

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 (1*S*/3*R*)-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 CoQ^{29,30}₁₀ 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 Lipoxygenases 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, plasmenyl/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 ($\epsilon = 415$ 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, obatoclax,

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 assayed 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. **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).

Figure 1

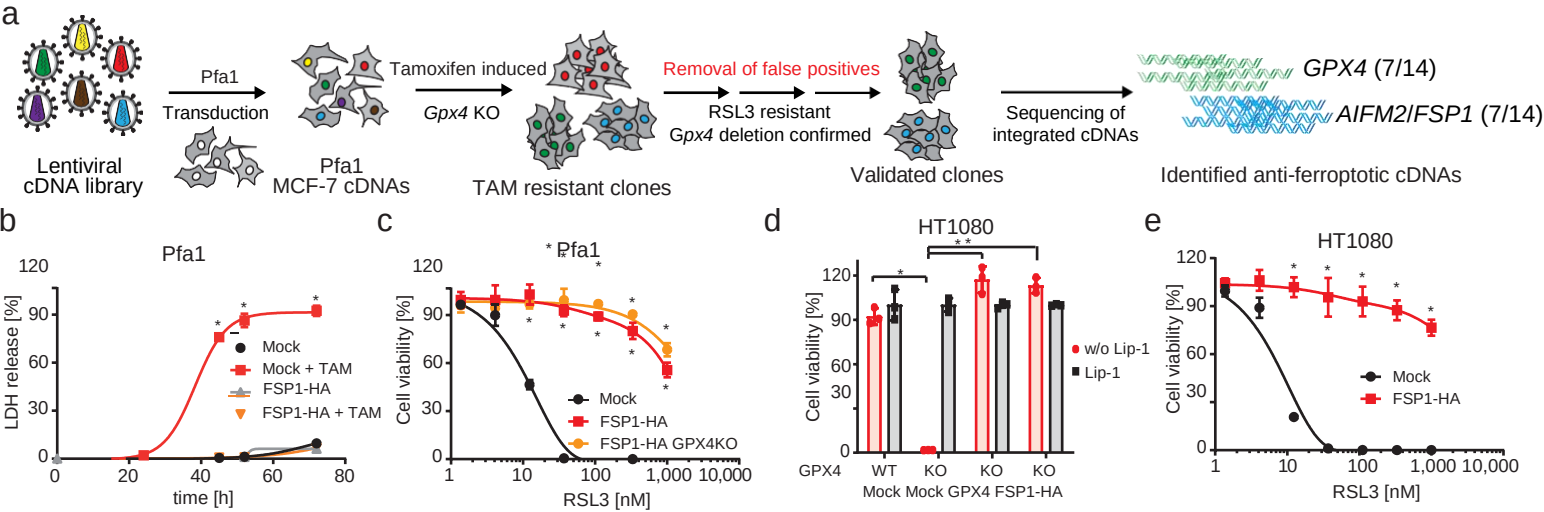
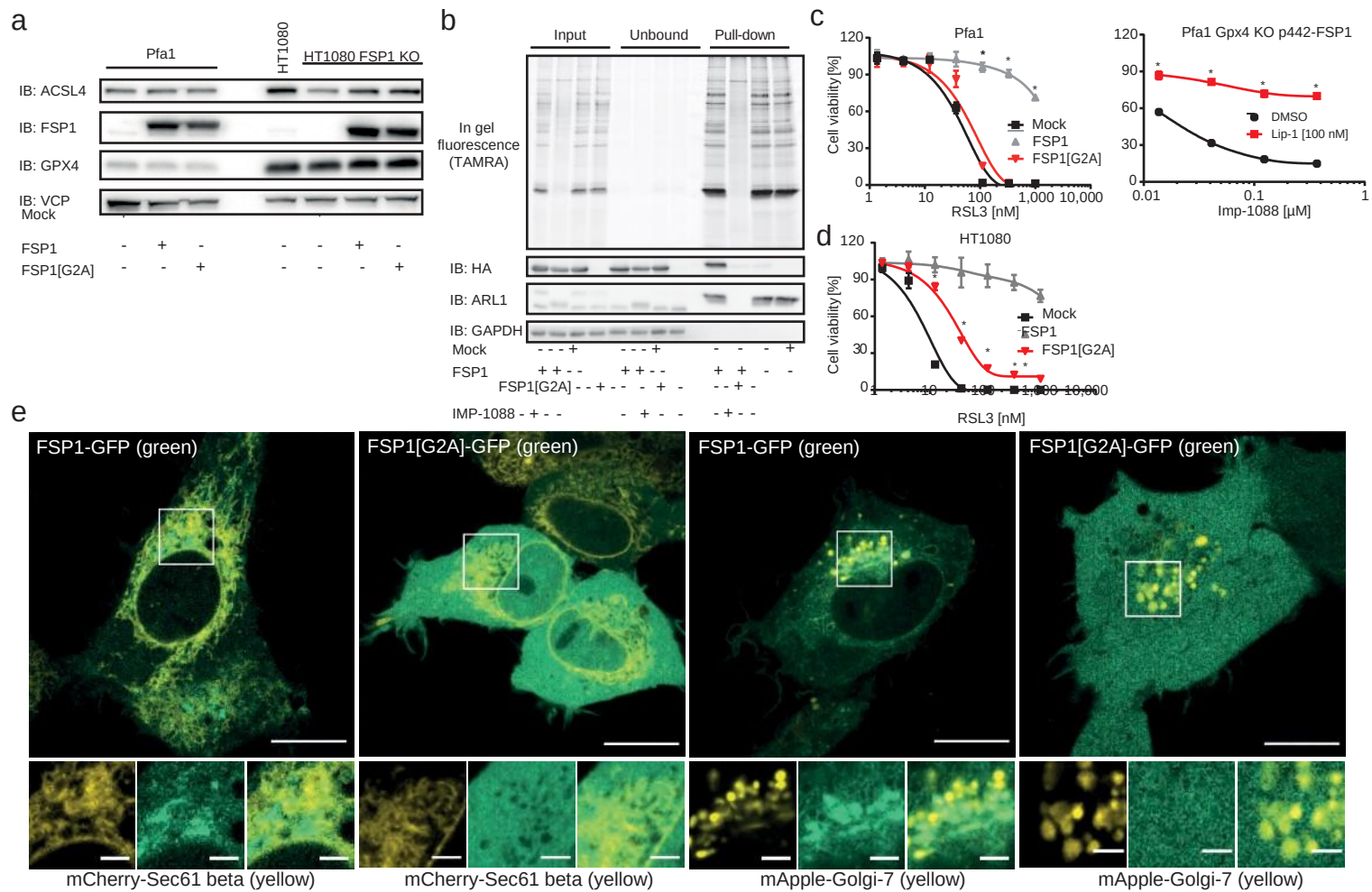


Figure 2



a

Normalized To Mode

Alexa Fluor 488-A

- Mock
- Mock + RSL3
- FSP1-HA
- FSP1-HA + RSL3

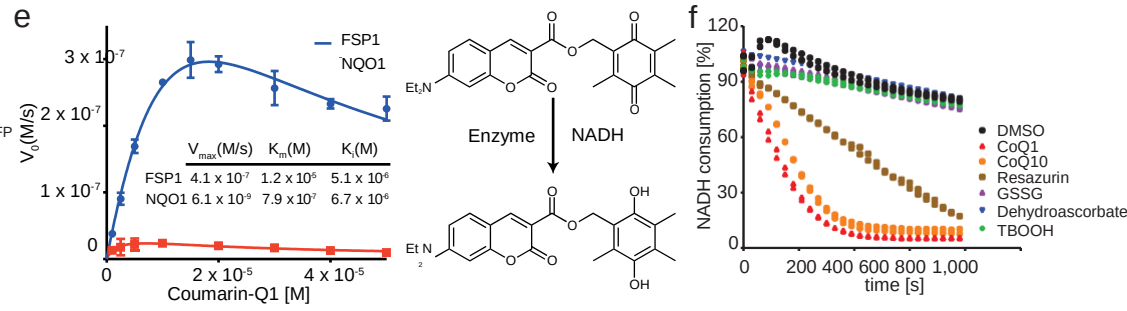
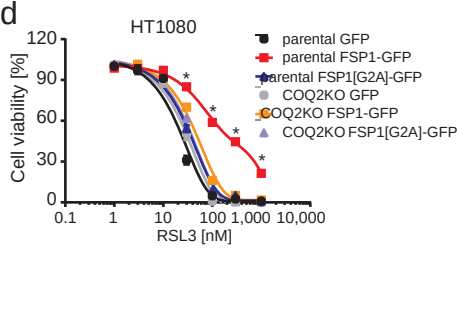
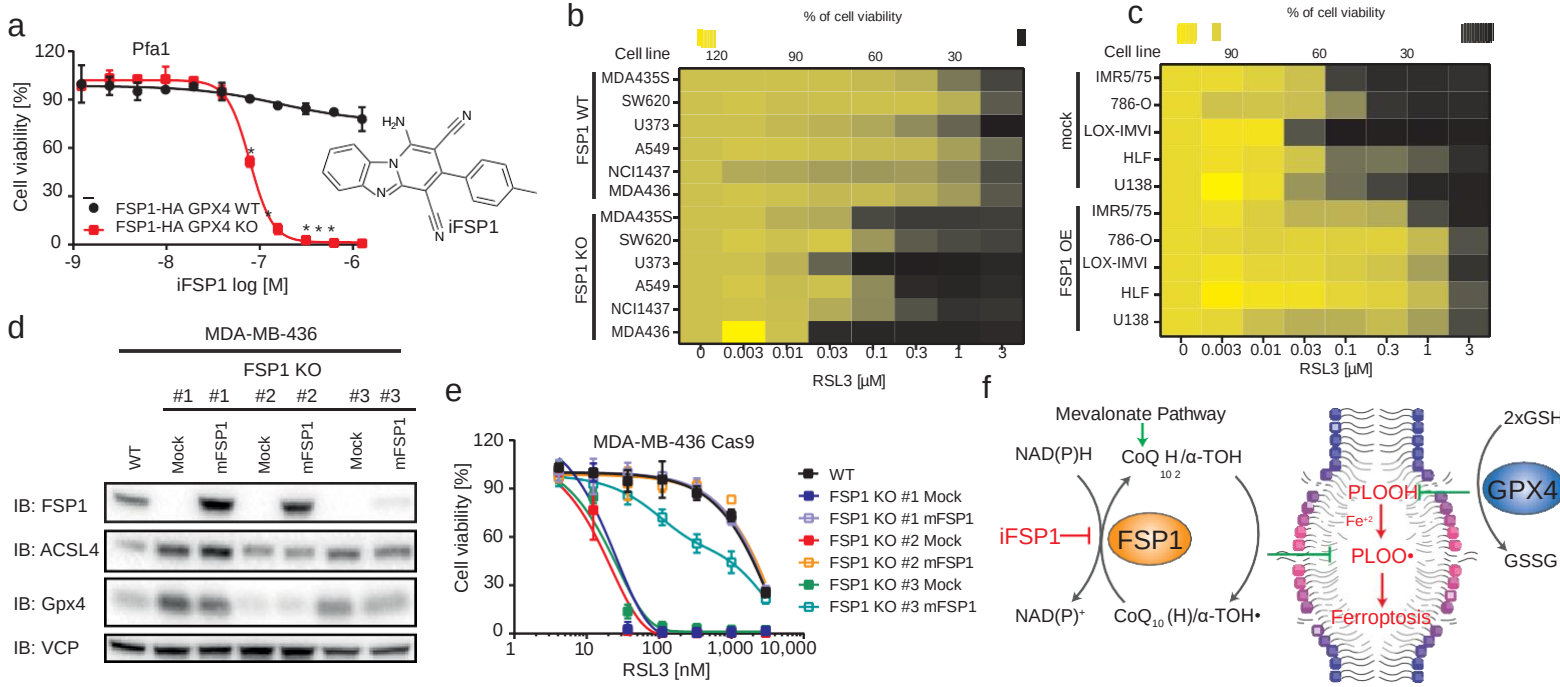
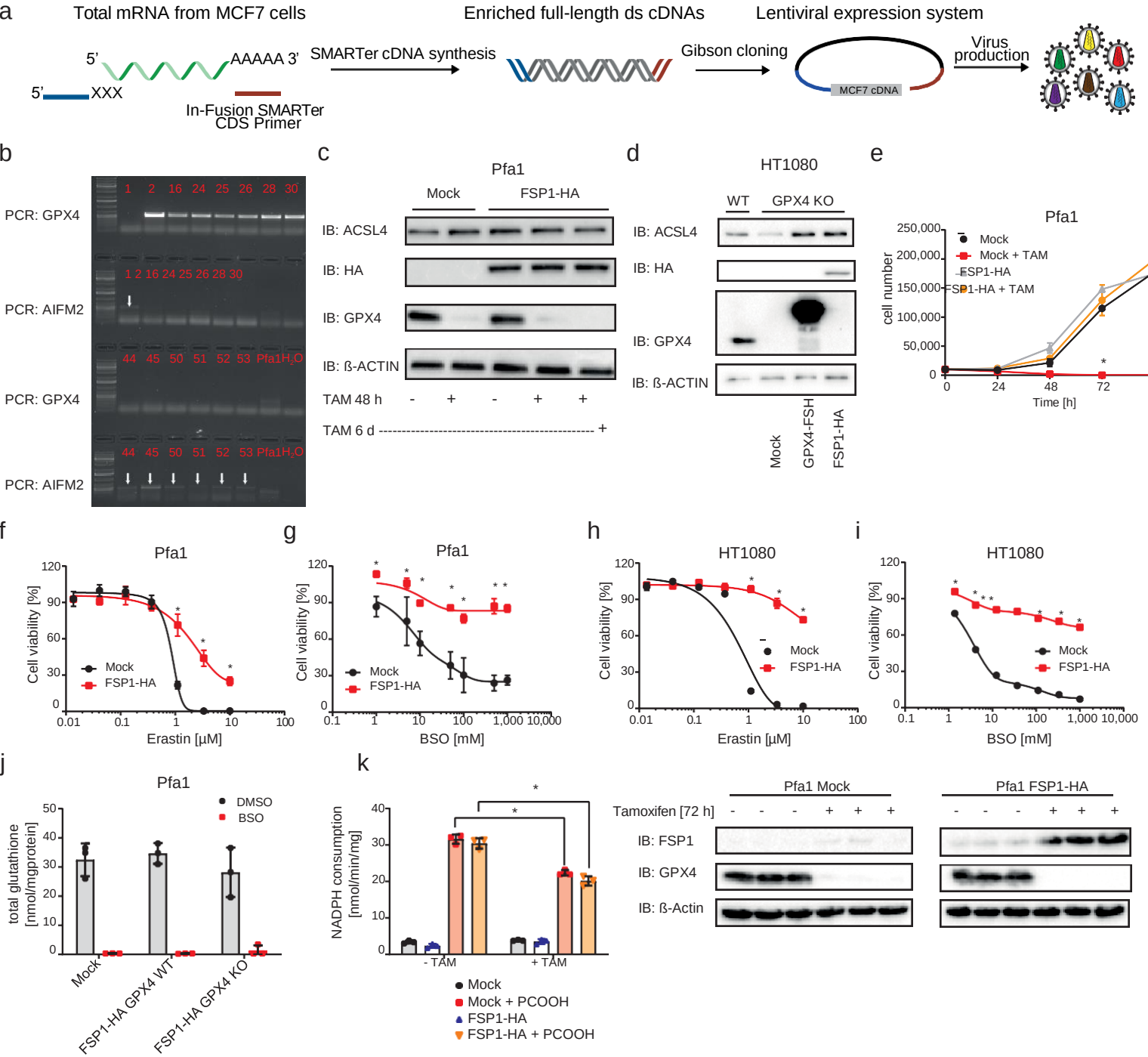


