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Methods and materials

Analysis of public datasets

Total RNA expression profiling data from 12 ovarian cancer (OV) patients and 57 healthy donors were obtained from the GSE66957 dataset available in the Gene Expression Omnibus (GEO) database. In addition, The Cancer Genome Atlas (TCGA) OV and colon adenocarcinoma (COAD) datasets were retrieved from the UCSC Xena platform (https://xenabrowser.net).

Cell culture

The colorectal cancer (CRC) cell lines RKO and SW620 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The OV cell lines SKOV3 and A2780 were kindly provided by Dr. Yihua WU (Zhejiang University School of Medicine, Hangzhou, China). The human embryonic kidney 293T (HEK293T) cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The normal human colonic epithelial cell line NCM460 was obtained from EK-Bioscience (Shanghai, China).

The RKO, SKOV3, A2780, and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, NY, USA), while SW620 and NCM460 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, NY, USA). All media were supplemented with 10% (volume fraction) fetal bovine serum (FBS) (C04001-500, Vivacell, Shanghai, China) and 1% (volume fraction) penicillin/streptomycin (BL505A, Biosharp, Hefei, China). Cells were dissociated using 0.03% (0.3 g/L) trypsin containing ethylenediaminetetraacetic acid (EDTA) and maintained in a cell culture incubator at 37 °C with 5% CO_2. Plasmids were transfected into CRC and OV cell lines using the Neofect™ DNA transfection reagent kit (TF201201, Neofect, Beijing, China) following the manufacturer's protocol, while HEK293T cells were transfected using LipoD293 (SL100668, SignaGen, Changzhou, China).

Generation of TAF1 knockout (KO) cells

In order to generate TATA-box binding protein-associated factor 1 (TAF1)-knockout (KO) cells, the pLentiCRISPR v2-TAF1 plasmid was co-transfected with two lentiviral packaging plasmids, psPAX2 and pMD2.G, into HEK293T cells using LipoD293 according to the manufacturer's instructions. After 48 h, the culture medium containing viral particles was collected and used to infect RKO, SW620, SKOV3, and A2780 cell lines for 24 h. Following infection, puromycin was added to the culture medium for selection. After two weeks of selection, surviving cells were individually plated into 96-well plates. These subclones were expanded and screened for TAF1 KO by sequencing and immunoblotting (IB), and cells that did not exhibit the KO were considered Mock cells. The single guide RNA (sgRNA) sequence used for the KO was listed in Table 1.

Table 1 CRISPR sgRNA sequence used in this study

Target	Sequence
TAF1	CCGAAGATACCAGCAGACGA

CRISPR: clustered regularly interspaced short palindromic repeats; sgRNA: single guide RNA; TAF1: TATA-box binding protein-associated factor 1.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from the indicated cells using TRIzol (15596018, Invitrogen, CA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using HiScript II reverse transcriptase (R223-01, Vazyme, Nanjing, China), followed by qPCR using SYBR qPCR Master Mix (Q711-02, Vazyme, Nanjing, China). The gene-specific primers used for amplification were listed in Table 2

Table 2 qPCR primer sequences used in this study

Target	F	R
SLC7A11	GGACAAGAAACCCAGGTGGT	GCAGATTGCCAAGATCTCAAGT
GPX4	CAGTGAGGCAAGACCGAAGT	CCGAACTGGTTACACGGGAA
GAPDH	CCCTTCATTGACCTCAACTACATG	TGGGATTTCCATTGATGACAAGC

qPCR: quantitative real-time polymerase chain reaction; F: forward; R: reverse; SLC7A11: solute carrier family 7 member 11; GPX4: glutathione peroxidase 4; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Immunoblotting and immunoprecipitation

Cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) Lysis Buffer (P0013C, Beyotime, Shanghai, China) supplemented with phenylmethylsulfonyl fluoride (PMSF) (ST505, Beyotime, Shanghai, China) and a protease inhibitor cocktail (HY-K0010, MCE, NJ, USA). The lysates were sonicated on ice to ensure complete cell lysis and then centrifuged at 13 300 r/min for 10 min at 4 °C to collect the supernatant. The protein concentration was determined using the bicinchoninic acid (BCA) assay (23225, Thermo Scientific, MA, USA).

