Ferroptosis is a form of regulated cell death that is caused by the iron-dependent peroxidation of lipids. The glutathione-dependent lipid hydroperoxide-glutathione peroxidase 4 (GPX4) prevents ferroptosis by converting lipid hydroperoxides into non-toxic lipid alcohols. Ferroptosis has previously been implicated in the cell death that underlies several degenerative conditions, and induction of ferroptosis by the inhibition of GPX4 has emerged as a therapeutic strategy to trigger cancer cell death. However, sensitivity to GPX4 inhibitors varies greatly across cancer cell lines, which suggests that additional factors govern resistance to ferroptosis. Here, using a synthetic lethal CRISPR–Cas9 screen, we identify ferroptosis suppressor protein 1 (FSP1) (previously known as apoptosis-resistance factor mitochondrial 2 (AIFM2)) as a potent ferroptosis-resistance factor.

GPX4 is considered to be the primary enzyme that prevents ferroptosis. The resistance of some cancer cell lines to GPX4 inhibitors led us to search for additional protective pathways. To identify ferroptosis-resistance genes, we performed a synthetic lethal CRISPR–Cas9 screen using a sublibrary of single-guide RNAs (sgRNAs) targeting genes related to apoptosis and cancer in U-2 OS osteosarcoma cells that were treated with the GPX4 inhibitor RSL3 (hereafter, RSL3) (Fig. 1a). This screen revealed a substantial dis-enrichment of sgRNAs targeting FSP1 (currently known as AIFM2) in the cells treated with RSL3 (Fig. 1b, c, Extended Data Fig. 1a, Supplementary Table 1), which was rescued by expression of untagged FSP1 (Extended Data Fig. 1c,d). In contrast to previous reports, the overexpression of FSP1 did not induce apoptosis (Extended Data Fig. 1e, f) and activation of p53 did not increase FSP1 expression (Extended Data Fig. 1g). FSP1 knockout cells displayed increased sensitivity to additional ferroptosis inducers, including the GPX4 inhibitor ML162 and the system xc\(^-\) inhibitors erastin and deferoxamine (DFO) and by the radical-trapping antioxidants ferrostatin-1 (Fer1) and idebenone (Fig. 1h), but not by inhibitors of apoptosis or necroptosis (Extended Data Fig. 1i–l).

The viability of RSL3-treated FSP1KO cells was rescued by the iron chelator deferoxamine (DFO) and by the radical-trapping antioxidants ferrostatin-1 (Fer1) and idebenone (Fig. 1g), but not by inhibitors of apoptosis or necroptosis (Extended Data Fig. 1i–l).

FSP1 mediates resistance to ferroptosis in lung cancer cells in culture and in mouse tumour xenografts. Thus, our data identify FSP1 as a key component of a non-mitochondrial CoQ antioxidant system that acts in parallel to the canonical glutathione-based GPX4 pathway. These findings define a ferroptosis suppression pathway and indicate that pharmacological inhibition of FSP1 may provide an effective strategy to sensitize cancer cells to ferroptosis-inducing chemotherapeutic agents.
FSP1 cells (FSP1<sup>ACSL4<sup>KO</sup></sup>) restored resistance to RSL3 to an extent similar to that of knockouts of ACSL4 alone (ACSL4<sup>KO</sup>) (Fig. 1h, Extended Data Fig. 1n), consistent with the requirement for ACSL4-mediated incorporation of polyunsaturated fatty acids into phospholipids for ferroptosis<sup>15</sup>. Together, these findings demonstrate that FSP1 is a strong suppressor of ferroptosis.

**Plasma-membrane FSP1 blocks ferroptosis**

FSP1 contains a short N-terminal hydrophobic sequence and a canonical flavin adenine dinucleotide-dependent oxidoreductase domain (Extended Data Fig. 1o). FSP1 has previously been detected on lipid droplets<sup>10</sup>, suggesting that deletion of FSP1 does not inhibit system xc<sup>−</sup> or lipid droplets had no effect (Fig. 2i). Consistent with previous results in HT1080 cells<sup>11</sup>, the depletion of lipid droplets using inhibitors of the diacylglycerol acyltransferase enzymes (DGAT1 and DGAT2) did not affect ferroptosis sensitivity (Extended Data Fig. 5a–c), which provides support for the conclusion that lipid-droplet localization is not required for the FSP1-mediated suppression of ferroptosis. Thus, FSP1 plasma-membrane localization is necessary and sufficient to confer ferroptosis resistance.

**FSP1 reduces CoQ<sub>10</sub> to suppress ferroptosis**

Under basal conditions, the ratiometric fluorescent lipid peroxidation sensor BODIPY S81/591C11 exhibited similar levels of oxidation in control and FSP1<sup>KO</sup> cells (Fig. 3a, Extended Data Fig. 6a, b). However, a brief treatment with RSL3 strongly increased C11 oxidation in FSP1<sup>KO</sup> cells (Fig. 3a, Extended Data Fig. 6a, b). Consistent with previous results in HT1080 cells<sup>11</sup>, the depletion of lipid droplets using inhibitors of the diacylglycerol acyltransferase enzymes (DGAT1 and DGAT2) did not affect ferroptosis sensitivity (Extended Data Fig. 5a–c), which provides support for the conclusion that lipid-droplet localization is not required for the FSP1-mediated suppression of ferroptosis. Thus, FSP1 plasma-membrane localization is necessary and sufficient to confer ferroptosis resistance.

(Extended Data Fig. 2f), the fractionation of organelles in iodixanol (OptiPrep) gradients revealed that FSP1(G2A)–GFP was present at lower levels in fractions enriched in plasma membrane (Fig. 2f, Extended Data Fig. 2f). Together, these results indicate that the myristoylation of FSP1 mediates the recruitment of this protein to lipid droplets and the plasma membrane.

Expression of FSP1<sup>(WT)</sup>–GFP, but not of FSP1<sup>(G2A)</sup>–GFP, rescued the resistance of FSP1<sup>(KO)</sup> cells to RSL3 (Fig. 2h, Extended Data Fig. 3f), which indicates that FSP1 must be myristoylated to suppress ferroptosis. We generated fusion proteins that selectively target FSP1<sup>(G2A)</sup>–GFP to the endoplasmic reticulum (amino acids 100–134 of cytochrome b<sub>5</sub>; Cb5), the outer mitochondrial membrane (TOM20 signal sequence, TOM20(SS)), lipid droplets (PLIN2) and the plasma membrane (first 11 amino acids of LYN kinase; LYN11) (Extended Data Fig. 4a, b). Only the expression of FSP1 targeted to the plasma membrane (LYN11–FSP1<sup>(G2A)</sup>–GFP) was sufficient to restore ferroptosis resistance in FSP1<sup>(KO)</sup> cells (Fig. 2i, Extended Data Fig. 4c). By contrast, expression of FSP1<sup>(G2A)</sup>–GFP targeted to the endoplasmic reticulum, mitochondria or lipid droplets had no effect (Fig. 2i). Consistent with previous results in HT1080 cells<sup>11</sup>, the depletion of lipid droplets using inhibitors of the diacylglycerol acyltransferase enzymes (DGAT1 and DGAT2) did not affect ferroptosis sensitivity (Extended Data Fig. 5a–c), which provides support for the conclusion that lipid-droplet localization is not required for the FSP1-mediated suppression of ferroptosis. Thus, FSP1 plasma-membrane localization is necessary and sufficient to confer ferroptosis resistance.

FSP1(G2A)–GFP was observed in proximity to the plasma membrane by total internal reflection fluorescence (TIRF) microscopy cell death analysis of cells treated with 100 nM RSL3 over 48 h. g, Dose response of RSL3-induced cell death in the presence of inhibitors of ferroptosis (Fer1, 1μM; DFO, 100 μM; and idebenone, 10 μM). h, Dose response analysis of RSL3-induced cell death of the indicated cell lines. The ACSL4<sup>KO</sup> and ACSL4<sup>40</sup>FSP1<sup>KO</sup> lines shown were generated using ACSL4 sgRNA no. 1. In e, g, h, shading indicates 95% confidence intervals for the fitted curves and each data point is the average of three technical replicates. Panels are representative of two biological replicates, except for b and c, which were derived from a single screen.

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that contain polyunsaturated fatty acids (Extended Data Fig. 6f, g, Supplementary Table 3). Levels of phospholipids containing polyunsaturated fatty acids were decreased and the corresponding lyposphopholipids were increased (Extended Data Fig. 6f, g, Supplementary Table 3), a known lipidomic signature of ferroptosis that reflects the removal of oxidized polyunsaturated fatty acids from the sn-2 position of phospholipids. These results suggest that the loss of FSP1 results in increased phospholipid oxidation even when GPX4 is functional, and that FSP1 prevents lipid peroxidation through a mechanism that is distinct from glutathione-dependent protective pathways.

