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FSP1 is a glutathione-independent ferroptosis suppressor

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ABSTRACT

Ferroptosis is an iron-dependent form of necrotic cell death marked by oxidative damage to phospholipids^{1,2}. To date, ferroptosis has been believed to be restrained only by the phospholipid hydroperoxide (PLOOH)-reducing enzyme glutathione peroxidase 4 (GPX4)^{3,4} and radical-trapping antioxidants (RTAs)^{5,6}. The factors which underlie a given cell type's sensitivity to ferroptosis⁷ is, however, critical to understand the pathophysiological role of ferroptosis and how it may be exploited for cancer treatment. Although metabolic constraints⁸ and phospholipid composition^{9,10} contribute to ferroptosis sensitivity, no cell-autonomous mechanisms have been yet been identified that account for ferroptosis resistance. We undertook an expression cloning approach to identify genes able to complement GPX4 loss. These efforts uncovered the flavoprotein "apoptosis inducing factor mitochondria-associated 2 (AIFM2)" as a previously unrecognized anti-ferroptotic gene. AIFM2, hereafter renamed "ferroptosis-suppressor-protein 1" (FSP1), initially described as a pro-apoptotic gene¹¹, confers an unprecedented protection against ferroptosis elicited by GPX4 deletion. We further demonstrate that ferroptosis suppression by FSP1 is mediated via ubiquinone (CoQ₁₀): its reduced form ubiquinol traps lipid peroxyl radicals that mediate lipid peroxidation, while FSP1 catalyses its regeneration by using NAD(P)H. Pharmacological targeting of FSP1 strongly synergizes with GPX4 inhibitors to trigger ferroptosis in a number of cancer entities. Conclusively, FSP1/CoQ₁₀/NAD(P)H exists as a stand-alone parallel system, which co-operates with GPX4 and glutathione (GSH) to suppress phospholipid peroxidation (pLPO) and ferroptosis.

Results

Ferroptosis is controlled by the selenoenzyme GPX4^{3,4,12}. With the recognition that targeting ferroptosis may help eradicate therapy-resistant tumors¹³⁻¹⁵, there is mounting interest in understanding the mechanisms that underpin cell sensitivity to ferroptosis¹⁶. Although acyl-CoA synthetase long chain family member 4 (ACSL4) was identified as a pro-ferroptotic gene, whose expression determines ferroptosis sensitivity^{9,10}, inhibition of GPX4 fails to trigger ferroptosis in some cancer cell lines regardless of ACSL4 expression, suggesting alternative resistance mechanisms.

Genetic suppressor screen uncovers FSP1

To uncover these factors, we generated a cDNA expression library derived from the MCF7 ferroptosis-resistant cell line^{9,10} (Extended Data Fig. 1a), and screened for genes complementing GPX4 loss (Fig. 1a). Sequencing of 14 single cell clones identified 7 which express both *GPX4* and *AIFM2* (Extended Data Fig. 1b). AIFM2 is a flavoprotein originally described as a p53-responsive gene¹⁷. It was initially claimed to induce apoptosis based on sequence similarity to another initially postulated pro-apoptotic gene, apoptosis-inducing factor, mitochondria-associated, 1 (AIFM1)¹¹. To avoid further confusion, we thus recommend future reference to AIFM2 be made as “Ferroptosis-Suppressor-Protein 1 (FSP1)” (accompanying manuscript Besuker *et al.*). For validation, we stably expressed FSP1 in Pfa1¹⁸ and in human fibrosarcoma HT1080 cells (Extended Data Fig. 1c,d). FSP1 overexpressing cells were robustly protected against pharmacological and genetic ferroptosis inducers¹ and proliferated indefinitely (Fig. 1b-e; Extended Data Fig. 1e-i;

Supplementary information Video). To the best of our knowledge, this is the first enzymatic system complementing GPX4 loss¹⁸.

Its anti-ferroptotic function was found to be independent of cellular GSH levels, GPX4 activity, ACSL4 expression and oxidizable fatty acid content (Extended Data Fig. 1c,d,j,k; Extended Data Fig. 2), showing that FSP1 does not interfere with canonical ferroptosis mechanisms. Moreover, the protection conferred by FSP1 was specific to ferroptosis-inducing agents, and not cytotoxic compounds and/or pro-apoptotic conditions. Moreover, p53 status did not impact on FSP1 expression (Extended Data Fig. 3a-e). In contrast to FSP1, overexpression of AIFM1 failed to suppress ferroptosis (Extended Data Fig. 3f,g).

N-myristoylation of FSP1 is essential

Early efforts revealed that N-terminal tagging of FSP1 abolished its anti-ferroptotic activity. Indeed, the N-terminus of FSP1 contains a canonical myristoylation motif¹⁹, presumably facilitating its association with lipid bilayers²⁰. Expression of wildtype and a mutant form of FSP1 lacking the predicted myristoylation site [G2A] in Pfa1 and HT1080 cells (Fig. 2a), and tagging with an alkyne-functionalized myristic acid mimetic (YnMyr) enabled the specific enrichment of only wildtype FSP1. This enrichment was abolished with both the G2A mutation and the pan-N-myristoyl transferase inhibitor IMP-1088²¹ (Fig. 2b). Myristoylation of FSP1 appears to be essential for its anti-ferroptotic role as the G2A mutant and wildtype FSP1 expressing cells treated with IMP-1088 abrogated ferroptosis resistance (Fig. 2c,d; Extended Data Fig. 3 h,i). Intrigued by these findings, we assessed the subcellular distribution of both the wildtype and G2A mutant FSP1 using C-terminally tagged fusion proteins. Although FSP1-GFP localized to an unspecified

perinuclear membrane compartment, it also partially overlapped with ER and Golgi markers (Fig. 2e; Extended Data Fig. 4a). In contrast, FSP1[G2A]-GFP was distributed throughout the cell, suggesting that ferroptosis is perhaps driven in a specific subcellular region. A more sophisticated investigation of the subcellular localization of FSP1 is provided by Besuker *et al.*, revealing a striking role for plasma membrane-targeted FSP1 in ferroptosis suppression.

