Phospholipid nanoparticle preparation and treatment

Lipid sources. Phospholipids were purchased from Avanti Polar Lipids: C18:0-20:4 PE (850804C), C18(plasm)-20:4 PE (852804), C18(plasm)-18:1 PE (852758P), C18(plasm)-22:6 PE (852806), C18:0-20:4 PC (850469C), C18(plasm)-20:4 PC (852469), C18(plasm)-18:1 PC (852467C), C18(plasm)-22:6 PC (852472), C16(-O-)-20:4 PC (878113C). All lipids were produced at purity > 99%.

Nanoparticle preparation. The representative procedure for preparing liposomes is described as following: C18(Plasm)-22:6 PE (2 mg, 1 mg/mL in chloroform) was transferred to a 4 mL amber borosilicate vial equipped with a PTFE lined cap. The solvent was evaporated until completely dry. A Branson sonicator was filled with reverse-osmosis water and heated to 65-70 °C, at which point the vial was partially submerged in the bath and milli-Q deionized water (2 mL) was added to re-suspend the lipid film. This solution was sonicated for approximately 2 min, allowed to cool to room temperature, sonicated again for 2 min, and re-cooled to room temperature prior to characterization. The final liposome solution was stored at 4 °C and protected from light until further use. This procedure was repeated for all other phospholipid species.

Nanoparticle characterizations. To characterize the lipid-containing nanoparticles, hydrodynamic size and polydispersity were measured using dynamic light scattering (Malvern ZS90 Particle Analyzer, $\lambda = 633$ nm, material/dispersant RI 1.590/1.330). Zeta potential measurements were also acquired with the Malvern ZS90, using laser Doppler electrophoresis. Polystyrene semi-micro cuvettes for the Malvern Zetasizer were purchased from VWR, and DTS1070 folded capillary cells were purchased directly from Malvern. Particle solutions were diluted in milli-Q water in polystyrene semi-micro cuvettes (VWR) or DTS1070 folded capillary cuvettes (Malvern) to produce samples for characterization on the Malvern SZ90.

Liposome	Diameter (number mean, nm)	Polydispersity index
C18(Plasm) - 20:4 PC	91.5 ± 52.4	0.633 ± 0.17
C18(Plasm) - 20:4 PE	100.0 ± 27.6	0.246 ± 0.10
C18(Plasm) - 22:6 PC	156.3 ± 150	0.756 ± 0.10
C18(Plasm) - 22:6 PE	173.6 ± 8.6	0.408 ± 0.05
C18(Plasm) - 18:1 PC	77.7 ± 35.9	0.353 ± 0.01
C18(Plasm) - 18:1 PE	84.4 ± 27.7	0.298 ± 0.08
18:0 20:4 PC	72.5 ± 6.3	0.558 ± 0.02
18:0 20:4 PE	202.7 ± 120.4	0.379 ± 0.08
C16 - 20:4 PC*	-	-

Table 1. Diameter and polydispersity of liposomes. The average and standard deviation of three technical repeats are provided. *C16 – 20:4 PC lipids did not self-assemble into measurable particles. We note that no aggregates were observed, enabling us to conclude the lipids were uniformly dispersed throughout the aqueous solution.

Nanoparticle treatment. The average molar concentration of phospholipid nanoparticles is ~1.3 mM, and these nanoparticles are used at 1:65 dilution in culture media to reach final concentrations of 20 μM. For viability assays, cells were pre-treated with lipid nanoparticles for 24 h prior to be splitted for compound treatment. Water used for generating phospholipid nanoparticles was used as vehicle control. For acute lipid treatment and lipid peroxidation imaging, lipid nanoparticles

were applied to the cells at the same time as ML210 or 1 hour later as indicated in the figure legends.