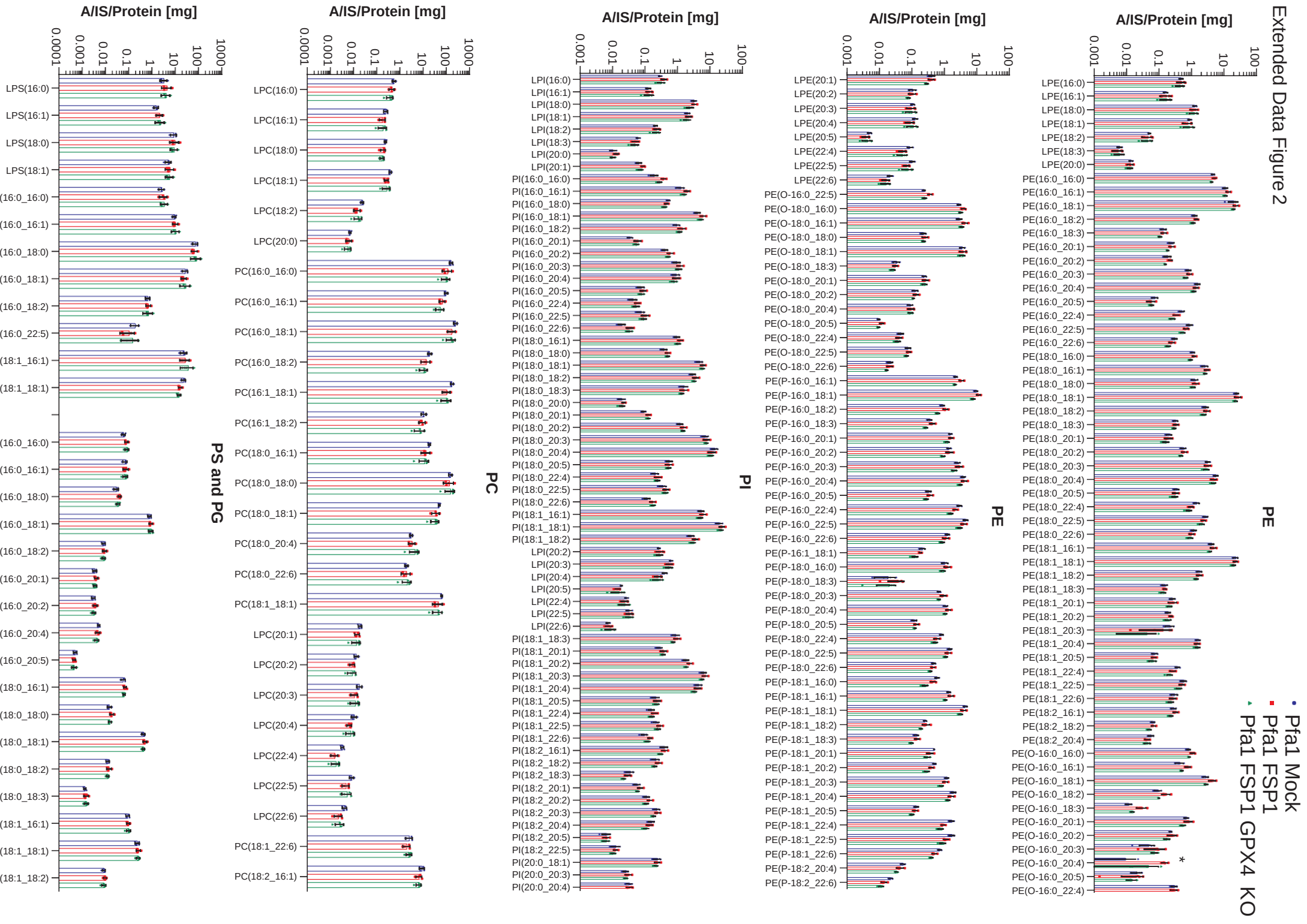
Figure 4



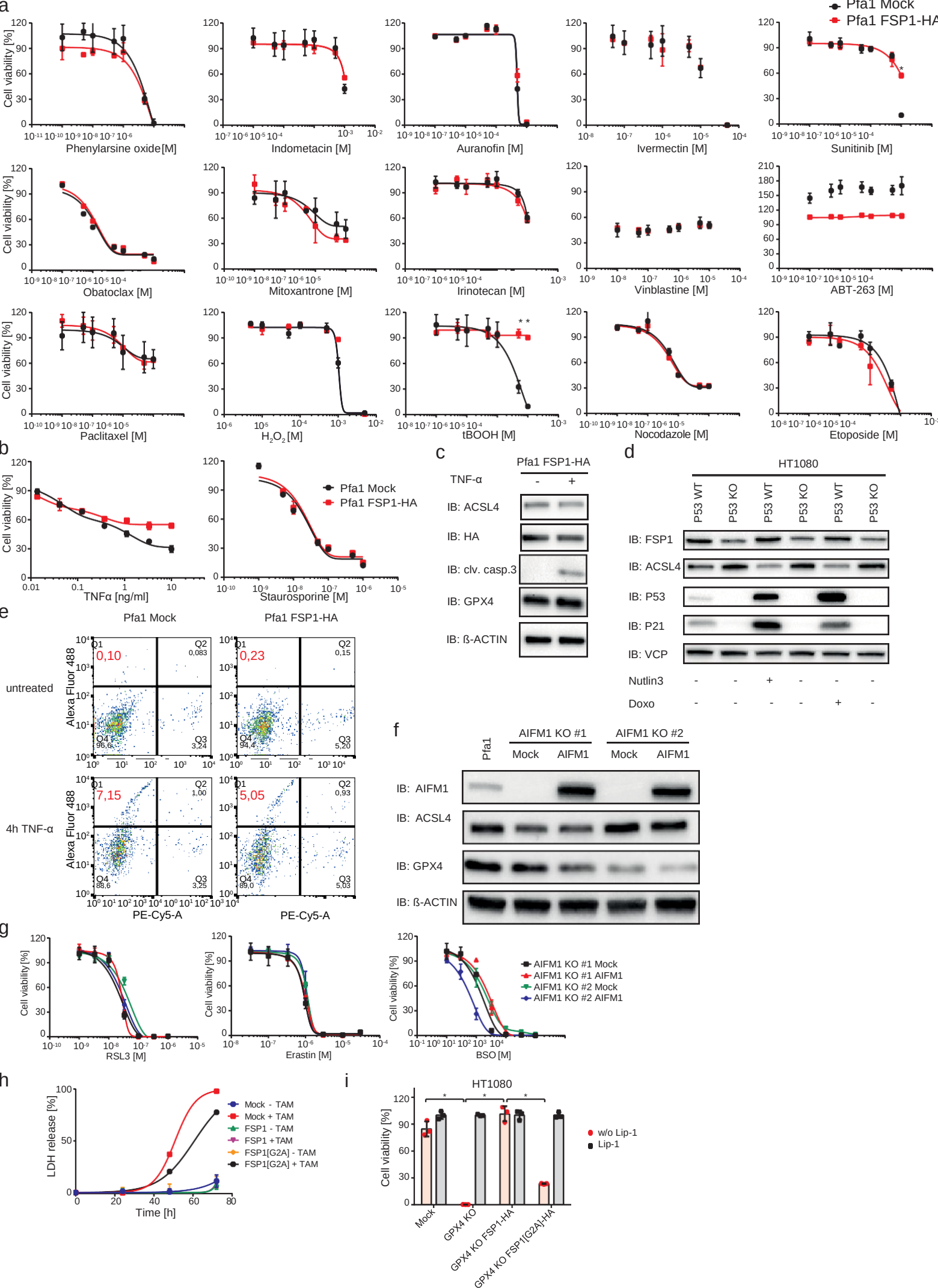
Extended Data Figure 1



Extended Data Figure 2

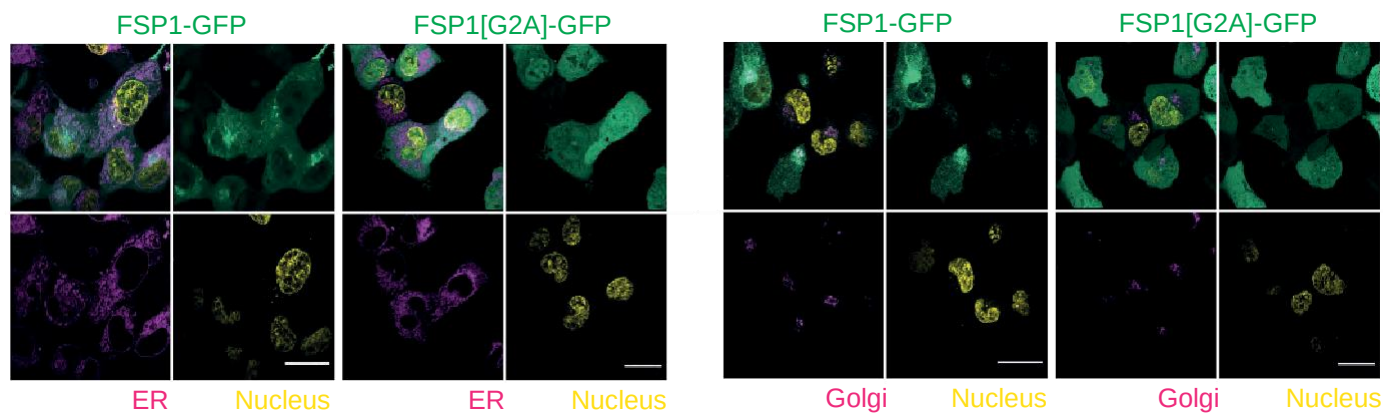


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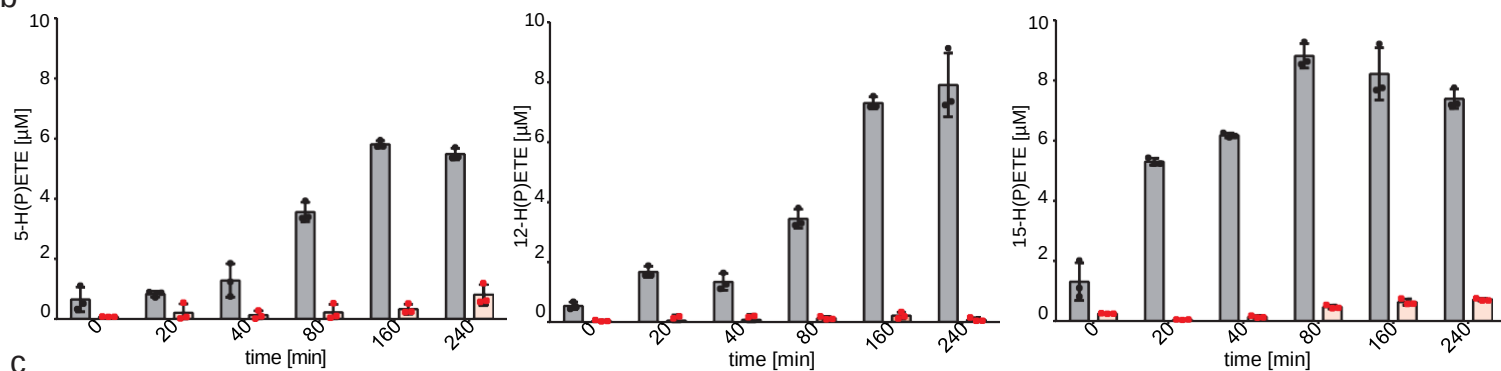


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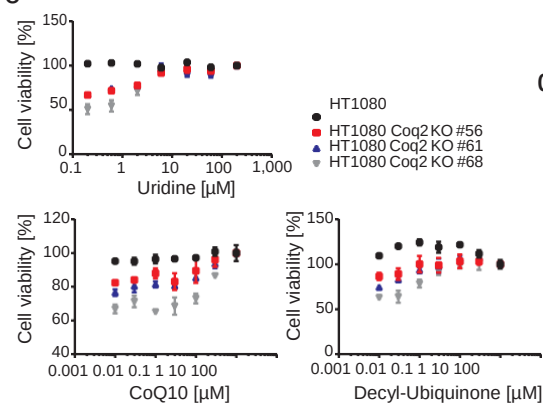
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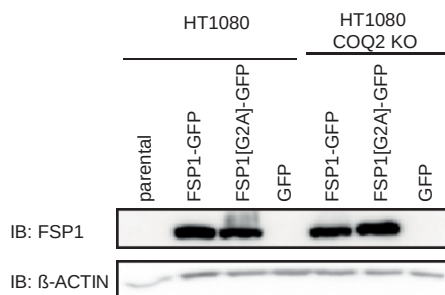
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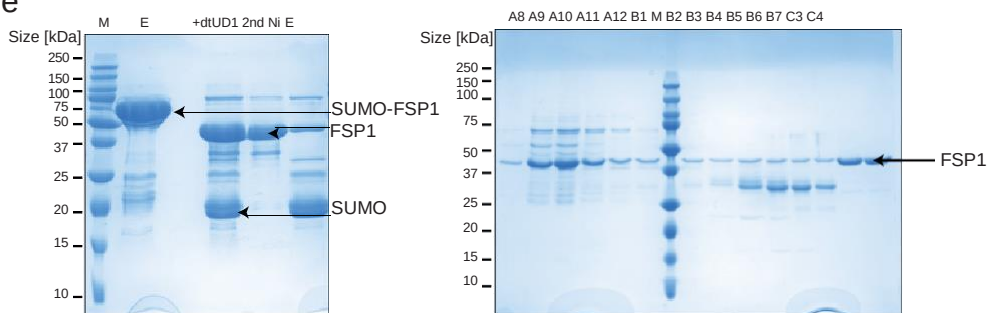
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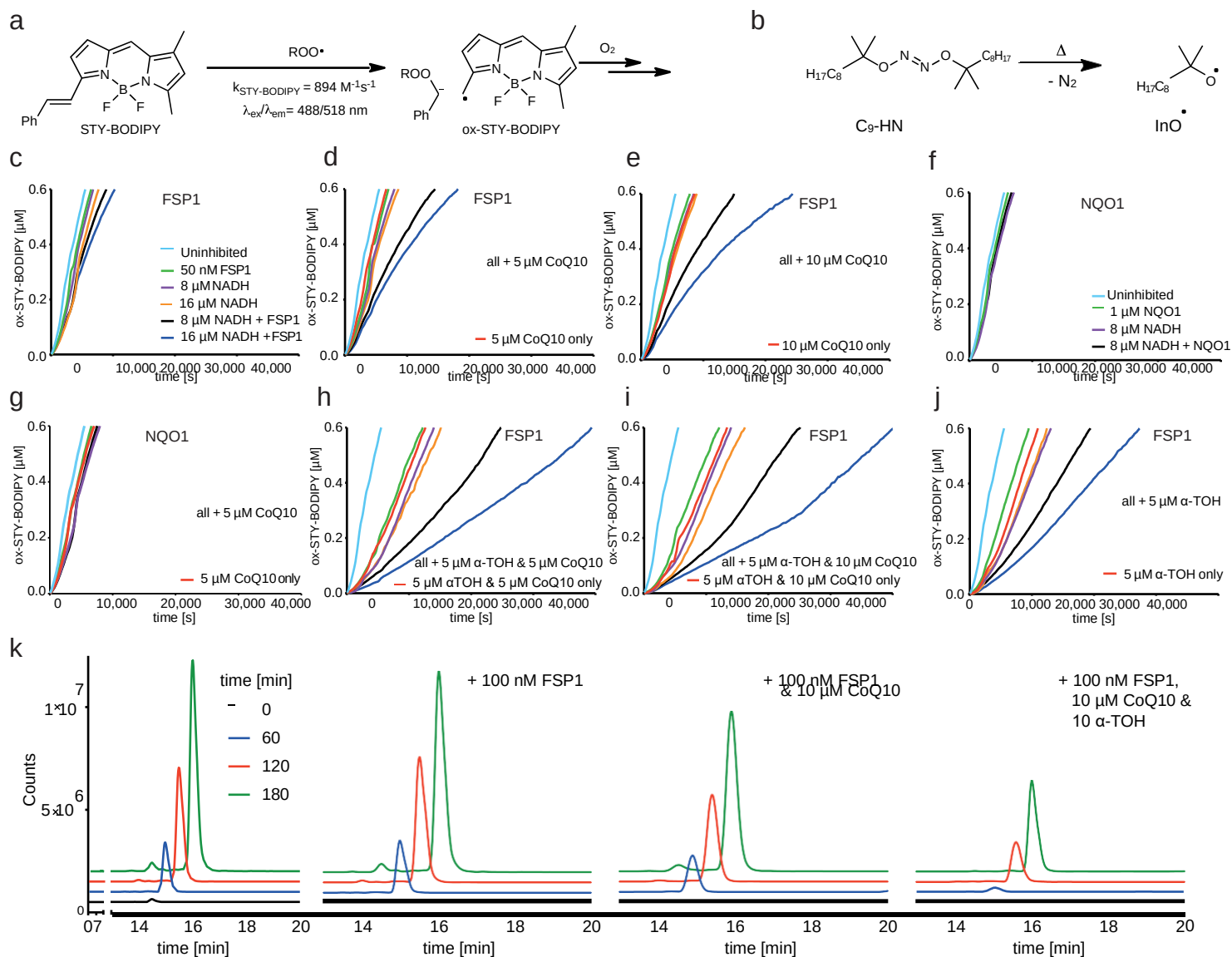
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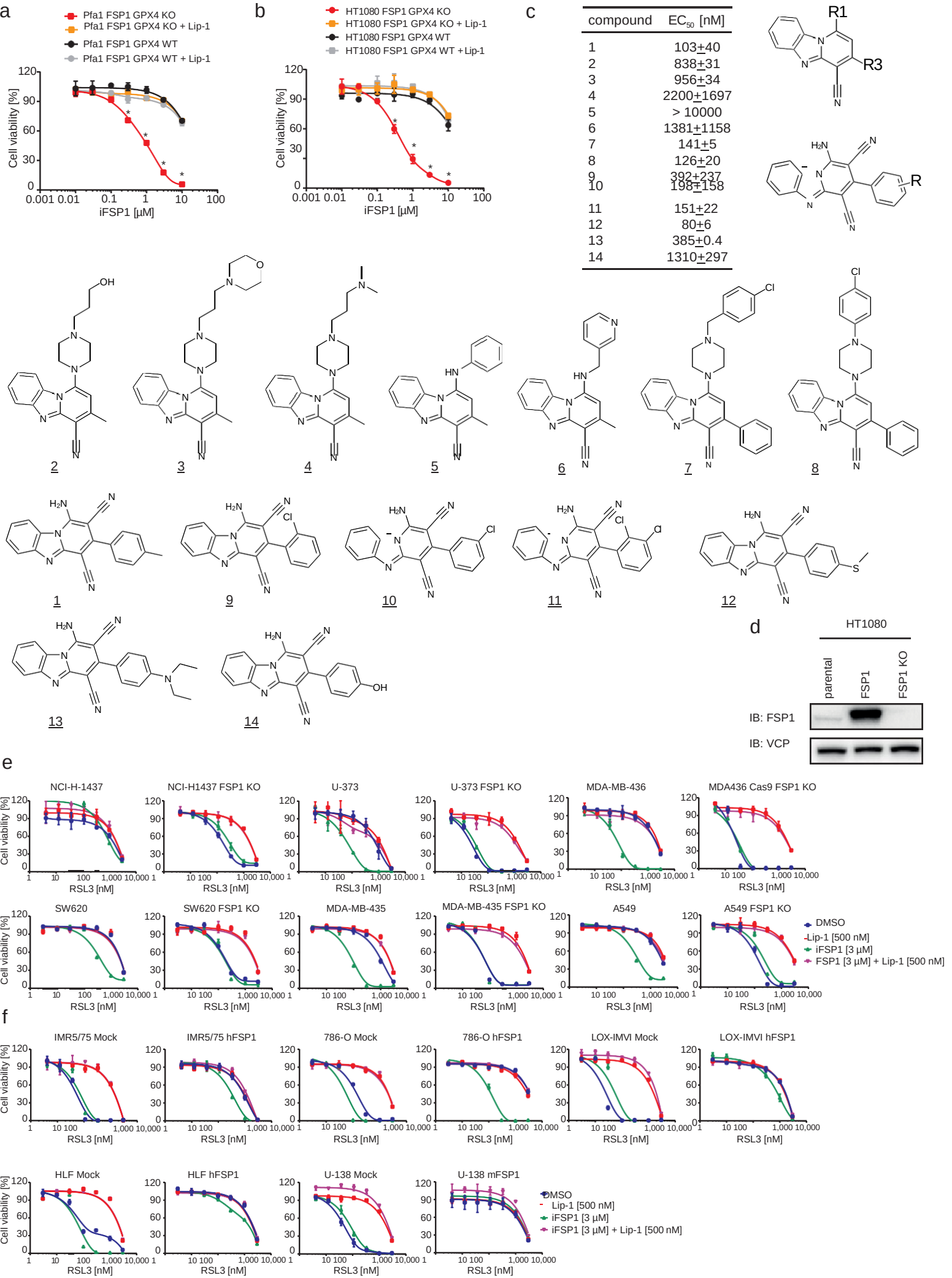
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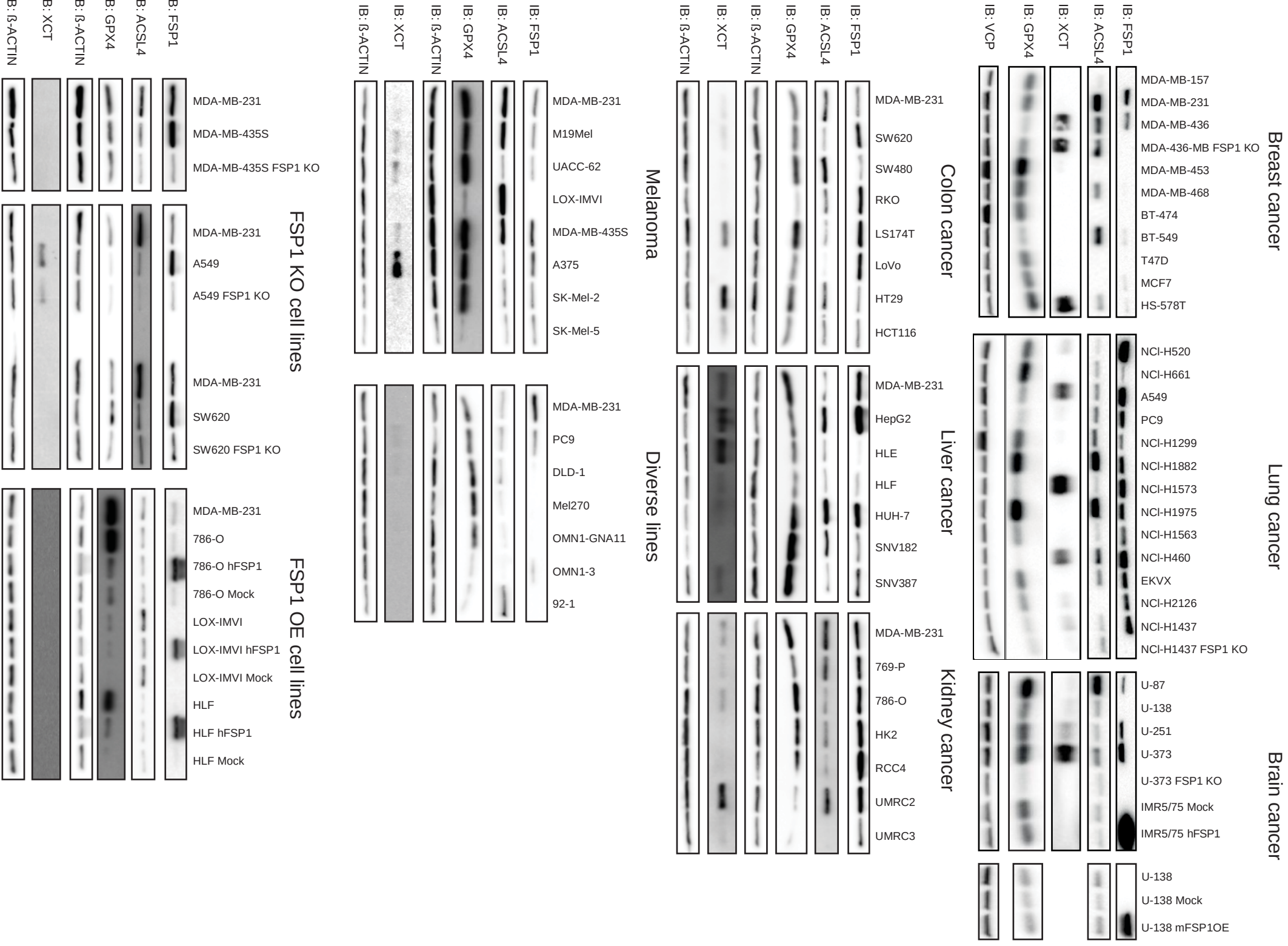


Extended Data Figure 5



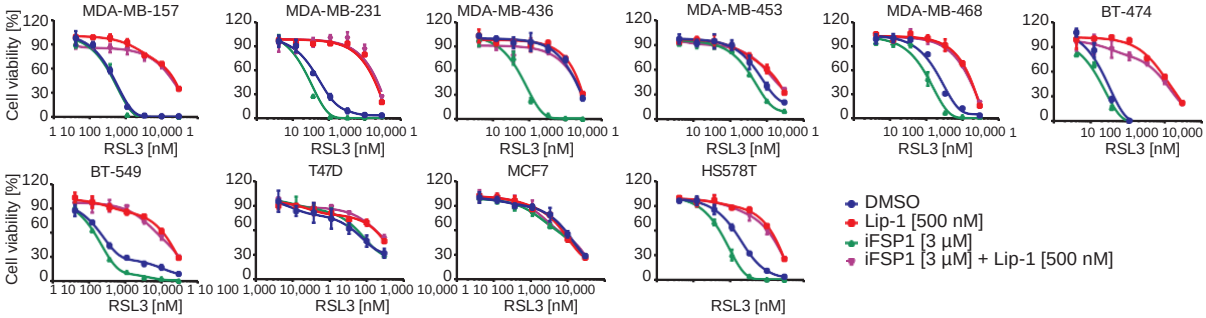
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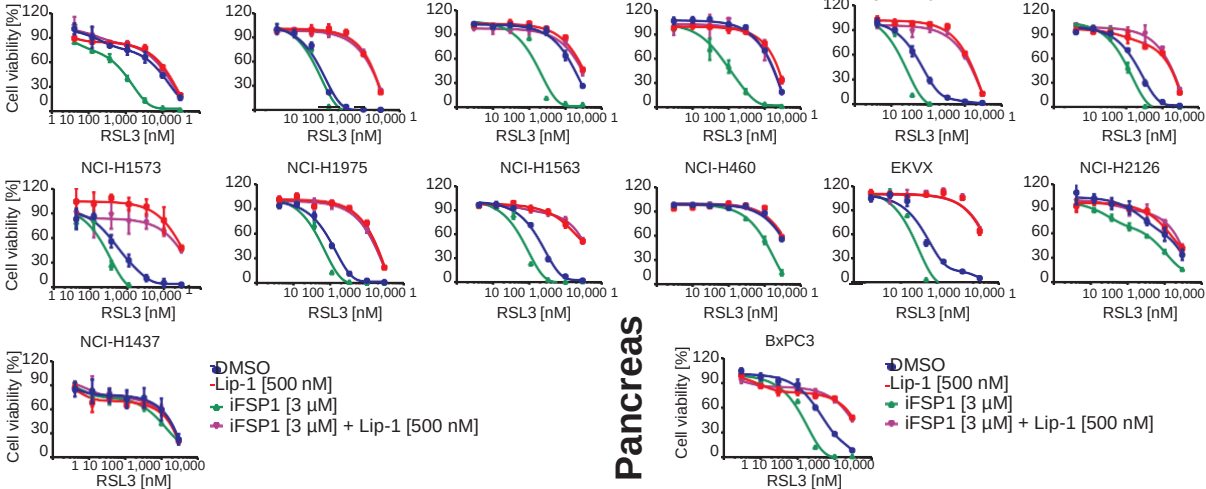


