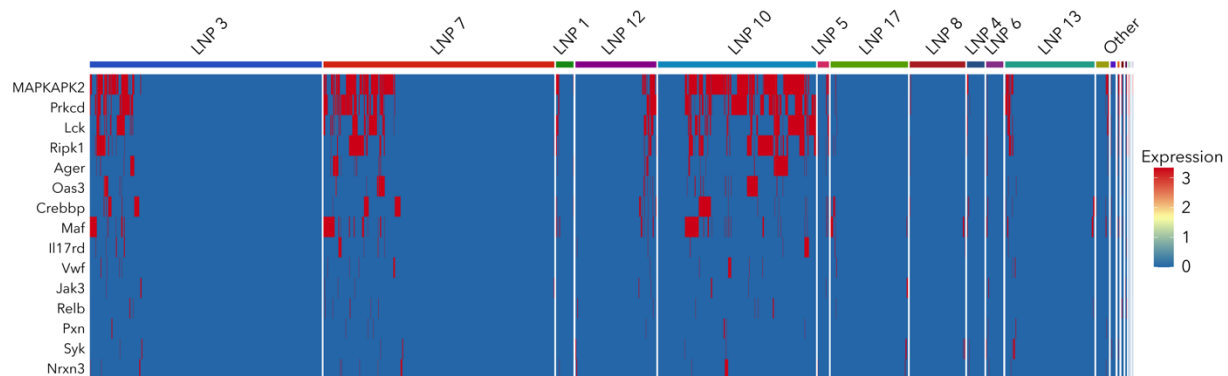
Supplementary Fig. 15. A comparison of normalized barcode delivery at the single-cell level split by LNP component. This analysis was done for each single cell containing LNP components (a) cKK-E12 (n= 5831 cells) or cKK-E15 (n = 1216 cells), (b) C₁₈ PEG2K (n = 2084 cells) or C₁₄ PEG2K (n = 4963 cells), (c) 20a-hydroxycholesterol (n = 2516 cells) or cholesterol (n = 4531 cells). (d) A comparison of the performance of each LNP ratio used in the screen, at the single-cell level. The three ratios used were 35:46.5:2.5:16 (n = 3643 cells), 45:39.5:2.5:13 (n = 1014 cells), and 50:35:2.5:12.5 (n = 2390 cells). In all cases, data is plotted as mean $\pm$ standard error mean. Statistical analyses for (a) – (c) were conducted using an unpaired t-test with Welch’s correction. Statistical analyses for (d) were conducted using a one-way ANOVA with Tukey’s multiple comparison test to compare different LNP ratios. ns (p > 0.05, not shown), * (p < 0.05), ** (p < 0.01), *** (p < 0.001), **** (p < 0.0001).