FSP1 prevents lipid peroxidation

Since ferroptosis is ultimately driven by pLPO, we stained Pfa1 cells with C11-BODIPY 581/591 to find that FSP1 overexpression blunted lipid peroxidation induced by (1S/3R)-RSL3 (RSL3) (Fig. 3a). Moreover, specific pLPO products were markedly lower in *Gpx4* knockout (KO) FSP1 overexpressing cells (Fig. 3b; Extended Data Fig. 4b). Since members of the AIF family were shown to possess NADH:ubiquinone oxidoreductase activity²², we hypothesized that FSP1 suppresses pLPO by regenerating lipophilic RTAs using NAD(P)H. The reduced form of coenzyme Q10 (CoQ₁₀-H₂) was reported to be a good RTA in phospholipids and lipoproteins²³, yet considered to be of limited value outside mitochondria because an efficient recycling mechanism is *hitherto* unknown. To investigate a possible link between FSP1 and CoQ₁₀-H₂, we generated CoQ₁₀-deficient HT1080 cells by deleting 4-hydroxybenzoate polyprenyltransferase (COQ2), the enzyme which catalyzing the first step in CoQ₁₀ biosynthesis (Fig. 3c). CoQ₁₀-deprived cells proliferated normally when supplemented with uridine, CoQ₁₀ or decyl-ubiquinone (Extended Data Fig. 4c). Importantly, while FSP1 overexpression in parental HT1080 cells suppressed ferroptosis, it failed to do so in COQ2 KO cells (Fig. 3d; Extended Data Fig. 4d). Consistent with earlier data showing that purified FSP1 reduces ubiquinone analogs of variable chain lengths²², heterologously

expressed FSP1 (Extended Data Fig. 4e) catalyzed the reduction of a ubiquinone analog with an appended coumarin fluorophore. This enabled the determination of kinetic parameters for FSP1, which revealed a relatively low K_m , and much higher V_{max} compared to related oxidoreductases (e.g. NQO1), along with typically observed substrate inhibition (Fig. 3e). Importantly, neither dehydroascorbate, oxidized glutathione, nor tert-butyl hydroperoxide (TBOOH) were FSP1 substrates (Fig. 3f).

To further interrogate our hypothesis that FSP1 suppresses pLPO by reducing CoQ₁₀, we carried out co-oxidations of egg phosphatidylcholine and STY-BODIPY²⁴ using a lipophilic alkoxyl radical generator (Extended Data Fig. 5a,b). Therein, neither FSP1 alone nor the combination of FSP1 and its reducing co-substrate, NAD(P)H, were able to suppress pLPO effectively (Extended Data Fig. 5c), while addition of CoQ₁₀ retarded the autoxidation in a dose-dependent manner (Extended Data Fig. 5d,e). These results imply that, through FSP1, CoQ₁₀ aids in importing the reducing equivalents from NAD(P)H into the lipid bilayer to inhibit propagation of lipid peroxidation. NQO1 was unable to serve in the same capacity as FSP1 in these assays (Extended Data Fig. 5f,g). Since CoQ₁₀ is readily autoxidized and suffers from poor dynamics within the lipid bilayer²⁵, we wondered if α -tocopherol (α -TOH) may also contribute to the protection observed by FSP1/CoQ₁₀. Thus, following its reaction with a lipid-derived peroxy radical, α -TOH could be regenerated by reduced CoQ₁₀, or even directly *in vitro* by FSP1 without the need for CoQ₁₀ (Extended Data Fig. 5h-j). Direct monitoring of phospholipid hydroperoxide formation in linoleate-rich liposomes corroborated the results of the co-oxidations, showing substantial FSP1-catalyzed suppression of pLPO which was further enhanced in the presence of both CoQ₁₀ and α -TOH (Extended Data Fig. 5k).

Loss of FSP1 sensitizes to ferroptosis

Motivated by the strong protective effect provided by FSP1 and the possibility to maintain cells in the absence of GPX4, we envisioned that a counter-screen of FSP1 overexpressing cells in a GPX4 KO/WT background overexpressing FSP1 would be useful for the discovery of inhibitors of FSP1. We screened approximately 10,000 drug-like compounds⁴, which led to the identification of iFSP1 as a potent FSP1 inhibitor (Fig. 4a). iFSP1 selectively induced ferroptosis in GPX4 KO Pfa1 and HT1080 cells overexpressing FSP1 (Extended Data Fig. 6a,b). Preliminary structure-activity relationship studies have yet to identify compounds with substantial improvement over iFSP1 (Extended Data Fig. 6c).

To determine if FSP1 could serve as a ferroptosis suppressor in cancer, we generated a monoclonal antibody against human FSP1 (Extended Data Fig. 6d), and explored its expression along with the main ferroptosis players in a panel of human cancer cell lines of different origins (Extended Data Fig. 7). Indeed, FSP1 was expressed in the majority of tumour cell lines, and iFSP1 treatment robustly sensitized them to RSL3-induced ferroptosis (Extended Data Fig. 8). We then generated FSP1 KO and FSP1 overexpressing cells from a selection of these cell lines (Fig. 4b,c; Extended Data Fig. 7) and compared the effects of pharmacological inhibition (iFSP1) versus FSP1 KO towards ferroptosis sensitisation. Expectedly, genetic FSP1 deletion was superior to small molecule inhibition, while iFSP1 treatment in the FSP1 KO background had no additive effect to RSL3 (Extended Data Fig. 6e,f). Notably, a few cells sensitive to RSL3 could not be re-sensitized by iFSP1 when FSP1 was overexpressed. This may be due to drug metabolism and excretion, and merit further investigation (Extended Data Fig. 6f). Detailed experiments demonstrated that

the FSP1 KO in MDA-MB-436 cells lowered their resistance to RSL3-induced ferroptosis, while mFSP1 re-expression restored ferroptosis resistance (Fig. 4d,e). Analysis of the cancer dependency map (DepMap - <https://depmap.org/portal/>) revealed that lower expression of FSP1 correlates with an increased GPX4 dependency in a panel of 559 cancer cell lines (Extended Data Fig 9a). Additionally, FSP1 expression directly correlated with resistance to ferroptosis inducers RSL3, ML162 and ML210 in a panel of 860 cancer cell lines (<https://portals.broadinstitute.org/ctrp>) (Extended Data Fig. 9b). No synergistic cell death was detected with cisplatin or other known cytotoxic compounds (Extended Data Fig. 9c,d), suggesting that FSP1 inhibition selectively sensitizes cells to ferroptosis inducers. This finding is particularly important since therapy-resistant tumours only respond to complete elimination of GPX4 activity; minute amounts are sufficient to sustain cell viability²⁶. Moreover, pharmacological targeting of GPX4 may only achieve partial anti-tumour effects. In fact, in mice bearing human xenografts, Besuker *et al.* demonstrate that the growth of H460 tumours can only be slowed by concomitant deletion of *GPX4* and *FSP1*, whereas GPX4 single KO tumours grow normally.