Extended Data Figure 8

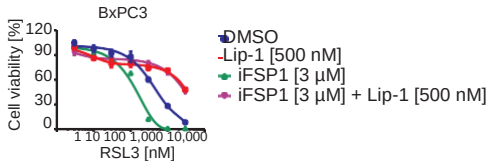
Breast



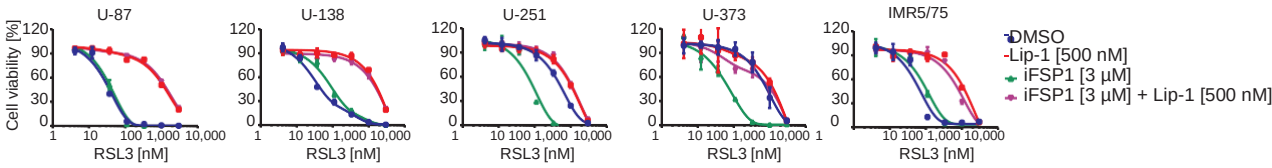
Lung



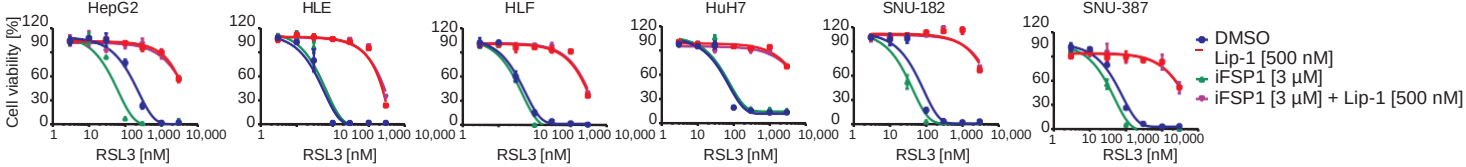
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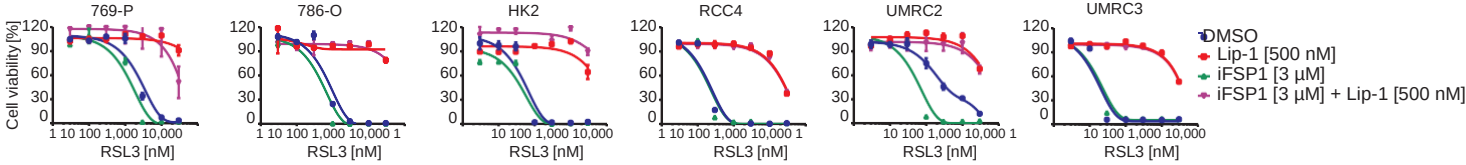
Brain



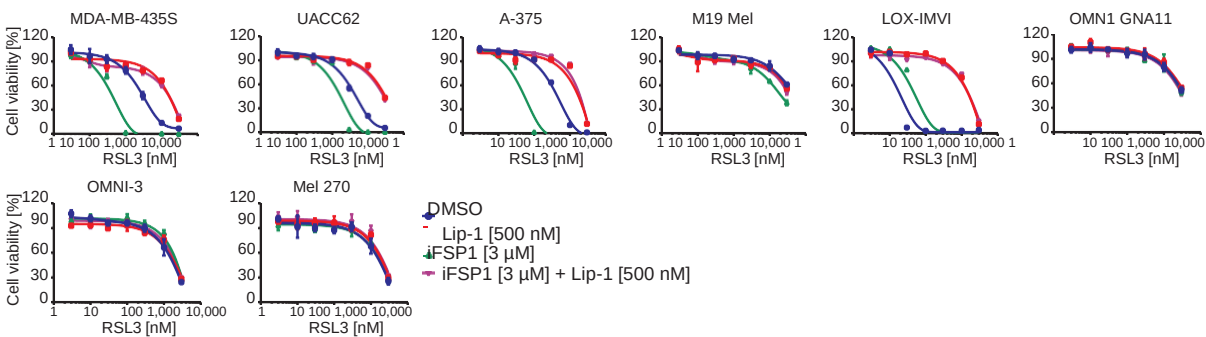
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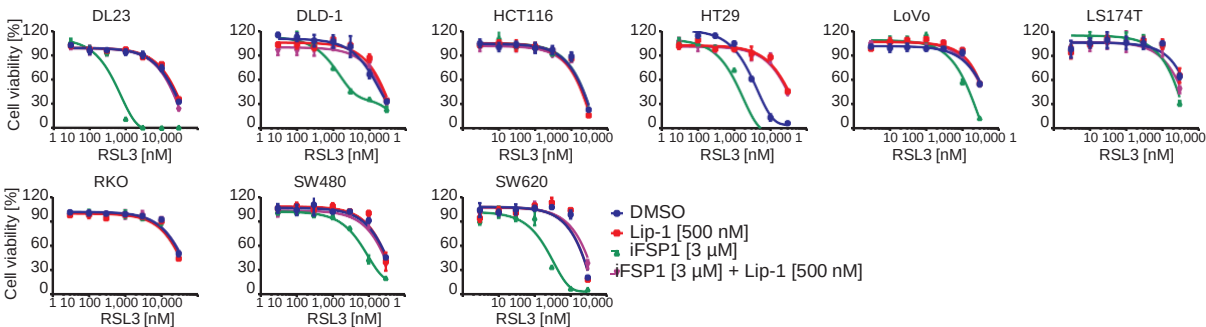
Kidney



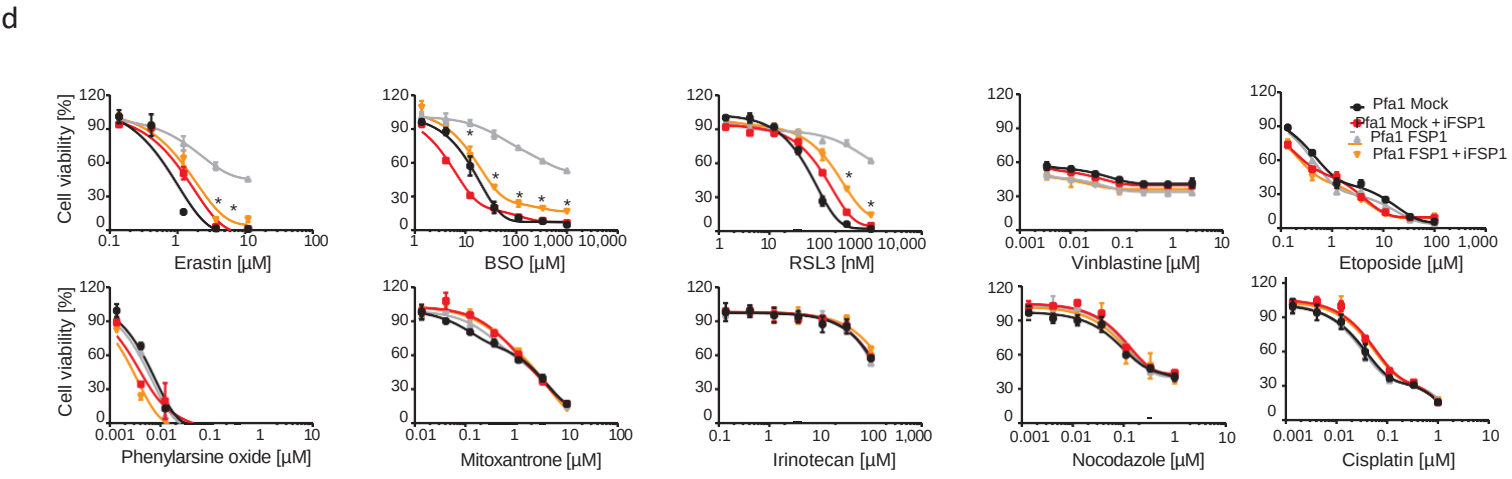
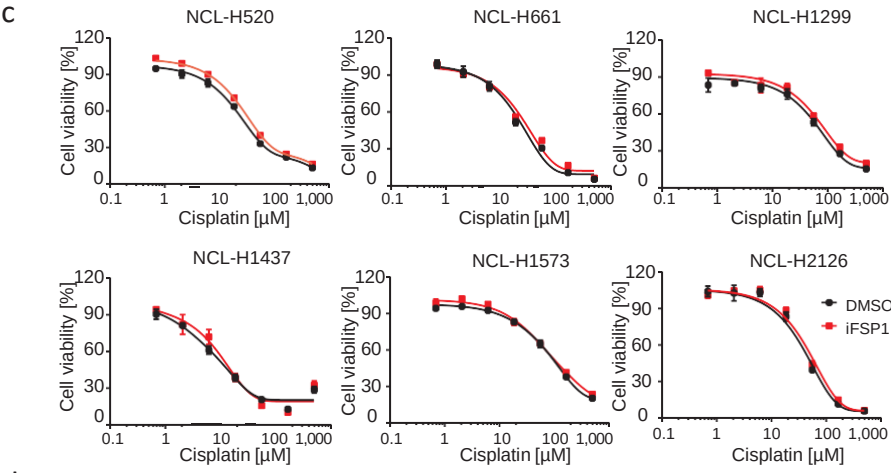
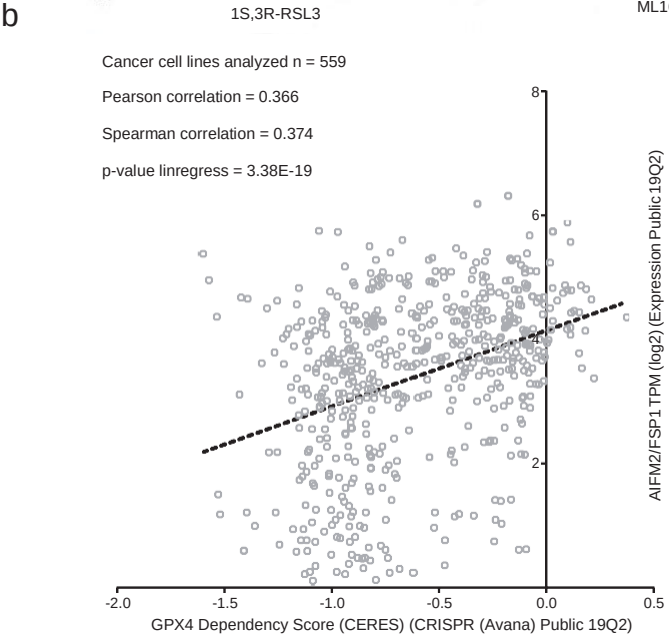
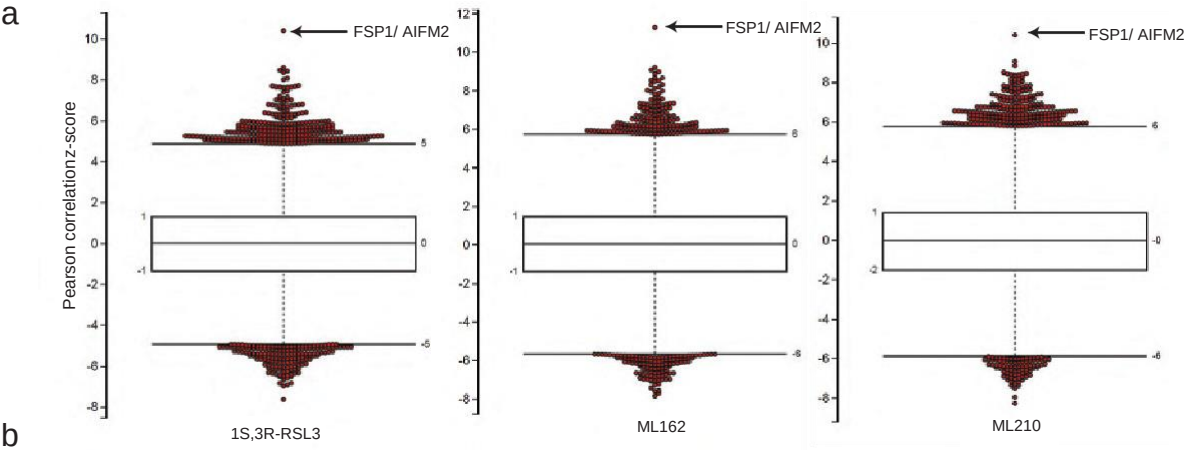
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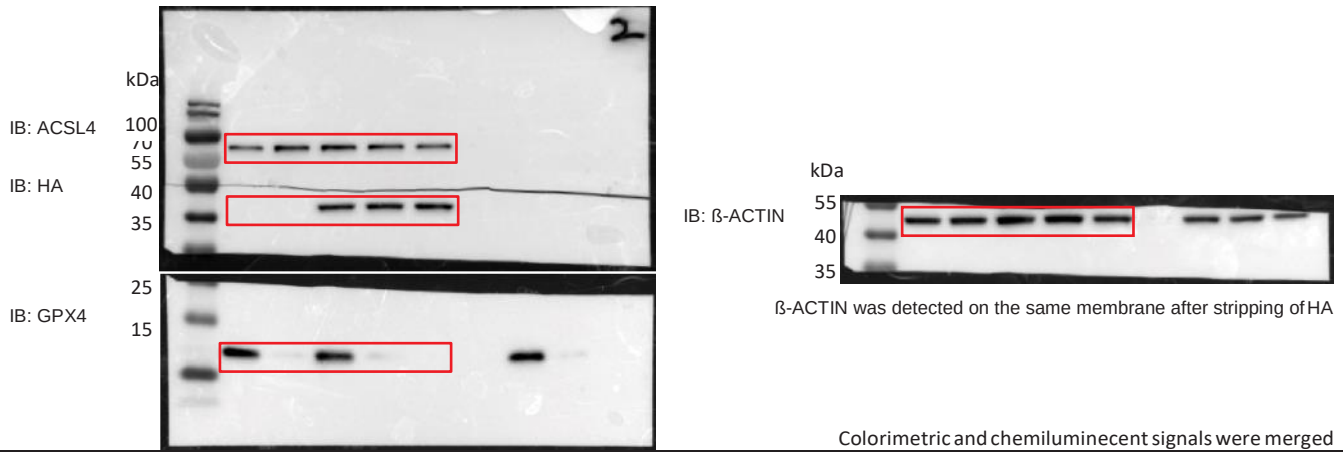
Intestine



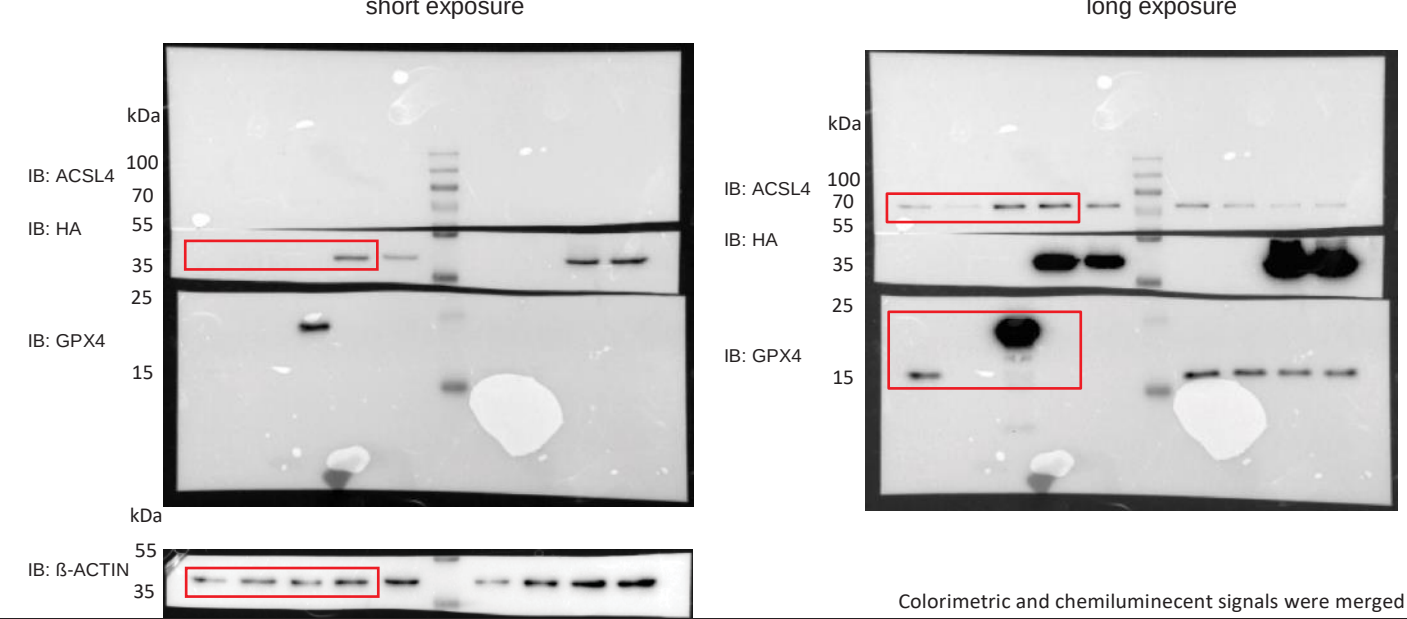
Extended Data Figure 9



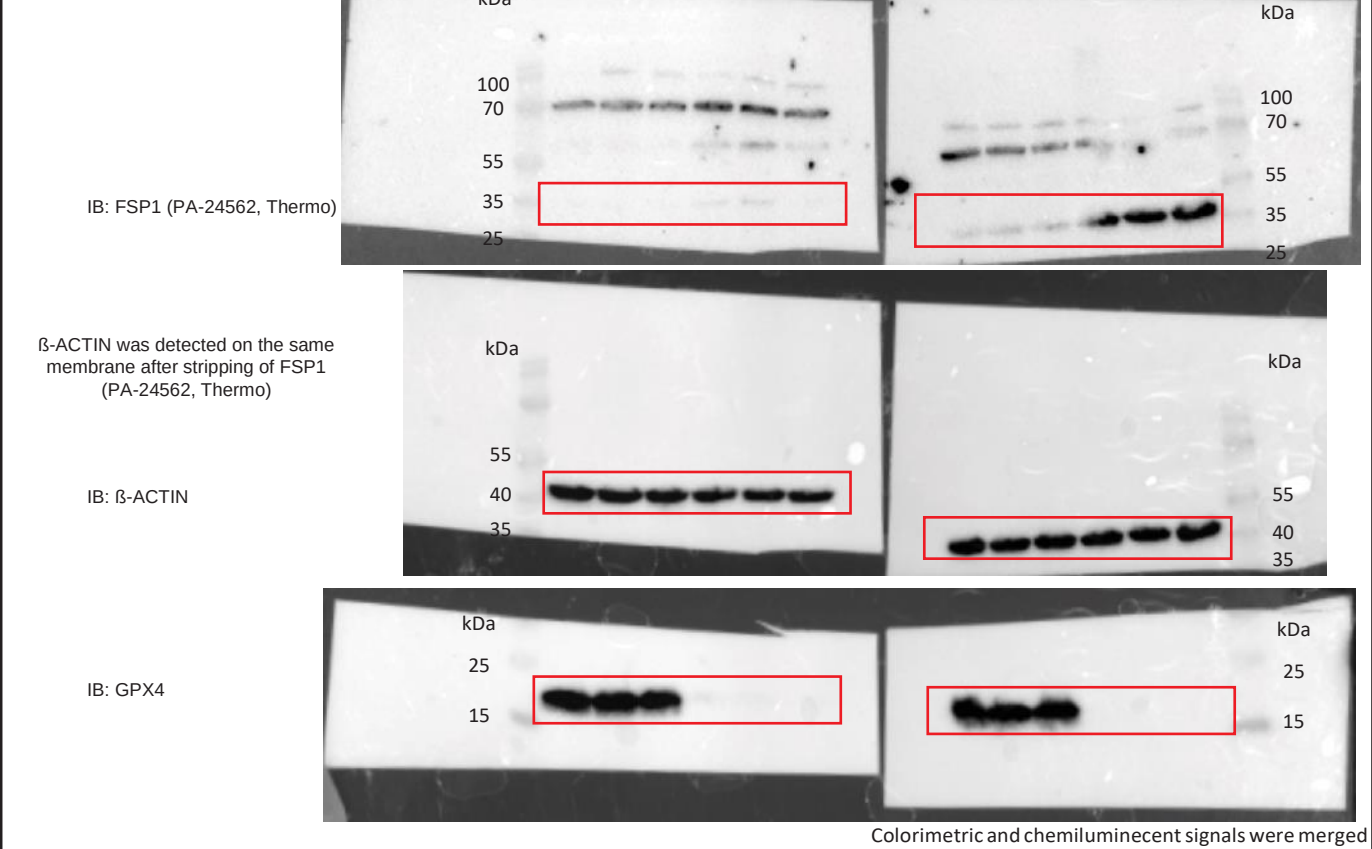
Extended Data Fig. 1c



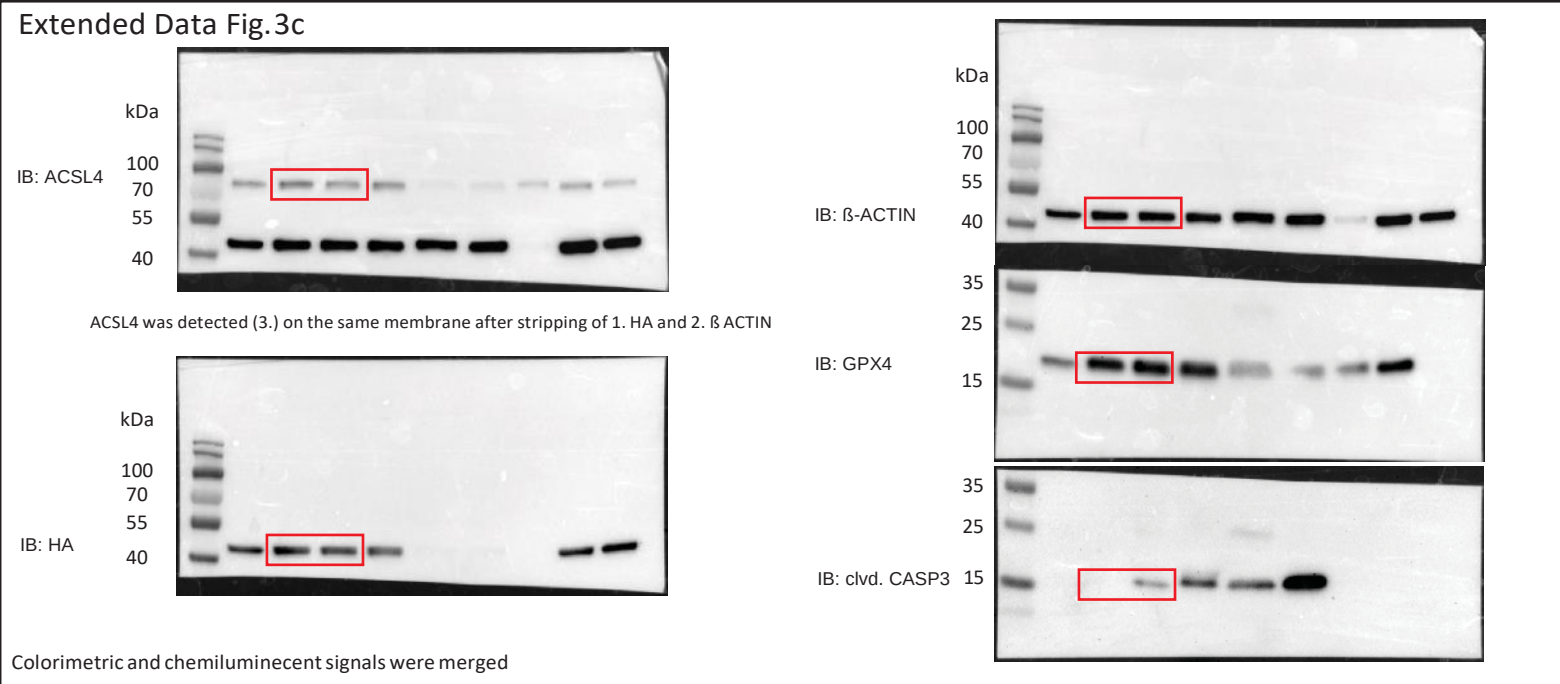
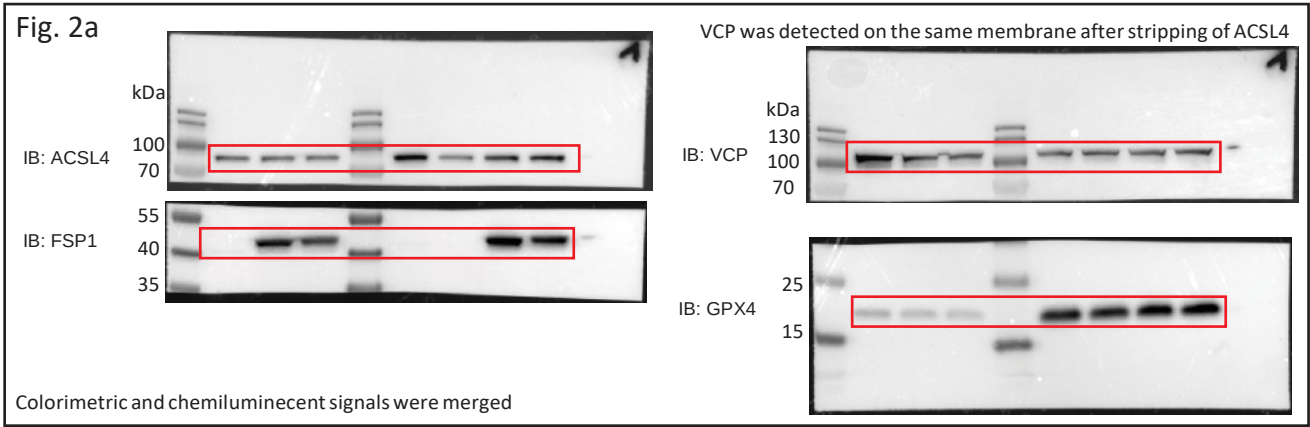
Extended Data Fig. 1d