FSP1 functions as an NADH-dependent CoQ oxidoreductase in vitro. Reduced CoQ can act as a radical-trapping antioxidant, and idebenone—a soluble analogue of CoQ—is sufficient to suppress lipid peroxidation (Extended Data Fig. 7a) and ferroptosis (Fig. 1g). Previous studies have detected high levels of CoQ in non-mitochondrial compartments, including the plasma membrane, but the function of this molecule in these compartments remains unclear. To examine the role of FSP1 CoQ oxidoreductase activity in suppressing ferroptosis, we mutated a conserved glutamate residue (E313 in AIF or E156 in FSP1) that is required for the binding of AIF to its cofactor, flavin adenine dinucleotide (Extended Data Fig. 7b, c). Mutation of E156 in FSP1 (FSP1(E156A)–GFP) did not affect FSP1–GFP expression or localization (Extended Data Figs. 3f, 7d, e) but greatly impaired FSP1-mediated reduction of coenzyme Q, and resazurin in vitro (Extended Data Fig. 7f–h) and abolished the ability of FSP1–GFP to rescue the resistance of FSP1KO cells to RSL3 (Fig. 3b). Consistent with these findings, the expression of FSP1(WT)–GFP, but not of FSP1(E156A)–GFP, increased the ratio of reduced-to-oxidized CoQ (Fig. 3c). Acute reduction of cellular CoQ levels by the inhibition of the CoQ biosynthesis enzyme COQ2 with 4-chlorobenzoic acid (4-CBA) strongly sensitized control cells and—to a lesser extent—FSP1KO cells to RSL3-induced ferroptosis (Fig. 3d, e, Extended Data Fig. 8a). Treatment with 4-CBA also suppressed the ability of FSP1(WT)–GFP to rescue FSP1KO cells (Extended Data Fig. 8b). A similar degree of sensitization to RSL3 was observed after knock out of COQ2 in control, but not in FSP1KO cells (Fig. 3f, g, Extended Data Fig. 8c) and COQ2KO cells exhibited increased C11 oxidation after treatment with RSL3 that was suppressed by DFO and by idebenone (Extended Data Fig. 8d, e). These data indicate that FSP1 and the CoQ synthesis machinery function in the same pathway to suppress lipid peroxidation and ferroptosis.

Deletion of NQO1, a quinone and CoQ oxidoreductase that has previously been proposed to function in ferroptosis, did not affect sensitivity to RSL3, but cells that lack both FSP1 and NQO1 (FSP1KO NQO1KO) were more sensitive than FSP1KO cells (Extended Data Fig. 39a–c). NQO1–GFP did not rescue ferroptosis resistance in FSP1KO cells to the same extent as did FSP1–GFP (Extended Data Fig. 9d–g), even when targeted to the plasma membrane (LYN1–NQO1–GFP) (Extended Data Fig. 9h, i). These results indicate that FSP1 is unique in its ability to suppress ferroptosis through the reduction of CoQ.

**FSP1 in cancer ferroptosis resistance**

The Cancer Therapeutics Response Portal (CTRP) reports correlations between gene expression and drug resistance for over 800 cancer cell lines. Data mined from the CTRP indicate that FSP1 expression positively correlates with resistance to multiple GPX4 inhibitors (RSL3, ML210 and ML162) (Fig. 4a, b, Extended Data Fig. 10a, b, Supplementary Table 4)—even more so than the system x− component and erastin target SLC7A11. Thus, FSP1 is a biomarker of ferroptosis resistance in many types of cancer. Consistent with the correlations observed in the CTRP, lung cancer cell lines that express low levels of FSP1 were the most sensitive to RSL3 and cell lines that express high levels of FSP1 were the most resistant (Fig. 4b, Extended Data Fig. 10c). Knock-out of FSP1 in the highly resistant H460 cell line resulted in a notable, approximately 100-fold sensitization to RSL3 (Fig. 4d, Extended Data Fig. 10d, e) and overexpression of FSP1–GFP in sensitive H1703 and H446 cells increased resistance to RSL3 by about 10–20 fold (Fig. 4e, Extended Data Fig. 10f–i).

To examine the possibility that the inhibition of FSP1 could be a clinically relevant approach to sensitize tumours to ferroptosis-activating chemotherapies, we used ferroptosis-resistant H460 lung cancer cells in a preclinical tumour xenograft mouse model. Owing to the poor
Fig. 3 | FSP1 suppresses lipid peroxidation by reducing CoQ. a, Control and FSP1KO cells treated with 250 nM RSL3 for 75 min were labelled with BODIPY 581/591 C11 and fixed before imaging. Ox., oxidized; Non-ox., non-oxidized. Images are representative of at least 30 cells imaged for each treatment condition. Scale bar, 20 μm. b, Dose response of RSL3-induced cell death of FSP1KO cells that express the indicated inducible FSP1–GFP constructs. c, Reduced-to-oxidized CoQ ratio in FSP1KO and FSP1KO cells that express the indicated FSP1–GFP constructs. Data represent mean ± s.d. of n = 6 biological replicates. *P = 0.0178, NS, not significant (P > 0.99), by one-way analysis of variance (ANOVA). d, e, Dose response of RSL3-induced death of control (d) and FSP1KO (e) cells pretreated for 24 h with 3 mM 4-CBA. f, g, Dose response of RSL3-induced cell death of COQ2KO (f) and FSP1KO/COQ2KO (g) cells. In h, d–g, shading indicates 95% confidence intervals for the fitted curves and each data point is the average of three technical replicates. All figures are representative of two biological replicates.

Fig. 4 | FSP1 mediates ferroptosis resistance in lung cancer. a, b, A high level of expression of FSP1 is correlated with resistance to GPX4 inhibitors in non-haematopoetic cancer cells. Plotted data were mined from the CTRP database, which contains correlation coefficients between gene expression and drug sensitivity for 907 cancer cell lines treated with 545 compounds. a, Correlation between FSP1 expression and resistance to individual compounds. b, Correlation between expression levels of individual genes and resistance to RSL3. Plotted values are z-scored Pearson’s correlation coefficients. c, Dose response of RSL3-induced cell death of the indicated cell lines. d, Dose response of RSL3-induced cell death of control and FSP1KO H460 cells. e, Dose response of RSL3-induced cell death of FSP1–GFP H1703 cells. f, Time-lapse analysis of cell death of GPX4KO and GPX4KO/FSP1KOH460 cells in the presence and absence of 1 μM Fer1. g, GPX4KO/FSP1KO H460 tumour xenograft cells were initiated in immune-deficient SCID mice (n = 16). Following 5 days of daily Fer1 injections (2 mg kg−1 body weight) to allow the cell lines to develop tumours, one set of mice (n = 8) continued to receive daily Fer1 injections and a second set (n = 8) received vehicle injections for the remaining 17 days. The distribution of fold changes in sizes of individual tumours during the treatment is shown. GPX4KO/FSP1KO (−) Fer1, n = 7; GPX4KO/FSP1KO (+) Fer1, n = 8. Box plots indicate median, 25th and 75th percentiles, and minima and maxima of the distributions. Day 15, *P = 0.0397; day 17, **P = 0.0187; day 18, *P = 0.0025; day 21, **P = 0.0327 by two-tailed t-test. h, Model illustrating the mechanism by which FSP1 suppresses ferroptosis. In e–h, shading indicates 95% confidence intervals for the fitted curves and each data point is the average of three technical replicates. Panels c–f are representative of two biological replicates.
irrespective of Fer1: Extended Data Fig. 10k). Fer1 withdrawal resulted in a significant reduction in the growth of the GPX4KO FSP1KO tumours (Fig. 4g). These data demonstrate that FSP1 maintains the growth of H460 lung cancer tumours in vivo when GPX4 is inactivated. To determine whether the growth of FSP1KO tumours can be inhibited by blocking cystine import, we treated H460 cells withimidazole–ketone–erastin (IKE), a system xc− inhibitor that can induce ferroptosis in vivo.21 Although U-2 OS and H460 FSP1KO cells exhibited increased sensitivity to IKE in cell culture (Extended Data Fig. 10i, m), IKE did not inhibit the growth of wild-type H460 and H460 FSP1KO tumour xenografts (Extended Data Fig. 10n, o). Because cells can overcome the effects of cystine depletion through the use of alternative pathways to generate glutathione,22 our results underscore the need for GPX4 inhibitors that are efficacious in vivo.

Ferroptosis has emerged as a potential cause of cell death in degenerative diseases and as a promising strategy to induce the death of cancer cells that are resistant to other therapies.23,24 Our studies and those of a companion paper25 identify FSP1 as a potent ferroptosis suppressor that operates in parallel to the canonical glutathione-dependent GPX4 pathway. FSP1KO mice are viable and display no obvious mutant phenotypes,23 consistent with the compensatory suppression of lipid peroxidation by GPX4. Mechanistically, our data support a model in which myristoylation targets FSP1 to the plasma membrane where it mediates the NADH-dependent reduction of CoQ, which functions as a radical-trapping antioxidant that suppresses the propagation of lipid peroxides (Fig. 4h). Our data also reveal that a fundamental role of non-mitochondrial CoQ is to function as an antioxidant that prevents lipid damage, and consequently ferroptosis. Localization of FSP1 at lipid droplets is not required for protection from ferroptosis. One possibility is that the FSP1-mediated regulation of lipophilic radical-trapping antioxidants in lipid droplets is important for the maintenance of lipid quality during prolonged periods of lipid storage, similar to the function of CoQ and tocopherol in preventing the oxidation of circulating lipoprotein particles.26,27 Finally, our findings indicate that FSP1 expression is important for predicting the efficacy of ferroptosis-inducing drugs in cancers and highlight the potential for FSP1 inhibitors as a strategy to overcome ferroptosis resistance in multiple types of cancer.