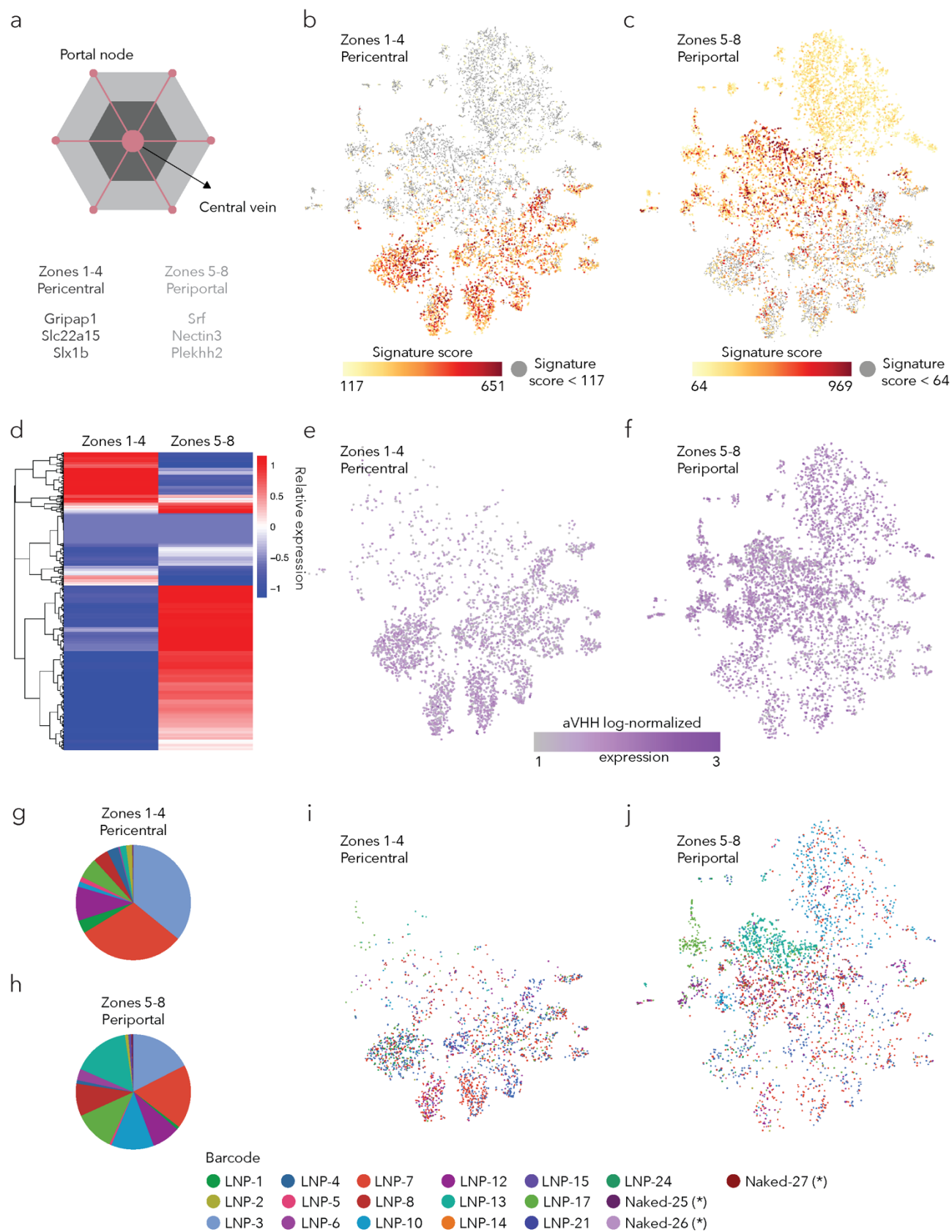
Supplementary Fig. 16. Violin plots representing the normalized barcode distributions of each LNP component at the cell population level. Violin plots for barcode distributions are shown for (a) cKK-E12, (b) cKK-E15, (c) 20a-hydroxycholesterol, (d) cholesterol, (e) C₁₈ PEG2K, and (f) C₁₄ PEG2K.



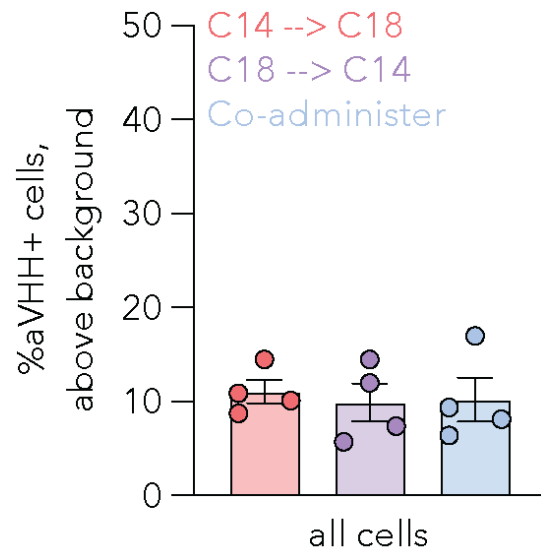
Supplementary Fig. 17. Chemically distinct LNPs exhibit different tropism within the liver microenvironment. (a) When confirmed with our traditional sequencing methods, LNP-3, LNP-7, LNP-10, and LNP-12 were among LNPs with the highest normalized barcode expression. All other LNPs are shown in gray, and naked barcodes are shown in black. Flow cytometry data from a confirmation experiment using LNP-10 and LNP-12 for (b) CD31⁺ endothelial cells, (c) CD68⁺ Kupffer cells, (d) CD11c⁺ dendritic cells, (e) CD19⁺ B cells, and (f) CD31⁻ CD45⁻ hepatocytes. In (b) – (f), n = 4 mice per group were used and data is plotted as mean ± standard error mean. Statistical analyses were conducted using an unpaired t-test. ns (p > 0.05, not shown), * (p < 0.05), ** (p < 0.01), *** (p < 0.001), **** (p < 0.0001).