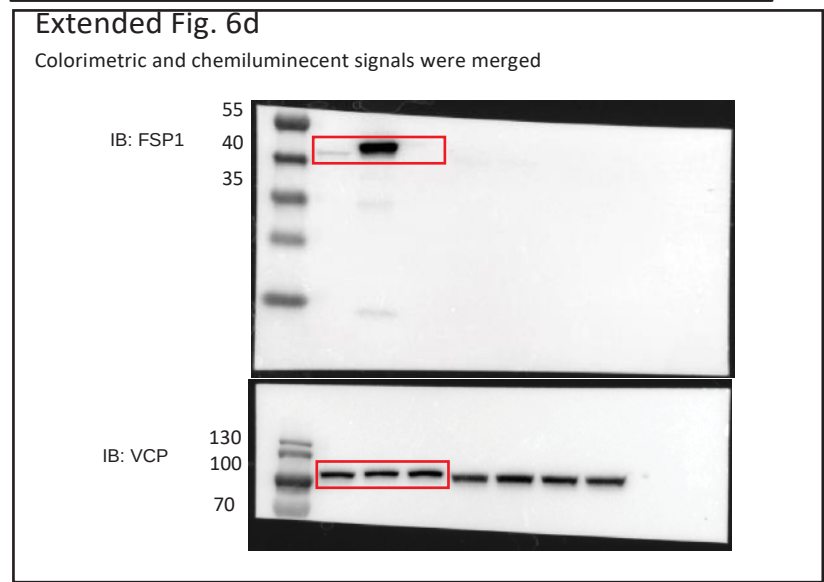
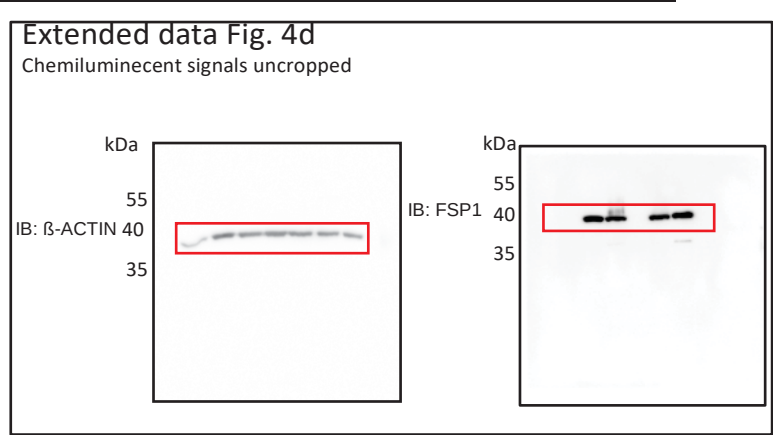
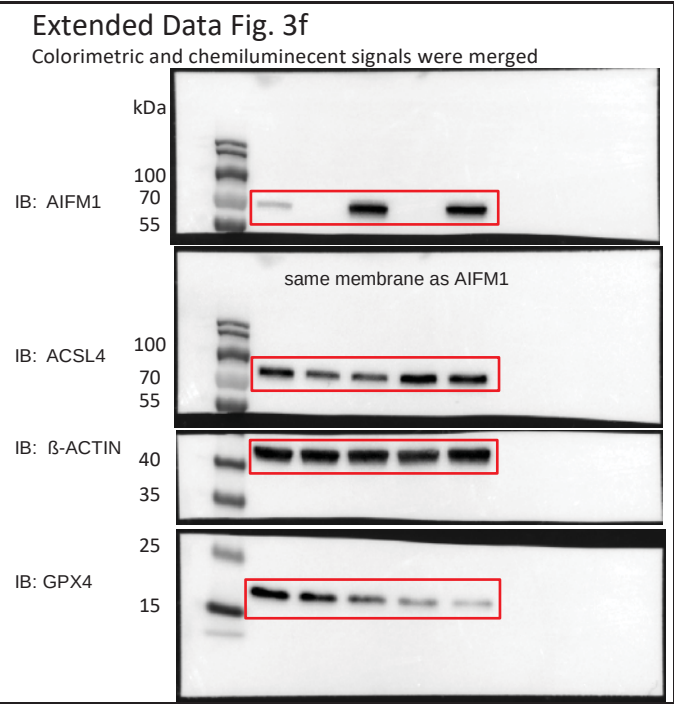
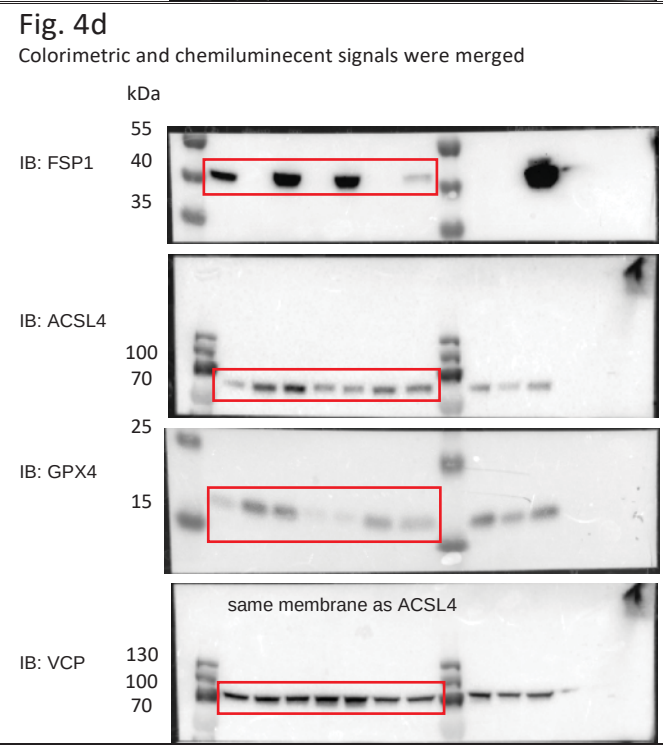
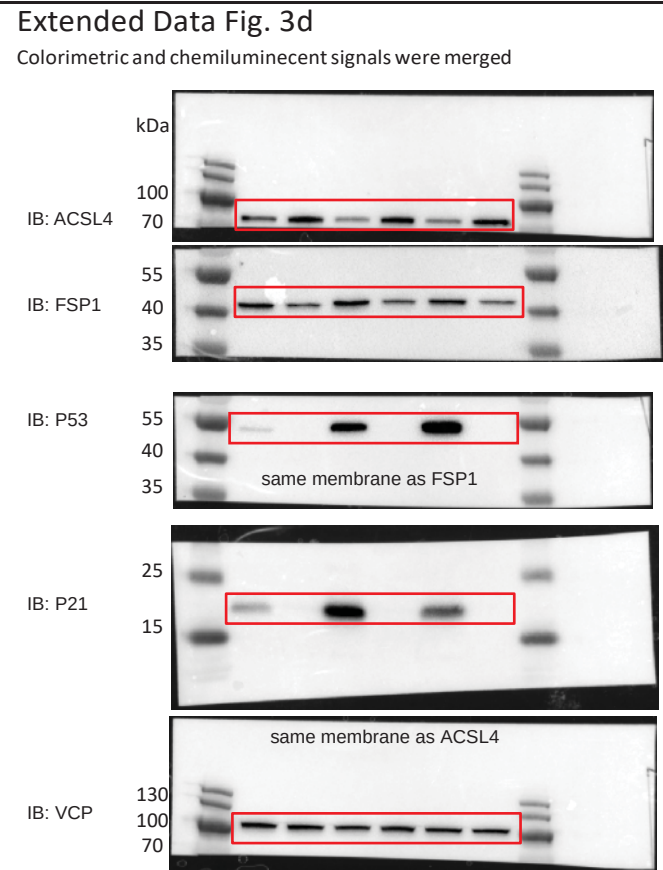
Extended Data Fig. 1k



Red boxes always indicate the cropped areas that were used in the figure (please note that the chemiluminescent signal alone was used for crop outs).

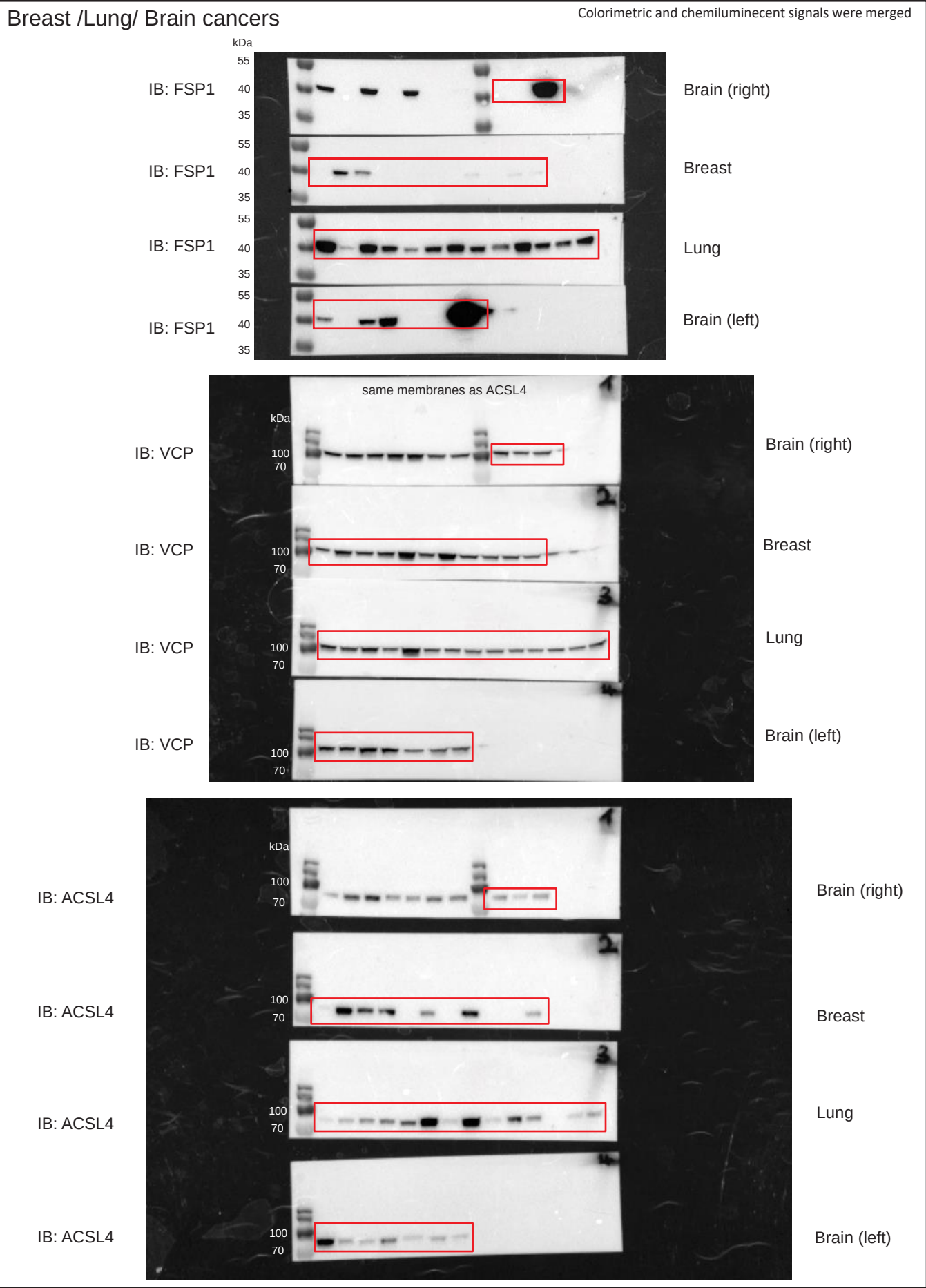


Red boxes always indicate the cropped areas that were used in the figure (please note that the chemiluminescent signal alone was used for crop outs).



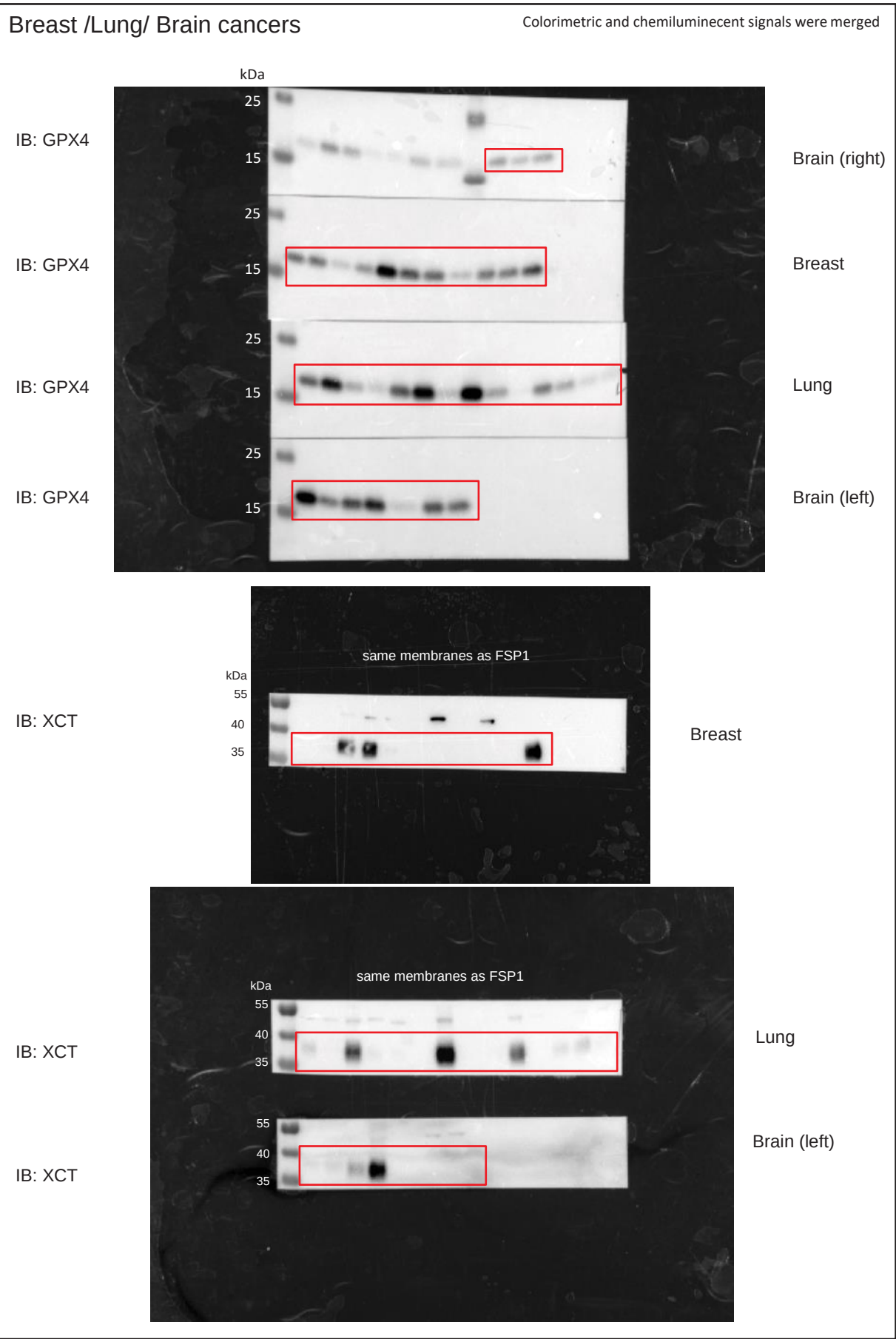
Extended Data Fig. 7

Red boxes always indicate the cropped areas that were used in the figure (please note that the chemiluminescent signal alone was used for crop outs).



Extended Data Fig. 7

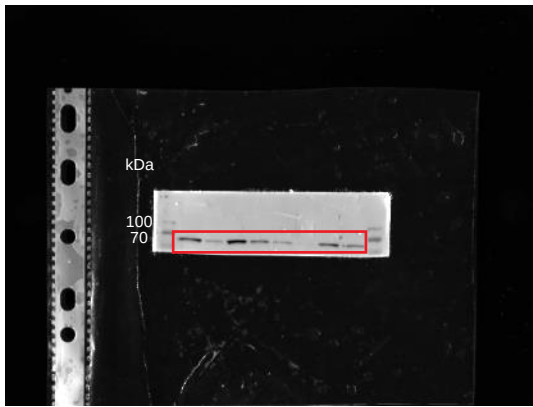
Red boxes always indicate the cropped areas that were used in the figure (please note that the chemiluminescent signal alone was used for crop outs).



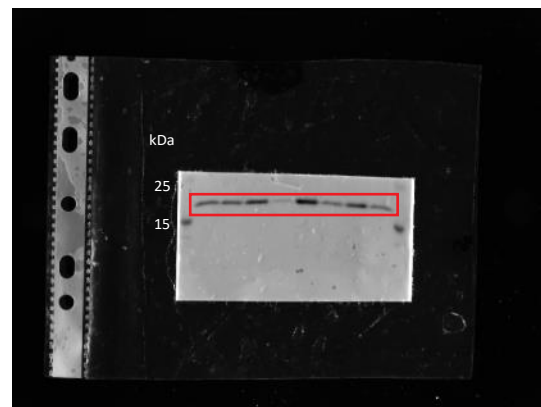
Colon cancer

Colorimetric and chemiluminescent signals were merged

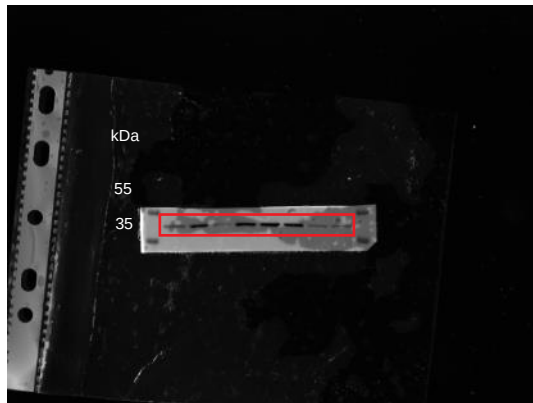
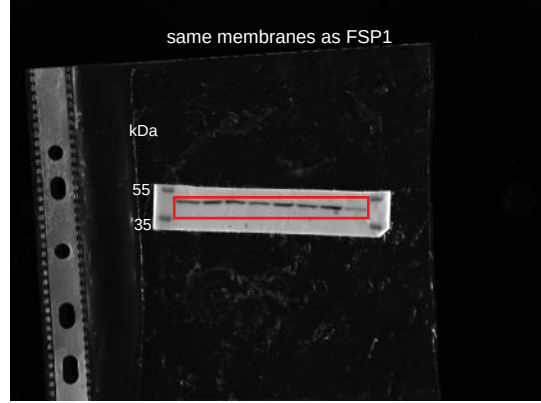
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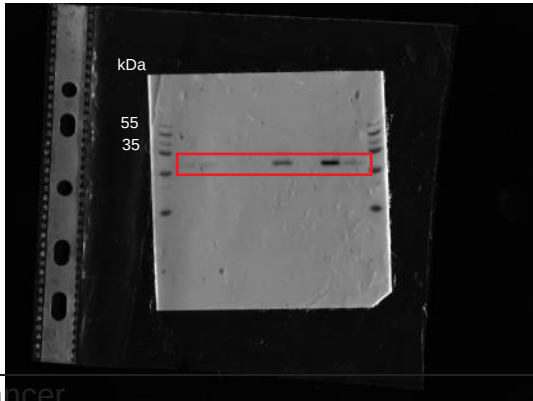
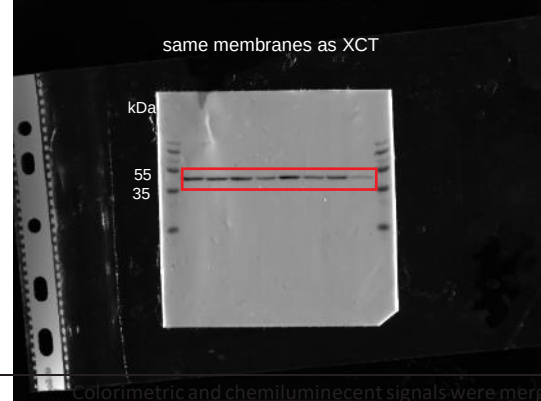
IB: GPX4