Online content
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Methods

No statistical methods were used to predetermine sample size. The experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment.

Cell lines and culture conditions

U-2 OS T-Rex FlipIn cells, a gift from D. Durocher, and U-2 OS Tet-On cells (Clontech) were cultured in DMEM containing 4.5 g/l glucose and l-glutamine (Corning). NCI-H460, NCI-H2291, NCI-H1703 and NCI-H446 cells (ATCC) were cultured in RPMI1640 containing high glucose, l-glutamine and HEPES (ATCC). U-2 OS COQ2KO cells were grown in DMEM supplemented with 200 μM uridine and FSP1KO COQ2KO cells were grown in DMEM supplemented with 200 μM uridine and 1 μg/ml Fer1. NCI-H460 GPX4KO lines and FSP1KO GPX4KO lines were generated containing 2 μg/ml blasticidin and isolation of single clones using cloning rings. NCI-H460 GPX4KO lines and FSP1KO GPX4KO lines were grown in DMEM supplemented with 200 μM uridine and 1 μg/ml Fer1. All media were supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), and all cell lines were grown at 37°C with 5% CO₂. All cell lines were tested for mycoplasma and were not authenticated.

Generation of doxycycline-inducible cell lines

U-2 OS expression lines were generated by transfection of U-2 OS T-Rex FlipIn cells with pOG44 Flip-Recombine system plasmid (Thermo Fisher Scientific) and pcDNA5/FRT/TO plasmid at a 9:1:1 ratio, followed by selection in 0.5 μg/ml hygromycin. NCI-H1703 and NCI-H446 expression lines were generated by infection with pLenti CMV TetR Blast virus (716-1) (Addgene plasmid no. 17492) in the presence of 8 μg/ml polybrene (Sigma-Aldrich), followed by selection in medium containing 2 μg/ml blasticidin for NCI-H1703 cells and 0.5 μg/ml blasticidin for NCI-H446 cells. TetR cells were subsequently infected with pLenti CMV/TO Hygro DEST virus (670-1) (Addgene plasmid no. 17293) containing the FSP1–GFP construct and were selected in medium containing 250 μg/ml hygromycin. FSP1–GFP–expressing cells were enriched by fluorescence-activated cell sorting of the GFP-positive populations.

Generation of CRISPR–Cas9 genome-edited cell lines

For the CRISPR–Cas9 synthetic lethal screen, U-2 OS Tet-On lines stably expressing Cas9 were generated by infection with lentivirus-Cas9-Blast, a gift from F. Zhang (Addgene plasmid no. 52962) and cells were selected in medium containing 1 μg/ml blasticidin. Active Cas9 expression was validated by flow cytometry analysis following infection with a self-cutting mCherry plasmid, which expresses mCherry and an sgRNA targeting the mCherry gene. U-2 OS FSP1KO lines were generated using CRISPR–Cas9 technology by transfection with pSpCas9(2B)A-Puro (PX459), a gift from F. Zhang (Addgene plasmid no. 52962) and cells were selected in medium containing 1 μg/ml blasticidin. Cas9 expression was validated by flow cytometry analysis following infection with a self-cutting mCherry plasmid, which expresses mCherry and an sgRNA targeting the mCherry gene. U-2 OS FSP1KO lines were generated using CRISPR–Cas9 technology by transfection with pSpCas9(2B)A-Puro (PX459), a gift from F. Zhang (Addgene plasmid no. 48139), followed by selection in medium containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific) and sterile-filtered.

Plasmids

Cloning of all expression plasmids and the HaloTag donor plasmid was performed using restriction enzyme-independent fragment insertion by polymerase incomplete primer extension. To generate the FSP1–HaloTag knock-in donor plasmid, 800-base-pair homology arms flanking the FSP1 stop codon were amplified from U-2 OS genomic DNA and inserted in frame 5′ and 3′ to the linker–TEV–HaloTag sequence in pUC57 (a gift from R. Tjian). The protoscaler adjacent motif site that corresponds to FSP1 sgRNA guide 3 was subsequently mutated in the donor sequence using mutagenesis primers to prevent cutting of the integrated donor sequence by Cas9. FSP1–WT–GFP was generated by insertion of FSP1–GFP in pDEST47 into pcDNAs/FRT/TO, and FSP1(G2A)–GFP and FSP1(E156A)–GFP were subsequently generated using site-directed mutagenesis. TOM20(5S)–FSP1(G2A)–GFP and LYN11–FSP1(G2A)–GFP were generated by insertion of the signal sequence of TOM20 and the first 11 amino acids of LYN kinase, respectively, at the N terminus of FSP1(G2A)–GFP. FSP1–GFP–PLIN2 and FSP1(G2A)–GFP–Cbx5 were generated by insertion of the full-length sequence for PLIN2 and amino acids 100–134 of cytochrome b5, respectively, at the C terminus of FSP1(G2A)–GFP. LYN11–mCherry–FRB was generated by replacement of GFP in LYN11–GFP–FRB with the sequence for mCherry. BFP–Sec61 was a kind gift from G. Voeltz. FSP1–GFP in pLenti CMV/TO Hygro DEST (Addgene plasmid no. 17293) was generated by insertion of FSP1–GFP into pENTR1A, followed by Gateway recombination cloning (Thermo Fisher Scientific). NQO1–GFP was generated by PCR amplification of NQO1 from U-2 OS cDNA and insertion into pcDNA5/FRT/TO encoding GFP. LYN11–NQO1–GFP was generated by insertion of amino acids 1–11 to LYN at the N terminus of NQO1–GFP. For protein expression, FSP1(5S) and FSP1(E156A) lacking the ATG start codon were inserted into the pT-His6–TEV vector (Addgene plasmid no. 29653), C-terminal to the His6–TEV tag. LentiCas9–Blast was developed by the Zhang laboratory.

Plasmid transfections were performed in U-2 OS cells with Fugene6 (Promega) transfection reagent. Virus was produced by cotransfection of HEK293T cells with GAG, POL and pLenti expression plasmids at a 1:1:1 w/w ratio, using the X-tremeGENE HP (Roche) transfection reagent. Medium containing secreted virus was collected after 48 h and sterile-filtered.

CRISPR guide RNA (sgRNA) sequences targeting FSP1, ACSL4, NQO1, GPX4 and COQ2 were designed using the CRISPR design tool developed by the Zhang laboratory, available online (http://crispr.mit.edu/). The oligonucleotide sequences preceding the protospacer motif were: FSP1 guide 1, 5′ caccggAAATCGGGACCTCGACG 3′; FSP1 guide 2, 5′ caccggCGCATTCACCGAAGCT 3′; FSP1 guide 3, 5′ caccggTGAGGAGCTCTC-TACCTTTGA 3′; ACSL4 guide 1, 5′ caccggTGAAATCATCCATTGCGCC 3′; ACSL4 guide 2, 5′ caccggCGCATTCACCGAAGCT 3′; NQO1 guide 1, 5′ caccggTTTGAGCAGCTCCAGAAGC 3′; NQO1 guide 2, 5′ caccggCGCATTCACCGAAGCT 3′; COQ2 guide, 5′ caccggCGCATTCACCGAAGCT 3′; GPX4 guide, 5′ caccggCGCATTCACCGAAGCT 3′.

Nucleotides in lowercase show the overhangs introduced into oligonucleotides that are necessary for cloning into the BbsI restriction site of vector PX459 or BsmBI site of lentivector v2.2.1.

Chemicals and reagents

Reagents used in this study include: RSL3 (Cayman Chemical), Fer1 (Cayman Chemical), idebenone (Cayman Chemical), DFO (Cayman Chemical), doxycycline (Sigma), erastin2 (also known as compound 35MEW28) (synthesized by Acme), ML162 (Cayman Chemical), ZVAD(Ome)–FMK (Cayman Chemical), necrostatin-1 (Cayman Chemical), puromycin (Thermo Fisher Scientific), nutlin-3 (Cayman Chemical), CellEvent caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific), etoposide (Sigma-Aldrich), rotenone (Sigma-Aldrich), blasticidin (Thermo Scientific), 10% fetal bovine serum (FBS, Thermo Fisher Scientific), and sterile-filtered.

Generation of CRISPR–Cas9 genome-edited cell lines

For the CRISPR–Cas9 synthetic lethal screen, U-2 OS Tet-On lines stably expressing Cas9 were generated by infection with lentivirus-Cas9-Blast, a gift from F. Zhang (Addgene plasmid no. 52962) and cells were selected in medium containing 1 μg/ml blasticidin. Cas9 expression was validated by flow cytometry analysis following infection with a self-cutting mCherry plasmid, which expresses mCherry and an sgRNA targeting the mCherry gene.