RNA extraction and qRT-PCR analysis

Cells were plated onto 6-well plates at a density of 200,000 cells per well. Total RNA was extracted using RNeasy Micro Kit (Qiagen, Cat. no 74004) and cDNA was prepared using QuantiTect reverse transcription kit (Qiagen, Cat. no. 205311). Quantitative real-time RT-PCR was performed using SYBR Green I Master Mix (Roche, Cat. no 04887352001) and the ABI Prism 7900 sequence detector (Applied Biosystems). Relative mRNA expression was calculated by the ΔΔCT method with GAPDH as reference. Primer sequences are listed below:

Gene	Primer	Sequence
GAPDH	forward	GTCTCCTCTGACTTCAACAGCG
GAPDH	reverse	ACCACCCTGTTGCTGTAGCCAA
PEX3	forward	AATTCCGAGAGCCTCACAGCTC
PEX3	reverse	GACCCGCAAAAGAACAACCAGC
AGPS	forward	GGCTGGCATAACAGGACAAGAG
AGPS	reverse	CTTCATGCCTGATGCGCGAGTA
AGPAT3	forward	CTGCTGGTCGGCTTTGTCTT
AGPAT3	reverse	TCCAGAGTGAGTAGGCGAGG
B2M	forward	CTCCGTGGCCTTAGCTGTG
B2M	reverse	TTTGGAGTACGCTGGATAGCCT

Genetic perturbations

Lentivirus production and cell infection. Lentiviruses were generated from lentiviral constructs including ones used for cDNA, sgRNA or shRNA expression in HEK-293T packaging cells.

FUGENE6 (Promega) or Lipofectamine 2000 (Life Technologies) was used as transfection reagents to deliver plasmids to cells following manufacturer's instructions. Second generation packaging plasmids, including pMD2.G and pPAX2, was used for lentiviral production. Lentivirus titer was briefly assessed with Lenti-X Go-Stix Plus (TakaraBio). Target cells were infected with lentiviruses in the presence of 5 μg/mL of polybrene (Millipore). Depending on the vector, infected cells were selected with 2 μg/mL of puromycin, 8 μg/mL of Blasticidin S, or 200 μg/mL of Hygromycin B and propagated for further analysis. Cells transduced with doxycycline-inducible constructs were treated with 1 μg/ml of doxycycline (Sigma-Aldrich) for 7-14 days prior to gene-knockout/expression validation using immunoblotting.

CRISPR-mediated individual gene editing. For CRISPR/Cas9-mediated genome-editing, cells were engineered for Cas9 expression with the pLX-311-Cas9 vector (Addgene 96924), which contains the blasticidin S-resistance gene driven by the SV40 promoter and the SpCas9 gene driven by the EF1α promoter. sgRNA sequences were cloned into the pLV709 doxycycline-inducible or pXPR_BRD050 constitutive sgRNA expression vectors. In various instances, CRISPR/Cas9-mediated genome-editing was also performed using lentiviral transduction of cells with lentiCRISPRv2-puro vectors containing constitutive expression of Cas9 and the respective sgRNA. LentiCRISPRv2 vectors was a gift from Feng Zhang (Addgene plasmid # 52961).

Sequences for sgRNAs used are listed below:

Gene	sgRNA	Target sequence
PEX3	sgRNA-1	CATGCTTCCAACACTGAGAG
PEX3	sgRNA-2	GTCTCGAGGAGAAAGTCCAC
GPX4	sgRNA-1	CTTGGCGGAAAACTCGTGCA
PEX10	sgRNA-1	AGGACGAGTACTACCGCGGT
PEX10	sgRNA-2	GAGGGCACATGTATCCGCGA

PEX12	sgRNA-1	GAGACCCGCTCTTCAGCATG
PEX12	sgRNA-2	GCTTAAAGAGAATTGTAATG
AGPS	sgRNA-1	GGGAGCCTGTCACATCACCA
AGPS	sgRNA-2	GAGAACCCGCAGCCTCCGCC
FAR1	sgRNA-1	ACAGACACCACAAGAGCGAG
FAR1	sgRNA-2	TACAGGAACAAGATCTGCAA
AGPAT3	sgRNA-1	CTTCCCAAAGCGCTCTACCG
AGPAT3	sgRNA-2	TACCACGTCCAGCCGATGAG
TMEM189	sgRNA-1	CCAACACCGAGTATGACGAG
TMEM189	sgRNA-2	ACCAACACCGAGTATGACGA
TMEM189	sgRNA-3	ATGCAGAAGTAGGTCTCGTG
ACSL4	sgRNA-1	GCATCATCACTCCCTTAGGT
SOD1	sgRNA-1	CCTCTATCCAGAAAACACGG
SOD1	sgRNA-2	TATCTCCAAACTCATGAACA
SOD1	sgRNA-3	AATGTGACTGCTGACAAAGA
CAT	sgRNA-1	ACATCTGAAGGATCCGGACA
CAT	sgRNA-2	CTGGATGTAAAAAGTCCAGG