IB: FSP1

IB: β -ACTIN

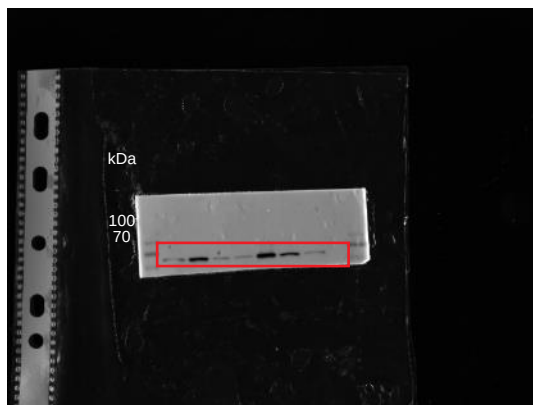
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IB: β -ACTIN

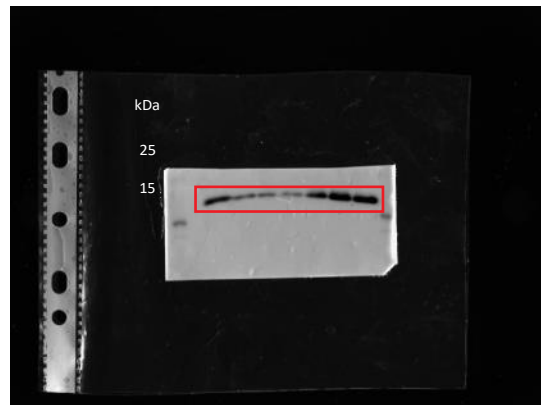
Liver cancer

Colorimetric and chemiluminescent signals were merged

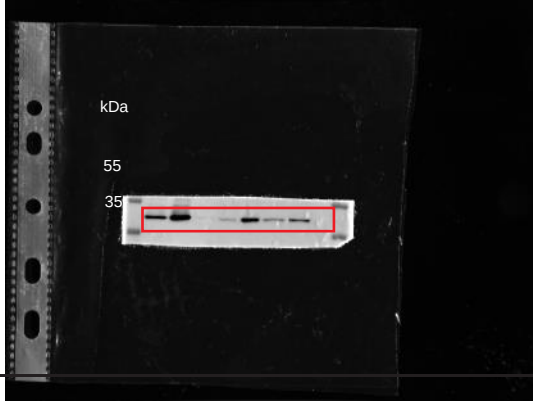
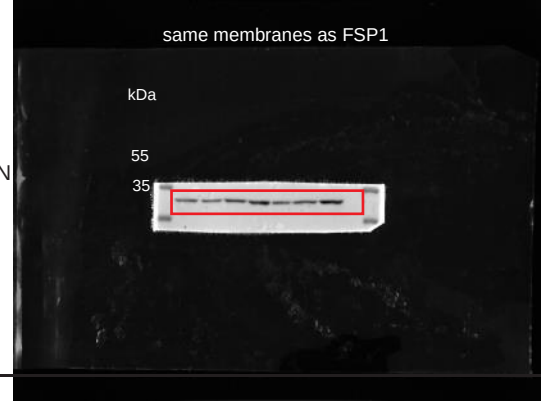
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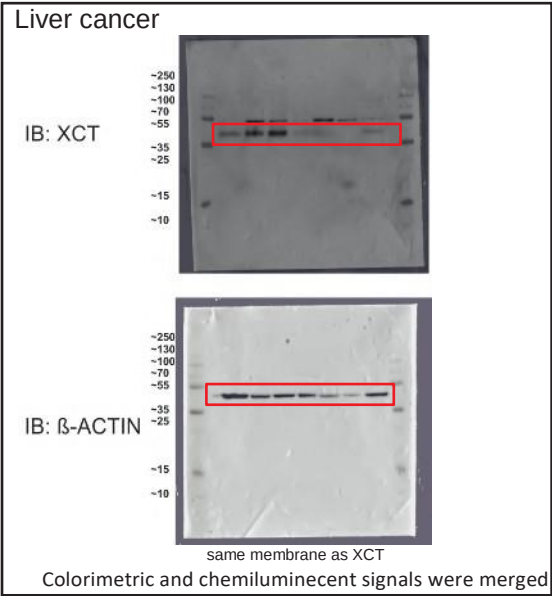
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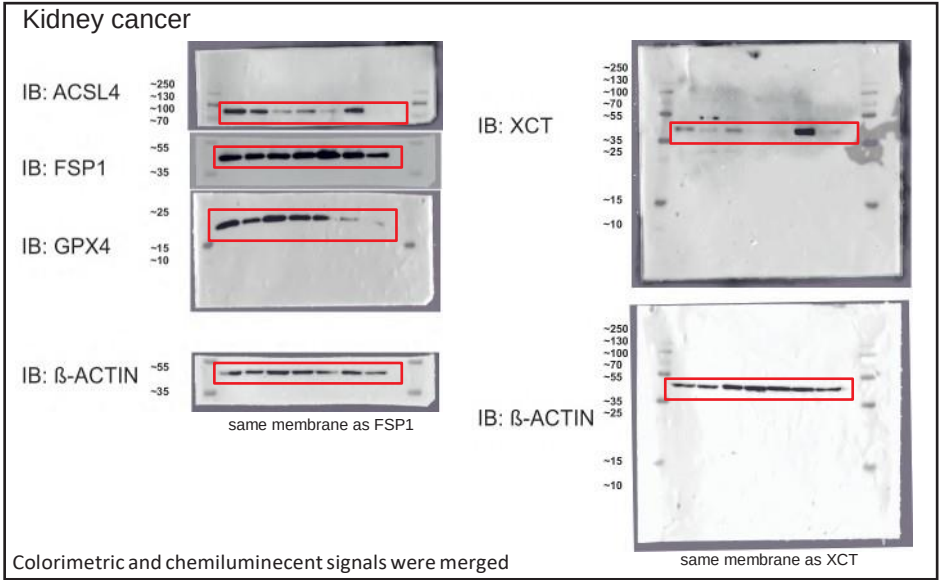
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IB: β -ACTIN

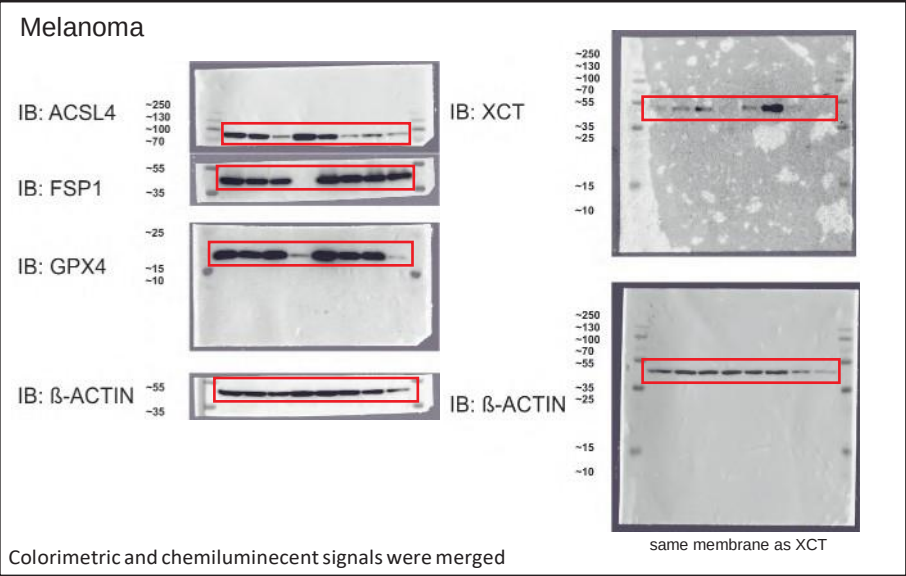
Extended Data Fig. 7



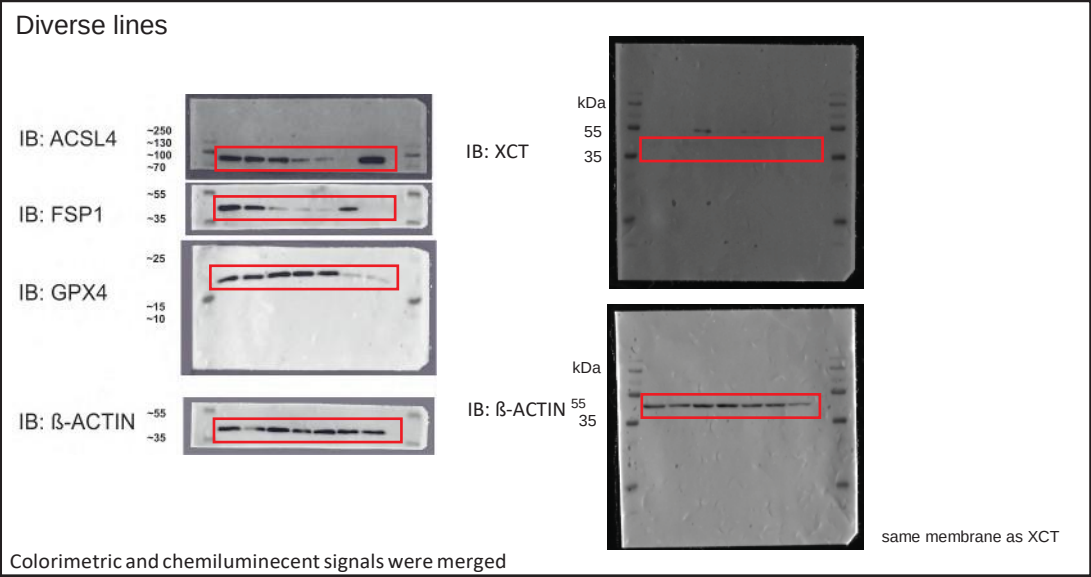
Extended Data Fig. 7



Extended Data Fig. 7

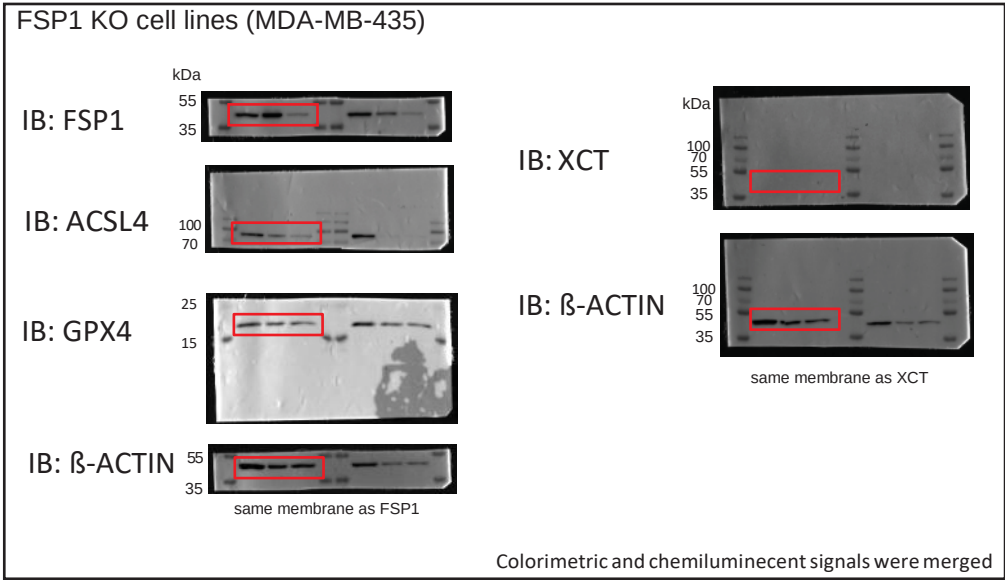


Extended Data Fig. 7

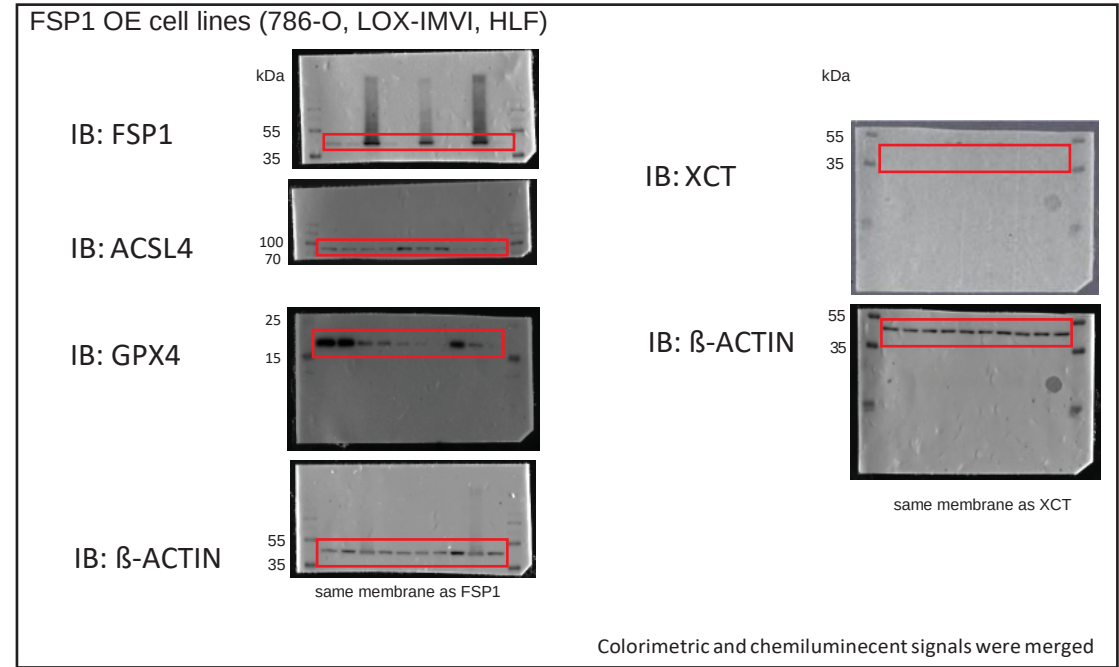
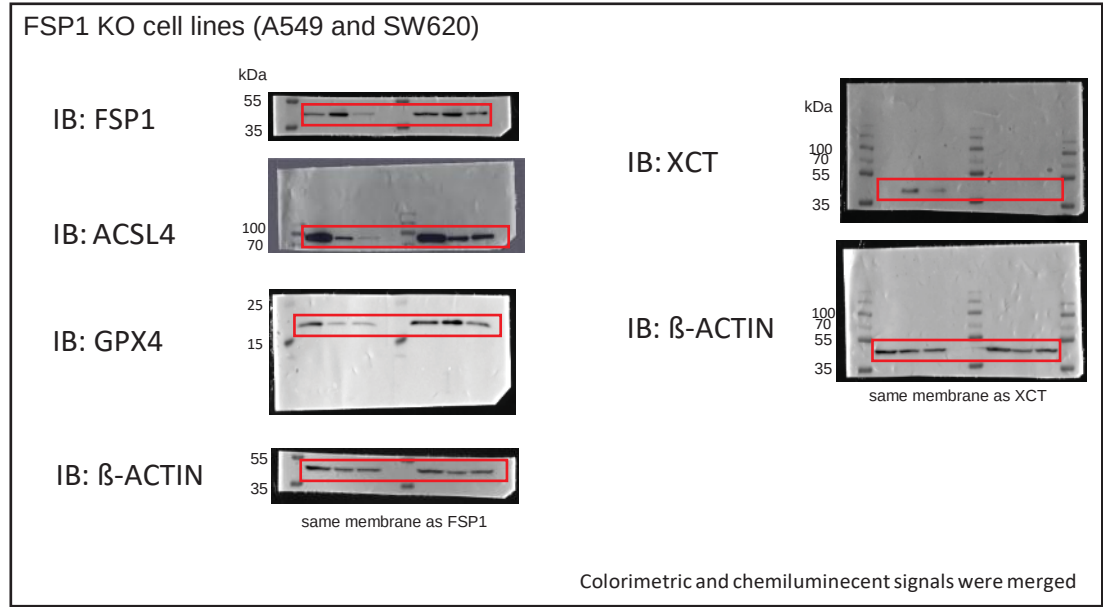


Extended Data Fig. 7

Red boxes always indicate the cropped areas that were used in the figure (please note that the chemiluminescent signal alone was used for crop outs).



Extended Data Fig. 7



ONLINE METHODS

Chemicals. Lipid standards were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). HPLC grade solvents were from Thermo Fisher Scientific (Hemel Hempstead, Hertfordshire, UK). All other chemicals and reagents were from Sigma-Aldrich, unless stated otherwise.

Cell Lines. 4-hydroxytamoxifen (TAM)-inducible *Gpx4*^{-/-} murine immortalized fibroblasts (Pfa1) were reported previously¹. These cells harbour two *loxP*-flanked *Gpx4* alleles and stably express TAM inducible Cre recombinase allowing the genetic deletion of *Gpx4* at will. Human fibrosarcoma (HT1080) cells, human hepatocellular carcinoma (HepG2, HLE, HLF, HUH-7, SNV182, SNV387 (HLE, HLF, HUH-7, SNV182 and SNV387), human lung cancer cells (NCI-H1299, NCI-H1437, NCI-1882, NCI-H1563, NCI-H1573, NCI-H1975, NCI-H2126, NCI-H520, NCI-H661, A549, PC9, EKVX, NCI-H460), human glioblastoma cells (U-87, U-251, U138, U-373MG, kind gift from Dr. Reinhard Zeidler, Helmholtz Zentrum München), human neuroblastoma cells (IMR5/75 (kind gift from Dr. Frank Westermann, DKFZ Heidelberg), human breast cancer cells (MDA-MB-157, MDA-MB-231, MDA-MB-436, MDA-MB-453, MDA-MB-468, BT474, BT-549, MCF7, T-47D, HS-578T), human colon cancer cells (SW620, SW480, RKO, LS174T, LoVo, HT29, HCT116, DLD-1), human kidney cancer cells (769-P, 786-O, LOX-IMVI, HK2, RCC4, UMRC2, UMRC3), human melanoma and uveal melanoma (M19Mel, UACC-62, LOX-IMVI, MDA-MB-435S, A375, SK-Mel-2, SK-Mel-5, Mel270, OMN1-GNA11, OMN1-3 and 92-1) were purchased from ATCC unless stated otherwise and cultured according to ATCC guidelines. MDA-MB-435 and U-373 MG are mentioned to be commonly misidentified cell lines. In the case of MDA-MB-435 (SAMN03151832) we confirmed it as MDA-MB-435S, which is the proven melanoma line. U-373 MG (in-house stock) itself was not reported to be misidentified but is rather seen in some cases to be the main contaminant of other cell lines. All cells were regularly tested for mycoplasma contamination.

Assessment of lipid peroxidation using C11-BODIPY (581/591). 150,000 cells per well were seeded on 6-well dishes (Nunc) one day prior to the experiment. On the next day, cells were treated with the indicated concentration of (1*S*,3*R*)-RSL3 to induce ferroptosis. Cells were

incubated with C11-BODIPY (581/591) (1 μ M) for 30 min at 37°C before they were harvested by trypsinisation. Subsequently, cells were resuspended in 500 μ L of fresh PBS (DPBS, Gibco) strained through a 35 μ M cell strainer (Falcon tube with cell strainer CAP) and analyzed using the 488-nm laser of flow cytometer (FACS Canto II, BD Biosciences) for excitation. Data was collected from the FL1 detector (C11-BODIPY) with a 502LP and 530/30 BP filter. At least 10,000 events were analyzed per sample. Data was analyzed using FlowJo Software.

Cell viability assays. Aquabluer method: Cells were seeded on 96-well plates (2,500 cells per well) and treated with the compounds (RSL3[1S3R], erastin, L-buthionine sulfoximine [BSO], dimethyl sulfoxide [DMSO], tert-butylhydroperoxide [tBOOH], H₂O₂, phenylarsine oxide [PAO], indomethacin, auranofin, ivermectin, sunitinib, obatoclox, mitoxantrone, irinotecan, vinblastine, ABT-263, nocodazole, etoposide, paclitaxel, TNF α , staurosporine, cisplatin, Imp-1088, uridine, CoQ10, decyl-ubiquinone, iFSP-1 and/or liproxstatin-1) after plating. Cell viability was assessed 24 h (unless stated otherwise) after treatment using AquaBluer as an indicator of viable cells according to the manufacturer's recommendations (MultiTarget Pharmaceuticals, LLC). LDH release method: Pfa1 cells and derived cell clones were seeded on 6-well plates (30,000 cells per well) and treated with 1 μ M TAM to induce the knockout of *Gpx4*. Cell death was quantified by measuring released lactate dehydrogenase (LDH) activity after different time points using the Cytotoxicity Detection Kit (LDH) according to the manufacturer's instructions (Roche Diagnostics, Germany). Determination of cell numbers: 10,000 Pfa1 p442-Mock or Pfa1 p442-FSP1-HA cells were seeded on 24-wells in triplicates. After addition of 1 μ M TAM the cell number was determined every 24h up to 96 h using a Neubauer improved chamber. Cells without TAM treatment were used as a control.

Screening of novel FSP1 inhibitors. Pfa1 FSP1 and Pfa1 GPX4KO FSP1 cells were seeded on separate 384-well plates (500 cells per well) and screened with a library of small molecule inhibitor compounds as reported earlier². Cell viability of the different cell lines was assessed 48 h after treatment using the AquaBluer method. Compounds harbouring selective lethality in Pfa1 GPX4 KO FSP1 overexpressing cells were then validated in cell- and target-based assays.