U-2 OS FSP1KO lines were generated using CRISPR–Cas9 technology by transfection with pSpCas9(2B)A-Puro (PX459), a gift from F. Zhang (Addgene plasmid no. 52962) and cells were selected in medium containing 1 μg/ml puromycin and isolation of individual clones using cloning rings. U-2 OS COQ2KO and FSP1KO COQ2KO, FSP1KO ACSL4KO and FSP1KO NQO1KO lines were generated by cotransfecting an FSP1KO plasmid (FSP1 sgRNA guide 1, described in ‘Plasmids’) with PX459 plasmids encoding the appropriate guides, together with pcDNA3.1/Hygro+ (Thermo Fisher Scientific) at a 20:1 w/w ratio, selection in medium containing 500 μg/ml puromycin, and isolation of individual clones using cloning rings. U-2 OS FSP1–HaloTag knock-in lines were generated by infection of U-2 OS T-Rex Flip-In cells with the donor plasmid pUC57 (described in ‘Plasmids’) and PX459 encoding FSP1 sgRNA guide 3 at a 2:1 w/w ratio in medium containing 1 μM SCR7 non-homologous end joining inhibitor (Xcress Biosciences) for 48 h, followed by selection in medium containing 1 μg/ml puromycin.

NCI-H460 FSP1KO lines were generated by infection with lentivirus-Cas9 v2-Blast (Addgene plasmid no. 83489) virus, selection in medium containing 2 μg/ml blasticidin and isolation of single clones using cloning rings. NCI-H460 GPX4KO lines and FSP1KO GPX4KO lines were generated by infection with lentivirus-Cas9 v2-Hygro (Addgene plasmid no. 98291) virus, selection in medium containing 250 μg/ml hygromycin, and isolation of single clones using cloning rings.
**Cell death analysis**

Cells were plated in triplicate at a density of 2,000–3,000 cells per well in black 96-well plates (Corning) 48 h before start of imaging. To induce expression of FSP1, cells were treated with 10 ng/ml doxycycline at the time of plating. After 48 h, the medium was replaced with fresh medium containing 30 nM SYTOX Green Dead Cell Stain, doxycycline (if needed) and the indicated drugs. The plates were immediately transferred to an IncuCyte Zoom imaging system (Essen Biosciences) enclosed in an incubator set to 37 °C and 5% CO2. Three images per well were captured in the green and phase channels every 1 or 2 h over a 48 h period, and the ratio of SYTOX Green-positive objects (dead cells) to phase objects (total cells) was quantified using Zoom image analysis software (Essen Biosciences). For each treatment condition, the SYTOX-to-phase-object ratio was plotted against the 48 h imaging interval, the average SYTOX counting was calculated, and the average SYTOX counting was plotted as a function of time.

**Western blotting**

Cells were washed twice with PBS, lysed in 1% SDS, sonicated for 10 s, and incubated for 5 min at 100 °C. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific), and equal amounts of protein by weight were combined with 1× Laemmli buffer, separated on 4–20% polyacrylamide gradient gels (Bio-Rad Laboratories) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Membranes were washed in PBS with 0.1% Tween-20 (PBST) and blocked in PBST containing 5% (w/v) dried milk. Membranes were incubated for 24 h in PBST containing 5% bovine serum albumin (BSA) (Sigma-Aldrich) and primary antibodies. After washing with PBST, membranes were incubated at room temperature for 30 min in 5% BSA and PBST containing fluorescent secondary antibodies. Immunoblots were imaged on a LI-COR imager (LI-COR Biosciences).

The following blotting reagents and antibodies were used: anti-PN2 (Abgent), anti-AIFM2 (ProteinTech Group and Santa Cruz Biotechnology), anti-α-tubulin (Cell Signaling Technology and Santa Cruz Biotechnology), anti-GPX4 (Abcam), anti-ACSL4 (Sigma-Aldrich), anti-GFP (ProteinTech Group), anti-NQO1 (ProteinTech Group), anti-GAPDH (EMD Millipore), anti-RAS (Cell Biosciences), anti-MDR1 (Cell Signaling Technology), anti-p21 (Cell Signaling Technology), anti-rabbit IRDye800 conjugated secondary (LI-COR Biosciences) and anti-mouse Alexa Fluor 680 conjugated secondary (Invitrogen).

**Fluorescence microscopy**

For fluorescence microscopy of PLIN2 and FSP1–GFP in fixed cells, cells grown on coverslips were treated with 200 μM oleate–BSA complex for 24 h, washed 3× with PBS, fixed for 15 min in PBS containing 4% (w/v) paraformaldehyde and washed 3× again with PBS. Cells were permeabilized for 15 min with blocking solution (1% BSA and PBS) containing 0.01% digitonin, washed 3× and incubated in blocking solution for an additional 15 min. Cells were incubated with anti-PLIN2 antibody in blocking solution (1:500 dilution) for 2 h at room temperature, washed 3× and incubated for 1 h in blocking solution containing anti-rabbit secondary antibody conjugated to Alexa Fluor 594 (1:500 dilution) (Thermo Fisher Scientific). After additional 3× washes, coverslips were mounted on glass slides using Fluoromount G (Southern Biotech). For fluorescence microscopy of FSP1–GFP and LYN–mCherry–FRB, cells were fixed in PBS containing 4% (w/v) paraformaldehyde and washed 3× with PBS before mounting.

For live-cell microscopy, cells were grown in 4-well or 8-well Laboratory-Tek II Chambered Coverglass (Thermo Fisher Scientific) imaging chambers. To image lipid droplets, cells were incubated for 24 h with 1 μM BODIPY 558/568 C12 (Thermo Fisher Scientific) or 100 μM AutoDOT before imaging. To image the cell membrane, cells were incubated with 5 μg/ml CellMask Deep Red for 30 min, and the medium was replaced before imaging. To image mitochondria, cells were incubated with 100 nM MitoTracker Orange CMTMRos or MitoTracker Green FM for 15 min. For imaging that required prior transfection, cells were transiently transfected with the indicated plasmids in 6-well plates using Fugene6, incubated for 48 h and seeded in Laboratory-Tek II chambers before imaging. To image FSP1–Halo Tag, cells were incubated with 100 nM GFP dye for 30 min, washed 3× with PBS and imaged in fresh medium.

Cells were imaged using a Deltavision Elite widefield epifluorescence deconvolution microscope (GE Healthcare) equipped with a 60× oil immersion objective (Olympus), used for DAPI, FITC, Tx-Red and Cy5 filters. For live-cell microscopy, cells were imaged in an enclosure heated to 37 °C and exposed to a continuous perfusion of a gas mixture containing 5% CO2, 2% O2 and 74% N2 (BioBlend, Praxair). Z-stacks of 0.2-μm slices totalling 4–6 μm in thickness were acquired for deconvolution using SoftWoRx software (GE Life Sciences). Single deconvolved slices for each channel were analysed and merged using ImageJ (http://imagej.nih.gov/ij/) and Fiji.

**Lipid droplet fractionation**

Ten 15-cm plates of U-2 OS cells expressing inducible FSP1–GFP were induced with 10 ng/ml doxycycline for 48 h. Cells were collected by scraping into PBS and centrifuged for 10 min at 500g. Cell pellets were resuspended in cold hypotonic lysis medium (HLM, 20 mM Tris-HCl pH 7.4 and 1 mM EDTA) supplemented with 1× complete, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich), incubated on ice for 10 min, and centrifuged at 1,000g for 10 min. The supernatant was subsequently transferred to Ultra-Clear ultracentrifuge tubes (Beckman-Coulter), diluted with 60% sucrose and HLM to a final concentration of 20% sucrose and HLM, and overlayed by 4 ml of 5% sucrose and HLM followed by 4 ml of HLM. Overlaid samples were centrifuged for 30 min at 15,000g in an ultracentrifuge using a SW-41 swinging bucket rotor (Beckman-Coulter). Bouyant fractions were collected using a tube slicer (Beckman-Coulter), additional fractions were pipetted from the top of the sucrose gradient in 1-ml increments, and pellets were resuspended in 1 ml HLM. One hundred microliters of 10% PBS was added to each fraction, yielding a final concentration of 1% PBS. Samples were then sonicated for 15 s and incubated for 10 min at 65 °C. Bouyant fractions were incubated at 37 °C for 1 h and sonicated every 20 min, followed by a final incubation at 65 °C for 10 min.
Plasma-membrane subdomains were separated using a continuous OptiPrep gradient as previously described. Six 15-cm plates of cells expressing inducible FSP1–GFP were incubated with 10 ng/ml doxycycline for 48 h and collected by scraping into PBS, centrifuged for 10 min at 500g and resuspended in 1 ml of base buffer (20 mM Tris–HCl pH 7.8 and 250 mM sucrose) supplemented with 1 mM MgCl₂, 1 mM CaCl₂, and 1× Complete, Mini, EDTA-free Protease Inhibitor Cocktail. Cells were passed 40× through a 1.5× 22-gauge needle and centrifuged at 1,000g for 10 min. The supernatant was retained, and the pellet was resuspended in an additional 1 ml base buffer containing 1 mM MgCl₂ and 1 mM CaCl₂. The resuspended pellet was passed 40× through a 22-gauge needle, centrifuged at 1,000g for 10 min and the supernatant was combined with 1 ml of supernatant from the previous step to make 2 ml in total. OptiPrep mixing solution was prepared by combining 60% OptiPrep stock solution with buffer containing 120 mM Tris–HCl pH 7.8 and 250 mM sucrose in a 5:1 v/v ratio. Two milliliters OptiPrep mixing solution was combined with 2 ml of sample supernatant from the previous centrifugation steps to yield 4 ml of a sample containing 25% OptiPrep. This OptiPrep-mixed sample was gently pipetted under 8 ml of a continuous 5–20% OptiPrep gradient prepared in base buffer in an UltraClear tube. The loaded sample was subsequently centrifuged for 90 min at 52,000 × g using a SW41 swinging bucket rotor. After centrifugation, individual 0.67-ml fractions were collected by pipetting from the top of the gradient and analysed by western blot. The plasma-membrane-localized proteins RAS and MDRI were used as markers of plasma-membrane fractions.

