Supplementary Information

USP29 and SMURF1 orchestrate FSP1-mediated ferroptosis suppression to facilitate chemoresistance in gastric cancer

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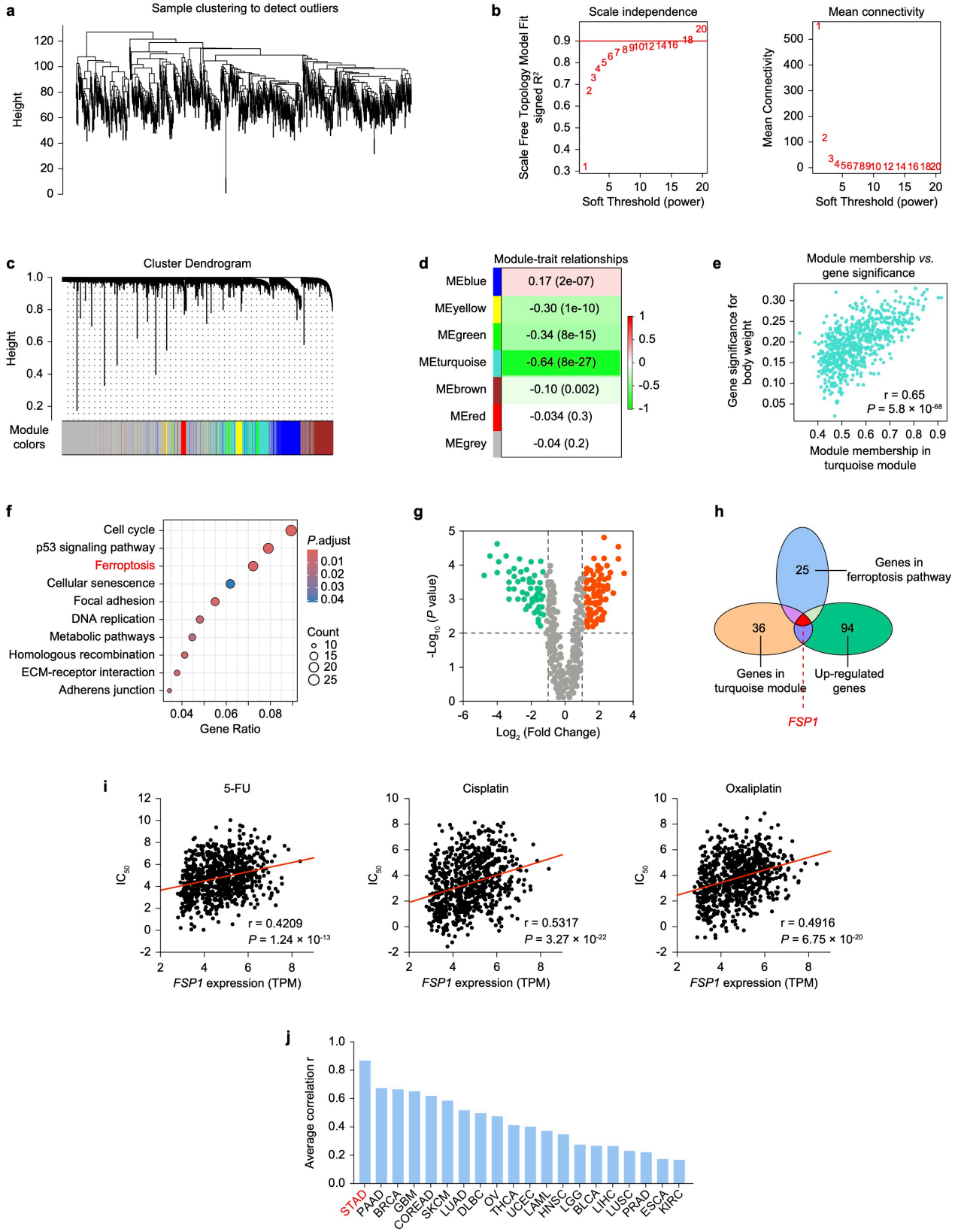
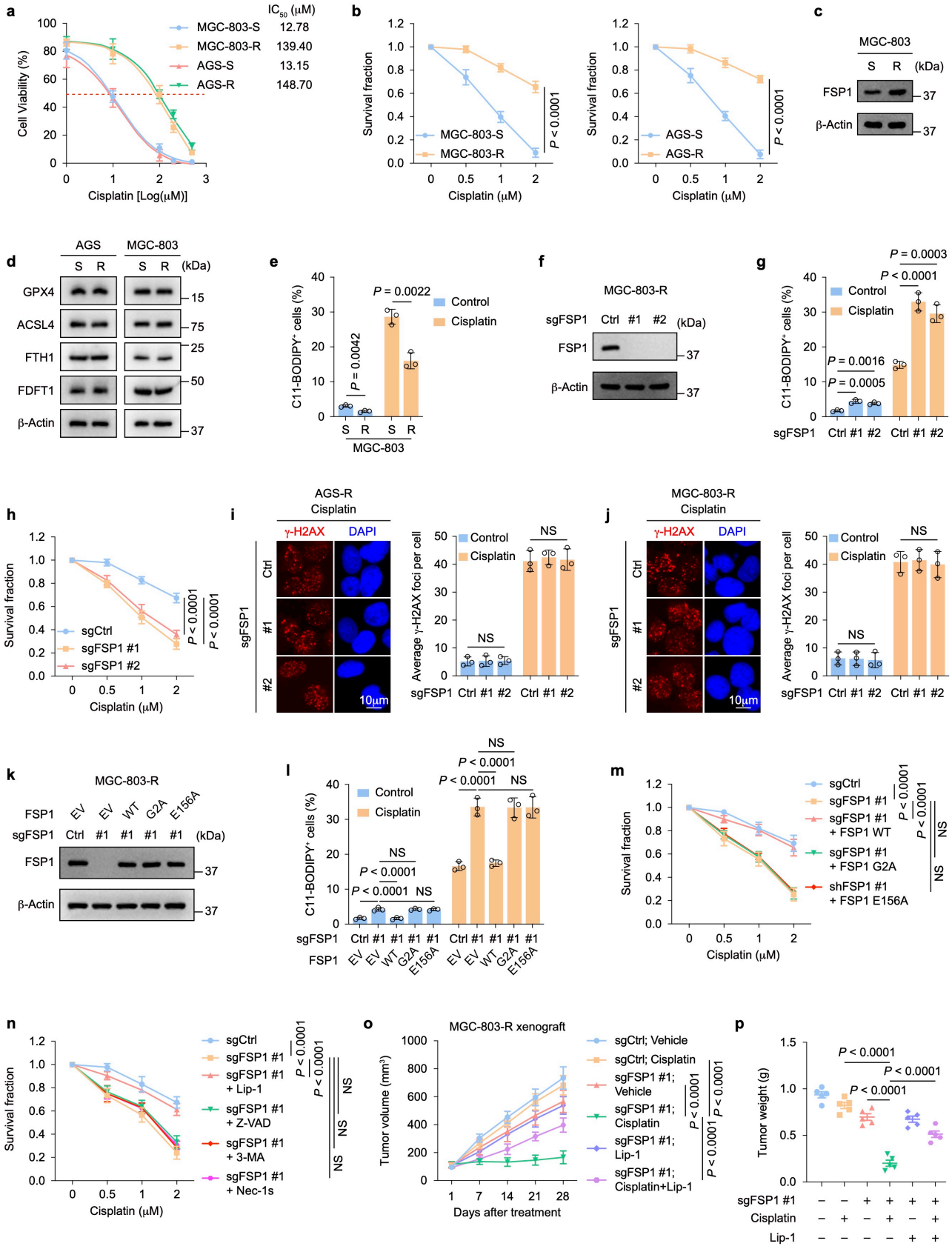
Supplementary Fig. S1