shRNA-mediated gene knockdown. For shRNA-mediated RNA interference, shRNAs targeting the genes of interest were pre-cloned into constitutive shRNA expression vectors pLKO.1 or pLKO-TRC005 by the Broad Institute Genetic Perturbation Platform. *TMEM189* shRNAs in pTRIPZ doxycycline-inducible vectors were purchased from Horizon Discovery (RHS4740-EG387521).

Gene	shRNA	Sequence
FAR1	shRNA-1	CTTTGTGGTTAGTCTGTGTTA

FAR1	shRNA-2	CCAGGATGGATTGATAACTTT
FAR1	shRNA-3	GCTGTTCAGTTAAATGTGATT
FAR1	shRNA-4	GCAGTGTATCTGGAGTATGTT
GNPAT	shRNA-1	GCCAAGACATTGACTCCTAAA
GNPAT	shRNA-2	CCAGAAAGATTCTCTCTGAAA
GNPAT	shRNA-3	GCCATACAAGTGACTACGAAA
GNPAT	shRNA-4	CCTCTCAATGTTATGATGTAT
AGPAT3	shRNA-1	GCTACGGAAACCAAGAGTTTA
AGPAT3	shRNA-2	GCGCTCCAGGAGATATATAAT
AGPAT3	shRNA-3	GACATGTGCGTGAGGAGATTT
AGPAT3	shRNA-4	TCGCAGACTGATAGGAGTAAC
TMEM189	shRNA-1	AGGGGTCTAGACTTGAGCA
TMEM189	shRNA-2	TATTTGATCTTCTGGGCCC
TMEM189	shRNA-3	TGAGCAGGAGGTTCCAGCC

siRNA-mediated transient gene knockdown. For siRNA experiments, iCell Cardiomyocytes[2] and cardiac progenitors (3 days post-plating) were transfected with 50 nM of ON-TARGETplus non-targeting siRNA, (Dharmacon Inc., Cat. no. D-001810-01-05), *Peroxin 3* siRNA (Santa Cruz Biotechnology, Cat. no. SC-95091), ON-TARGETplus Human *AGPS* (8540) siRNA - SMARTpool, (Dharmacon Inc. # L-009267-00-0005), using TransIT-TKO transfection reagent (Mirus Bio, Cat. No. MIR2150).

cDNA expression. cDNAs in pReceiver-Lv244-mCherry-hygro lentiviral vectors for constitutive expression were purchased from GeneCopoeia as listed below. For cDNA-mediated rescue experiments, the sequences of mouse *Agps, Agpat3, Far1, Pex3* and *Pex10* cDNAs were verified to be not targeted by at least one human gene-targeting sgRNA. Cells infected with pLv244

lentiviruses were selected with 2 μg/mL of puromycin 24 hours post-infection for another 96 hours. cDNA expression efficiency was confirmed with immunoblotting.