Determination of intracellular GSH levels. Cells were seeded on a 6-well plate (1×10^5 cells per well) and cultured for 24 h. Cells were trypsinized, washed three times with ice-cold PBS and extracted with 5% trichloroacetic acid. Ether was used to subsequently remove the trichloroacetic acid. Total glutathione content in the aqueous layer was measured using an enzymatic method, based on the catalytic action of glutathione in the reduction of 5,50-dithiobis (2-nitrobenzoic acid) by the glutathione reductase system³. To this end, 500 μ L of the sample were mixed with 1.2 mL assay buffer (0.2 M K_2HPO_4 , 0.2 M KH_2PO_4 , 10 mM EDTA, (pH 7.2)) containing 4 mg/mL DNTP, 1 IU glutathione reductase (GR) and 3.62 mg/mL NADPH/ H^+ . GSH content was defined by the intensity of colour change from DTNB to TNB within a period of 5 min at 412 nm absorbance. To calculate GSH content per mg protein, cells were treated after TCA extraction with 0.5 M NaOH overnight for cell membrane destruction and protein release. Protein quantification was performed using the Pierce BCA Protein Assay Kit (Thermo Scientific) as described in manufacturer's protocol.

GPX4-specific activity assay. GPX4 activity was assessed using a GPX4-specific substrate in an enzyme-coupled test in crude whole cell lysates as previously described⁴. Cells were seeded on 15 cm plates grown to ~80% confluency before they were harvested by trypsinization. Cell pellets were washed twice with PBS and stored at $-80^\circ C$ to preserve enzymatic activity. Cell pellets were resuspended in 100 μ L lysis buffer (100 mM KH_2PO_4/K_2HPO_4 (pH 7.4), 150 mM KCl, 0.05% CHAPS, 2 mM β -mercaptoethanol, 1% protease inhibitors cocktail). After 15 min incubation in lysis buffer on ice, homogenate was centrifuged ($4^\circ C$, 10 min, $18,400 \times g$) and the supernatant was transferred to a fresh tube. For activity measurement, 50 μ L of protein supernatant from cells were added to a reaction mix consisting of 1 mL assay buffer (100 mM Tris (pH 7.8), 5 mM EDTA, 0.1% Triton X-100 (peroxide free)), 3 mM GSH NADPH/ H^+ , 3 mM GSH, 0.6 U/mL glutathione reductase and 20-30 μ M phosphatidylcholine hydroperoxide (PCOOH). GPX4 activity was determined by measuring the spectrometric decrease of 200 μ M NADPH/ H^+ at 340 nm which is consumed by GR during reduction of GSSG formed by GPX4. For normalization, protein concentration of the samples was measured using the Pierce 660 nm Protein Assay Reagent

(Thermo Fisher) according to the manufacturer's instructions. Measurements were performed in a SpectraMax microplate reader (Molecular Device GmbH).

Preparation of lentiviral cDNA library. For the full-length cDNA libraries, MCF7 cells were washed with PBS, lysed with RNA later (Ambion) and stored at -80°C. Total RNA was extracted following the RNeasy Mini Kit Extraction (Qiagen) standard protocol. Subsequently, mRNA was purified using the Dynabeads mRNA Purification kit (Invitrogen). A total of 100 ng of mRNA was used for cDNA library construction using In-Fusion SMARTer Directional cDNA Library Construction Kit (Clontech). The SMARTer V Oligo sequence linked to the 3' In-Fusion SMARTer CDS Primer sequence by a *SpeI* restriction site (AAGCAGTGGTATCAACTAGTCTCATCGTACCCG) was cloned in the p442 plasmid. After *SpeI* digestion, this plasmid was used to clone SMARTer ds cDNA.

Global cDNA overexpression screen for new anti-ferroptotic genes. For virus production, HEK 293T cells were plated on 15 cm dishes and transfected with 6 µg of DNA using X-tremeGENE™ HP DNA Transfection Reagent (Roche). Supernatants were collected 48 hours after transfection. Pfa1 cells were seeded on 10 x 15 cm plates (200,000 cells/plate) and 0.5 mL virus supernatant was added. A control plate without virus was included. After 48 hours 1 µM TAM was added in order to knockout *Gpx4*. After one week, surviving single cell clones were transferred to the 96 well format. Surviving colonies were split at low confluence and treated with 100 nM of RSL3 to further select for ferroptosis-resistant cell clones. To confirm the deletion of the *Gpx4* gene in the surviving colonies, two *Gpx4*-deletion specific PCRs were performed on gDNA of resistant clones (data not shown). The first PCR amplifying the region between exons 5 and 7 (CTCCCAGTGCCATCAAATGG; GGGGCACACACTTGTAGGG; 326 bp). The second PCR amplifying a region between introns 5 and 6 (AGGTAAGTGGCATACTAGTGTGG; TAGGGAGATAACTCGTTCAGACG; 237bp).

Identification of human cDNAs in ferroptosis-resistant single cell clones. For the identification of the respective cDNAs, genomic DNA was isolated from surviving single cell clones by phenol:chloroform extraction. PCR amplification of the p442-cDNA inserts was carried out using

p442 backbone specific primers (p442_seq_fwd: CCGAGCTCTATAAAAGAGCTCACAACC; p442_seq_rev: ATCGGGCTGCAGGAATTGCT). PCR products were purified and sequenced. Samples that produced poor quality sequence were reanalysed using a nested PCR approach. Therefore, PCR products from an initial PCR reaction (p442_Lib-scr_Fw: CCTGCTTCTCGCTTCTGTTCG; p442_Lib-scr_Rv: ACACCGGCCTTATTCCAAGC) were amplified using the p442 backbone specific primers (p442_seq_fwd: CCGAGCTCTATAAAAGAGCTCACAACC; p442_seq_rev: ATCGGGCTGCAGGAATTGCT). After the identification of *hGPX4* and *hFSP1* cDNAs from untargeted PCR reactions, all ferroptosis resistant cell clones with positive *Gpx4* deletion were reanalysed using gene-specific primers for *hGPX4* (TAACCTGGACAAGTACCGGGG, ACGCTGGATTTTCGGGTCTGC; 549 or 571 bp depending on the GPX4 splice variant) and *hFSP1* (ACAACTTCCGGCAGGGGC, GCTGTTGATCTTGATGCCGG; 524 bp).

Analysis of ubiquinone levels by LC-MS. For CoQ10 quantifications, 10^6 cells were washed twice with PBS and pellets re-suspended in 100 μ L of MilliQ water. 500 μ L of hexane and 400 μ L of internal standard (10 μ M of ubiquinone Q9 in methanol) were added to the cell suspension. Cells were lysed by sonication, vortexed and centrifuged at 15,000 rpm for 2 min. The upper phase was transferred to a new tube, and the lower phase re-extracted with 0.5 mL of hexane. The combined extracts were dried at 35°C under a stream of N_2 . The residue was dissolved in 150 μ L of hexane, centrifuged and the hexane phase was transferred to a Strata SI-1 Silica column (55 μ m, 70A, 100 mg/1 mL, Phenomenex). After completing sample absorption, the column was washed once with 750 μ L hexane and once with 750 μ L hexane/acetic acid ethyl ester (18/1, v/v). The column was blown out and ubiquinones were eluted with 750 μ L hexane/acetic acid ethyl ester (9/1, v/v). The fraction was collected and evaporated to dryness at 45°C under a stream of nitrogen. For LC/MS analysis the dry eluates were re-dissolved in 50 μ L isopropanol and centrifuged 15,000 rpm for 2 min. 20 μ L of the dissolved supernatants were transferred into sampler-vials and 5 μ L were applied to an C8 column assembly @40°C (Acclaim C8 pre-column, 5 μ m particles, 10 $\times$ 2 mm; Acclaim RSLC C8 column (2.2 μ m particles, 50 $\times$ 2.1 mm) and isocratically eluted for 30 min. Ammonium acetate (5 mM) in acetonitrile/H₂O (95/5, v/v) was used as mobile phase with a flow rate of 350 μ L/min. The eluent was directed to the ESI source of a Q Exactive

mass spectrometer (QE-MS) from 10 min to 29 min after sample injection. LC/MS analysis was performed on a Thermo Scientific Dionex Ultimate 3000 UHPLC system hyphenated with a QE-MS equipped with a HESI probe (Thermo Scientific, Bremen, Germany). The data was acquired using tSIM in positive mode with: range - m/z 812.66 and m/z 880.72; resolution - 70,000; microscans - 1; AGC-Target – $1e6$; maximum Injection Time - 400 ms; and the follow HESI Source Parameters: heath gas (30); auxiliary gas (10); sweep gas (3); spray voltage (2.5 kV); Capillary temperature (320°C); S-lens RF level (55.0) and Aux Gas Heater temperature (120°C). Peaks corresponding to the calculated ion masses (± 7 mMU) were integrated using TraceFinder software (Thermo Scientific, Bremen, Germany).

Enhanced resolution confocal microscopy. Briefly, 20,000 HT1080 cells expressing FSP1-GFP or FSP1[G2A]-GFP were seeded into a 8 chamber cover glass slide and allowed to attach overnight. ER and Golgi were stained with ER tracker red and BODIPY™ FL C5-Ceramide, respectively according to manufacturer protocols. Subsequently, cells were washed and live cell images were acquired using FluoroBrite-DMEM imaging media. For enhanced resolution confocal microscopy using genetically encoded ER (mCherry-Sec61 beta) and Golgi (mApple-Golgi-7) markers 20,000 HT1080 cells expressing FSP1-GFP or FSP1[G2A]-GFP (infected with lentiviral particles containing p442-mCherry-Sec61 beta or p442-mApple-Golgi-7) were seeded into a 8 chamber cover glass slide and allowed to attach overnight. Images were acquired on an inverse Nikon TiE microscope, equipped with an image-scanning based RCM-unit (confocal.nl). As excitation source a multiline laser system Skyra (Cobolt) and as detection a sCMOS camera Zyla 4.2P (Andor) has been used. All measurements have been performed using a 100x/1.49 APO TIRF oil objective. Images were corrected for camera offset and were post-processed with a median filter (kernel: 3x3 px). Laser power was set in a range between 30-100 μ W.

Preparation of lentiviral particles for overexpression of genes. HEK 293T cells were used to produce replication-incompetent lentiviral particles pseudotyped with the ecotropic envelope protein of the murine leukaemia virus (MLV) or the pantropic envelope protein VSV-G. A third generation lentiviral packaging system consisting of a transfer plasmid (lentiCas9-Blast (Addgene

#52962), p442-Mock, p442-GPX4-FSH, p442-FSP1-HA, p442-FSP1[G2A]-HA, p442-FSP1-GFP, p442-FSP1[G2A]-GFP, p442-GFP, p442-AIFM1, p442-mCherry-Sec61 (cloned from Addgene plasmid #49155) beta and p442-mApple-Golgi-7 (cloned from Addgene plasmid #54907)), pEcoEnv-IRES-puro (ecotropic particles) or pMD2.G (pantropic particles), pMDLg_pRRE and pRSV_Rev was co-lipofected into HEK 293T cells. Viral particle containing cell culture supernatants were harvested 48 and 72 h after transfection and used to transduce the cell line of interest.

CRISPR/Cas9-mediated gene knockout. Single sgRNA guides (Table 1) were designed to target critical exons of the genes to be inactivated. Guides were cloned into the *BbsI*-digested pKLV-U6gRNA(*BbsI*)-PGKpuro2aBFP vector (Addgene #50946) or the *BsmBI*-digested lentiCRISPR v2 blast and lentiCRISPR v2 puro vectors (Addgene #98293, #98290).

Table 1. Sequence of sgRNA guides used to generate the different knockout cell lines

Parental cell line	Cell line name	Lentiviral backbone	Target gene	sgRNA
HT1080_Cas9	HT1080 FSP1 KO	pKLV-U6gRNA(<i>BbsI</i>)-PGKpuro2aBFP	<i>FSP1</i>	GGAGATGGGGTCCCAGGTCT, GGTCTTTTGGCGAACCTG
HT1080_Cas9	HT1080 Gpx4 KO	pKLV-U6gRNA(<i>BbsI</i>)-PGKpuro2aBFP	<i>GPX4</i>	GGCCTTGCAGTGC GCGTCCC, GATACGCTGAGTGTGGTTTG
HT1080_Cas9	HT1080 p53 KO	pKLV-U6gRNA(<i>BbsI</i>)-PGKpuro2aBFP	<i>P53</i>	CCTGGGGGCAGCTCGTGGTG, CAGGGGAGTACTGTAGGAAG
HT1080_Cas9	HT1080 Coq2 KO	pKLV-U6gRNA(<i>BbsI</i>)-PGKpuro2aBFP	<i>COQ2</i>	CTCCTTCGCCCTGGCGCGTG, TAGTCTGCCAGGCTGGGCGG
MDA436_Cas9	MDA436 FSP1 KO	pKLV-U6gRNA(<i>BbsI</i>)-PGKpuro2aBFP	<i>FSP1</i>	GGAGATGGGGTCCCAGGTCT, GGTCTTTTGGCGAACCTG

Pfa1_Cas9	Pfa1 AIFM1 KO	pKLV-U6gRNA(BbsI)-PGKpuro2aBFP	<i>Aifm1</i>	GATCCCGAGCCCGGATGGATC, GGTCGCATGTATGGCAGTTC
O-786	O-786 GPX4 KO	lentiCRISPR v2 blast, lentiCRISPR v2 puro	<i>GPX4</i>	GCGTGTGCATCGTCACCAACG, GCACGCCCGATACGCTGAGTG
U-373	U-373 FSP1 KO	lentiCRISPR v2 blast, lentiCRISPR v2 puro	<i>FSP1</i>	GCGTGTGCATCGTCACCAACG, GCACGCCCGATACGCTGAGTG
NCI-H1437	NCI-H1437 FSP1 KO	lentiCRISPR v2 blast, lentiCRISPR v2 puro	<i>FSP1</i>	GCGTGTGCATCGTCACCAACG, GCACGCCCGATACGCTGAGTG
MDA-MB-435	MDA-MB-435 FSP1 KO	lentiCRISPR v2 blast, lentiCRISPR v2 puro	<i>FSP1</i>	GCGTGTGCATCGTCACCAACG, GCACGCCCGATACGCTGAGTG
A549	A549 FSP1 KO	lentiCRISPR v2 blast, lentiCRISPR v2 puro	<i>FSP1</i>	GCGTGTGCATCGTCACCAACG, GCACGCCCGATACGCTGAGTG
SW620	SW620 FSP1 KO	lentiCRISPR v2 blast, lentiCRISPR v2 puro	<i>FSP1</i>	GCGTGTGCATCGTCACCAACG, GCACGCCCGATACGCTGAGTG

Transient expression of the CRISPR/Cas9 system. HT1080 cells were infected with VSV-G pseudotyped lentiviral particles containing the transfer plasmid lentiCas9-Blast. 200,000 HT1080_Cas9 cells were seeded on a 6-well plate and cultured for 24 h. On the next day, cells were co-transfected with the desired sgRNA expressing plasmids (pKLV-U6gRNA-PGKpuro2aBFP) using the X-tremeGENE HP agent according to the manufacturer's recommendations (Roche Diagnostics, Mannheim, Germany). Two days after transfection, cells were plated on 15 cm dishes to allow colony formation. Single cell clones were picked and genotypes analysed by PCR using gene locus specific primers. Knockout clones were identified by sequencing out-of-frame mutations or, if possible, Western blot analysis was used to confirm successful gene deletion.

Stable expression of the CRISPR/Cas9 System. Pfa1_Cas9 cells were generated by transducing Pfa1 cells with lentiCas9-Blast containing ecotropic lentiviral particles. MDA436_Cas9 cells were

generated by transducing MDA-MB-436 cells with LentiCas9-Blast containing pantropic lentiviral particles. Pfa1_Cas9 and MDA436_Cas9 cells were used to generate Pfa1 AIFM1 KO and MDA436 FSP1 KO cells by lentiviral infection with particles containing the desired sgRNA expressing plasmids (pKLV-U6gRNA(sgRNA)-PGKpuro2ABFP). Two days after infection, MDA436_Cas9 cells were selected with puromycin, while Pfa1_Cas9 cells were sorted on a BD Bioscience FACSARIA II using Blue Fluorescent Protein (BFP) as a marker. Single cell clones analysis was performed as mentioned for the transient CRISPR/Cas9 method. Human cancer cell lines (O-786, U-373MG, NCI-H1437, MDA-MB-435, A549, SW620) were transduced with VSV-G coated lentiviral particles containing lentiCRISPR v2 blast/puro targeting *GPX4* or *FSP1*. 48 h after infection cells were selected with both blasticidin and puromycin to generate a pool of *GPX4* and *FSP1* KO cells. Cell pools were analysed by Western blot to confirm successful gene knockout.

Determination of apoptosis using AnnexinV/PI staining. 200,000 Pfa1 p442-Mock and Pfa1 p442-FSP1 cells were seeded on each well of a 6-well plate. On the next day, cells were treated with TNF α (10 ng/mL) for 4 h and stained according to the manufacturers protocol (eBioscience™ Annexin V Apoptosis Detection Kit FITC). Subsequently, they were analysed on a BD FACSCANTO II instrument using the AlexaFluor 488 filter for the Fit-C labeled AnnexinV antibody and the PE-Cy5 filter for PI staining.

Production of purified recombinant human FSP1. The *FSP1* gene was cloned in a small ubiquitin-like modifier (SUMO) fusion expression plasmid (pETSUMO) using *NcoI/NotI* restriction sites. For protein expression the autoinduction medium ZYM5052 was used as previously described ⁵. The cultures were incubated in a shaker at 37 °C and the optical density (OD) was measured in regular intervals. Upon reaching an OD of approximately 1, the temperature was lowered and the culture incubated overnight at 18 °C for optimal yield. Bacterial cell cultures were spun down at 4000 rpm, 4°C, for 30 min. The supernatant was discarded, and cell pellets were subsequently resuspended in 30 mL lysis buffer (300 mM NaCl, 50 mM TRIS, 20 mM Imidazol, 10 mM β -mercaptoethanol, pH = 8) additionally containing 1 mL AEBSF (1 mg/mL), 1 mL DNase (1 mg/mL), and lysozyme. Lysis was performed with a Bandelin sonicator for 4 cycles with 3 min sonication

at 50 % power and 3 min resting on ice. Lysate was subsequently spun down at 27,000 rpm, 4 °C for 45 min. The supernatant was collected, and the pH adjusted to 8. Afterwards, protein was purified employing affinity chromatography using a Ni-NTA system (Bio-Rad). Column was washed in buffer (300 nM NaCl, 50 mM Tris, 10 mM β -mercaptoethanol, pH = 8). Sumo-tag was cleaved on column before elution by addition of dTUD1 Sumo-protease. Protein was eluted using the same buffer. Size-exclusion chromatography (SEC) was performed using HiLoad 16/60 Superdex 75 at room temperature. The chromatography was performed in TBS (50 mM Tris, 150 mM NaCl, 0.5 mM β -mercaptoethanol, pH=8).

Enrichment of YnMyr labeled proteins. Pfa1 cells stably expressing p442-FSP1-HA, p442-FSP1[G2A]-HA or p442-Mock were seeded in triplicate in 100 mm dishes one day before the start of the experiment. Cells were fed with 40 μ M alkynyl myristic acid analogue (tetradecynoic acid, YnMyr) for 24 h before lysis in 1X PBS containing 1% Triton-X and 0.1% SDS and 1X cOmplete™ EDTA-free protease inhibitor cocktail (Roche). Pharmacological inhibition of *N*-myristoyltransferase (NMT) in WT-FSP1-HA cells was achieved by addition of 1 μ M IMP-1088⁶ one hour before addition of the YnMyr probe. 100 μ g of protein were ligated to azido-TAMRA-Biotin (AzTB) capture reagent by copper(I)-catalysed alkyne-azide cycloaddition (CuAAC) or click-chemistry as described previously⁷. 6 μ L of click reagent mixture (1 μ L of 10 mM capture reagent (AzTB), 2 μ L of 50 mM CuSO₄, 2 μ L of 50 mM tris(2-carboxyethyl)phosphine (TCEP) and 1 μ L of 10 mM Tris(benzyltriazolylmethyl)amine (TBTA)) were added to each sample and the incubated for 1 h at RT with gentle mixing. The click reaction was quenched with 5 mM EDTA and proteins were precipitated by adding 2 sample volumes water, 4 volumes methanol and 1 volume chloroform and centrifuging at 6,000 $\times g$ for 5 min. Precipitated protein was washed once with methanol and the pellet re-suspended in 10 μ L PBS containing 2% SDS and 10 mM DTT. Once dissolved, the samples were diluted 10-fold with PBS to 1 mg/mL and 0.2% SDS. For the affinity enrichment, 30 μ L Dynabeads™ MyOne™ Streptavidin C1 magnetic bead slurry (Invitrogen) were pre-washed twice with PBS containing 0.2% SDS before incubation with the 75 μ g of protein from each samples for 2 h at RT with gentle mixing. The rest was kept as the input sample fraction. After the incubation, the supernatant was collected as the unbound fraction and the beads washed

three times with PBS containing 0.2% SDS. The pull-down fraction was eluted in 20 μ L of 2x protein loading buffer for 15 min at 95 °C. The three fractions were run side by side in a 13.5% SDS-PAGE gel (10 μ g per lane were loaded for input and unbound fractions and the equivalent of 60 μ g (6X concentrated) was loaded for the pull-down sample). In-gel fluorescence was scanned using a Typhoon™ FLA 9500 imager (GE Healthcare Life Sciences). For TAMRA, 532/575 nm excitation/emission wavelengths were used. Gels were then transferred to nitrocellulose membranes using wet transfer for 1 h at 100 V. Quality of the transfer was assessed by Ponceau staining before cropping the membranes at 25 kDa and 37 kDa marker bands. Membranes were blocked in 5% semi skimmed milk in TBST and incubated with primary antibodies (GAPDH, HA tag or ARL1) overnight at 4 °C. Endogenously expressed ADP ribosylation factor like GTPase 1 (ARL1), a known myristoylated protein, was used as positive control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as loading control. Membranes were washed with TBST for 10 min three times and incubated with the corresponding secondary antibody (1:10000 dilution) for 1 h at RT. The unbound secondary antibody was washed away with TBST (10 min, 3 times) and membranes were developed with 200 μ L Luminata™ Crescendo Western HRP substrate (Merck) and imaged using an ImageQuant™ LAS 4000 scanner (GE Healthcare Life Sciences).