CRISPR–Cas9 synthetic lethal screen
The CRISPR–Cas9 screen was performed as previously described. The ‘Apoptosis and Cancer’ sublibrary of sgRNAs comprising 31,324 elements—including 29,824 sgRNAs targeting 3,015 genes (about 10 sgRNAs per gene) and 1,500 negative-control sgRNAs—was used. To to click chemistry with TAMRA–azide–PEG–biotin.

Enrichment of N-myristoylated proteins
YnMr-labelled proteins in cell lysates were conjugated to TAMRA–azide–PEG–biotin using click chemistry as described in ‘Click chemistry and in-gel fluorescence’. After protein precipitation in cold methanol, the pellet was resuspended in 80 μl PBS containing 1% SDS and PBS and 1× EDTA-free complete protease inhibitor. Equal amounts of protein by weight were diluted to 0.1% SDS and PBS and subjected to click chemistry with TAMRA–azide–PEG–biotin.

Lipidic profiling using liquid chromatography–tandem mass spectrometry
Cas9 and FSP1 U-2 OS cells grown to near confluence were lysed in 10 cm plates were scraped into PBS, centrifuged at 500g for 5 min, and processed as previously described. After addition of internal standards (10 nmol of dodecylglycerol and 10 nmol of pentadecanoic acid), lipids were extracted in a 4 ml solution of 2:1 chloroform:methanol:PBS. The organic and aqueous layers were separated by centrifugation at 1,000g for 5 min. Following the collection of the organic layer, the remaining organic material in the aqueous layer was acidified by addition of 0.1% formic acid and re-extracted with 2 ml of chloroform. Extracts were combined, dried down under a stream of nitrogen and then resolubilized in 120 μl of chloroform. Ten microliters of sample was analysed by single reaction monitoring-based liquid chromatography–mass spectrometry. Liquid chromatography separation was performed using a Luna reverse-phase C5 column, and mass spectrometry analysis was performed using an Agilent 6400 triple quadrupole (QQQ)–liquid chromatography–mass spectrometry instrument. Metabolites were quantified by integrating the AUC, and the values were normalized to the internal standards.
Glutathione measurements
The day before the experiment, $2 \times 10^5$ Cas9-ctl and FSP1KO U-2 OS cells per well were seeded into 6-well dishes. Cells were treated with DMSO (vehicle), erastin2 (1 μM) for 6 or RSL3 (250 nM) for 1 h. Cells were collected by scraping and prepared for measurement of glutathione (GSH + GSSG) using the Cayman Chemical Glutathione Assay Kit (Cayman Chemical) according to the manufacturer’s protocol. The GSH and GSSG concentrations were calculated using a standard curve and normalized to the total protein level in each sample. Three independent biological replicates were performed for each condition.

**BODIPY 581/591 C11 analysis**
The day before the experiment, $2 \times 10^4$ U-2 OS cells per well were seeded into 6-well dishes containing a 22-mm² glass coverslip in each well. Cells were treated with DMSO (vehicle) or RSL3 (250 nM) for 75 min. At the end of the treatment, the treatment medium was removed and cells were washed once with HBSS. Cells were then labelled in 1 ml HBSS containing 5 μM BODIPY 581/591 C11 and incubated at 37°C for 10 min. The label mixture was removed and 1 ml of fresh HBSS was added to the cells. The coverslip was transferred to a glass microscope slide onto which 25 μl of fresh HBSS had been applied. Confocal imaging and quantification of BODIPY 581/591 C11 were performed as previously described on two independent biological replicates per treatment. Using ImageJ, each nucleus was attributed two regions of interest (ROI), one perinuclear and one plasma membrane-localized. Red and green fluorescence values were quantified for each ROI and corrected for background by subtracting the red or green fluorescence in cell-free areas. The BODIPY 581/591 C11 value was calculated as the ratio of the green fluorescence (which indicates oxidized probe) to total (green + red, which indicates total reduced plus oxidized probe) fluorescence.

**Tumour xenograft growth studies**
For Fer1 withdrawal experiments, tumour xenografts were established by injection of GPX4KO and GPX4KO FSP1KO H460 cells into the flank of male C.B17 SCID mice, 6 weeks of age (Taconic Farms) ($n = 8$). In brief, cells were washed with PBS, trypsinized and collected in serum-containing medium. Collected cells were then washed with serum-free medium once and resuspended in serum-free medium at a concentration of $2 \times 10^4$ cells/μl. One hundred microlitres of cells ($2 \times 10^5$ cells) were injected per mouse. Fer1 was prepared at a concentration of 0.2 mg/ml in 18:1:1 v/v PBS:ethanol:PEG40. Mice were injected intraperitoneally with Fer1 daily (2 mg kg$^{-1}$ body weight), and tumour size was measured once every 2 days in each mouse for an additional 17 days. Mice not included in the analysis were gated using the same forward scatter threshold across all samples. Apoptotic cells were identified using the same forward scatter threshold and processing the results. The analytical column was a 150-mm ×4.6-mm C18 column with 5-μm spherical particles connected to a Autosampler, Model 5310 column oven and ESA CouloChem III detector. The EZChrom Elite software (Agilent) was used for monitoring output of the HPLC system equipped with a Model 5100 quaternary pump, Model 5210 autosampler, Model 5310 column oven and ESA CoulChem III detector. The EZChrom Elite software (Agilent) was used for monitoring output signal and processing the results. The analytical column was a 150-mm × 4.6 mm C18 column with 5-μm spherical particles connected to a Security Guard equipped with a C18 cartridge (4-mm × 3-mm).

**CoQ measurements**
CoQ measurements were performed as previously described. To simultaneously measure the reduced and oxidized form of CoQ, a cold butylhydroxytoluene (BHT) solution was added to prevent auto-oxidation at the beginning of sample extraction. One hundred microlitres of a cold BHT-in-propanol solution (5 mg/ml) and 600 μl of cold 1-propanol were added to each tube containing cells in the frozen state. Immediately after this, the mixture was subjected to sonication for 2 min. Subsequently, 100 μl of cold coenzyme Q$_4$ solution (2 μg/ml), which was used as internal standard, was added, and the mixture was vortex-mixed for 1 min. It was then centrifuged for 10 min at 3,500 rpm and 1°C, and the propanol organic supernatant layer was transferred to an autosampler vial. One-hundred-microlitre aliquots of the 1-propanol extract were immediately analysed, and the reduced and oxidized CoQ levels were determined using high-performance liquid chromatography (HPLC). HPLC analysis was performed using an automated Hitachi Chromat system equipped with a Model 5110 quaternary pump, Model 5210 autosampler, Model 5310 column oven and ESA CoulChem III detector. The EZChrom Elite software (Agilent) was used for monitoring output signal and processing the results. The analytical column was a 150-mm × 4.6 mm C18 column with 5-μm spherical particles connected to a Security Guard equipped with a C18 cartridge (4-mm × 3-mm).