Species	Gene	Transcript	Catalog number
mouse	*Agps*	NM_172666.3	EX-Mm35936-Lv244-GS
mouse	*Agpat3*	NM_053014.3	EX-Mm06713-Lv244-GS
mouse	*Far1/Mlstd2*	NM_027379.3	EX-Mm09165-Lv244
mouse	*Pex3*	NM_019961.3	EX-Mm07693-Lv244
mouse	*Pex10*	NM_001042407.1	EX-Mm26952-Lv244-GS
human	*TMEM189*	NM_199129.2	EX-W0118-Lv244
	Empty vector	pReceiver-Lv244	EX-NEG-Lv244

Site-directed mutagenesis. Mutagenesis was performed on the pReceiver-Lv244-m*Agpat3*-mCherry-hygro vector using the Q5 site-directed mutagenesis kit (New England Biolabs, E0554) following the manufacturer's instructions to generate the E176A point mutation. The following primers were used in the PCR reaction: m*Agpat3*-E176A-forward, ctc ctg tac tgc g**cc**a gga aca cgc ttc; m*Agpat3*-E176A-reverse, gac tac cca gag tac atg tgg ttt. Mutated constructs were verified by Sanger sequencing.

Single cell cloning of CRISPR knockout cells. GPX4$^{-/-}$ single-cell 786-O clones were established and validated in our prior study[14]. For OVCAR-8 cells, *GPX4*$^{-/-}$ single-cell clones (SCC) were generated from *GPX4*-sg1 expressing cells, *AGPS*$^{-/-}$ SCC were generated from *AGPS*-sg2 expressing cells, and *FAR1*$^{-/-}$ SCC were generated from *FAR1*-sg1 expressing cells. Briefly, puromycin-selected polyclonal *GPX4*-sg1, *AGPS*-sg2, or *FAR1*-sg1 expressing cells were seeded in 96-well tissue culture-treated plates at 1 cell per well by FACS. Every 3 days for 2-3 weeks, media was added or replaced and wells were monitored to ensure a single monoclonal growth. Clones were expanded and relative expression levels of the target proteins were validated by

immunoblotting. *GPX4*-sg1 cells were cultured in medium containing 2.5 μM ferrostatin-1 unless otherwise indicated.

Generation of double knockout cells. To generate cells with double knockout of *GPX4* and each of the peroxisome/ether-lipid biosynthesis genes, *GPX4*$^{-/-}$ SCC2 OVCAR-8 cells were transfected with blasticidin-resistant sgRNA expression vectors containing sgNC, *AGPS*-sg1, *AGPS*-sg2, *FAR1*-sg2, *PEX3*-sg1, *PEX3*-sg2, *PEX10*-sg1, or *PEX10*-sg2. Cells were selected with 8 μg/ml of blasticidin 48 hours post infection for another 4 days. Target gene knockout efficiency in each cell line was validated by immunoblotting.

To generate cells with double knockout of *ACSL4* and each of the peroxisome/ether-lipid biosynthesis genes, cancer cells expressing blasticidin-resistant *AGPS*-sg2, *FAR1*-sg2, *PEX3*-sg1, or *PEX10*-sg1 were infected with lentivirus from doxycycline-inducible pLV706-*ACSL4*-sgRNA1-puromycin construct. Cells were selected with puromycin 24 hours post infection for another 96 hours. Target gene knockout efficiency in each cell line was validated by immunoblotting.

Supplementary Information

Gene-list Network Enrichment Analysis (GeLiNEA)

Background. Unbiased computational methods to interpret experiment results are needed to avoid the influences of an investigator's "experience". Gene-set enrichment analysis[28] (GSEA) is a popular choice[87]; to work well it generally prefers a larger number of genes. GSEA can be extended to include network connections between genes using a network-rewiring randomization algorithm. One such method, Network Enrichment Analysis[88], evaluates the number of connections between a gene list of interest and predefined gene sets representing biological concepts with a goal to identify a gene set that best represents the gene list. While such an approach is more powerful than GSEA, network rewiring is not computationally efficient, and can be replaced with the analytical approach of random graphs with given expected degrees (number of connections in a network)[89]. Graph rewiring, in both simulated and an analytical form, breaks down the structure of gene sets and thus creates a bias that leads to unrealistically low p-values.

Approach. In contrast to graph-rewiring approaches, our null model randomizes gene-list membership while keeping the underlying network intact. To avoid any potential bias, we require that gene-list randomization preserves degree distribution of nodes in the gene list, i.e., when randomizing, the node can only be reshuffled to a node of the same degree (or a similar-degree node for high-degree nodes). A similar approach, in the context of counting connections within a gene list, was adopted by Rossin and collaborators[90], using simulations to determine the significance. Instead, we have derived an analytical solution for the null-model distribution and can thus obtain precise quantities even for very small p-values.