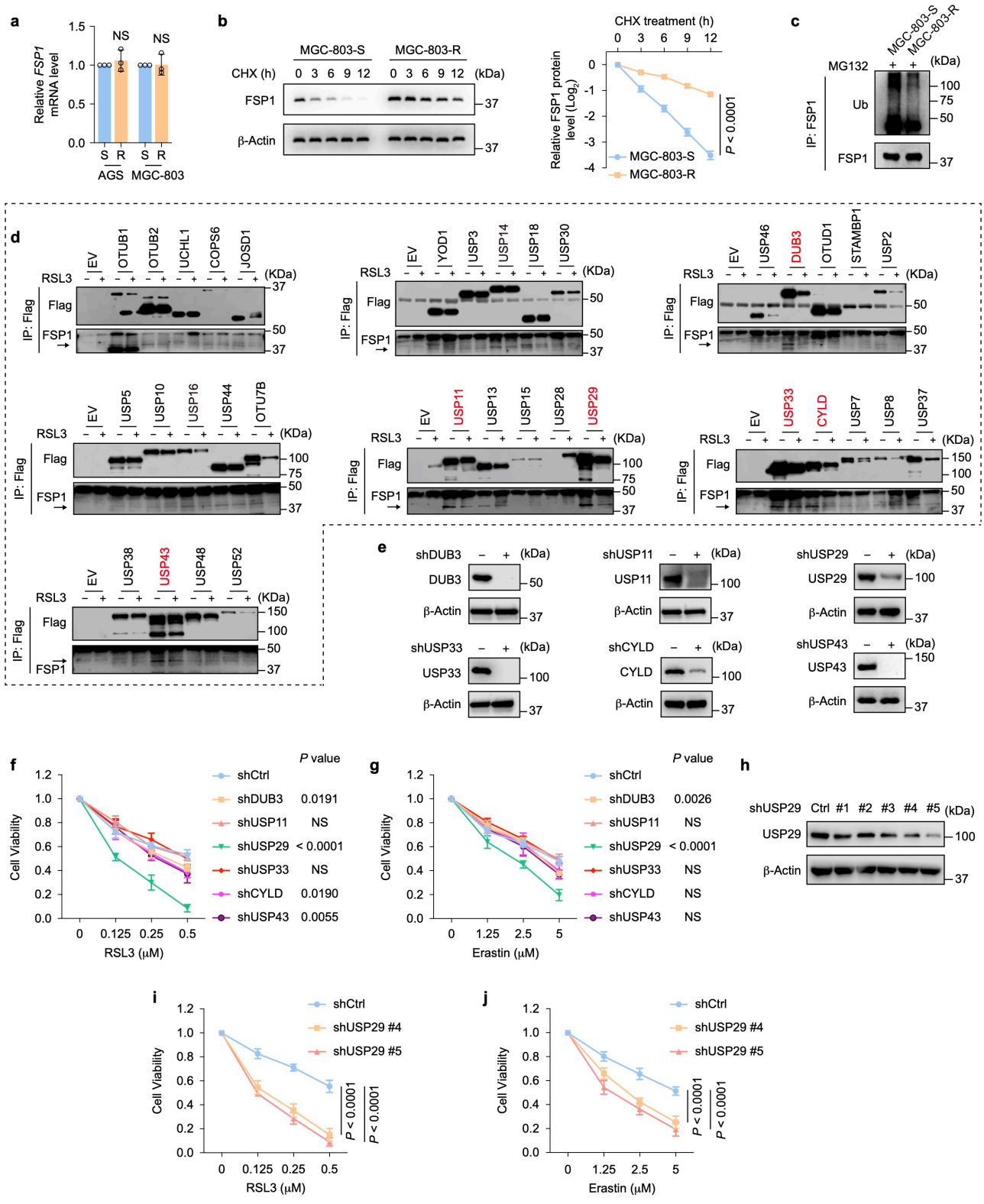
Supplementary Fig. S1 | FSP1 mediated ferroptosis contributes to chemoresistance in cancer. **a** Sample clustering to detect outliers, all the samples were in the clusters. **b** Threshold beta value calculation (WGCNA output). The left panel shows the Scale-Free Topology (SFT) Index in Y-axis as a function of the Soft Threshold in X-axis. The graph is reaching a saturation point at threshold beta value = 18. The right panel shows the Mean Connectivity in Y-axis as a function of the Soft Threshold in X-axis. **c** Gene dendrogram obtained by average linkage hierarchical clustering. The color row underneath the dendrogram shows the module assignment determined by the Dynamic Tree Cut. **d** Correlation between modules and traits. The left number in each cell refers to the correlation coefficient of each module in the trait, and the right number is the corresponding *P* value. **e** Scatter plot of gene significance versus the module membership in the turquoise module. **f** Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of genes in the turquoise module. **g** Volcano plot of gene change for cisplatin resistant group versus sensitive group. Red, upregulation; green, downregulation. **h** Venn diagram of genes in turquoise module, upregulation genes for cisplatin resistant group versus sensitive group, and genes in ferroptosis pathway. **i** Correlation of IC_{50} with FSP1 expression in cells treated with the chemotherapeutics of 5-FU, cisplatin, or oxaliplatin. Data are from Genomics of Drug Sensitivity in Cancer (https://www.cancerrxgene.org) database. *P* values are indicated and determined by Pearson correlation test. **j** Average correlation r values of chemotherapeutics of 5-FU, cisplatin, and oxaliplatin IC_{50} with FSP1 expression in the indicated cancer type. Source data are provided as Source Data file.