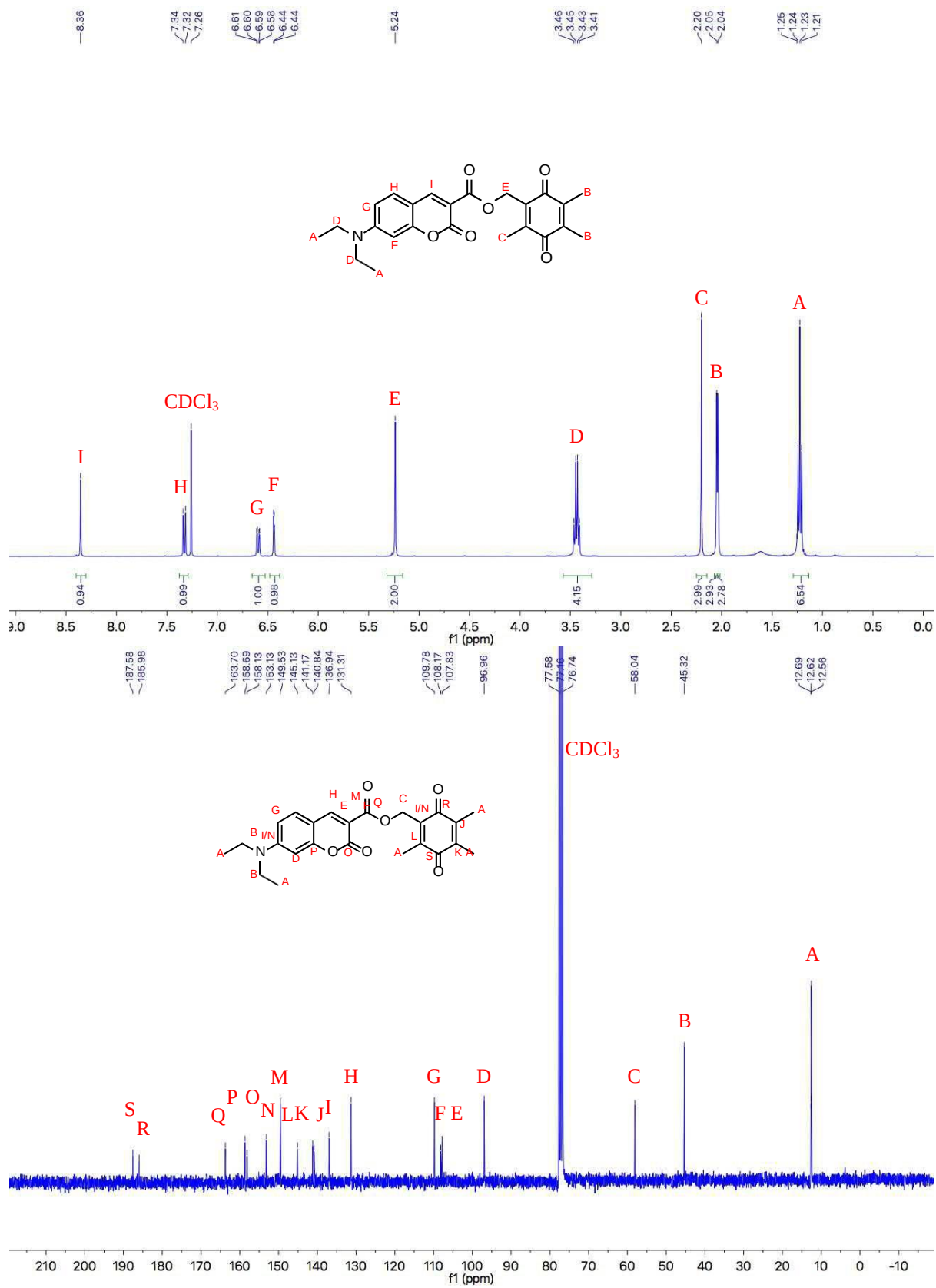
Immunoblotting. Western blot analysis of cell lysates were performed as described previously¹, by using antibodies against GPX4 (1:1000; no. ab125066, Abcam), ACSL4 (1:200; no. sc-271800, Santa Cruz), β -ACTIN (1:10000; no. A5441, Sigma), VCP (1:2000; ab11433, Abcam), FSP1 (1:1000; PA5-24562, Thermo, Extended Data Fig. 1e), FSP1 (1:10; Rat IgG2a monoclonal antibody raised against recombinant human FSP1 protein, clone 6D8-11, developed in-house), XCT (1:10; Rat IgG2a monoclonal antibody raised against a N-terminal peptide of hXCT, clone 3A12-1-1, developed in-house), P53 (1:1000; p53 Antibody #9282, Cell Signaling), P21 (1:1000; p21 Waf1/Cip1 (12D1) Rabbit mAb, Cell Signaling), cleaved caspase 3 (1:1000; cleaved caspase3 antibody (D175), Cell Signaling), HA tag (Rat IgG1 Anti-HA High affinity 3F10, Roche), ARL1 (1:500, no. 16012-1-AP, Proteintech).

Generation of monoclonal antibodies. Rat monoclonal antibodies against human FSP1 were generated by immunization with purified SUMO-his-tagged FSP1 protein and antibodies against human XCT1 were generated using ovalbumin-coupled N-terminal peptide GRPLSLGNKEPPGQEKVQLK (aa 22-41). Animals were injected subcutaneously and intraperitoneally with a mixture of 50 µg protein or 40 µg peptide, 5 nmol CpG (Tib Molbiol, Berlin, Germany) and an equal volume of incomplete Freund's adjuvant. 6 weeks later a booster injection was performed without Freund's adjuvant. Spleen cells were fused with P3X63Ag8.653 myeloma cells using standard procedures. Hybridoma supernatants were screened in a solid-phase enzyme-linked immunosorbent assay (ELISA) for binding to the respective antigen and positive supernatants were further validated by Western blot analysis. Hybridoma cells from selected supernatants were subcloned twice by limiting dilution to obtain stable monoclonal cell lines. Experiments were performed with hybridoma supernatant FSP1 (AIFM2) clone 6D8 (rat IgG2a) and GRLP-H (XCT1) clone 3A12 (rat IgG2a).

FSP1 activity assay. NADH consumption was measured at 340 nm using 100 µl of enzyme reactions in TBS buffer (50 mM TRIS pH8, 250 mM NaCl) on a 96-well Nunc cell culture plate. Enzyme reactions contained 1 µM of human recombinant FSP1 protein, 500 µM NADH (freshly prepared in TBS buffer) and 200 µM of different substrate candidates (i.e. CoQ1, CoQ10, Resazurin, oxidized glutathione, tert-Butyl hydroperoxide or dehydroascorbate). A SpectraMax M5 Microplate Reader was used to determine the absorbance at 340 nm every 30 sec. Reactions without NADH/ without enzyme were used to normalize the results.

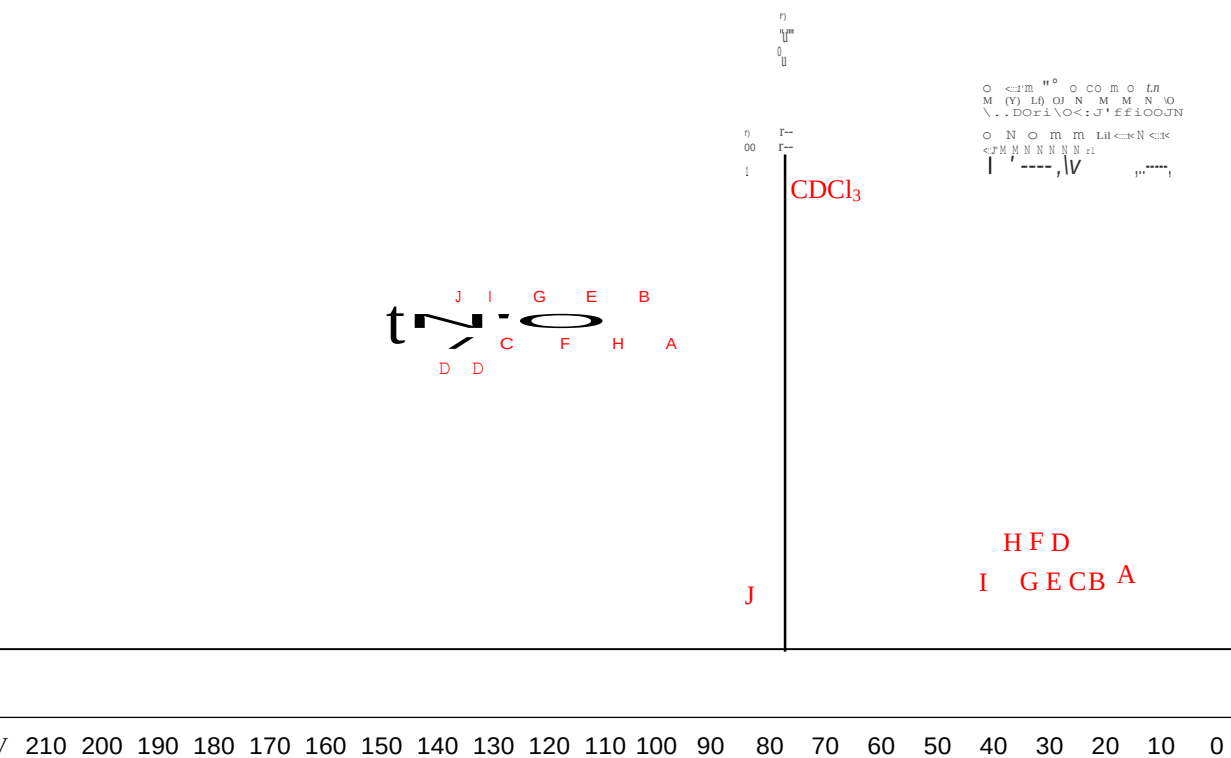
Synthesis of coumarin-quinone conjugate: ((2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)methyl 7-(diethylamino)-2-oxo-2H-chromene-3-carboxylate). 7-(Diethylamino)-2-oxo-2H-chromene-3-carboxylic acid (1.0 mmol), DCC (1.1 mmol) and DMAP (0.1 mmol) were added to a solution of DCM (3.5 mL). To this, was added 2-(hydroxymethyl)-3,5,6-trimethylcyclohexa-2,5-diene-1,4-dione⁸ (1.0 mmol) and the solution was stirred overnight at room temperature. The product precipitated out, it was filtered, washed with ethanol and dried thoroughly. Yellow powder, 83%. ¹H NMR (400 MHz, CDCl₃) δ 8.36 (s, 1H), 7.33 (d, *J* = 9.0 Hz, 1H), 6.59 (dd, *J* = 9.0,

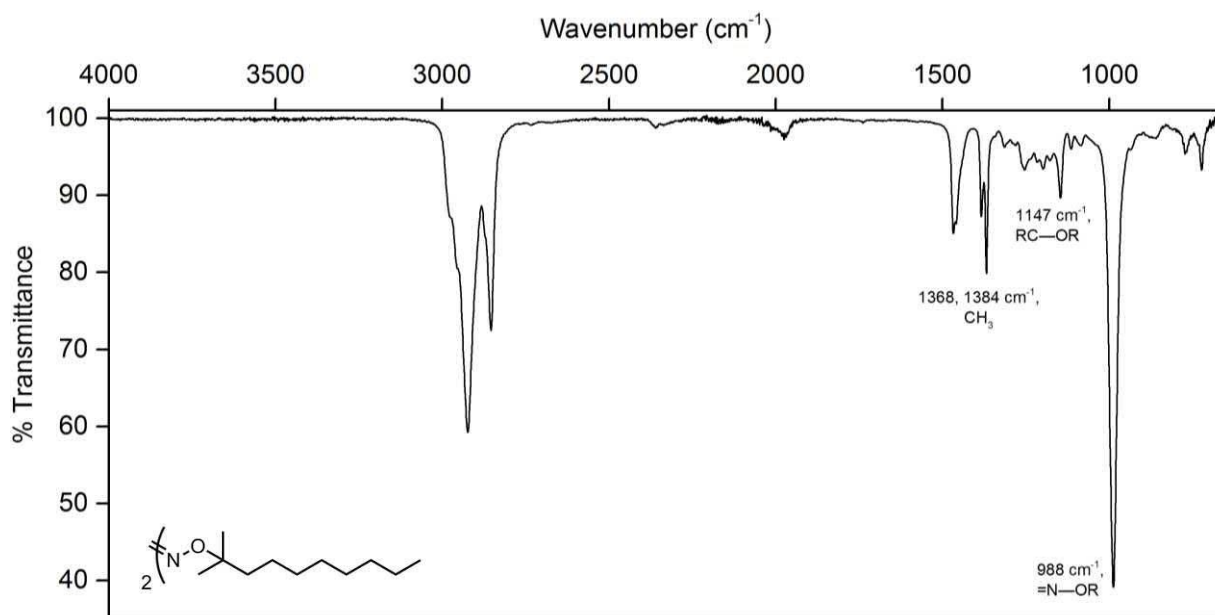
2.5 Hz, 1H), 6.44 (d, $J = 2.4$ Hz, 1H), 5.24 (s, 2H), 3.44 (q, $J = 7.1$ Hz, 4H), 2.20 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 1.23 (t, $J = 7.1$ Hz, 6H). ^{13}C NMR (76 MHz, CDCl_3) δ 187.58, 185.98, 163.70, 158.69, 158.13, 153.13, 149.53, 145.13, 141.17, 140.84, 136.94, 131.31, 109.78, 108.17, 107.83, 96.96, 58.04, 45.32, 12.69, 12.62, 12.56. HRMS: m/z Calc: $\text{C}_{24}\text{H}_{25}\text{NO}_6$: 423.1682 Found: 423.1677.



Synthesis of C9-Hyponitrite: ((E)-1,2-bis((2-methyldecan-2-yl)oxy)diazene). *2-Iodo-2-methyldecan-2-ol*. An oven dried 25 mL round-bottom flask and stir bar were cooled under N₂ atmosphere, wrapped completely in foil, charged with concentrated HI (6.7 mL, *ca.* 2 eq) and then cooled in an ice bath. Lithium iodide (3.36g, 25 mmol, 1 eq) was added in portions (caution: exotherm/fumes with powdered LiI; beads are preferred). A solution of 2-methyldecan-2-ol⁹ (4.31 g, 25 mmol, 1 eq.) in methylene chloride (5 mL) was added slowly by syringe with vigorous stirring under nitrogen atmosphere. The ice bath was then removed and the biphasic mixture stirred for about 10 min, at which point the alcohol was completely consumed by ¹H NMR. The mixture was carefully transferred to separatory funnel and the bottom aqueous layer cautiously extracted twice with methylene chloride (10 mL). The combined organic layers were washed once with 1:1 brine:water containing drops of sodium bisulfite solution until the mixture was mostly colourless. The organic extracts were fully protected from light while drying over Na₂SO₄, filtering and concentrating on a rotary evaporator followed by hovac (flask backfilled with nitrogen) to yield **2a** as a pale oil (6.63 g, 94%). The product was pure by ¹H NMR without distillation and was stored at -20°C in a foil-wrapped vial, ideally backfilled with nitrogen. ¹H NMR (300 MHz, CDCl₃) δ 1.92 (m, 6H), 1.65-1.22 (m, 14H), 0.88 (t, *J* = 6.9 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 53.27, 50.72, 38.20, 32.02, 29.65, 29.57, 29.43, 28.63, 22.81, 14.27. HRMS: *m/z* Calc: C₁₁H₂₃ (ESI: M-I)⁺ 155.1800; obsd 155.1817. *bis-2-methyldecan-2-yl hyponitrite (C9-HN)*. An oven dried 25 mL round-bottom flask and stir bar were cooled under N₂ atmosphere and charged with 2-iodo-2-methyldecan-2-ol (1.44g, 6 mmol) followed by degassed anhydrous methylene chloride (10 mL). The flask was completely wrapped in aluminium foil to exclude light and then cooled thoroughly in an ice bath. Freshly dried silver hyponitrite (1.24 g, 4.5 mmol) was added in three portions over 5 min with vigorous stirring. The ice bath was then removed and NMR analysis indicated consumption of the tertiary iodide usually within 10-20 min. The reaction mixture was then re-cooled in an ice bath and diluted with cold pentane (10 mL) before filtering over celite. The filtrate was concentrated on a rotary evaporator without heating followed by hovac for about 5 min while on ice. The residual oil was crystallized overnight from methanol (*ca.* 20 mL, N₂ purged) on dry ice. The majority of supernatant was then removed by syringe, discarded, and the solid washed with three portions of cold methanol in the same manner. The solid was allowed to thaw into a

small volume of methanol (*ca.* 5 mL) until a solution formed. The product was promptly isolated thereafter while being kept cold on ice at all times. The solution was extracted with three portions of cold pentane (5 mL), the combined pentane extracts washed once with 1:1 methanol:water, dried over Na₂SO₄, filtered, concentrated on a rotary evaporator without heating followed by hivaac to yield the product. The product when free from residual solvent is solid on ice and rapidly melts when allowed to warm to ambient temperature. The solid was aliquoted by Pasteur pipette into tared vials and the vials were stored at -78°C. Stocks up to 0.2 M were prepared in ethanol or chlorobenzene and held on ice or dry ice just prior to use. Clear wax, 24%. ¹H NMR (600 MHz, CDCl₃) δ 1.65 (m, 2H), 1.34 (s, 6H), 1.34-1.24 (m, 12H), 0.88 (t, *J* = 6.6 Hz, 3H). ¹³C NMR (151 MHz, CDCl₃) δ 83.42, 40.63, 32.03, 30.16, 29.69, 29.42, 25.94, 24.02, 22.82, 14.27. HRMS: *m/z* Calc: C₁₁H₂₃ (ESI: M-N₂O₂-C₁₁H₂₃)⁺ 155.1800; obsd 155.1788. FTIR (ATR) ν : 988, 1147, 1368, 1384, 1466, 2854, 2923 cm⁻¹.





Reduction of a coumarin-quinone conjugate by FSP1. The kinetic parameters were determined by the addition of varying amounts of Coumarin-Q1 probe to 50 nM FSP1 and 200 μ M NADH in TBS (pH 7.4) at 37°C. The initial rates of the reaction were obtained by monitoring the increase in the fluorescence upon the reduction of the quinone to the hydroquinone on a plate reader (ex/em = 415/470 nm). The kinetic parameters were obtained by fitting the data to a standard substrate inhibition model in GraphPad Prism. Each experiment was performed independently three times, error bars represent SD from the mean.

Inhibited autoxidation of egg-PC liposomes. To a 96-well polypropylene plate (Nunc) was added EggPC liposomes (1 mM) and STY-BODIPY (1 μ M) in TBS at pH 7.4. This was followed by the addition of either FSP1 (50 nM), NADH (8 or 16 μ M), CoQ10 (5 or 10 μ M), α TOH (5 or 10 μ M) or a combination thereof. The plate was incubated for 10 min at 37°C in the BioTek Synergy H1 plate reader followed by a vigorous mixing protocol for 5 min. The plate was ejected from the plate reader and the autoxidation was initiated by the addition of 3 μ L C₉-HN (0.2 mM) in EtOH, followed by another mixing protocol for 5 min. Data was acquired by excitation of STY-BODIPY at 488 nm and emission was measured at 518 nm. The data was transformed into [ox-STY-BODIPY] by taking the raw RFU values and dividing them by the response factor of 7.49×10^{10} . The data shown is an average of three independent experiments.

LC-MS analysis of 5-H(P)ETE/12-H(P)ETE/15-H(P)ETE formation. Mock and FSP1 overexpressing Pfa1 cells were plated on a 10-cm plate and allowed to adhere overnight. On the next day, media was removed, the cells were washed with PBS and suspended in media containing 0.2 μ M RSL3 and 40 μ M AA. The cells were harvested using a cell scraper at appropriate time points and flash frozen in liquid nitrogen. After thawing, the cells were treated with 5 mL of MeOH containing the internal standard, 1 mg/mL BHT and 1.5 mg/mL triphenylphosphine, followed by sonication to lyse the cells. BHT was added in order to suppress any air oxidation of the PUFAs present in the sample. The mixture was left on a shaker for 30 min at room temperature to ensure complete reduction of HPETEs. 10 mL of chloroform and 1 mL of 0.9% NaCl solution was added followed by shaking for 1h to ensure maximal extraction of lipids. The tubes were centrifuged to separate the organic and aqueous layers. The lipids were recovered by removing the bottom chloroform layer. The mixture was re-extracted using 5 mL chloroform and the two organic layers were combined. The combined chloroform layers were dried in a vacuum dryer at 20°C. The lipids were re-suspended in 500 μ L MeOH containing BHT, followed by the addition of 500 μ L KOH (1.5%) solution and incubated at 60°C for 30 min to saponify all the phospholipids. After incubation, the solution was acidified using 500 μ L HCl (2 M) solution. The mixture was purified using the C-18 solid phase extraction columns (Thermo Scientific) as per manufacturer's protocol. The eluent in methanol was evaporated to dryness and resuspended in 100 μ L methanol. Aliquots (5 μ L) were injected on a Waters Acquity C₁₈ column (2.1 mm $\times$ 50 mm, 1.7- μ m particle size) in a Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) system. H(P)ETEs were eluted isocratically with a mixture of acetonitrile, methanol, water and acetic acid (42:25:33:0.007) and detected using MS/MS analysis. Eicosanoids were detected in the negative ion mode by multiple reaction monitoring of m/z 333 $\rightarrow$ 235 for PGB₂, m/z 319 $\rightarrow$ 115 for 5-HETE, m/z 319 $\rightarrow$ 179 for 12-HETE, and m/z 319 $\rightarrow$ 219 for 15-HETE. Each experiment was performed independently three times, error bars represent SD from the mean.

Determination of PLPC-OOH from autoxidation of soy lecithin liposomes. Autoxidations of 13.3 mM soy-lecithin liposomes were carried out at 37°C and initiated using C₉-HN (1 mM). The

autoxidations were inhibited using NADH (25 μ M) and FSP1 (100 nM) in the presence of either CoQ10 (10 μ M), α TOH (10 μ M) or a combination thereof. Aliquots (250 μ L) of the autoxidation mixtures were taken every hour and diluted using a solution of 1% BHT in MeOH (500 μ L) in order to lyse the liposome particles. The BHT was added in order to prevent further autoxidation of PUFAs. Aliquots (10 μ L) were injected on a ThermoFisher Hypersil Gold C₁₈ column (4.6 mm $\times$ 250 mm, 5.0- μ m particle size) in a Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) system. PLOOH were eluted with a gradient of 1.0 mL/min gradually changing to 1.1 mL/min at 12 min. The solvent system changed from 15:85 $\rightarrow$ 1:99 for the mixture of 10 mM NH₄OAc (in H₂O) and methanol (15:85), respectively. The overall run time was 20 min and PLOOH were detected in positive ion mode by multiple reaction monitoring m/z 790 $\rightarrow$ 184.