Our data unambiguously establish the NADH/FSP1/CoQ₁₀ relay as a potent suppressor of pLPO and ferroptosis (Fig. 4f). As such, phospholipid redox homeostasis can be disassociated from the GSH/GPX4 axis, and can be further exploited pharmacologically to efficiently sensitize cancer cells to ferroptosis inducers. Our discovery explains why NAD(P)H²⁷ and defects in the mevalonate pathway through loss of ubiquinone^{13,28} converge on FSP1 and thereby predict sensitivity to ferroptosis. Furthermore, our data provide a compelling case for the long-debated antioxidant

role of extra-mitochondrial CoQ₁₀^{29,30} and advocates that its beneficial effects should be ultimately rationalized alongside FSP1.

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DATA AVAILABILITY STATEMENT

For immunoblot source data, see Supplementary Fig. 1. Raw data for all experiments are available as Source Data to the relevant figures.

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Competing interests

The authors declare no competing interests.

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Contributions

M.C., J.P.F.A. and S.D. conceived the study and wrote the manuscript. M.A. and V.O.D performed (oxi)lipidomics analysis and data interpretation. S.D., B.P., E.P., I.W., F.P.F., J.P.F.A., T.V., V.M., I.I., K.B., M.S., M.R., T.N.X.S. and M.C.D.S. performed *in vitro* experiments. R.S and D.A.P. performed functional characterization of recombinant FSP1. S.D., F.P.F., D.A.P, J.P.F.A. and M.C. performed evaluation and interpretation of the *in vitro* data. A.M. and G.M.P. expressed and purified recombinant FSP1. C.S. provided TNBC cell lines. A.F. and A.S. helped in generating monoclonal antibodies. B.P and J.W. carried out screening of FSP1 inhibitors and related SAR studies. W.S. and A.S performed LC-MS analysis of ubiquinone content. A.G.G. and E.W.T. studied myristoylation of FSP1. A.K., M.S., F.P.F. and J.P.F.A. performed enhanced microscopy experiments. All authors read and agreed on the content of the paper.

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References

- 1 Dixon, S. J. *et al.* Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* **149**, 1060-1072, doi:10.1016/j.cell.2012.03.042 (2012).
- 2 Conrad, M., Angeli, J. P., Vandenabeele, P. & Stockwell, B. R. Regulated necrosis: disease relevance and therapeutic opportunities. *Nat Rev Drug Discov* **15**, 348-366, doi:10.1038/nrd.2015.6 (2016).
- 3 Yang, W. S. *et al.* Regulation of ferroptotic cancer cell death by GPX4. *Cell* **156**, 317-331, doi:10.1016/j.cell.2013.12.010 (2014).
- 4 Friedmann Angeli, J. P. *et al.* Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. *Nat Cell Biol* **16**, 1180-1191, doi:10.1038/ncb3064 (2014).
- 5 Zilka, O. *et al.* On the Mechanism of Cytoprotection by Ferrostatin-1 and Liproxstatin-1 and the Role of Lipid Peroxidation in Ferroptotic Cell Death. *ACS Cent Sci* **3**, 232-243, doi:10.1021/acscentsci.7b00028 (2017).
- 6 Shah, R., Shchepinov, M. S. & Pratt, D. A. Resolving the Role of Lipoygenases in the Initiation and Execution of Ferroptosis. *ACS Cent Sci* **4**, 387-396, doi:10.1021/acscentsci.7b00589 (2018).
- 7 Stockwell, B. R. *et al.* Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell* **171**, 273-285, doi:10.1016/j.cell.2017.09.021 (2017).
- 8 Tarangelo, A. *et al.* p53 Suppresses Metabolic Stress-Induced Ferroptosis in Cancer Cells. *Cell Rep* **22**, 569-575, doi:10.1016/j.celrep.2017.12.077 (2018).
- 9 Kagan, V. E. *et al.* Oxidized arachidonic and adrenic PEs navigate cells to ferroptosis. *Nat Chem Biol* **13**, 81-90, doi:10.1038/nchembio.2238 (2017).
- 10 Doll, S. *et al.* ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition. *Nat Chem Biol* **13**, 91-98, doi:10.1038/nchembio.2239 (2017).
- 11 Wu, M., Xu, L. G., Li, X., Zhai, Z. & Shu, H. B. AMID, an apoptosis-inducing factor-homologous mitochondrion-associated protein, induces caspase-independent apoptosis. *J Biol Chem* **277**, 25617-25623, doi:10.1074/jbc.M202285200 (2002).
- 12 Ingold, I. *et al.* Selenium Utilization by GPX4 Is Required to Prevent Hydroperoxide-Induced Ferroptosis. *Cell* **172**, 409-422 e421, doi:10.1016/j.cell.2017.11.048 (2018).
- 13 Viswanathan, V. S. *et al.* Dependency of a therapy-resistant state of cancer cells on a lipid peroxidase pathway. *Nature* **547**, 453-457, doi:10.1038/nature23007 (2017).
- 14 Hangauer, M. J. *et al.* Drug-tolerant persister cancer cells are vulnerable to GPX4 inhibition. *Nature* **551**, 247-250, doi:10.1038/nature24297 (2017).
- 15 Tsoi, J. *et al.* Multi-stage Differentiation Defines Melanoma Subtypes with Differential Vulnerability to Drug-Induced Iron-Dependent Oxidative Stress. *Cancer Cell* **33**, 890-904 e895, doi:10.1016/j.ccell.2018.03.017 (2018).
- 16 Angeli, J. P. F., Shah, R., Pratt, D. A. & Conrad, M. Ferroptosis Inhibition: Mechanisms and Opportunities. *Trends Pharmacol Sci* **38**, 489-498, doi:10.1016/j.tips.2017.02.005 (2017).
- 17 Horikoshi, N., Cong, J., Kley, N. & Shenk, T. Isolation of differentially expressed cDNAs from p53-dependent apoptotic cells: activation of the human homologue of the Drosophila peroxidase gene. *Biochem Biophys Res Commun* **261**, 864-869, doi:10.1006/bbrc.1999.1123 (1999).
- 18 Seiler, A. *et al.* Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. *Cell Metab* **8**, 237-248, doi:10.1016/j.cmet.2008.07.005 (2008).
- 19 Eisenhaber, F. *et al.* Prediction of lipid posttranslational modifications and localization signals from protein sequences: big-Pi, NMT and PTS1. *Nucleic Acids Res* **31**, 3631-3634 (2003).