Supplementary Fig. S2



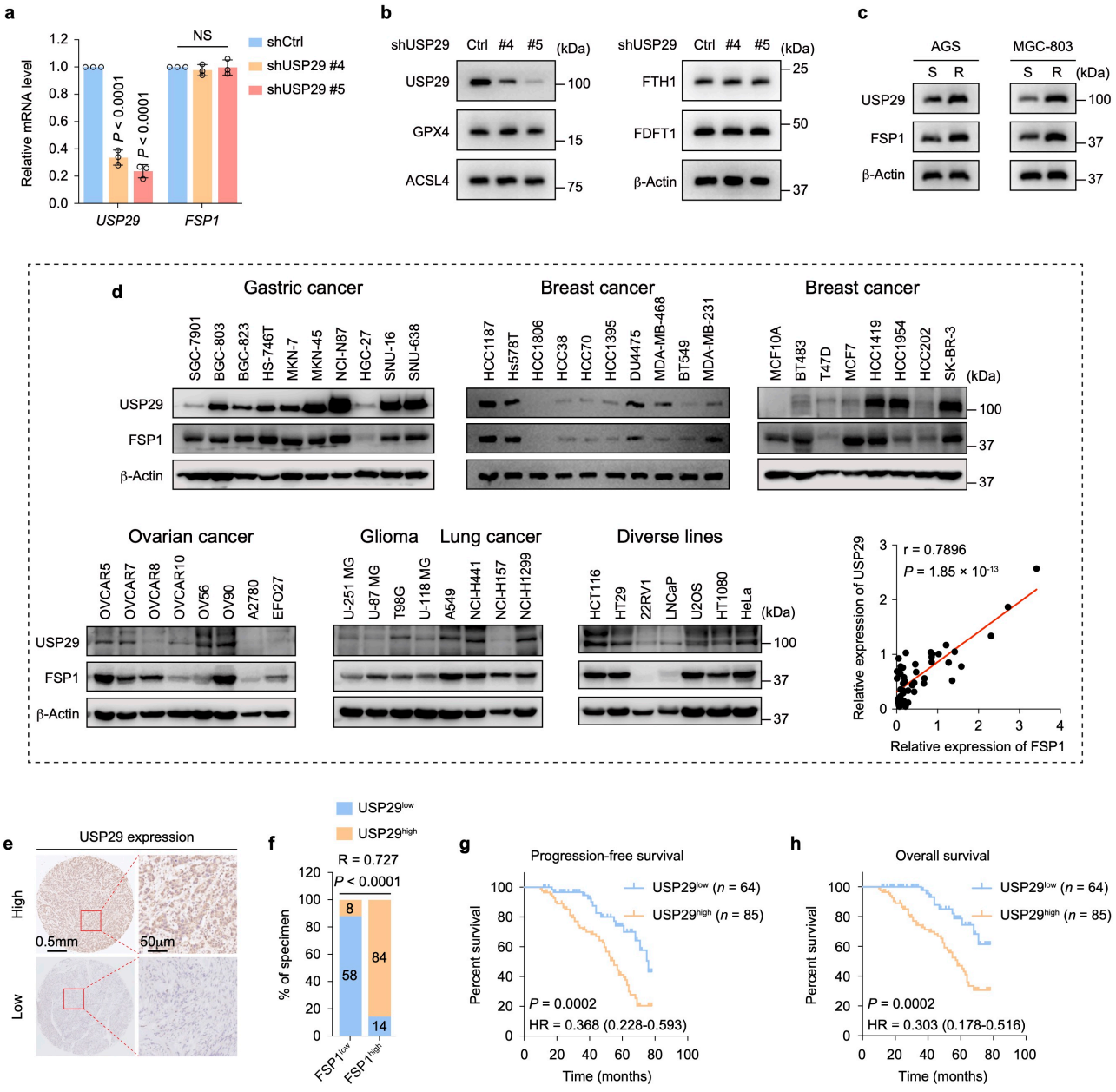
Supplementary Fig. S2 | FSP1 enables chemoresistance in GC via ferroptosis inhibition. **a** IC_{50} of cisplatin in the indicated cells. **b** Survival fraction of cells exposed to cisplatin at the indicated doses for two weeks. **c** Immunoblot of FSP1 in MGC-803 cisplatin sensitive (S) and resistant (R) cells. **d** Immunoblots showing the indicated ferroptosis factors in the indicated cells. **e** Percentage of C11-BODIPY-positive cells in the indicated MGC-803 cells with/without 24-hour cisplatin (2 μM) treatment. **f** Immunoblot of FSP1 in MGC-803-R cells stably expressing control or two independent FSP1 sgRNAs. **g** Percentage of C11-BODIPY-positive cells in cells from (**f**) with/without 24-hour cisplatin (2 μM) treatment. **h** Survival fraction of control or FSP1-knockout MGC-803-R cells exposed to cisplatin at the indicated doses for two weeks. **i, j** Representative micrographs (left) and quantitation (right) for γ-H2AX foci formation in the indicated cells after cisplatin treatment. Scale bars are indicated. **k** Immunoblots of FSP1 in MGC-803-R cells stably expressing the indicated vectors. **l** Percentage of C11-BODIPY-positive cells in cells from (**k**) with/without 24-hour cisplatin (2 μM) treatment. **m** Survival fraction of cells from (**k**) exposed to cisplatin at the indicated doses for two weeks. **n** Survival fraction of control or FSP1-knockout MGC-803-R cells exposed to cisplatin at the indicated doses combining with/without the indicated reagents for two weeks. **o** Growth curves of the MGC-803-R xenograft tumor models with the indicated treatment. **p** Quantification of the weight of the tumors in different groups of the xenograft tumor models. Experiment was repeated 3 times, and representative blots are presented in **c**, **d**, **f**, **k**. Data shown represent mean ± SD from three independent experiments in **a**, **b**, **e**, **g**-**j**, **l**-**n,** or from one representative experiment of 5 mice per group in **o**, **p**. *P* values are determined by 2-way ANOVA in **b**, **h**, **m**-**o**, unpaired 2-sided Student's *t* test in **e**, or 1-way ANOVA in **g**, **i**, **j**, **l**, **p**. The survival fraction was calculated as the ratio of colony-forming cells with the indicated treatment to those in the untreated control group. NS, not significant. Source data are provided as Source Data file.