**Apoptosis activation assay**
Cells grown in 6-cm plates were washed with PBS, trypsinized and centrifuged for 5 min at 500g. Cell pellets were resuspended in PBS containing 5% FBS and 5 μM CellEvent caspase-3/7 Green Detection Reagent and were incubated for 30 min at 37°C. Cells were analysed on a LSRFortessa (Becton Dickinson) flow cytometer, and the raw data were processed using the FlowJo software package (TreeStar). Apoptotic cells were gated using the same forward scatter threshold across all samples, and FITC fluorescence of the gated populations was determined.
Protein purification and activity assays
Expression vectors were transformed into Rosetta DE3 competent cells (EMD Millipore) and LB cultures were inoculated for overnight growth at 37 °C while shaking. The following day, the cultures were diluted 1:10 into 500 ml of LB and allowed to grow to an optical density at 600 nm (OD$_{600}$) of 0.5, at which point the incubator was set to start cooling to 20 °C. The cultures were grown further to an OD$_{600}$ of 0.7 and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight. Bacterial pellets were resuspended in 2 ml of cold lysis buffer containing 50 mM potassium phosphate pH 8.0, 300 mM potassium chloride, and 30 mM imidazole, supplemented with 1× Complete, Mini, EDTA-free Protease Inhibitor Cocktail. The resuspended cells were sonicated 5× on ice at 50% power for 10 s, with 2-min incubations on ice in between sonications, and were centrifuged at 20,000g for 15 min at 4 °C. The supernatant was combined with 200 μl of Ni-NTA agarose beads (Thermo Fisher Scientific) washed 3× with lysis buffer, and the supernatant–bead mixture was rotated for 1 h at 4 °C. The beads were subsequently washed 5× with cold lysis buffer, and bound proteins were eluted by incubating beads for 15 min in 500 μl of cold lysis buffer containing 250 mM imidazole while rotating. The eluted proteins were dialysed into PBS containing 10% glycerol and snap-frozen in liquid N$_2$. Protein concentration was determined by measuring the absorbance at 280 nm.

To measure NADH oxidation kinetics, recombinant FSP1 was combined with 500 μM NADH and 200 μM coenzyme Q$_1$ in a total volume of 100 μl PBS. A reduction in absorbance at 340 nm, corresponding to NADH oxidation, was determined over the course of 1 h. To measure resazurin reduction kinetics, recombinant FSP1 was combined with 500 μM NADH and 500 μM resazurin in a total volume of 100 μl PBS. Fluorescence (emission at 590 nm) corresponding to reduced resazurin was determined over the course of 1 h. All measurements were taken using a SpectraMax i3 Multi-Mode Platform plate reader (Molecular Devices).

Analysis of the CTRP dataset
Data for significant correlations between FSP1 gene expression and resistance to RSL3, ML162 and ML210 were downloaded from the CTRP v2 website\textsuperscript{18}. Data for non-haematopoietic cancer cells was extracted from the v21.data.gex_global_analysis.txt table and plotted using Prism.

Statistical analysis and reproducibility
All figures, including western blots, dose–response curves and enzymatic activity assays panels are representative of two biological replicates unless stated otherwise. Images are representative of at least $n = 10$ imaged cells. $P$ values for pairwise comparisons were calculated using the two tailed t-test. For comparison across multiple experimental groups, $P$ values were calculated using one-way ANOVA, and adjusted using Bonferroni correction for multiple comparisons. For Fig. 4a, b and Extended Data Fig. 10a, b, the normalized z-scored Pearson correlation coefficients were obtained from CTRP v2 (https://portals.broadinstitute.org/ctrp/). For xenograft experiments, all mice were randomized following tumour–cell injection into treatment groups. Outliers were identified using the Grubbs method, and were removed from analyses. To compare between groups of mice in each time point, $P$ values were calculated using the unpaired, two way t-test.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability
All data that support the conclusions in this manuscript are available from the corresponding author upon reasonable request. Raw data for Fig. 1 are provided in Supplementary Table 1. Raw data for Fig. 3 are provided in Supplementary Table 3. Raw data for Fig. 4 are provided in Supplementary Table 4, and are publicly available from the Cancer Cell Line Encyclopedia (https://portals.broadinstitute.org/ccle) and CTRP databases.

Code availability
The casTLE statistical framework software for analysis of data from the CRISPR screen can be accessed at www.bitbucket.org/dmorgens/castle/. Bowtie software can be accessed at www.bowtie-bio.sourceforge.net/bowtie2/index.shtml. MATLAB image analysis software to analyse lipid droplet distributions can be obtained at www.droplet-proteome.org.

Author contributions
K.B. and J.A.O. conceived the project and designed the experiments. J.A.O. and K.B. wrote the manuscript. All authors read and edited the manuscript. K.B. performed the majority of the experiments. Z.L. and M.A.R. performed and analysed the CRISPR screen with guidance from M.C.B. K.B. prepared samples and R.Z. performed the TIRF microscopy. B.F. performed the lipidomics, and P.H.T. measured CoQ levels and redox state. J.H. performed the click chemistry myristoylation experiments. S.J.D. and L.M. performed the glutathione measurements and C11 experiments. J.M.H. generated the overexpression and knockout lung cancer lines and analysed ferropotosis in these lines. D.K.N., J.M.H., B.F., and M.A.R. performed the xenograft experiments. T.J.M. and B.T. synthesized IKE. J.H. performed the click chemistry myristoylation experiments. S.J.D. and L.M. performed the glutathione measurements and C11 experiments. J.M.H. generated the overexpression and knockout lung cancer lines and analysed ferropotosis in these lines. D.K.N., J.M.H., B.F., and M.A.R. performed the xenograft experiments. T.J.M. and B.T. synthesized IKE.

Competing interests
J.A.O. is a member of the scientific advisory board for Ferro Therapeutics.

Additional information
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**Extended Data Fig. 1 | Synthetic lethal screen coverage and validation.**

**a**, Distribution of counts across all sgRNA elements from the CRISPR–Cas9 screen. **b**, Western blot of control and FSP1KO cells. **c, d**, Western blot analysis (c) and dose response of RSL3-induced death (d) of FSP1KO cells that express doxycycline-inducible, untagged FSP1. **e**, Time-lapse analysis of cell death of FSP1KO cells that express inducible, untagged FSP1. **f**, Flow cytometric analysis of caspase 3/7 activity in FSP1 KO cells that express inducible, untagged FSP1, treated with doxycycline for 48 h. As a positive control, non-induced cells were treated with 50 μM etoposide for 24 h before analysis. **g**, Western blot analysis of lysates from control cells treated with 10 μM nutlin-3 for 48 h. **h**, Dose response of ML162 and erastin2-induced cell death. **i, j**, Dose response of rotenone-induced death of control (i) and FSP1KO (j) cells. **k, l**, Dose response of hydrogen-peroxide-induced death of control (k) and FSP1KO (l) cells. **m**, Dose response of RSL3-induced cell death in the presence of inhibitors of apoptosis (ZVAD(OMe)-FMK, 10 μM) and necroptosis (necrostatin-1, 1 μM). **n**, Western blot analysis of lysates from ACSL4KO and FSP1KO ACSL4KO cells. **o**, Schematic of domains present in AIF and FSP1. In **d, i–m**, shading indicates 95% confidence intervals for the fitted curves and each data point is the average of three technical replicates. Panels are representative of two biological replicates, except panels **c–e** and **k, l**, which show single experiments.
Extended Data Fig. 2 | Subcellular distribution of FSP1. a, Inducible FSP1–GFP cells were transiently transfected with LYN11–mCherry–FRB for 24 h, induced with doxycycline for 48 h and fixed before imaging. b, FSP1–GFP cells were treated with 200 μM oleate for 24 h to induce lipid droplets and treated with 100 μM AutoDOT to label lipid droplets before imaging. c, Line intensity plots showing colocalization between FSP1–HaloTag and organelle markers. d–f, Confocal and TIRF microscopy of FSP1–HaloTag (d), and inducible FSP1(WT)–GFP (e) and FSP1(G2A)–GFP (f) cells. g, FSP1–HaloTag cells were transiently transfected with BFP–Sec61 for 48 h before imaging to label the endoplasmic reticulum. h, FSP1–HaloTag cells were incubated with 100 nM MitoTracker Green FM to label mitochondria. i, Plasma-membrane subdomains from control cells were enriched by OptiPrep gradient centrifugation. Endo., endogenous FSP1. Western blot is representative of two biological replicates. Images are representative of at least n = 10 imaged cells. Scale bars, 10 μm.
Extended Data Fig. 3 | Myristoylation and lipid droplet localization of FSP1.

**a**, Schematic showing the procedure for metabolic labelling of cells with the myristate-alkyne YnMyr and conjugation of YnMyr-labelled proteins with TAMRA-azide-PEG-biotin using click chemistry.

**b**, Analysis of FSP1 myristoylation in buoyant fractions enriched in lipid droplets, by streptavidin enrichment of YnMyr-labelled proteins, click chemistry and SDS–PAGE. Cells were treated with 200 μM oleate to induce lipid droplets and with 100 μM YnMyr or 100 μM myristate for 24 h.

**c**, FSP1–GFP was induced with doxycycline for 24 h and cells were incubated with 100 μM YnMyr for an additional 24 h to label proteins in the presence or absence of 75 μM emetine. YnMyr-labelled proteins were affinity-purified and analysed by click chemistry and SDS–PAGE.

**d**, Buoyant fractions enriched in lipid droplets, from cells expressing inducible FSP1–GFP, were isolated by sucrose gradient fractionation and analysed by western blot. Endo., endogenous FSP1. **e**, Inducible FSP1–GFP cells were treated with 200 μM oleate in the presence or absence of 10 μM NMT inhibitor, fixed and stained with anti-PLIN2 antibody before imaging. Images are representative of at least n = 10 imaged cells. Scale bar, 10 μm.