- 20 Borgese, N., Aggujaro, D., Carrera, P., Pietrini, G. & Bassetti, M. A role for N-myristoylation in protein targeting: NADH-cytochrome b5 reductase requires myristic acid for association with outer mitochondrial but not ER membranes. *J Cell Biol* **135**, 1501-1513 (1996).
- 21 Mousnier, A. *et al.* Fragment-derived inhibitors of human N-myristoyltransferase block capsid assembly and replication of the common cold virus. *Nat Chem* **10**, 599-606, doi:10.1038/s41557-018-0039-2 (2018).
- 22 Elguindy, M. M. & Nakamaru-Ogiso, E. Apoptosis-inducing Factor (AIF) and Its Family Member Protein, AMID, Are Rotenone-sensitive NADH:Ubiquinone Oxidoreductases (NDH-2). *J Biol Chem* **290**, 20815-20826, doi:10.1074/jbc.M115.641498 (2015).
- 23 Frei, B., Kim, M. C. & Ames, B. N. Ubiquinol-10 is an effective lipid-soluble antioxidant at physiological concentrations. *Proc Natl Acad Sci U S A* **87**, 4879-4883 (1990).
- 24 Haidasz, E. A., Van Kessel, A. T. & Pratt, D. A. A Continuous Visible Light Spectrophotometric Approach To Accurately Determine the Reactivity of Radical-Trapping Antioxidants. *J Org Chem* **81**, 737-744, doi:10.1021/acs.joc.5b02183 (2016).
- 25 Niki, E. Mechanisms and dynamics of antioxidant action of ubiquinol. *Mol Aspects Med* **18 Suppl**, S63-70 (1997).
- 26 Mannes, A. M., Seiler, A., Bosello, V., Maiorino, M. & Conrad, M. Cysteine mutant of mammalian GPx4 rescues cell death induced by disruption of the wild-type selenoenzyme. *FASEB J* **25**, 2135-2144, doi:10.1096/fj.10-177147 (2011).
- 27 Shimada, K., Hayano, M., Pagano, N. C. & Stockwell, B. R. Cell-Line Selectivity Improves the Predictive Power of Pharmacogenomic Analyses and Helps Identify NADPH as Biomarker for Ferroptosis Sensitivity. *Cell Chem Biol* **23**, 225-235, doi:10.1016/j.chembiol.2015.11.016 (2016).
- 28 Shimada, K. *et al.* Global survey of cell death mechanisms reveals metabolic regulation of ferroptosis. *Nat Chem Biol* **12**, 497-503, doi:10.1038/nchembio.2079 (2016).
- 29 Morre, D. J. & Morre, D. M. Non-mitochondrial coenzyme Q. *Biofactors* **37**, 355-360, doi:10.1002/biof.156 (2011).
- 30 Nyquist, S. E., Barr, R. & Morre, D. J. Ubiquinone from rat liver Golgi apparatus fractions. *Biochim Biophys Acta* **208**, 532-534, doi:10.1016/0304-4165(70)90228-x (1970).
- 31 Rees, M. G. *et al.* Correlating chemical sensitivity and basal gene expression reveals mechanism of action. *Nat Chem Biol* **12**, 109-116, doi:10.1038/nchembio.1986 (2016).
- 32 Seashore-Ludlow, B. *et al.* Harnessing Connectivity in a Large-Scale Small-Molecule Sensitivity Dataset. *Cancer Discov* **5**, 1210-1223, doi:10.1158/2159-8290.CD-15-0235 (2015).
- 33 Basu, A. *et al.* An interactive resource to identify cancer genetic and lineage dependencies targeted by small molecules. *Cell* **154**, 1151-1161, doi:10.1016/j.cell.2013.08.003 (2013).

Figure legends

Figure 1 | Identification and validation of FSP1 as a robust ferroptosis suppressor. **a**, Scheme depicting the identification of AIFM2/FSP1 as a yet-unrecognized ferroptosis suppressor, using double-selection with 4-hydroxytamoxifen (TAM)-induced *Gpx4* knockout (KO) followed by RSL3-mediated elimination of false-positive cell clones. Surviving single cell clones were analysed by Sanger sequencing. **b**, Cell death induced by TAM was measured by lactate dehydrogenase (LDH) release of Pfa1 cells stably expressing Mock and FSP-HA using supernatants collected at the indicated time points in a 96-well plate. **c-e**, Dose-dependent toxicity of (1*S*, 3*R*)-RSL3 (RSL3)-induced cell death of Pfa1 cells (WT or KO for GPX4) expressing Mock or FSP1-HA (c), HT1080 cells expressing Mock or FSP1 (d), and HT1080 WT and HT1080 GPX4 KO cells overexpressing mock, hGPX4-FSH or FSP1-HA treated with and without 200 nM liproxstatin-1 (Lip-1)(e). Cell viability was assessed 24 h (b,c) or 72 h (d) thereafter using Aquabluer. Data represents the mean \pm s.d. of $n = 3$ wells of a 96-well plate from 1 representative of two (b), three (c-e) independent experiments, * $P < 0.0001$ (two-way ANOVA).