Supplementary Fig. S3



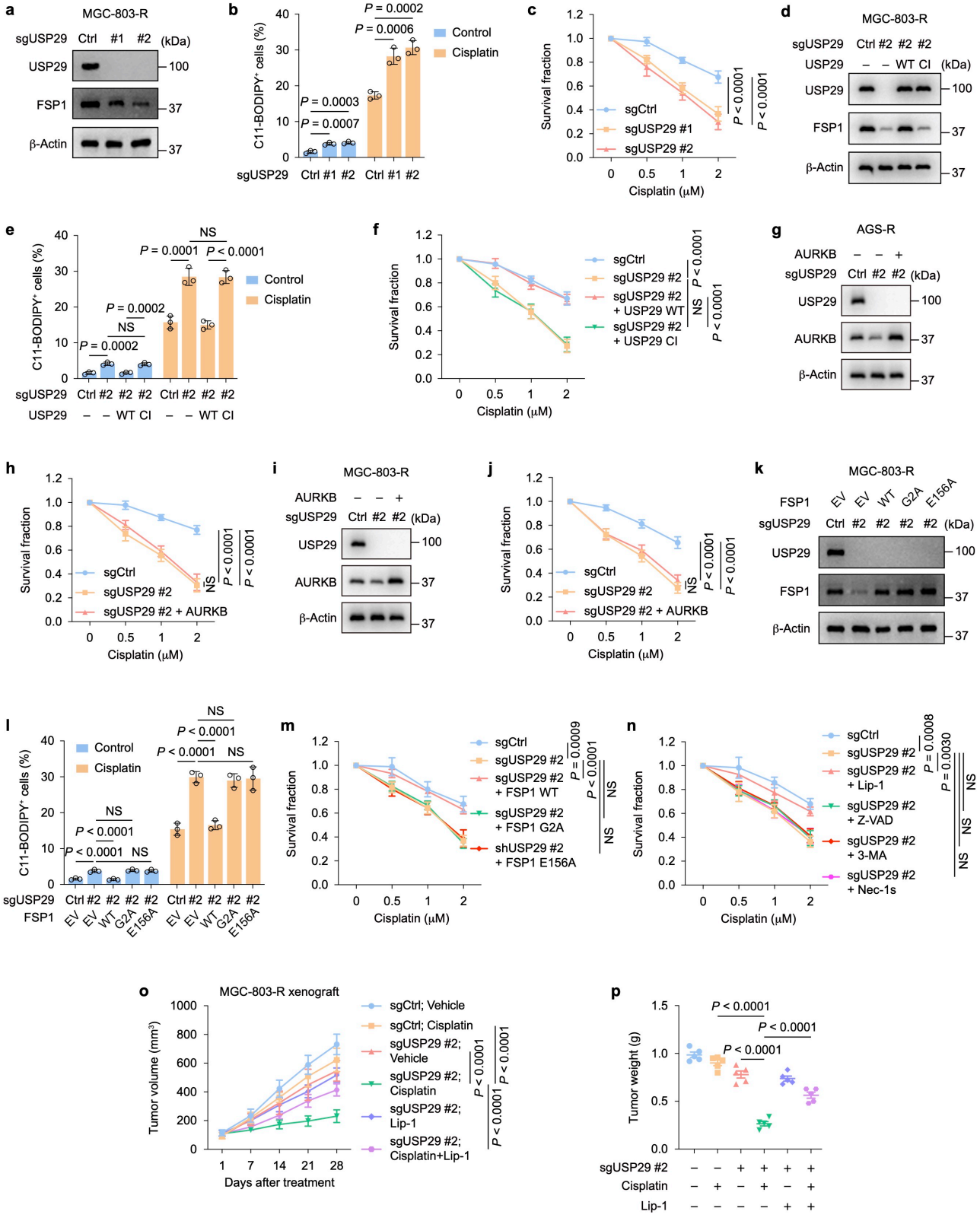
Supplementary Fig. S3 | Identification USP29 binding FSP1 to regulate ferroptosis. **a** *FSP1* mRNA expression level in the indicated cells. **b** FSP1 protein stability in cisplatin sensitive or resistant MGC-803 cells. **c** Ubiquitination of FSP1 in cisplatin sensitive or resistant MGC-803 cells. **d** HEK293T cells were transfected the indicated deubiquitinases (DUBs) with/without RSL3 treatment. After 48 h, cells were lysed and subjected to immunoprecipitation with Flag beads. The immunoprecipitates were then blotted with the indicated antibodies. **e** Immunoblots of DUB3, USP11, USP29, USP33, CYLD, and USP43 in HT1080 cells after infected with lentiviruses encoding control shRNA or the corresponding five mixed different targeting shRNAs. **f**, **g** Cells from (**e**) were treated with RSL3 (**f**) or erastin (**g**) for 72 h and then cell viability was determined. **h** Immunoblot of USP29 in HT1080 cells stably expressing control or five independent USP29 shRNAs. **i**, **j** Cells from (**h**) were treated with RSL3 (**i**) or erastin (**j**) for 72 h and then cell viability was determined. Experiment was repeated 3 times, and representative blots are presented in **b**-**e**, **h**. Data shown represent mean ± SD from three independent experiments in **a**, **b**, **f**, **g**, **i**, **j**. *P* values are determined by unpaired 2-sided Student's *t* test in **a**, 2-way ANOVA in **b**, **f**, **g**, **i**, **j**. NS, not significant. Source data are provided as Source Data file.