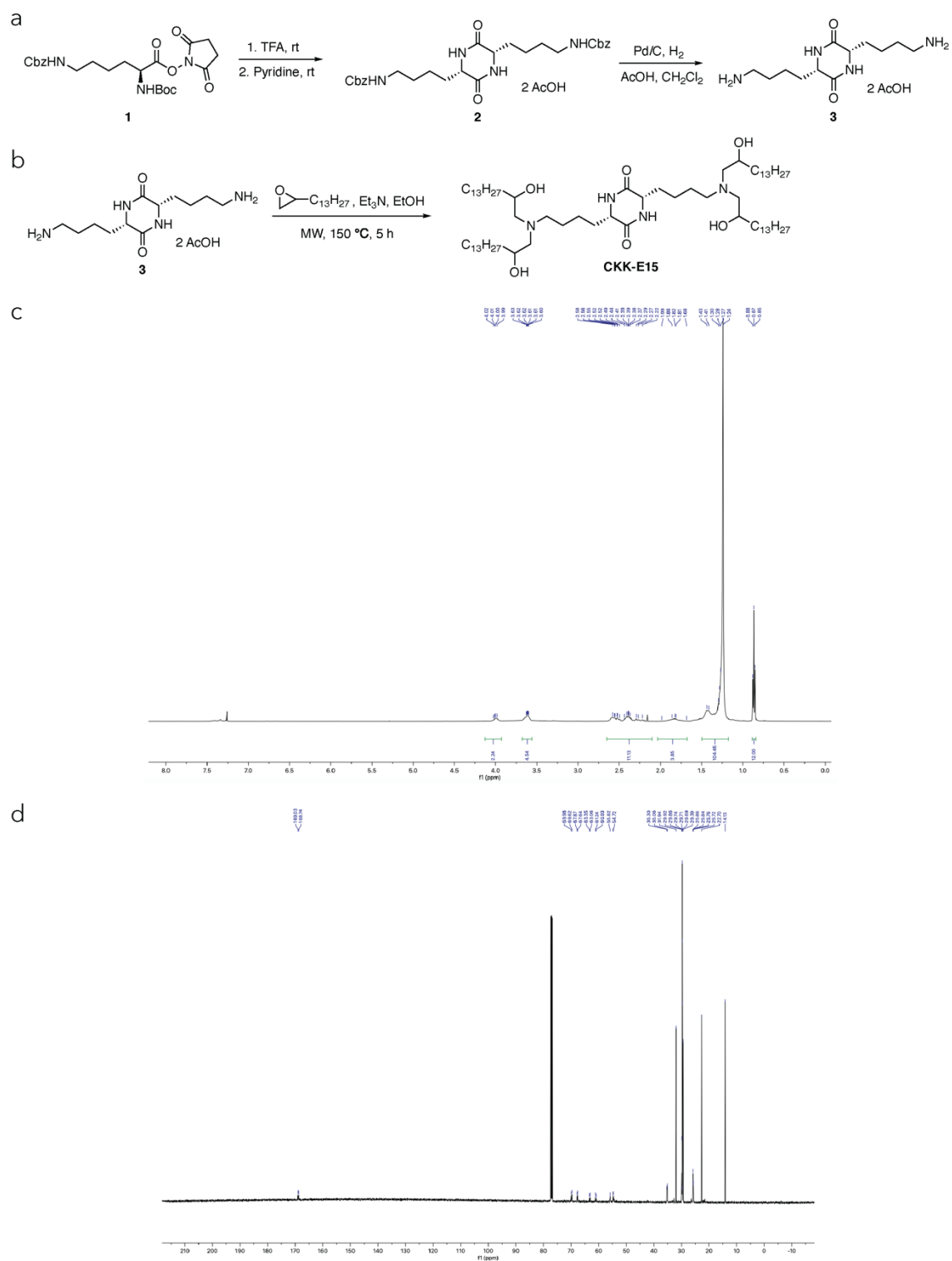
Supplementary Fig. 18. Gene expression for 15 immune-related genes in each single cell that receives each LNP shown.