Figure 2 | N-myristoylation of FSP1 is important for its anti-ferroptotic function. **a**, Immunoblotting against ACSL4, FSP1, GPX4 and VCP of Pfa1 cells stably expressing Mock, FSP1-HA or FSP1[G2A]-HA (left), parental HT1080 cells and HT1080 FSP1 KO cells stably Mock, FSP1 or FSP1[G2A] (right). Immunoblot pictures represent crop outs from the chemiluminescent signal files. For gel source data showing the overlap of colorimetric and chemiluminescent signals, see Supplementary Figure 1. **b**, Specific enrichment of myristoylated proteins using metabolic

labeling with YnMyr myristate analogue followed by click chemistry to AzTB (Pfa1 FSP1-HA, Pfa1 FSP1-HA + IMP-1088, Pfa1 FSP1[G2A]-HA, Pfa1 Mock). TAMRA in-gel fluorescence showing labelling of myristoylated proteins. FSP1 was specifically enriched with YnMyr and the enrichment was prevented by the pan-myristoylation inhibitor IMP-1088 as well as the FSP1[G2A] mutant, demonstrated by immunoblot analysis (HA antibody). Endogenously expressed ADP ribosylation factor like GTPase 1 (ARL1), served as positive control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control. Immunoblot pictures represent crop outs from the chemiluminescent signal files. For gel source data showing the Cy5 ladder and chemiluminescent signals separately, see Supplementary Figure 1. **c**, (left) Dose-dependent toxicity of RSL3 in Pfa1 cells stably expressing Mock, FSP1-HA or FSP1[G2A]-HA. The FSP1[G2A] mutant failed to prevent RSL3-induced ferroptosis. (right) Inhibition of myristoylation (IMP-1088) in FSP1 overexpressing *Gpx4* KO Pfa1 cells induced cell death in a dose-dependent manner, which was prevented by the ferroptosis inhibitor liproxstatin-1 (Lip-1). **d**, RSL3-induced cell death of HT1080 FSP1 KO cells stably expressing Mock, FSP1 or FSP1[G2A]. Cell viability was assessed after 24 h using Aquabluer (c,d). Data represents mean \pm s.d. of $n = 4$ (c, left) or $n = 3$ wells (c, right; d) of a 96-well plate from one representative of three (c,d) independent experiments, * $P < 0.0001$ (two-way ANOVA). **e**, Enhanced resolution confocal microscopy of HT1080 cells (FSP1-GFP or FSP1[G2A]-GFP) overexpressing mCherry-Sec61 beta (ER localization) or mApple-Golgi-7 (Golgi localization). GFP is displayed in green, while mCherry and mApple fluorescence are pseudo-colored in yellow. Scale bars indicate 10 μ m and 2 μ m in the overview and magnified images, respectively.

Figure 3 | FSP1 protects from unrestrained lipid peroxidation. **a**, Flow cytometry analysis of RSL3-induced (300 nM for 3 h) BODIPY 581/591 C11 oxidation in Pfa1 cells overexpressing Mock or FSP1-HA. Data shows one representative of two independently performed experiments. **b**, Heat map showing the representation of mono-oxidized phospholipid species (PE, phosphatidylethanolamines; PC, phosphatidylcholine) in Pfa1 Mock and Pfa1 FSP1-HA treated with or without 4-hydroxytamoxifen (TAM) for 48 h. For heatmap illustration, samples ($n = 6$) were averaged and normalized to cell number (1×10^6 cells). Each lipid species was normalized to its maximum level detected. Experiment was performed independently twice. Abbreviations (a, acyl; e, plasmanyl; p, plasmeryl/plasmalogen). **c**, LC/MS relative quantification of ubiquinone CoQ₁₀ ([M+NH₄]⁺ m/z: 880.7177, RT 22.8 min) in HT1080 parental and HT1080 COQ2-KO clones. Ubiquinone 9 ([M+NH₄]⁺ m/z: 812.6551, RT 12.3 min) was used as internal standard. **d**, Dose-dependent toxicity of RSL3 in HT1080 parental and COQ2-KO cells overexpressing FSP1-GFP, FSP1[G2A] or GFP. Cell viability was assessed after 24 h using Aquabluer. **e**, Kinetic parameters for the reduction of coumarin-quinone conjugate by FSP1 (50 nM, blue) and NQO1 (50 nM, red) in TBS (10 mM, pH 7.4) in the presence of NADH (200 μ M) at 37°C. Initial rates were determined from the fluorescence of the product hydroquinone ($\epsilon_x = 415$ nm, $\epsilon_m = 470$ nm). The data was fit to a standard substrate inhibition model and represent mean \pm SD. **f**, NADH consumption assay (340 nm) in TBS buffer using recombinant purified hFSP1 in combination with different electron acceptor molecules (ubiquinone-1 (CoQ₁), ubiquinone-10 (CoQ₁₀), resazurin, oxidized glutathione (GSSG), dehydroascorbate, tert-butyl hydroperoxide (TBOOH)). Data shows $n = 2$ of one representative of three independent experiments (f). Data shows mean \pm s.d. of $n = 4$ (d) or $n =$

3 (c,e) wells of a 96-well plate from one representative of three (e,f) or one (c,d) independent experiments, * $P < 0.0001$ one-way ANOVA (c) and two-way ANOVA (d).

Figure 4 | FSP1 inhibition sensitizes tumor cells to ferroptosis. **a**, Chemical structure of iFSP1. Dose-dependent toxicity of iFSP1 of Pfa1 WT and GPX4 KO cells overexpressing FSP1-HA. **b,c**, Heatmaps depicting the dose-dependent toxicity of RSL3 in a panel of genetically engineered human cancer cell lines (FSP1 KO (b); FSP1 overexpressing, OE (c); for detailed cell viability assays including iFSP1 and liproxstatin-1 treatments please refer to Extended data Fig. 6e,f). **d**, Immunoblot analysis of FSP1, ACSL4, GPX4 and VCP of parental MDA-MB-436 cells and three independent FSP1 KO clones (KO #1-3) overexpressing Mock or murine FSP1 (mFSP1). Immunoblot pictures represent crop outs from the chemiluminescent signal files. For gel source data showing the overlap of colorimetric and chemiluminescent signals, see Supplementary Figure 1. **e**, Dose-dependent toxicity of RSL3 of the cell lines depicted in (d). Expression of FSP1 restored resistance to RSL3-induced ferroptosis in all three clones. **f**, Graphical abstract depicting the anti-ferroptotic function of FSP1 as a glutathione-independent suppressor of phospholipid peroxidation by inhibition of lipid radical-mediated autoxidation ($\text{PLO}\cdot/\text{OO}\cdot$) of lipid bilayers. Data shows mean \pm s.d. of $n = 3$ wells of a 96-well plate from one representative of one (a), two (b,c,e) independent experiments, * $P < 0.01$ (two-way ANOVA).