Supplementary Fig. S4



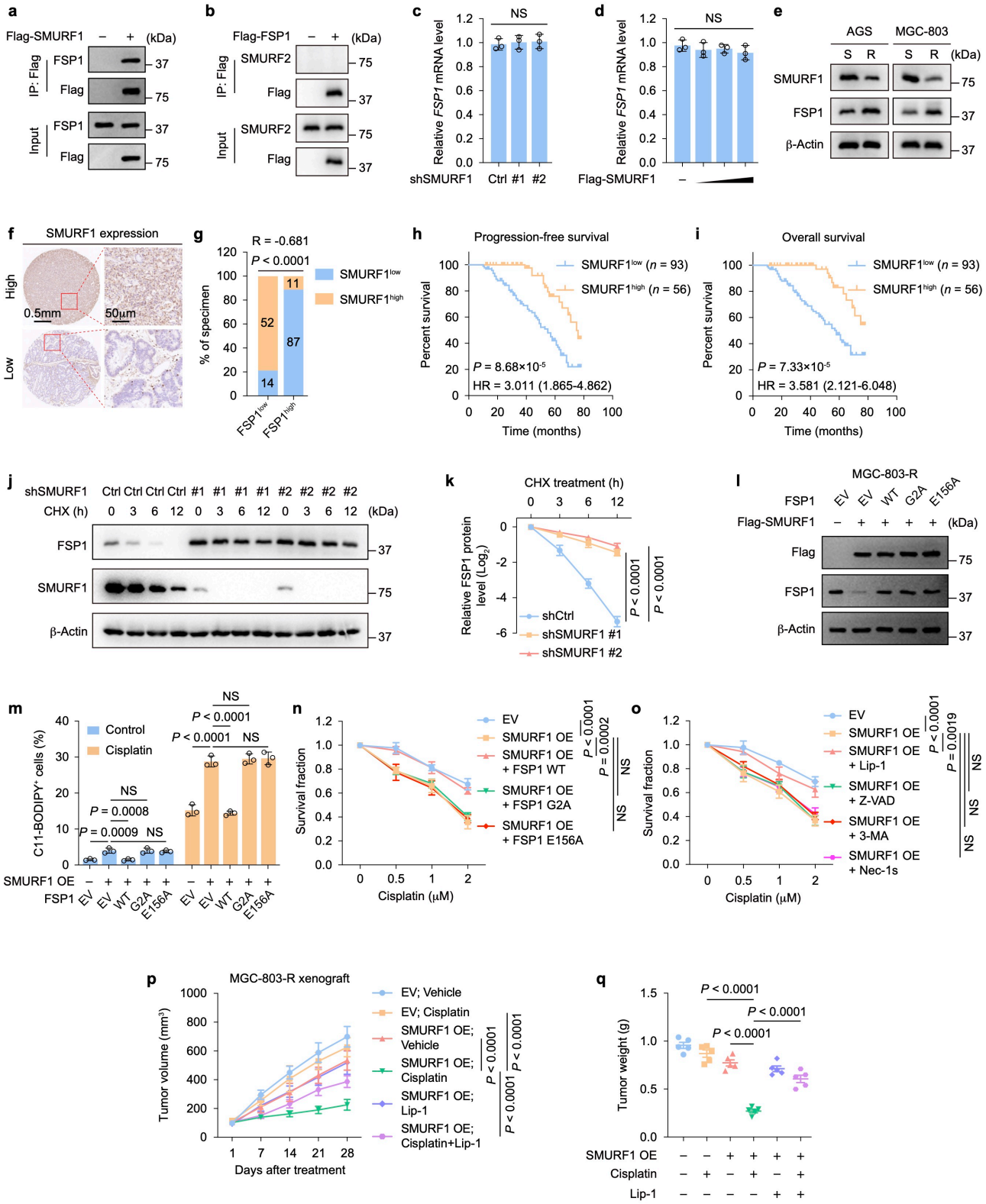
Supplementary Fig. S4 | USP29 positively correlates with FSP1. **a** Relative *USP29* or *FSP1* mRNA level after transduced with lentivirus carrying control or two independent USP29 shRNAs. **b** Protein levels of the indicated ferroptosis factors after transduced with lentivirus carrying control or two independent USP29 shRNAs. **c** Immunoblots of USP29 and FSP1 in the indicated cells. **d** Immunoblots and correlation of USP29 and FSP1 in different cell lines. **e**, **f** Representative images of USP29 IHC staining (**e**) and correlation analysis of USP29 and FSP1 (**f**) in the GC cohort (n = 164). Representative IHC staining of FSP1 are shown in **Fig. 1c**. Scale bars are indicated. **g**, **h** Progression-free survival (**g**) and overall survival (**h**) analysis of the GC cohort with low or high USP29 expression. Experiment was repeated 3 times, and representative blots are presented in **b**-**d**. P values are determined by 1-way ANOVA in **a**, linear regression in **d**, 2-sided χ^2 test in **f**, or 2-sided log-rank (Mantel-Cox) test in **g**, **h**. Source data are provided as Source Data file.

Supplementary Fig. S5



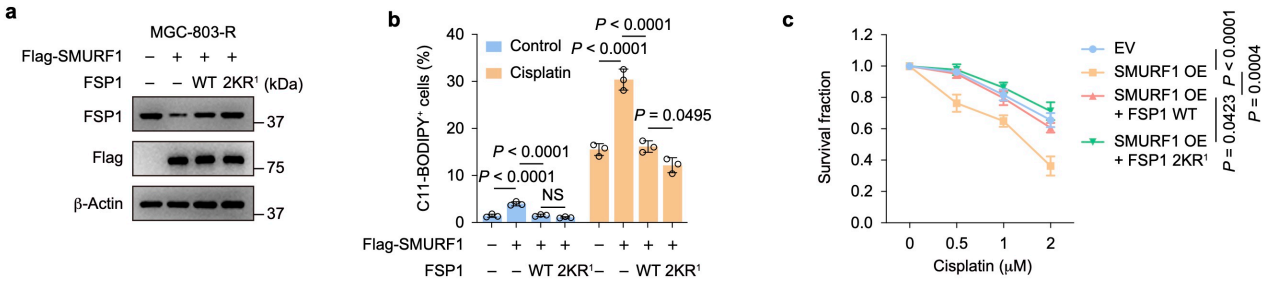
Supplementary Fig. S5 | USP29 attenuates ferroptosis via FSP1 to facilitate GC chemoresistance. **a** Immunoblots of FSP1 and USP29 in MGC-803-R cells stably expressing control or two independent USP29 sgRNAs. **b** Percentage of C11-BODIPY-positive cells in cells from (**a**) with/without 24-hour cisplatin (2 μM) treatment. **c** Survival fraction of cells from (**a**) exposed to cisplatin at the indicated dose for two weeks. **d** Immunoblots of FSP1 and USP29 in MGC-803-R cells stably expressing control or USP29 sgRNA transfected with USP29 WT or CI vector. **e** Percentage of C11-BODIPY-positive cells in cells from (**d**) with/without 24-hour cisplatin (2 μM) treatment. **f** Survival fraction of cells from (**d**) exposed to cisplatin at the indicated dose for two weeks. **g-j** Immunoblots of USP29 and AURKB, and colony formation assay treated with cisplatin for AGS-R (**g**, **h**) and MGC-803-R (**i**, **j**) cells stably expressing the indicated vector. **k** Immunoblots of FSP1 and USP29 in MGC-803-R cells stably expressing the indicated vectors. **l** Percentage of C11-BODIPY-positive cells in cells from (**k**) with/without 24-hour cisplatin (2 μM) treatment. **m** Survival fraction of cells from (**k**) exposed to cisplatin at the indicated dose for two weeks. **n** Survival fraction of control or USP29-knockout MGC-803-R cells exposed to cisplatin at the indicated dose combining with/without the indicated reagents for two weeks. **o** Growth curves of the MGC-803-R xenograft tumor models with the indicated treatment. **p** Quantification of the weight of the tumors in different groups of the xenograft tumor models. Experiment was repeated 3 times, and representative blots are presented in **a**, **d**, **g**, **i**, **k**. Data shown represent mean ± SD from three independent experiments in **b**, **c**, **e**, **f**, **h**, **j**, **l-n**, or from one representative experiment of 5 mice per group in **o**, **p**. *P* values are determined by 1-way ANOVA **b**, **e**, **l**, **p**, or 2-way ANOVA in **c**, **f**, **h**, **j**, **m-o**. The survival fraction was calculated as the ratio of colony-forming cells with the indicated treatment to those in the untreated control group. NS, not significant. Source data are provided as Source Data file.