Live cell microscopy. Cells were seeded on 8-well μ -slides (Ibidi, Germany) at a density of 20,000 cells per well and incubated overnight. On the next day, propidium iodide and Hoechst dye (1 μ g/mL) was added to each well and ferroptosis was induced by adding 250 nM RSL3. Live cell microscopy was performed using an Axio Observer Z1 imaging system (Visitron Systems, Puchheim, Germany), equipped with LED excitation light (pE-4000, CoolLED, Andover, UK) for fluorescence illumination. For Hoechst and PI excitation, the LED modules 385 nm and 550 nm (at 5% and 10% output power; exposure time 400 ms) and fluorescence filters ET-DAPI and ET-CY3 (Chroma, US) were used. Additionally, phase contrast images (exposure time 10 ms) were generated. Microscopic images were obtained using a 20x air objective (EC Plan-Neofluar 20x/0,5 Ph2 and acquired with a CCD camera (CoolSnap ES2, Photometrics, Tucson, AZ, USA) and the imaging software VisiView (Visitron Systems, Puchheim, Germany). During imaging, the cells were maintained at 37°C and 5% CO₂ by using a temperature controlled incubation chamber (ibidi heating system with ibidi gas incubation system, Ibidi, Germany).

Lipid extraction of cell pellets. Cell pellets were resuspended in 1 mL ice cold PBS, pH 7.4, containing 100 μ M diethylenetriaminepentaacetic acid (DTPA) and 100 μ M butylated hydroxytoluene (BHT). For oxPL analysis, 5 ng of DMPE and DMPC was added to each sample before extraction as internal standards. For phospholipid classes analysis, samples were spiked

with 5 μ L of Splash Lipidomix internal standards including d7-phosphatidylcholine (PC) (15:0/18:1); d7-phosphatidylethanolamine (PE) (15:0/18:1); d7-phosphatidylserine (PS) (15:0/18:1); d7-phosphatidylglycerol (PG) (15:0/18:1); d7-phosphatidylinositol (PI) (15:0/18:1); d7-phosphatidic acid (PA) (15:0/18:1); d7-lysophosphatidylcholine (LPC) (18:1); d7-lysophosphatidylethanolamine (LPE) (18:1). Lipids were extracted by adding 2.5 mL of methanol and 1.25 mL of chloroform to 1 mL of sample, followed by 1 min vortex and incubation on ice for 15 min. Then, 1.25 mL of chloroform and 1.25 mL of water was added. After vortexing and centrifugation (400g, 5 min), lipids were recovered in the bottom chloroform layer. The chloroform layer was dried, dissolved in methanol, and stored at -80°C before analysis by LC-MS/MS.

LC-MS/MS targeted analysis of oxPLs and truncated-PLs. Lipid extracts were separated using reverse-phase HPLC on a Luna 3 μ m C18 150 $\times$ 2-mm column (Phenomenex, Torrance, CA) with a gradient of 50 – 100 % B over 10 min followed by 30 min at 100 % B (A, 1 mM ammonium acetate, methanol/acetonitrile/water/, 60:20:20, v/v/v; B, 1 mM ammonium acetate, methanol) with a flow rate of 200 $\mu\text{L min}^{-1}$. Products were analysed in MRM mode, on a 6500 Q-Trap (Sciex, Cheshire, United Kingdom), monitoring transitions from the parent ion to daughter ion (dwell 75 ms) with TEM 500 $^{\circ}\text{C}$, GS1 40, GS2 30, CUR 35, IS – 4500 V, DP – 50 V, EP – 10 V, CE – 38 V and CXP at – 11 V. The area under the curve for the parent ion to daughter ion was integrated and normalized to the internal standard for lipid species described in Table 2. All oxPLs and truncated-PLs shown herein were previously identified, with all structures confirmed using MS/MS^{10,11}.

Table 2. Internal standard for lipid species

Analyte	Mass transition		Analyte	Mass transition	
	Q1 (m/z)	Q3 (m/z)		Q1 (m/z)	Q3 (m/z)
PE(14:0_14:0)	634.5	227.2	PE(16:0p_22:5(2O))	780.6	361.2
PC(14:0_14:0)	662.5	227.2	PE(18:0a_HETE)	782.6	319.2
PE(18:0a_5`-oxo-valeroyl)	578.4	115.1	PE(16:0a_22:4(O))	782.6	347.2
PC (18:1a_5`-oxo-valeroyl)	604.4	115.1	PE(18:1p_HDoHE)-1	788.6	343.2
PC(18:0a_5`-oxo-valeroyl)	606.4	115.1	PE(18:1p_HDoHE)-2	788.6	343.2

PE(18:0a_9'-oxo-nonanoyl)	634.5	171.1	PE(18:0p_HDoHE)	790.6	343.2
PC(18:0a_9'-oxo-nonanoyl)	662.5	171.1	PE(18:1p_22:4(O))	792.6	347.2
PE(16:0p_20:5(O))	736.6	317.2	PE(18:0p_22:5(O))	792.6	345.2
PE(16:0p_HETE)	738.6	319.2	PE(20:0p_HETE)	794.6	319.2
PE(16:0p_HDoHE)-1	762.6	343.2	PE(16:0a_HDoHE)	804.7	343.2
PE(16:0p_HDoHE)-2	762.6	343.2	PE(18:0a_HDoHE)	806.7	343.2
PE(18:1p_HETE)	764.6	319.2	PE(18:0a_22:5(O))	808.7	345.2
PE(16:0e_22:5(O))	766.6	345.2	PE(18:1a_22:4(O))	808.7	347.2
PE(16:0p_22:4(O))	766.6	347.2	PC(18:0a_HETE)	810.7	319.2
PE(18:1a_HDoHE)	778.6	343.2			

MRM parameters in negative mode used for measuring oxPLs and truncated-PLs. Lipids were measured using a 6500 QTrap as outlined in Methods. Where more than one oxPLs with the same structure is listed, this denotes additional molecular species with different retention times.

LC-MS/MS targeted analysis of phospholipid classes. The LC-MS/MS analysis was performed using a Shimadzu Nexera UHPLC system, a column oven set at 35°C, and a QTRAP® 6500 system (Sciex, Cheshire, United Kingdom). Chromatographic separation of lipid extracts (10 µL) was achieved on a Waters XBridge Amide HILIC column (4.6x150 mm, 3.5 µm). A programmed 24 min gradient was used with the following steps: 94% solvent A (1 mM ammonium acetate, acetonitrile/water, 95:5, v/v, pH 8.2) for 6 min, solvent B (1 mM ammonium acetate, acetonitrile/water, 50:50, v/v, pH 8.2) from 6 to 25% for 4 min, followed by solvent B from 25 to 98% B for 1 min and from 98 to 100% B for 2 min, holding at 100% for 5.6 min and equilibration for 5.4 min with solvent A at 99.9%. Initial flow rate was set at 0.7 mL min⁻¹ and increased to 1.5 mL min⁻¹ following 13.5 min and hold for 9.5 min. Products were analysed in scheduled multiple reaction monitoring (MRM) mode, with 60 s MRM detection window and target scan time of 0.5 s. MS settings were as follows: TEM 550 °C, GS1 50, GS2 60, CUR 30, IS – 4500 V, DP – 120 V, EP – 10 V and CXP at – 11 V. The area under the curve for the parent ion to daughter ion transition for each species was integrated and normalized to the relevant internal standard (IS) for each lipid class (Table 3).

Table 3. MRM parameters in negative mode used for measuring PE, LPE, PC, LPC, PG, PI and PS species.

Analyte	Mass transition		Analyte	Mass transition	
	Q1 (m/z)	Q3 (m/z)		Q1 (m/z)	Q3 (m/z)

18:1(d7) LPC +AcO _IS	587,409	288,296	PE(P-18:0_16:0)	702,500	255,233
LPC(16:0) +AcO	554,346	255,233	PE(P-18:0_18:3)	724,500	277,217
LPC(16:1) +AcO	552,331	253,217	PE(P-18:0_20:3)	752,600	305,249
LPC(18:0) +AcO	582,378	283,264	PE(P-18:0_20:4)	750,500	303,233
LPC(18:1) +AcO	580,362	281,249	PE(P-18:0_20:5)	748,500	301,217
LPC(18:2) +AcO	578,346	279,233	PE(P-18:0_22:4)	778,600	331,264
LPC(20:0) +AcO	610,409	311,300	PE(P-18:0_22:5)	776,600	329,249
15:0/18:1(d7) PC _IS	811,625	288,298	PE(P-18:0_22:6)	774,500	327,233
PC(16:0_16:0) +AcO	792,576	255,233	PE(P-18:1_16:0)	700,500	255,233
PC(16:0_16:1) +AcO	790,560	253,217	PE(P-18:1_16:1)	698,500	253,217
PC(16:0_18:1) +AcO	818,592	281,249	PE(P-18:1_18:1)	726,500	281,249
PC(16:1_18:2) +AcO	814,560	253,217	PE(P-18:1_18:2)	724,500	279,233
PC(16:1_18:1) +AcO	816,576	281,249	PE(P-18:1_18:3)	722,500	277,217
PC(16:1_18:2) +AcO	814,560	253,217	PE(P-18:1_20:1)	754,600	309,280
PC(18:0_16:1) +AcO	818,592	253,217	PE(P-18:1_20:2)	752,600	307,264
PC(18:0_18:0) +AcO	848,639	283,264	PE(P-18:1_20:3)	750,500	305,249
PC(18:0_18:1) +AcO	846,623	281,249	PE(P-18:1_20:4)	748,500	303,233
PC(18:0_22:6) +AcO	892,607	327,233	PE(P-18:1_20:5)	746,500	301,217
PC(18:1_18:1) +AcO	844,607	281,249	PE(P-18:1_22:4)	776,600	331,264
LPC(20:1) +AcO	608,393	309,280	PE(P-18:1_22:5)	774,500	329,249
LPC(20:2) +AcO	606,378	307,264	PE(P-18:1_22:6)	772,500	327,233
LPC(20:3) +AcO	604,362	305,249	PE(P-18:2_20:4)	746,500	303,233
LPC(20:4) +AcO	602,346	303,233	PE(P-18:2_22:6)	770,500	327,233
LPC(22:4) +AcO	630,364	331,264	15:0/18:1(d7) PG _IS	740,552	288,298
LPC(22:5) +AcO	628,362	329,249	PG(16:0_16:0)	721,503	255,233
LPC(22:6) +AcO	626,346	327,233	PG(16:0_16:1)	719,487	253,217
PC(18:1_22:6) +AcO	890,592	327,233	PG(16:0_18:0)	749,534	283,264
PC(18:2_16:1) +AcO	814,560	279,233	PG(16:0_18:1)	747,518	281,249
18:1(d7) LPE _IS	485,343	288,298	PG(16:0_18:2)	745,503	279,233
LPE(16:0)	452,278	255,233	PG(16:0_20:1)	775,549	309,280
LPE(16:1)	450,263	253,217	PG(16:0_20:2)	773,534	307,264
LPE(18:0)	480,310	283,264	PG(16:0_20:4)	769,503	303,233
LPE(18:1)	478,293	281,249	PG(16:0_20:5)	767,487	301,217
LPE(18:2)	476,278	279,233	PG(18:0_16:1)	747,518	253,217
LPE(18:3)	474,263	277,217	PG(18:0_18:0)	777,565	283,264
LPE(20:0)	508,341	311,300	PG(18:0_18:1)	775,549	281,249
LPE(20:1)	506,325	309,280	PG(18:0_18:2)	773,534	279,233
LPE(20:2)	504,310	307,264	PG(18:0_18:3)	771,518	277,217
LPE(20:3)	502,294	305,249	PG(18:1_16:1)	745,503	281,249
LPE(20:4)	500,278	303,233	PG(18:1_18:1)	773,534	281,249
LPE(20:5)	498,263	301,217	PG(18:1_18:2)	771,518	279,233

LPE(22:4)	528,310	331,264	LPI(17:1)_IS	583,300	267,400
LPE(22:5)	526,294	329,249	LPI(16:0)	571,289	255,233
LPE(22:6)	524,278	327,233	LPI(16:1)	569,273	253,217
15:0/18:1(d7) PE_IS	709,557	288,298	LPI(18:0)	599,320	283,264
PE(16:0_16:0)	690,508	255,233	LPI(18:1)	597,305	281,249
PE(16:0_16:1)	688,492	253,217	LPI(18:2)	595,289	279,233
PE(16:0_18:1)	716,524	281,249	LPI(18:3)	593,273	277,217
PE(16:0_18:2)	714,508	279,233	LPI(20:0)	627,352	311,300
PE(16:0_18:3)	712,492	277,217	LPI(20:1)	625,336	309,280
PE(16:0_20:1)	744,555	309,280	LPI(20:2)	623,32	307,264
PE(16:0_20:2)	742,539	307,264	LPI(20:3)	621,305	305,249
PE(16:0_20:3)	740,524	305,249	LPI(20:4)	619,289	303,233
PE(16:0_20:4)	738,508	303,233	LPI(20:5)	617,273	301,217
PE(16:0_20:5)	736,492	301,217	LPI(22:4)	647,32	331,264
PE(16:0_22:4)	766,539	331,264	LPI(22:5)	645,305	329,249
PE(16:0_22:5)	764,524	329,249	LPI(22:6)	643,289	327,233
PE(16:0_22:6)	762,508	327,233	15:0/18:1(d7) PI_IS	828,56	288,298
PE(18:0_16:0)	718,539	283,264	PI(16:0_16:0)	809,518	255,233
PE(18:0_16:1)	716,524	283,264	PI(16:0_16:1)	807,503	253,217
PE(18:0_18:0)	746,570	283,264	PI(16:0_18:0)	837,55	283,264
PE(18:0_18:1)	744,555	281,249	PI(16:0_18:1)	835,534	281,249
PE(18:0_18:2)	742,539	279,233	PI(16:0_18:2)	833,518	279,233
PE(18:0_18:3)	740,524	277,217	PI(16:0_20:1)	863,565	309,28
PE(18:0_20:1)	772,586	309,280	PI(16:0_20:2)	861,55	307,264
PE(18:0_20:2)	770,570	307,264	PI(16:0_20:3)	859,534	305,249
PE(18:0_20:3)	768,555	305,249	PI(16:0_20:4)	857,518	303,233
PE(18:0_20:4)	766,539	303,233	PI(16:0_20:5)	855,503	301,217
PE(18:0_20:5)	764,524	301,217	PI(16:0_22:4)	885,55	331,264
PE(18:0_22:4)	794,570	331,264	PI(16:0_22:5)	883,534	329,249
PE(18:0_22:5)	792,555	329,249	PI(16:0_22:6)	881,518	327,233
PE(18:0_22:6)	790,539	327,233	PI(18:0_16:1)	835,534	253,217
PE(18:1_16:1)	714,508	281,249	PI(18:0_18:0)	865,581	283,264
PE(18:1_18:1)	742,539	281,249	PI(18:0_18:1)	863,565	281,249
PE(18:1_18:2)	740,524	279,233	PI(18:0_18:2)	861,55	279,233
PE(18:1_18:3)	738,508	277,217	PI(18:0_18:3)	859,534	277,217
PE(18:1_20:1)	770,570	309,280	PI(18:0_20:0)	893,612	283,264
PE(18:1_20:2)	768,555	307,264	PI(18:0_20:1)	891,597	309,28
PE(18:1_20:3)	766,539	305,249	PI(18:0_20:2)	889,581	307,264
PE(18:1_20:4)	764,524	303,233	PI(18:0_20:3)	887,565	305,249
PE(18:1_20:5)	762,508	301,217	PI(18:0_20:4)	885,55	283,3
PE(18:1_22:4)	792,555	331,264	PI(18:0_20:5)	883,534	301,217

PE(18:1_22:5)	790,539	329,249	PI(18:0_22:4)	913,581	331,264
PE(18:1_22:6)	788,524	327,233	PI(18:0_22:5)	911,565	329,249
PE(18:2_16:1)	712,492	279,233	PI(18:0_22:6)	909,55	327,233
PE(18:2_18:2)	738,508	279,233	PI(18:1_16:1)	833,518	281,249
PE(18:2_20:4)	762,508	303,233	PI(18:1_18:1)	861,55	281,249
PE(O-16:0_16:0)	676,529	255,233	PI(18:1_18:2)	859,534	279,233
PE(O-16:0_16:1)	674,513	253,217	PI(18:1_18:3)	857,518	277,217
PE(O-16:0_18:1)	704,56	283,264	PI(18:1_20:1)	889,581	309,28
PE(O-16:0_18:2)	702,544	281,249	PI(18:1_20:2)	887,565	307,264
PE(O-16:0_18:3)	700,529	279,233	PI(18:1_20:3)	885,55	305,249
PE(O-16:0_20:1)	698,513	277,217	PI(18:1_20:4)	883,534	303,233
PE(O-16:0_20:2)	730,576	309,28	PI(18:1_20:5)	881,518	301,217
PE(O-16:0_20:3)	728,56	307,264	PI(18:1_22:4)	911,565	331,264
PE(O-16:0_20:4)	724,529	303,233	PI(18:1_22:5)	909,55	329,249
PE(O-16:0_20:5)	722,513	301,217	PI(18:1_22:6)	907,534	327,233
PE(O-16:0_22:4)	752,56	331,264	PI(18:2_16:1)	831,503	279,233
PE(O-16:0_22:5)	750,544	329,249	PI(18:2_18:2)	857,518	279,233
PE(O-18:0_16:0)	704,56	255,233	PI(18:2_18:3)	855,503	277,217
PE(O-18:0_16:1)	702,544	253,217	PI(18:2_20:1)	887,565	309,28
PE(O-18:0_18:0)	732,591	283,264	PI(18:2_20:2)	885,55	307,264
PE(O-18:0_18:1)	730,576	281,249	PI(18:2_20:3)	883,534	305,249
PE(O-18:0_18:3)	726,544	277,217	PI(18:2_20:4)	881,518	303,233
PE(O-18:0_20:1)	758,607	309,28	PI(18:2_20:5)	879,503	301,217
PE(O-18:0_20:2)	756,591	307,264	PI(18:2_22:5)	907,534	329,249
PE(O-18:0_20:4)	752,56	303,233	PI(20:0_18:1)	891,597	281,249
PE(O-18:0_20:5)	750,544	301,217	PI(20:0_20:3)	915,597	305,249
PE(O-18:0_22:4)	780,591	331,264	PI(20:0_20:4)	913,581	303,233
PE(O-18:0_22:5)	778,576	329,249	LPS(17:1)_IS	508,56	267,2
PE(O-18:0_22:6)	776,56	327,233	LPS(16:0)	496,268	255,2
PE(P-16:0_16:1)	672,5	253,217	LPS(16:1)	494,252	253,2
PE(P-16:0_18:1)	700,5	281,249	LPS(18:0)	524,299	283,264
PE(P-16:0_18:2)	698,5	279,233	LPS(18:1)	522,284	281,2
PE(P-16:0_18:3)	696,5	277,217	15:0/18:1(d7) PS_IS	753,547	241,5
PE(P-16:0_20:1)	728,6	309,28	PS(16:0_16:0)	734,498	255,233
PE(P-16:0_20:2)	726,5	307,264	PS(16:0_16:1)	732,482	253,217
PE(P-16:0_20:3)	724,5	305,249	PS(16:0_18:0)	760,513	283,264
PE(P-16:0_20:4)	722,5	303,233	PS(16:0_18:1)	760,513	281,249
PE(P-16:0_20:5)	720,5	301,217	PS(16:0_18:2)	758,498	279,233
PE(P-16:0_22:4)	750,5	331,264	PS(16:0_22:5)	808,513	329,249
PE(P-16:0_22:5)	748,5	329,249	PS(18:1_16:1)	758,498	281,249
PE(P-16:0_22:6)	746,5	327,233	PS(18:1_18:1)	786,529	281,249

PE(P-16:1_18:1)	698,5	281,249	
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Lipids were measured using a 6500 QTrap. Lipids labelled as standards were used for quantification.

Heatmaps. For generation of heatmaps, samples with $n \geq 3$ were averaged and normalized to cell number (1×10^6 cells) or total protein content. Next, log10 was applied. R's heatmap package was used to generate the heatmap plots. The Euclidean metric was chosen to establish the treatments' relationships depicted as clusters¹². The clusters are aggregated following the "shortest distance" rule. Lipids are color-coded by PL class.

Data presentation and statistical analyses. Data are presented as mean $\pm$ s.d. unless stated otherwise. As a general rule for the cell-based experiments, the graphs show the mean $\pm$ s.d. of $n = x$ wells (x values are given in the figure legends) representative of a single experiment performed independently y times (y value is given in figure legends) for reproducibility. Statistical analysis was performed using GraphPad Prism 6.0 and 8.0 software.

References

- 1 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).
- 2 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).
- 3 Tietze, F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* **27**, 502-522 (1969).
- 4 Roveri, A., Maiorino, M. & Ursini, F. Enzymatic and immunological measurements of soluble and membrane-bound phospholipid-hydroperoxide glutathione peroxidase. *Methods Enzymol* **233**, 202-212 (1994).
- 5 Studier, F. W. Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* **41**, 207-234 (2005).
- 6 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).
- 7 Thinon, E. *et al.* Global profiling of co- and post-translationally N-myristoylated proteomes in human cells. *Nat Commun* **5**, 4919, doi:10.1038/ncomms5919 (2014).
- 8 Giraud, L. G. A. Diels-Alder Trapping of ortho-Quinone Methides. A New Entry to Substituted Xanthene-1,4-diones. *Synthesis* **8**, 1153-1160, doi:10.1055/s-1998-2116 (1998).

- 9 Dryzhakov, M., Hellal, M., Wolf, E., Falk, F. C. & Moran, J. Nitro-Assisted Bronsted Acid Catalysis: Application to a Challenging Catalytic Azidation. *J Am Chem Soc* **137**, 9555-9558, doi:10.1021/jacs.5b06055 (2015).
- 10 Aldrovandi, M. *et al.* Specific oxygenation of plasma membrane phospholipids by *Pseudomonas aeruginosa* lipoxygenase induces structural and functional alterations in mammalian cells. *Biochim Biophys Acta Mol Cell Biol Lipids* **1863**, 152-164, doi:10.1016/j.bbalip.2017.11.005 (2018).
- 11 Slatter, D. A. *et al.* Mapping the Human Platelet Lipidome Reveals Cytosolic Phospholipase A2 as a Regulator of Mitochondrial Bioenergetics during Activation. *Cell Metab* **23**, 930-944, doi:10.1016/j.cmet.2016.04.001 (2016).
- 12 Kolde, R. Pheatmap: pretty heatmaps. (2012).