Extended Data Figure 1 | Identification and characterization of FSP1 as an anti-ferroptotic gene.

a, Scheme depicting the generation of a lentiviral cDNA overexpression library using the total mRNA from MCF7 cells. **b**, Genomic PCRs of 14 (remaining clones after removal of false positives) Pfa1 cell clones using human specific primers amplifying the human cDNAs of *GPX4* (571 bp) or *AIFM2* (524 bp). The clones 2, 16, 24, 25, 26, 28 and 30 showed positive PCR results for *GPX4* (571 bp). Clones 1, 44, 45, 50, 51, 52 and 53 were positive for *AIFM2* (524 bp) as indicated by the red arrows. Data shows one of $n = 3$ independent experiments. **c**, Immunoblot analysis of ACSL4, HA-tag, GPX4 and β -ACTIN of Pfa1 cells stably expressing Mock or FSP1-HA. *Gpx4* deletion was induced via administration of 4-hydroxytamoxifen (TAM) for the indicated time. **d**, Immunoblot analysis of ACSL4, HA, GPX4 and β -ACTIN of HT1080 (WT) and HT1080 GPX4 KO cells stably expressing Mock, GPX4-FSH or FSP1-HA. **e**, Proliferation of Pfa1 Mock and Pfa1 FSP1-HA cells treated with or without TAM. Cell numbers were assessed every 24 h using a Neubauer haemocytometer. Data shows mean \pm s.d. of $n = 3$ wells of a 24-well plate from one representative of two independent experiments. **f,g**, Dose-dependent toxicity of Erastin and L-buthionine sulfoximine (BSO) (g) induced cell death of Pfa1 cells expressing Mock or FSP1-HA. **h,i**, Dose-dependent toxicity of Erastin and L-buthionine sulfoximine (BSO), an inhibitor of γ -glutamyl-cysteine ligase, (i) induced cell death of HT1080 cells expressing Mock or FSP1-HA. Cell viability was assessed 48 h (f,h) or 72 h (g,i) after treatments using Aquabluer. Data shows mean \pm s.d. of $n = 3$ wells of a 96-well plate from one representative of three (f-i) independent experiments, * $P < 0.01$ (two-way ANOVA). **j**, Measurement of total glutathione levels in Pfa1 Mock, FSP1 and FSP1 GPX4 KO cells treated with or without BSO. Data shows mean \pm s.d. of $n = 3$ wells of a 96-well plate from one representative of three independent experiments. **k**, (left)

Determination of the NADPH consumption by glutathione reductase as an indirect measure of the GPX4 activity. Phosphatidylcholine lipid hydroperoxide (PCOOH) was used as a GPX4-specific substrate. Cell lysates from Pfa1 Mock and FSP1-HA cells treated with or without TAM for 48 h were used for the assay as shown by the immunoblot (FSP1, GPX4, β -ACTIN) on the right. FSP1 was detected using the polyclonal antibody (PA5-24562). Data shows mean \pm s.d. of $n = 3$ wells of a 6-well plate from one representative of three independent experiments. Immunoblot pictures (c,d,k) represent crop outs from the chemiluminescent signal files. For gel source data (c,d,k) showing the overlap of colorimetric and chemiluminescent signals, see Supplementary Figure 1.

Extended Data Figure 2 | FSP1 expression does not change the phospholipid composition.

Lipidomic profile (only detectable phospholipid species of phosphatidylethanolamine PE, phosphatidylcholine PC, phosphatidylglycerol PG, phosphatidylinositol PI and phosphatidylserine PS including plasmeyl (O) and plasmany (P) lipids) of Pfa1 Mock, FSP1-HA and Gpx4 KO FSP1-HA cells. Data represents the mean values of area of analyte (A)/internal standard (IS)/ total protein (mg) of $n = 4$ replicates of one experiment performed independently three times. log₁₀ has been applied to better visualize and compare the abundance of the different phospholipid species in the samples, * $P < 0.05$ (multiple t test with Sidak-Bonferroni correction for multiple comparisons).

Extended Data Figure 3 | FSP1 is a highly specific anti-ferroptotic protein. a, Dose-dependent toxicity of phenylarsine oxide, indomethacin, auranofin, ivermectin, sunitinib, obatoclox,

mitoxantrone, irinotecan, vinblastine, ABT-263, nocodazole, etoposide, paclitaxel, H₂O₂ and tert-butyl hydroperoxide (tBOOH) of Pfa1 cells expressing Mock or FSP1-HA. Cell viability was assessed 24 h after treatment using Aquabluer. **b**, Dose-dependent toxicity of TNF- α and staurosporine of Mock and FSP1-HA expressing Pfa1 cells. Cell viability was assessed 24 h after treatment using Aquabluer. **c**, Immunoblot analysis (ACSL4, HA, cleaved caspase 3 (clv. Casp3), GPX4 and β -ACTIN) of Pfa1 FSP1-HA cells treated with or without TNF- α for 6 h. **d**, Immunoblot analysis of FSP1, ACSL4, P53, P21 and VCP of HT1080 p53 WT and KO (CRISPR CAS9 modified) cell lines treated with the MDM2 (MDM2 proto-oncogene) inhibitor Nutlin3 or the cytostatic compound doxorubicin (Doxo). Expression of FSP1 was not altered by Nutlin3 or Doxo treatment, while the expression of P53 and P21 was strongly induced in HT1080 P53 WT cells. Data shows one representative of $n = 3$ independent experiments. **e**, Flow cytometry analysis of annexin V/PI staining in Pfa1 cells expressing Mock or FSP1-HA treated with or without TNF- α for 4 h. No difference in the apoptotic activity was observed using the Alexa Fluor 488 /PE-Cy5 channels. Data shows one representative experiment of an experiment performed independently two times. **f**, Immunoblot analysis of AIFM1, ACSL4, GPX4 and β -ACTIN in two different Pfa1 AIFM1 KO cell clones overexpressing Mock or AIFM1. Data shows one representative of $n = 3$ independent experiments. **g**, Dose-dependent toxicity of RSL3, Erastin and L-buthionine sulfoximine (BSO) of Pfa1 AIFM1 KO cell clones (#1 and #2) overexpressing Mock or AIFM1. AIFM1 expression does not impact on ferroptosis sensitivity. Data shows the mean of $n = 3$ replicates of a representative experiment performed independently three times. **h**, Time-dependent lactate dehydrogenase (LDH) release of Pfa1 cells stably expressing Mock, FSP1-HA or FSP1[G2A] treated with TAM to induce GPX4 loss. Supernatants were collected from 6-well plates at different time