Supplementary Fig. S6



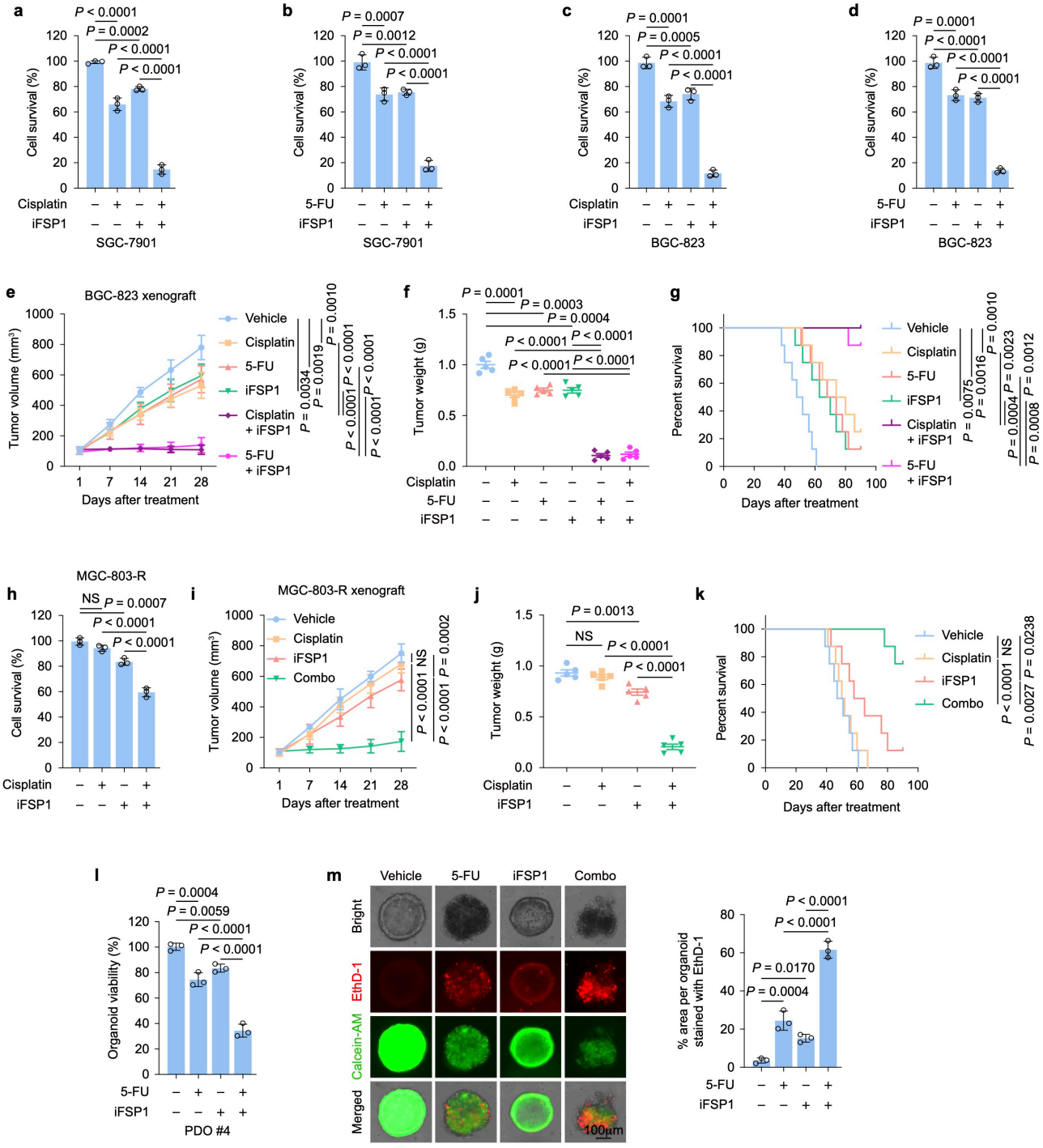
Supplementary Fig. S6 | SMURF1 boosts FSP1 degradation to increase chemosensitivity in GC. **a**, **b** Co-immunoprecipitation of FSP1 and SMURF1 (**a**) or SMURF2 (**b**) in HEK293T cells. **c**, **d** Relative *FSP1* mRNA levels in cells transfected with the indicated vectors. **e** Immunoblots of FSP1 and SMURF1 in the indicated cells. **f**, **g** Representative images of SMURF1 IHC staining (**f**) and correlation analysis of SMURF1 and FSP1 (**g**) in the GC cohort (n = 164). Representative IHC staining of FSP1 are shown in **Fig. 1c**. Scale bars are indicated. **h**, **i** Progression-free survival (**h**) and overall survival (**i**) analysis of the GC cohort with low or high SMURF1 expression. **j**, **k** FSP1 protein stability in cells transfected with the indicated vectors. **l** Immunoblots of Flag and FSP1 in MGC-803-R cells stably expressing the indicated vectors. **m** Percentage of C11-BODIPY-positive cells in cells from (**l**) with/without 24-hour cisplatin (2 μM) treatment. **n** Survival fraction of cells from (**l**) exposed to cisplatin at the indicated dose for two weeks. **o** Survival fraction of control or SMURF1-OE MGC-803-R cells exposed to cisplatin at the indicated dose combining with/without the indicated reagents for two weeks. **p** Growth curves of the MGC-803-R xenograft tumor models with the indicated treatment. **q** Quantification of the weight of the tumors in different groups of the xenograft tumor models. Experiment was repeated 3 times, and representative blots are presented in **a**, **b**, **e**, **j**, **l**. Data shown represent mean ± SD from three independent experiments **c**, **d**, **k**, **m**-**o**, or from one representative experiment of 5 mice per group in **p**, **q**. *P* values are determined by 1-way ANOVA in **c**, **d**, **m**, **q**, 2-sided χ^2 test in **g**, 2-sided log-rank (Mantel-Cox) test in **h**, **i**, or 2-way ANOVA in **k**, **n**-**p**. The survival fraction was calculated as the ratio of colony-forming cells with the indicated treatment to those in the untreated control group. NS, not significant. Source data are provided as Source Data file.