**f**, Western blot analysis of FSP1 KO cells induced for 48 h with doxycycline to express the indicated proteins. All panels are representative of two biological replicates.
Extended Data Fig. 4 | Targeting of FSP1 to subcellular compartments.

a, Western blot analysis of FSP1 KO cells induced for 48 h with doxycycline to express the indicated proteins. b, Live-cell microscopy of cells that express the indicated FSP1(G2A)–GFP constructs, incubated with 100 nM Mitotracker Orange to label mitochondria, 1 μM BODIPY 558/568 C12 to label lipid droplets or 5 μg ml⁻¹ Cell Mask to label the plasma membrane. To label the endoplasmic reticulum, cells were transiently transfected with BFP–Sec61 48 h before imaging. Images are representative of at least n = 10 imaged cells. Line intensity plots show colocalization between FSP1 and organelle markers. Scale bar, 10 μm.

b, Plasma-membrane subdomains from FSP1 KO cells that express inducible LYN11–FSP1(G2A)–GFP were enriched by OptiPrep gradient centrifugation. The densitometry plot indicates the distribution of overexpressed and endogenous proteins. Panels are representative of two biological replicates except for c, which shows a single experiment.
Extended Data Fig. 5 | Lipid droplets are not required for inhibition of ferroptosis by FSP1. a, Control cells were treated with inhibitors of DGAT1 (20 μM T863) and DGAT2 (10 μM PF-06424439) for 48 h, stained with 1 μM BODIPY 493/503 and imaged by fluorescence microscopy. The image is representative of $n = 50$ imaged fields. Scale bar, 10 μm. b, The size and number of lipid droplets were quantified from cells ($n > 5,000$) in a. c, Dose response of RSL3-induced cell death of control cells pretreated for 48 h with 20 μM T863 and 10 μM PF-06424439 before addition of RSL3. Each data point is the average of three technical replicates. All panels are representative of two biological replicates.
Extended Data Fig. 6 | Analysis of lipid peroxidation, glutathione and lipid levels in FSP1KO cells. a, b, Ratio of oxidized-to-total BODIPY 581/591 C11 from images in Fig. 3a, at the plasma membrane (a) or at internal membranes (b). Each data point represents an individual cell quantified in one of two biological replicates. For a, Cas9ctl DMSO, n = 34; Cas9ctl RSL3, n = 45; FSP1KO DMSO, n = 30; FSP1KO RSL3, n = 33; ***P < 0.001 by one-way ANOVA. For b, Cas9ctl DMSO, n = 33; Cas9ctl RSL3, n = 45; FSP1KO DMSO, n = 30; FSP1KO RSL3, n = 33; ***P < 0.001 by one-way ANOVA. Error bars show mean ± s.d.

c, Total intracellular glutathione (GSH + GSSG) levels in control and FSP1 KO were determined following treatment with 250 nM RSL3 or 1 μM erastin2. The graph shows mean ± s.d. of three biological replicates. n.s., FSP1KO DMSO versus RSL3, P = 0.7278; n.s., FSP1KO RSL3 versus Cas9 ctl RSL3, **P = 0.0072 by one-way ANOVA.

d, e, GSH and GSSG levels in control and FSP1KO cells were measured. Where indicated, cells were treated with 1 μM erastin2. The graph shows mean ± s.d. of three biological replicates. n.s., GSH P = 0.6269; n.s., GSSG P = 0.8284 by two-tailed t-test. f, The plot shows the average of the fold change in lipids measured in two FSP1KO cell lines generated using FSP1 sgRNA no. 1 and FSP1 sgRNA no. 2 (labelled KO1 and KO2, respectively), relative to control cells. Cas9ctl, n = 5; KO1, n = 4; KO2, n = 5 biological replicates (Supplementary Table 3). g, Levels of select lipid species in biological replicates of control and FSP1KO cells measured in f. The average values are indicated. 16:0 20:4 PE, **P = 0.0017; 18:0 20:4 PE, **P = 0.0011; 18:0 LPE, KO2 **P = 0.0036, KO1 **P = 0.0019; 16:0 LPE, KO2 *P = 0.0133 and KO1 *P = 0.0335 by two-tailed t-test.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Analysis of the FSP1 oxidoreductase mutant. a. FSP1KO cells were treated with 250 nM RSL3 and 10 μM idebenone or 50 μM DFO for 75 min, labelled with BODIPY 581/591 C11 and fixed before imaging. Images are representative of at least n = 10 cells imaged for each treatment condition. Scale bar, 20 μm. b. Sequence alignment showing residues conserved between AIF and FSP1. The arrow points to E313 in AIF (aligns to E156 in FSP1) that functions in binding to flavin adenine dinucleotide. c. Structural alignment between the crystal structure of mouse AIF (RCSB Protein Data Bank code (PDB) 1GV4) and the Phyre2-generated model of FSP1. d. Live-cell microscopy of FSP1KO cells expressing inducible FSP1(E156A)–GFP labelled with 5 μg ml⁻¹ Cell Mask. The image is representative of at least n = 10 imaged cells. Scale bar, 10 μm. e. Plasma-membrane subdomains from FSP1KO cells that express FSP1(E156A)–GFP were enriched by OptiPrep gradient centrifugation. f. SDS-PAGE and Coomassie brilliant blue stain of recombinant His–FSP1(WT) and His–FSP1(E156A) purified with Ni-NTA agarose beads. g. Reduction of resazurin by recombinant FSP1 in the presence of NADH. h. Oxidation of NADH by recombinant FSP1 in the presence of coenzyme Q. Panels g and h are representative of two biological replicates, and e shows a single experiment.
Extended Data Fig. 8 | Lipid peroxidation in CoQ-depleted cells. a, Total CoQ levels in control and FSP1\textsuperscript{KO} cells treated for 48 h with 3 mM 4-CBA. The graph shows mean ± s.d. of three biological replicates. ***P = 0.0007, *P = 0.0132 by two-tailed t-test. b, Dose response of RSL3-induced death of inducible FSP1–GFP cells pretreated for 48 h with 3 mM 4-CBA and doxycycline before addition of RSL3. Shading indicates 95% confidence intervals for the fitted curves and each data point is the average of three technical replicates. The panel is representative of two biological replicates. c, Genomic sequencing of the COQ2 gene in COQ2\textsuperscript{KO} and FSP1\textsuperscript{KO} COQ2\textsuperscript{KO} cells. The ATG start codon is boxed in the COQ2 consensus sequence. d, Control and COQ2\textsuperscript{KO} cells treated with 250 nM RSL3 for 3 h were labelled with BODIPY 581/591 C11 and fixed before imaging. e, COQ2\textsuperscript{KO} cells were treated with 250 nM RSL3 and 10 μM idebenone or 50 μM DFO for 3 h, labelled with BODIPY 581/591 C11 and fixed before imaging. In panels d, e, images are representative of at least n = 10 cells imaged for each treatment condition. Scale bars, 20 μm.
Extended Data Fig. 9 | Role of NQO1 in ferroptosis resistance. a, Western blot analysis of lysates from NQO1KO and NQO1KO FSP1KO cells. b, Dose response of RSL3-induced death of control and NQO1 KO cells. c, Dose response of RSL3-induced death of FSP1 KO and NQO1KO FSP1KO cells. Cells in b and c were generated using NQO1 sgRNA 1. d, Western blot analysis of lysates of FSP1KO cells that express doxycycline-inducible NQO1–GFP. e, Live-cell microscopy of inducible NQO1–GFP cells labelled with 5 μg ml⁻¹ Cell Mask. f, Plasma-membrane subdomains from FSP1KO cells that express NQO1–GFP were enriched by OptiPrep gradient centrifugation. g, Dose response of RSL3-induced death of FSP1KO cells expressing the indicated inducible constructs. h, Live-cell microscopy of FSP1KO cells that express inducible LYN11–NQO1–GFP labelled with 5 μg ml⁻¹ Cell Mask. i, Dose response of RSL3-induced death of FSP1KO cells that express the indicated inducible constructs. For panels b, c, g, i, shading indicates 95% confidence intervals for the fitted curves and each data point is the average of three technical replicates. Panels are representative of two biological replicates except for f and i, which show the results of single experiments. In e and h, the images are representative of at least n = 10 imaged cells. Scale bars, 10 μm.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | The role of FSP1 in cancer. a, b, A high level of expression of FSP1 is correlated with resistance to the GPX4 inhibitors ML210 (a) and ML162 (b) in non-haematopoietic cancer cells. Plotted data were mined from the CTRP database that contains correlation coefficients between gene expression and drug sensitivity for 907 cancer cell lines treated with 545 compounds. Plotted values are z-scored Pearson’s correlation coefficients. c, Western blot of FSP1 expression in a panel of lung cancer lines. d, Western blot of lysates from control and FSP1KO H460 cells. e, $EC_{50}$ RSL3 dose for the indicated H460 cell lines was calculated from the results in Fig. 1d. Bars indicate 95% confidence intervals. f, Western blot analysis of H446 cells that express doxycycline-inducible FSP1–GFP. g, Dose response of RSL3-induced death of control and FSP1–GFP H446 cells. h, Western blot analysis of H446 cells that express doxycycline-inducible FSP1–GFP. i, Dose response of IKE-induced death of control and FSP1–GFP H446 cells. j, Western blot analysis of GPX4KO and GPX4KO FSP1KO H460 cells. k, GPX4KO H460 tumour xenograft cells were initiated in immune-deficient SCID mice ($n = 16$). Following 5 days of daily Fer1 injections (2 mg kg$^{-1}$ body weight) to allow lines to develop tumours, 1 set of mice ($n = 8$) continued to receive daily Fer1 injections and a second set ($n = 8$) received vehicle injections for the remaining 17 days. The distribution of fold changes in sizes of individual tumours during the treatment is shown. GPX4KO (−) Fer1, $n = 7$; GPX4KO (+) Fer1, $n = 7$. l, Dose response of IKE-induced death of control and FSP1KO U-2 OS cells. m, Dose response of IKE-induced death of control and FSP1KO H460 cells. n, o, Control (n) and FSP1KO (o) H460 tumour xenografts were initiated in immune-deficient SCID mice ($n = 16$). After 10 days, each group of mice ($n = 8$) was injected daily with IKE or vehicle (40 mg kg$^{-1}$ body weight). The distribution of fold changes in sizes of individual tumours during the treatment is shown. Cas9ctl (−) IKE, $n = 4$; Cas9ctl (+) IKE, $n = 4$; FSP1KO (−) IKE, $n = 7$; FSP1KO (+) IKE, $n = 4$. In k, l, m, box plots show median, 25th and 75th percentiles, minima and maxima of the distributions. Panels are representative of two biological replicates except l, m, which show the results of single experiments. In i, l, m, shading indicates 95% confidence intervals for the fitted curves and each data point is the average of three technical replicates.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
- SoftWoRx V6.5.2 (GE Life Sciences), PHYRE2 Protein Fold Recognition Software V2.0 (www.sbg.bio.ac.uk/phyre2), BD FACSDiva V6.2 (bdbiosciences.com), SoftMax Pro V6.3 (moleculardevices.com), iQ3 live cell imaging software (Andor Technology), EZChrom Elite V3.2.0 (Agilent), Image Lab V6.0.1 (Bio-Rad Laboratories, Inc.)