points after TAM induction and assayed for LDH content in a 96-well plate. **i**, HT1080 WT and HT1080 GPX4 KO cells overexpressing mock, hGPX4-FSH, FSP1-HA or FSP1[G2A]-HA treated with and without 200 nM Liproxstatin-1 (Lip-1). Cell viability was assessed after 72 h using Aquabluer. Data shows mean \pm s.d. of $n = 3$ wells of a 96-well plate from one representative of three independent experiments (a, b, g, h, i), * $P < 0.01$ (two-way ANOVA). Immunoblot pictures (c,d,f) represent crop outs from the chemiluminescent signal files. For gel source data (c,d,f) showing the overlap of colorimetric and chemiluminescent signals, see Supplementary Figure 1.

Extended Data Figure 4 | FSP1 protects from unrestrained lipid peroxidation in a Coq2 dependent manner. **a**, Enhanced resolution confocal microscopy pictures demonstrating different localizations of FSP1-GFP and the FSP1[G2A]-GFP mutant in HT1080 cells. DAPI (yellow), GFP (green), ER-or Golgi-tracker (magenta) (Bars indicate 20 nm). Data shows one representative of $n = 3$ independently performed experiments. **b**, Formation of 5-H(P)ETE (MRM: 319 \rightarrow 115), 12-H(P)ETE (MRM: 319 \rightarrow 179) and 15-H(P)ETE (MRM: 319 \rightarrow 219) in either Mock (black) or FSP1-HA overexpressing (red) Pfa1 cells treated with 0.2 μ M RSL3 and 40 μ M arachidonic acid. Hydroperoxides were analyzed as their alcohols following reduction with PPh3 (triphenylphosphane) in methanol (Abbreviation: H(P)ETE, hydro(pero)xyeicosatetraenoic acid). Data shows the mean of biological triplicates from one representative of $n = 3$ independently performed experiments. **c**, Dose-dependent rescue of three independent HT1080 Coq2 KO cell clones (56, 61 and 68) by supplementation of the cell culture medium with uridine, CoQ₁₀ or decyl-ubiquinone. Cell viability was assed using the Aquabluer assay 48 h after treatment. Data

shows mean \pm s.d. of $n = 3$ wells of a 96-well plate performed once. **d**, Immunoblot analysis of FSP1 and β -ACTIN in HT1080 parental (left) and HT1080 COQ2-KO (#56) (right) overexpressing FSP1-GFP, FSP1[G2A] or GFP. Immunoblot pictures represent crop outs from the chemiluminescent signal files. For gel source data showing the uncropped chemiluminescent signals, see Supplementary Figure 1. For gel source data, see Supplementary Figure 1. **e**, SDS gels showing the different purification steps of recombinant FSP1 from bacterial cell lysates. (left) SDS gel of protein extracts after initial nickel affinity chromatography (E1), the SUMO-tag was cleaved in the eluate by addition of the SUMO protease (dtUD1) and a second round of nickel affinity chromatography was performed to remove the cleaved SUMO-tag as well as uncleaved SUMO-FSP1 and SUMO protease (E2). The flow through fraction was collected (2nd Ni). The SUMO-FSP1 fusion protein is visible around 55kDa, and FSP1 at 40,5 kDa. (right) SDS gel showing different fractions containing FSP1 40,5 kDa (A8-A12, B1-B7 and C3-C4) from size exclusion chromatography of FSP1 after the second nickel affinity chromatography. Fractions C3 and C4 were used for subsequent assays. One representative of at least three independent experiments.

Extended Data Figure 5 | FSP1 protects from lipid peroxidation by reducing RTAs. a,b, Co-oxidations of STY-BODIPY (1 μ M) (a) and the polyunsaturated lipids of (chicken) egg phosphatidylcholine liposomes (1 mM). The increase in fluorescence of ox-STY-BODIPY is monitored over the course of the autoxidation, which is initiated using C₉-HN (0.2 mM) (b). **c-j**, Representative autoxidations inhibited by either 50 nM FSP1 (green), 8 μ M NADH (purple), 16 μ M NADH (orange), 50 nM FSP1 and 8 μ M NADH (black) or 50 nM FSP1 and 16 μ M NADH (blue) (c). Analogous representative autoxidations inhibited to which CoQ₁₀ (d, e), α TOH and CoQ₁₀ (h,

i), or α TOH (j) were added. Recombinant NQO1 failed to suppress autoxidations in a similar way (f, g). **k**, 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxide (PLPC-OOH) produced from the autoxidation of soy lecithin liposomes (13.3 mM), inhibited by FSP1 alone, or in the presence of either 10 μ M CoQ₁₀ or 10 μ M α TOH and 10 μ M CoQ₁₀. PLPC-OOH was measured 0, 60, 120 and 180 min after autoxidation was induced using LC-MS (MRM: 790 \rightarrow 184). Data shows one of $n = 3$ representative experiments.