Supplementary Fig. S7



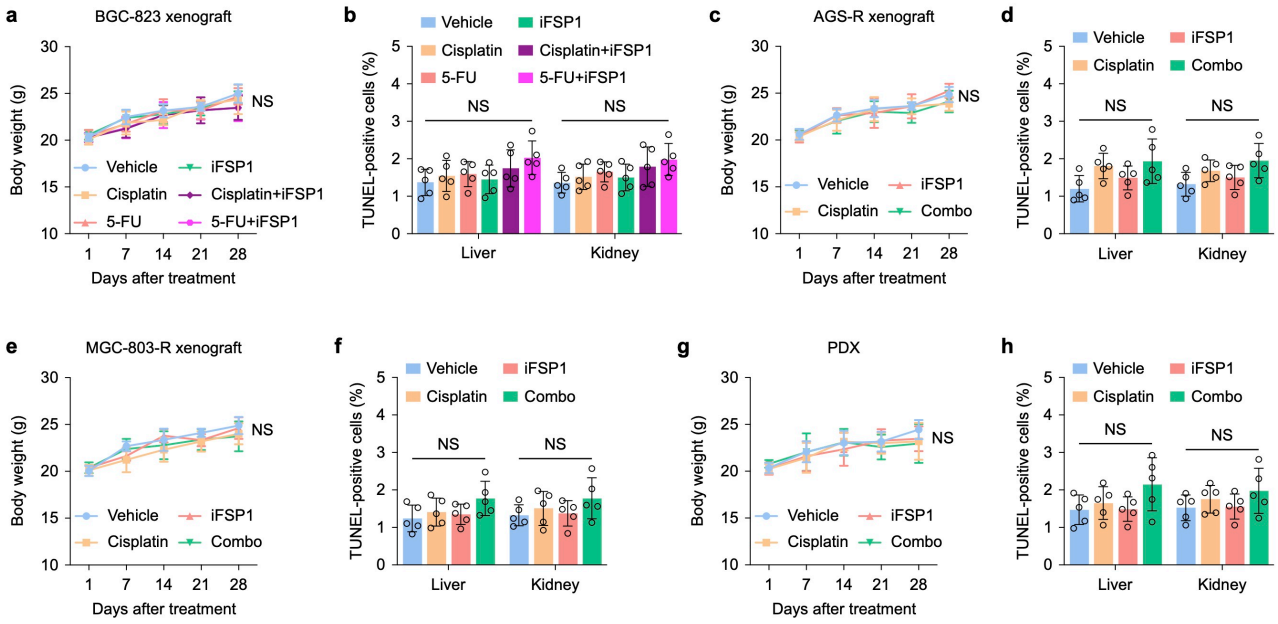
Supplementary Fig. S7 | SMURF1 increases ferroptosis and chemosensitivity in GC by promoting FSP1 degradation through Lys63 and Lys193. **a** Immunoblots of FSP1 and Flag in MGC-803-R cells transfected with the indicated vectors. **b** Percentage of C11-BODIPY-positive cells in cells from (**a**) with/without 24-hour cisplatin (2 μM) treatment. **c** Survival fraction of cells from (**a**) exposed to cisplatin at the indicated dose for two weeks. Experiment was repeated 3 times, and representative blots are presented in **a**. Data shown represent mean ± SD from three independent experiments in **b**, **c**. *P* values are determined by 1-way ANOVA in **b**, or 2-way ANOVA in **c**. The survival fraction was calculated as the ratio of colony-forming cells with the indicated treatment to those in the untreated control group. Source data are provided as Source Data file.

Supplementary Fig. S8



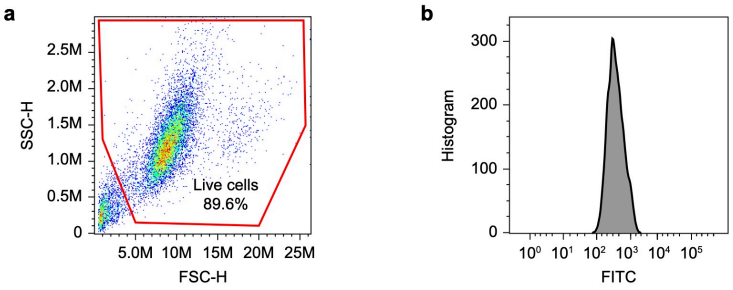
Supplementary Fig. S8 | Targeting FSP1 increases chemosensitivity and reverses chemoresistance in GC. **a-d** Cell survival of the indicated cells exposed to vehicle, cisplatin (20 nM), 5-FU (20 nM), iFSP1 (10 μM) or their combination for two weeks. **e** Growth curves of the BGC-823 xenograft tumor models with the indicated treatment. **f** Quantification of the weight of the tumors in different groups of the xenograft tumor models. **g** Survival curves of the BGC-823 xenograft tumor models. **h** Cell survival of MGC-803-R cells treated with vehicle, or cisplatin (1 μM), iFSP1 (10 μM) or their combination for two weeks. **i** Growth curves of the MGC-803-R xenograft tumor models with the indicated treatment. **j** Quantification of the weight of the tumors in different groups of the xenograft tumor models. **k** Survival curves of the MGC-803-R xenograft tumor models. **l** Organoid viability of PDO #4 with the indicated treatment. **m** Representative micrographs (left) and quantitation (right) of PDO #4 with the indicated treatment. Organoids were stained with Calcein-AM (live cells, green) and EthD-1 (dead cells, red) for 30 min and then fluorescent signals were captured. Scale bar is indicated. Data shown represent mean ± SD from three independent experiments in **a-d**, **h**, **l**, **m**, or from one representative experiment of 5 mice per group in **e**, **f**, **i**, **j**. Data shown come from one representative experiment of 8 mice per group in **g**, **k**. *P* values are determined by 1-way ANOVA in **a-d**, **f**, **h**, **j**, **l**, **m**, 2-way ANOVA in **e**, **i**, or 2-sided log-rank (Mantel-Cox) test in **g**, **k**. NS, not significant. Source data are provided as Source Data file.

Supplementary Fig. 9



Supplementary Fig. 9 | Combination of iFSP1 and chemotherapeutic agents is well tolerant. **a-h** body weight and quantification of TUNEL-positive cells in liver and kidney of BGC-823 (**a**, **b**), AGS-R (**c**, **d**), MGC-803-R (**e**, **f**), and PDX (**g**, **h**) xenograft tumor models with the indicated treatment. Data are shown as the mean ± SD from one representative experiment of 5 mice per group. *P* values are determined by 2-way ANOVA in **a**, **c**, **e**, **g**, or 1-way ANOVA in **b**, **d**, **f**, **h**. NS, not significant. Source data are provided as Source Data file.

Supplementary Fig. 10



Supplementary Fig. 10 | Gating strategy of Flow Cytometry in C11 BODIPY experiments. **a** Live cells were gated from FSC/SSC plot. **b** Plot histogram of live cells determined by FITC.

Supplementary Table 1. Characteristics of gastric cancer patients

Characteristics	Patients with FSP1 low expression (n = 66)	Patients with FSP1 high expression (n = 98)	P value
Age (yr)	65.5 ± 10.7	64.5 ± 13.3	0.274
Sex			0.659
Male	38	53	
Female	28	45	
BMI	21.38 ± 4.72	22.09 ± 3.53	0.486
ASA classification			0.984
I-II	60	89	
III	6	9	
Tumor size			0.742
≤ 5 cm	30	42	
> 5 cm	36	56	
Tumor differentiation			0.509
High	5	7	
Intermediate	34	42	
Low/undifferentiation	27	49	
cTNM stage			0.336
II	4	6	
III	50	82	
IV	12	10	
Neoadjuvant chemotherapy regimen			0.314
FLOFOX	12	28	
XELOX	8	10	
SOX	46	60	
Operation			0.237
Laparoscopic surgery	45	75	
Open surgery	21	23	
Multivisceral resection			0.665
Yes	15	22	
No	61	76	
Lymph nodes number	23.5 ± 11.2	25.0 ± 10.8	0.285
TRG			< 0.0001
I	39	20	
II	20	32	
III	7	46	

Data are shown as the mean ± SD or number where applicable. P values are determined by an unpaired, 2-tailed Student's t test or Fisher's exact test.