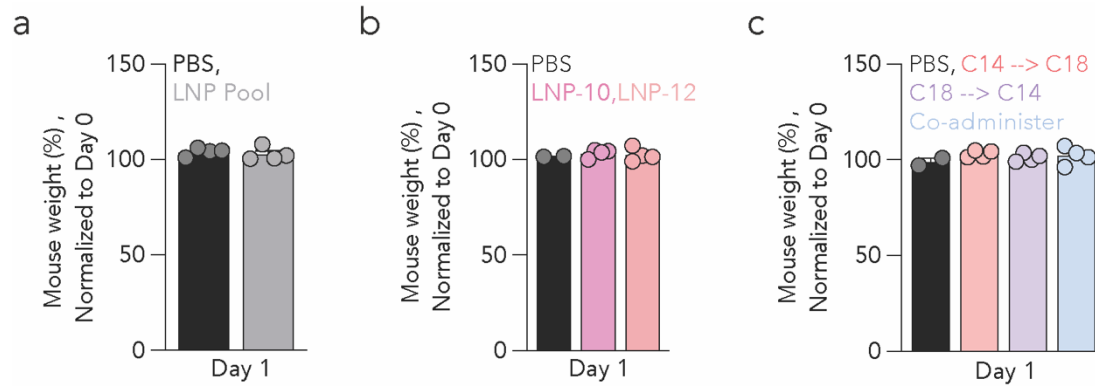
Supplementary Fig. 19. The impact of liver metabolic zonation on LNP delivery. (a) Cells close to the central vein are pericentral; those close to the portal node are periportal. (b) Cells within our single-cell data that have high signature scores for genes found in pericentral cells. (c) Cells within our single-cell data that have high signature scores for genes found in periportal cells. (d) Cells identified as pericentral or periportal have different gene expression profiles relative to each other. Log-normalized aVHH expression for each single cell overlaid on a t-SNE plot for (e) pericentral cells and (f) periportal cells. Percentage of each barcode found in cells expressing aVHH for (g) pericentral cells and (h) periportal cells. The most common barcode delivered by LNPs, for 24 chemically distinct LNPs, overlaid on the t-SNE plot in (i) pericentral cells and (j) periportal cells.



Supplementary Fig. 20. The impact of a C₁₄ PEG2K LNP on the functional delivery of a C₁₈ PEG2K LNP. Flow cytometry data for cells receiving a C₁₄ PEG2K LNP followed by a C₁₈ PEG2K LNP, a C₁₈ PEG2K LNP followed by a C₁₄ PEG2K LNP, or both administered at the same time. In all cases, n = 4 mice per group were used and data is plotted as mean $\pm$ standard error mean. Statistical analyses were conducted using a one-way ANOVA with Tukey's multiple comparison test to compare different conditions. ns (p > 0.05, not shown), * (p < 0.05), ** (p < 0.01), *** (p < 0.001), **** (p < 0.0001).



Supplementary Fig. 21. cKK-E15 synthesis pathway. (a,b) Synthesis pathway for cKK-E15 and intermediates, used as an ionizable lipid for the LNP screen. (c) ^1H -NMR and (d) ^{13}C NMR measurements for cKK-E15.



Supplementary Fig. 22. Mouse weights for experiments. (a) Changes in weight for mice treated with the control, 1X PBS, did not differ from changes in weight for mice treated with the LNP pool. (b) Changes in weight for mice treated with control, 1X PBS, did not differ significantly from changes in weight for mice treated with LNP-10 or LNP-12. (c) Changes in weight for mice treated with control, 1X PBS, did not differ significantly from changes in weight for mice treated with a C₁₄ PEG2K LNP followed by a C₁₈ PEG2K LNP, or a C₁₈ PEG2K LNP followed by a C₁₄ PEG2K LNP, or a C₁₄ PEG2K LNP and a C₁₈ PEG2K LNP co-administered. For the scRNA-Seq experiment, n = 4 mice per group were used for PBS-treated and LNP-treated mice. For the confirmation flow cytometry experiments shown in (b) and (c), n = 2 PBS-treated mice per group were used for gating and n = 4 mice per group were LNP treated. In all cases, data is plotted as mean $\pm$ standard error mean. Statistical analyses were conducted using an unpaired t-test for (a) and a one-way ANOVA with Tukey's multiple comparison test for (b) and (c). ns (p > 0.05, not shown), * (p < 0.05), ** (p < 0.01), *** (p < 0.001), **** (p < 0.0001).