Extended Data Figure 6 | Development of a FSP1-specific inhibitors (iFSP1) as ferroptosis sensitizer. a-b, Dose-dependent toxicity of iFSP1 (novel FSP1 inhibitor) in FSP1 overexpressing cells (Pfa1 (a); HT1080 (b)) with or without GPX4 loss. Treatment with the ferroptosis inhibitor liproxstatin-1 (Lip-1) (150 nM) protected GPX4 KO cells from iFSP1 induced ferroptosis. iFSP1 is only toxic to cells that depend solely (no GPX4 expression detectable) on FSP1 function. **c**, Efficacy of iFSP1 and structurally related analogues; EC₅₀ values (mean \pm s.d.) of iFSP1 (**1**) and its derivatives (**2** - **14**) calculated from experiments performed at least twice in triplicate are shown in the table with the corresponding chemical structures depicted below. Based on commercially available analogues a preliminary structure activity relationship (SAR) study revealed that substitution of the amino position (R1, R2) showed broad tolerability of aliphatic groups and that lipophilic substituents of the phenyl group at the 3-position (R3) in the ortho and meta positions were well tolerated. **d**, Immunoblot analysis of FSP1 and VCP in HT1080 parental as well as HT1080 FSP1 overexpressing and FSP1 KO cells. A self-made monoclonal antibody against human FSP1 was used. Immunoblot pictures represent crop outs from the chemiluminescent signal files. For gel source data showing the overlap of colorimetric and chemiluminescent signals, see

Supplementary Figure 1. **e**, Dose-dependent toxicity of RSL3 in a panel of genetically engineered (FSP1 KO) human cancer cell lines (NCI-H1437, NCI-H1437 FSP1 KO, U-373, U-373 FSP1 KO, MDA-MB-436, MDA-MB-436 FSP1 KO, SW620, SW620 FSP1 KO, MDA-MB-435S, MDA-MB-435S FSP1KO, A549, A549 FSP1 KO) treated with or without FSP1 inhibitor (iFSP1) and liproxstatin-1 (Lip-1). **f**, Dose-dependent toxicity of RSL3 in a panel of genetically modified (murine and human FSP1 overexpression) human cancer cell lines (IMR5/75 Mock, IMR5/75 hFSP1, 786-O Mock, 786-O hFSP1, LOX-IMVI Mock, LOX-IMVI hFSP1, HLF Mock, HLF hFSP1, U-138 Mock, U-138 mFSP1) treated with or without iFSP-1 and Lip-1. Data shows the mean \pm s.d. of $n = 3$ wells of a 96-well plate from one representative of three (a-c) or two (e, f) independent experiments, * $P < 0.0001$ (two-way ANOVA).

Extended Data Figure 7 | FSP1 is expressed in a wide range of cancer cell lines. **a**, Immunoblot analysis of the expression of key ferroptosis players including ACSL4, FSP1, GPX4 and XCT (SLC7A11) in a panel of cancer cell lines from different origins. In addition, genetically modified cancer cell lines carrying a knockout of FSP1 (MDA-436-MB FSP1 KO, NCI-H1437 FSP1 KO, U-373 FSP1 KO, MDA-MB-435S FSP1 KO, A549 FSP1 KO and SW620 FSP1 KO) as well as cell lines with lentiviral overexpression of FSP1 (IMR5/75 hFSP1, 786-O hFSP1, LOX-IMVI hFSP1 and HLF hFSP1) are shown. VCP (Valosin containing protein) or β -ACTIN served as loading control. MDA-MB-231 was used as reference to compare expression levels in between independent blots. Data shows one representative of two independent experiments. Immunoblot pictures represent crop outs from the chemiluminescent signal files. For gel source data showing the overlap of colorimetric and chemiluminescent signals, see Supplementary Figure 1.

Extended Data Figure 8 | iFSP1 sensitized cancer cell lines from different origins to RSL3-induced ferroptosis. Dose-dependent toxicity of RSL3 in a panel of human cancer cell lines from different origins (breast, lung, pancreas, brain, liver, kidney, skin, intestine) treated with or without FSP1 inhibitor (iFSP1) and liproxstatin-1 (Lip-1). Data shows the mean \pm s.d. of $n = 3$ wells of a 96-well plate from one representative of two independent experiments.

Extended Data Figure 9 | FSP1 expression directly correlated with resistance to ferroptosis and its inhibition selectively sensitizes cells to ferroptosis. **a**, Correlation of a panel of 860 cancer cell lines (<https://portals.broadinstitute.org/ctrp>)³¹⁻³³. The sensitivity to (1*S*,3*R*)-RSL3, ML162 and ML210 was correlated with gene expression. Genes were plotted according to their Pearson correlation score. FSP1 (AIFM2) was the highest ranking gene that correlated with resistance to (1*S*,3*R*)-RSL3 ($P=0.392$), ML162 ($P=0.424$) and ML210 ($P=0.398$). **b**, Dot plot depicting the correlation of a cell's dependency on GPX4 (CERES score of -1 means full dependency based on CRISPR/Cas9 KO screen) and the expression level of FSP1 (AIFM2) in a panel of 559 different cancer cell lines (DepMap - <https://depmap.org/portal/>). DepMap publishes its data under CC Attribution 4.0 license. Cell lines with high expression of FSP1 were found to be less dependent on GPX4 (Pearson correlation score of 0.366, p -value= $3.38E-19$). **c**, Dose-dependent toxicity of RSL3 in a panel of human lung cancer cells (NCI-H1437, NCI-H1299, NCI-H1573, NCI-H2126, NCI-H520, NCI-H661) treated with or without the FSP1 inhibitor iFSP-1 (5 μ M). Co-treatment of RSL3 and iFSP-1 improved the ferroptotic response of all cell lines except NCI-H1437. **d**, Dose-dependent toxicity of different cytotoxic compounds (Erastin, L-buthionine sulfoximine (BSO), RSL3, vinblastine, etoposide, phenylarsine oxide (PAO), mitoxantrone, irinotecan, nocodazole,

cisplatin) in Pfa1 Mock and FSP1 overexpressing cells treated with or without iFSP-1. The protective effect of FSP1 overexpression is lost upon iFSP-1 (5 μ M) treatment. Data shows the mean \pm s.d. of $n = 3$ wells of a 96-well plate from one representative of two independent experiments (c, d).