Data analysis
- casTLE statistical framework V1.0 (bitbucket.org/dmorgens/castle), ImageJ V1.8.0 (image.nih.gov/ij), MATLAB R2016b (mathworks.com), droplet detection and quantification software for MATLAB (Olzmann lab, www.dropletproteome.org), Prism V7 (GraphPad), Zoom Image Analysis Software 2016B (Essen Bioscience), BowTie 2 V2.3.4.3 (bowtie-bio.sourceforge.net/bowtie2/index.shtml), FlowJo V10 (TreeStar) (flowjo.com)

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Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data that support the conclusions in this manuscript are available from the corresponding author upon request. Raw data for figure 1 can be accessed in Supplementary Table 1. Raw data for figure 3 can be accessed in Supplementary Table 3. Raw data for figure 4 can be accessed in Supplementary Table 4 and are publicly available from the CTRP and CCLE databases (portals.broadinstitute.org).
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

### Sample size

No statistical tests were used to calculate sample size. In cases where statistics were derived, sample size was n=3 or more independent biological replicates. For measurement of lipid levels using mass spectrometry and measurement of reduced COQ levels using HPLC, sample sizes were n=5 and n=6 biological replicates, respectively, to account for expected variability due to sample preparation and noise from the instruments. For mouse xenograft experiments, sample size was n=8 for each treatment group to account for differences in tumor formation and growth, and to ensure recovery of a sufficient quantity of mice with successful xenografts of approved size at each time point of the study.

### Data exclusions

These criteria were established prior to performing the xenograft studies. In the ferrostatin-1 withdrawal experiments, animals not included in the analysis included mice that were sacrificed early due to sickness (n = 1 of GPX4KO (+) Fer1) and mice whose tumors were determined to be outliers according to the Grubbs statistical test using Prism (Graphpad) software (n = 1 of GPX4KO (-) Fer1 and n = 1 of GPX4KO/FSP1KO (-) Fer1). For the IKE injection experiments, animals not included in the analysis included mice that were sacrificed early due to development of exceedingly large tumors (n = 1 of Cas9 ctl (+) IKE, n = 3 of Cas9 ctl (-) IKE, n = 3 of FSP1KO (+) IKE and n = 1 of FSP1KO (-) IKE), mice in which tumors failed to initiate (n = 2 of Cas9 ctl (+) IKE), and mice whose tumors were determined to be statistical outliers according to the Grubbs test (n = 1 of Cas9 ctl (+) IKE, n = 1 of Cas9 ctl (-) IKE, n = 1 of FSP1KO (+) IKE).

### Replication

All attempts at replication were successful. Figures, including western blots, dose response curves and enzymatic activity assay panels are representative of two biological replicates except for the following, which show single experiments: plasma membrane fractionations in Extended Data Fig. 4c, 7e and 9f and cell death curves in Extended Data Fig. 1d,e, 1k,j, 9i and 10l,m. Images are representative of at least n = 10 imaged cells.

### Randomization

For the xenograft studies, following injection of H460 cells, the mice were randomly assigned into 2 treatment groups for the ferrostatin-1 withdrawal experiments and into 2 treatment groups for the IKE injection experiments.

### Blinding

Blinding was not possible because the experiments were performed by a single researcher.

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**Reporting for specific materials, systems and methods**

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | Antibodies            |
| ☑   | Eukaryotic cell lines |
| ☑   | Palaeontology         |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |
| ☑   | Clinical data         |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChIP-seq              |
| ☑   | Flow cytometry        |
| ☑   | MRI-based neuroimaging |

### Antibodies

**Antibodies used**

- Anti-Plin2 (Abgent, cat. #AP5118c), anti-AIFM2 (Proteintech Group, Inc. cat. #20886-1-AP and Santa Cruz Biotechnology, clone B-6, cat. #sc-377120), anti-α-tubulin (Cell Signaling Technology, Inc., cat. #2144 and Santa Cruz Biotechnology, clone B-7, cat. #sc-5286), anti-GPX4 (Abcam, cat. #ab41787), anti-ACSL4 (Sigma-Aldrich, cat. #SAB-2701949), anti-GFP (Proteintech Group, Inc., clone 1E10H7, cat. #66002-1-1), anti-NQO1 (Proteintech Group, Inc., cat. #1145-1-AP), anti-GAPDH (EMD Millipore, cat. #mab374), anti-RAS (Cell Biolabs, Inc. cat. #STA-400), anti-MDR1 (Cell Signaling Technology, Inc., clone D3H1Q, cat. #12683S), anti-p21 (Cell Signaling Technology, Inc., clone 1D1, cat. #2947).

**Validation**

Anti-AIFM2, anti-GPX4, anti-ACSL4, and anti-NQO1 were validated using genetic knockout of the endogenous genes with Cas9 and one or more targeted sgRNAs. Anti-Plin2, anti-α-tubulin, anti-GAPDH, anti-RAS, anti-MDR1 and anti-p21 were validated un in human cells by the manufacturer.
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

H460, H2291, H2172, H1703, H446 were purchased from ATCC (atcc.org). U-2 OS Flip-In cells were a gift from Dr. Daniel Durocher (Lunenfeld-Tenenbaum Research Institute).

Authentication

None of the cell lines used were authenticated.

Mycoplasma contamination

All cell lines are negative for mycoplasma contamination.

Commonly misidentified lines (See ICLAC register)

Cell lines used in the study are not flagged in the Register of Misidentified Cell Lines.

Animals and other organisms

Policy information about studies involving animals. ARRIVE guidelines recommended for reporting animal research

Laboratory animals

Male, C.B17 SCID mice, 6 weeks of age

Wild animals

The study did not involve wild animals.

Field-collected samples

The study did not involve field-collected samples.

Ethics oversight

All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committees (IACUC) of the University of California, Berkeley.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

• The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
• The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
• All plots are contour plots with outliers or pseudocolor plots.
• A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Cells grown in 6-cm plates were washed with PBS, trypsinized and centrifuged for 5 min at 500 x g. Cell pellets were resuspended in PBS containing 5% FBS and 5 μM CellEventTM Caspase-3/7 Green Detection Reagent, and were incubated for 30 min at 37°C prior to analysis.

Instrument

LSRFortessa (Becton-Dickinson)

Software

Data was collected using BD FACSDiva V6.2 (Becton-Dickinson) and analyzed using FlowJo V10 (TreeStar).

Cell population abundance

Cells were not sorted during the procedure.

Gating strategy

Apoptotic cells were gated using the same low FSC threshold (FSC- gate) across all samples and the FITC signals of the gated populations were determined.

• Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.