Let $G = (V, E)$ be a network with a node set $V = \{v_1, v_2, \ldots, v_n\}$ and an edge set E. Let $V^{(k)} \subset V$ be a set of nodes with degree k and let $n^{(k)}$ be the size of $V^{(k)}$. Let gene list L be an ordered list of pairwise distinct nodes and let $S \subset V$ be a gene set. Let $d(L, S) = |\{(u, v) \in E : u \in L \,\&\, v \in S\}|$ be the number of connections between L and S. Note that if both u and v belong to both the gene set S and the gene list L, the connection is counted twice. This is in agreement with our intuition as it gives higher weight to connections in the overlap between L and S. The definition of $d(L, S)$ implies its additivity, $d(L, S) = \sum_k d(L^{(k)}, S)$ where $L^{(k)}$ are all degree-k nodes from L. Sets $L^{(k)}$ are pairwise disjoint and their sizes are denoted by $m^{(k)}$; the degree distribution of L then is $m^{(0)}, m^{(1)}, \ldots, m^{(n-1)}$.

Our goal is to determine, for a given gene list L and a given gene set S, the significance (p-value) of the $d(L, S)$ under a null model of random gene lists with given degree distribution. Let $\mathcal{U}$ be a space of all gene lists with the same degree distribution as L; gene lists from $\mathcal{U}$ can be seen as realizations of degree-preserving randomization. We define a random variable $X = d(U, S), U \in \mathcal{U}$, representing the number of connections between a random gene list U and the

given gene set S. Due to additivity we can write $X = X^{(1)} + X^{(2)} + \ldots + X^{(n-1)}$, where the random variable $X^{(k)} = d(U \cap V^{(k)}, S)$ is the number of connections between a random set of $m^{(k)}$ nodes of degree k and the gene set S.

It is a routine practice in probability theory to associate a non-negative integral-valued random variable X with a generating function $F(x) = \sum f_i x^i$, where $f_i = P(X = i)$. The p-value of observing $d(L, S)$ connections then can be expressed as

$$p = P(X \geq d(L, S)) = \sum_{i \geq d(L,S)} f_i.$$

Since X is as a sum of independent (non-negative integral-valued) random variables $X^{(k)}$, its generating function can be written as a product of generating functions $F^{(k)}(x)$ associated with $X^{(k)}$,

$$F(x) = \prod_k F^{(k)}(x). \tag{1}$$

To derive generating functions $F^{(k)}(x)$ for $X^{(k)}$ we have to consider the combinatorics of randomly selecting $m^{(k)}$ nodes and counting their connections to S. In this case we work with bivariate generating functions and associate the x variable with the number of connections and the y variable with the number of selected nodes. If c_i denotes the number of connections between the i-th node of $V^{(k)}$ and the gene set S then the generating function for one node is $1 + x^{c_i} y$. The generating function $G^{(k)}(x, y)$ for the number of different ways to select m nodes of degree k that have exactly j connections to S thus is

$$G^{(k)}(x, y) = \prod_{i=1}^{n^{(k)}} (1 + x^{c_i} y) = \sum_{j,m} g_{j,m} x^j y^m = \sum_m G_m^{(k)}(x) y^m,$$

and the probability-generating function $F^{(k)}(x)$ can then be directly obtained from $G^{(k)}_{m^{(k)}}(x)$ by normalizing it with a combinatorial number $\binom{n^{(k)}}{m^{(k)}}$.

Application. Enrichments (reported in Figure 1 and Extended Data Figure 1) were computed using curated gene sets (C2) from MSigDB version 6.0 and high-confidence human protein-protein association network STRING version 10.5 (confidence score ≥ 0.7). Equation (1) was used to compute p-values and adjustment for multiple testing was done using the Benjamini-Hochberg correction method. For comparison, GSEA analysis was performed using the same input gene lists and MSigDB C2 gene sets using standard settings.