BMI: body mass index; ASA: American Society of Anesthesiologists; TRG: tumor regression grade.

Supplementary Table 2. Patient characteristics for PDO/PDX establishment in gastric cancer

Six patients for PDO establishment			
Characteristics	Number	Characteristics	Number
Gender		Distant metastases (M-stage)	
Female	3	M0	6
Male	3	Tumor stage (TNM)	
Age (yr, range)	42-64	II	2
Tumor location		III	4
Upper	2	Histopathology	
Lower	4	Tubular	6
Borrmann type		Histological differentiation	
II	3	Mild	3
III	3	Poor	3
Type of surgery		Perineural invasion	
Subtotal	4	Not exist	4
Total	2	Exist	2
Tumor infiltration (T-stage)		Vascular invasion	
T3	4	Not exist	3
T4	2	Exist	3
Lymph node metastases (N-stage)			
N1	2		
N2	3		
N3	1		

One patient for PDX establishment			
Gender	Female	Recurrent tumor location	Gastrointestinal anastomosis
Age (yr, range)	50-55	Recurrent tumor size	4.8 cm
TNM stage of initial tumor	IIIb	Recurrent tumor infiltration	T3
Histological differentiation of initial tumor	Poor	Recurrent tumor lymph node metastases	N3
Perineural invasion of initial tumor	Exist	Histological differentiation of recurrent tumor	Poor
Vascular invasion of initial tumor	Exist	Perineural invasion of recurrent tumor	Exist
Postoperative chemotherapy	XELOX	Vascular invasion of recurrent tumor	Exist
Chemotherapy cycles	5		
Recurrence time from first surgery	5 months		

Supplementary Table 3. Antibodies used in this study

Antibodies	Product number	Company
anti-4-HNE (1:100 for IHC)	BS-6313R	Invitrogen
anti-ACSL4 (1:1000 for WB)	A20414	Abclonal
anti-beta-Actin (1:3000 for WB)	JJ09-29	GeneTex
anti-CYLD (1:1000 for WB)	8462	Cell Signaling Technology
anti-DUB3 (1:1000 for WB)	26143-1-AP	Proteintech
anti-FDFT1 (1:1000 for WB)	13128-1-AP	Proteintech
anti-Flag (1:3000 for WB)	sc-166355	Santa Cruz
anti-FSP1 (1:200 for IHC, 1:1000 for WB)	20886-1-AP	Proteintech
anti-FTH1 (1:1000 for WB)	83428-1-RR	Proteintech
anti-gamma-H2AX (1:1000 for IF)	9718	Cell Signaling Technology
anti-GFP (1:3000 for WB)	sc-390394	Santa Cruz
anti-GPX4 (1:1000 for WB)	67763-1-Ig	Proteintech
anti-GST (1:3000 for WB)	66001-2-Ig	Proteintech
anti-PML (1:3000 for WB)	sc-966	Santa Cruz
anti-SMURF1 (1:100 for IHC, 1:1000 for WB)	ab57573	Abcam
anti-STUB1 (1:1000 for WB)	ab134064	Abcam
anti-TRIM33 (1:1000 for WB)	90051	Cell Signaling Technology
anti-Ub (1:3000 for WB)	sc-8017	Santa Cruz
anti-UBE4A (1:1000 for WB)	21548-1-AP	Proteintech
anti-UBE4B (1:1000 for WB)	ab97697	Abcam
anti-USP11 (1:1000 for WB)	ab109232	Abcam
anti-USP29 (1:100 for IHC, 1:1000 for WB)	27522-1-AP	Proteintech
anti-USP33 (1:1000 for WB)	20445-1-AP	Proteintech
anti-USP43 (1:1000 for WB)	PA5-55684	Invitrogen
anti-ZEB2 (1:1000 for WB)	14026-1-AP	Proteintech
ant-SMURF2 (1:1000 for WB)	ab313470	Abcam

Supplementary Table 4. shRNAs sequence used in this study

Gene symbol	shRNAs	Sequence (5'-3')
USP29	#1	CCCTCAATCAGTCTACAGAAT
USP29	#2	TGTGTGGAGTATCTTGGTGTA
USP29	#3	CTGGTGAAGAATAACGAGCAA
USP29	#4	GCAGTGTATTGAGGAGAGCAT
USP29	#5	CCACTTTAGAGATAGGGCAAT
DUB3	#1	GGAAATAAGTCGTATTGATAA
DUB3	#2	GGATCATTCTCGCCACCTTGA
DUB3	#3	GCCACCTTGAATAGTGGAAAC
DUB3	#4	GGCGGCACGAGAAGGTGAAAT
DUB3	#5	GCCTGTTTCACGATGTCCAAT
USP11	#1	CCCTCCCTTCTAGTCTTTATT
USP11	#2	CCGTGATGATATCTTCGTCTA
USP11	#3	CCGATTCTATTGGCCTAGTAT
USP11	#4	CCGTGACTACAACAACTCCTA
USP11	#5	CCGATTCTATTGGCCTAGTAT
USP33	#1	GTGGAATTTGTCAGCAGATAT
USP33	#2	CCTCCGGTTGTTCATGTTGAT
USP33	#3	CTGGATATAGAAGCGGATGAA
USP33	#4	GCAACAGTGATAGAGCAGAAA
USP33	#5	GCCTCAGAACATTTGGGATAA
CYLD	#1	TATATGTGCATGTACCAGAGT
CYLD	#2	CATCTTCAGATGTTGGTATCT
CYLD	#3	AAGAAGGTCGTGGTCAAGGT
CYLD	#4	CCCATTAAATTCAGTGTAGTT
CYLD	#5	GCAGCGTTACAGACAAACAAA
USP43	#1	CGTTCTTGATTCGGGAAGATA
USP43	#2	GCAGAGTTCAAGAATGCTGTT
USP43	#3	GCAGAGGGTGACAATGTGTAT
USP43	#4	CTTACCCAAGATGAAGTCTAA
USP43	#5	GACTTCCTCTACGACTTGTAT
FSP1	#1	GATTCTCTGCACCGGCATCAA
FSP1	#2	GTACGTGCTTGTGGTCATGAC
SMURF1	#1	CCACTTGTCTTATTTCCACTT
SMURF1	#2	CGATGGTTTGTTTGGTCCTTT

Supplementary Table 5. qRT-PCR primer sequence used in this study

Gene symbol	Primer	Sequence (5'-3')
ACTB	Forward	CATGTACGTTGCTATCCAGGC
ACTB	Reverse	CTCCTTAATGTCACGCACGAT
FSP1	Forward	AGACAGGGTTCGCCAAAAAGA
FSP1	Reverse	CAGGTCTATCCCCACTACTAGC
USP29	Forward	GAAACTCGGGCCTTCATTCAA
USP29	Reverse	CTGTGCTCTGGTTCCAATGG