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes (HATF00010, Merck Millipore, MA, USA), and blocked with 50 g/L skimmed milk. Membranes were incubated overnight at 4 °C with primary antibodies. After 1 h of incubation with secondary antibodies at room temperature, protein signals were detected using the Odyssey Imaging System (LI-COR, NE, USA).

For the immunoprecipitation (IP) of exogenously expressed FLAG-tagged, hemagglutinin (HA)-tagged, or green fluorescent protein (GFP)-tagged proteins, cells were harvested 48 h post-transfection and lysed in IP Lysis Buffer (P0013J, Beyotime, Shanghai, China), supplemented with phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail. The lysates were sonicated, centrifuged at 13 300 r/min for 10 min at 4 °C to obtain the supernatant, and then incubated overnight at 4 °C with anti-FLAG M2 magnetic beads (M8823, Sigma, MO, USA) or anti-HA magnetic beads (SAE0197, Sigma, MO, USA) in a rotating incubator. The immunoprecipitates were washed several times with tris-buffered saline (TBS) (ST661, Beyotime, Shanghai, China) and analyzed by western blotting (WB).

To detect the ubiquitination of FLAG-tagged glutathione peroxidase 4 (GPX4), cells were transfected for 48 h and then treated with MG132 (10 μmol/L; HY-13259, MCE, NJ, USA) for 4 h. Cells were then lysed in RIPA Lysis Buffer supplemented with PMSF and protease inhibitor cocktail. The lysates were incubated overnight at 4 °C with anti-FLAG M2 magnetic beads. Afterwards, the beads were washed several times with TBS and analyzed by WB.

All IB and IP experiments were performed independently at least three times, and the representative results are shown. Protein decay curves are presented as the mean±standard deviation (SD) of three independent experiments. The plasmids and antibodies used in this study were listed in Tables 3 and 4, respectively.

Table 3 Plasmids used in this study

Plasmid name	Vector	Application
TAF1 KO sgRNA	pLentiv-V2	CRISPR/Cas9
GFP-TAF1	pCS2+	Overexpression and IP
FLAG-TAF1	pCDNA3.1	Overexpression and IP
FLAG-GPX4	pCDNA3.1	Overexpression and IP
HA-GPX4	pCDH	Overexpression and IP
FLAG-TBP	pCDNA3.1	Overexpression and IP
FLAG-DUF	pCDNA3.1	Overexpression and IP
FLAG-ZINC	pCDNA3.1	Overexpression and IP
FLAG-BRD	pCDNA3.1	Overexpression and IP
HA-Ub	pCDNA3.1	Overexpression and IP
HA-Ub-K6	pCDNA3.1	Overexpression and IP

HA-Ub-K11	pCDNA3.1	Overexpression and IP
HA-Ub-K27	pCDNA3.1	Overexpression and IP
HA-Ub-K29	pCDNA3.1	Overexpression and IP
HA-Ub-K33	pCDNA3.1	Overexpression and IP
HA-Ub-K48	pCDNA3.1	Overexpression and IP
HA-Ub-K63	pCDNA3.1	Overexpression and IP

TAF1: TATA-box binding protein-associated factor 1; KO: knockout; sgRNA: single guide RNA; CRISPR: clustered regularly interspaced short palindromic repeats; Cas 9: CRISPR-associated protein 9; GFP: green fluorescent protein; GPX4: glutathione peroxidase 4. FLAG-tagged truncated TAF1 variants, each containing one of the following domains: the N-terminal kinase domain (FLAG-TBP (TBP: TATA-box binding protein)), the HAT domain (FLAG-DUF (DUF: domain of unknown function)), the E1/E2 domain (FLAG-ZINC (ZINC: zinc finger domain), or the C-terminal kinase domain (FLAG-BRD (BRD: bromodomain)). Hemagglutinin (HA)-tagged ubiquitin (Ub) mutants, each containing only a single lysine residue (K6, K11, K27, K29, K33, K48, and K63).

Table 4 Antibodies used in this study

Target	Clone	Company	Catalog number	Application
TAF1	Rabbit monoclonal	CST	12781S	WB
TAF1	Rabbit polyclonal	Proteintech	20260-1-AP	IHC
IREB2	Rabbit monoclonal	Abcam	ab181153	WB
ACSL4	Mouse monoclonal	Santa Cruz	sc-271800	WB
TP53	Rabbit monoclonal	CST	2527T	WB
FTL	Rabbit monoclonal	Abcam	ab109373	WB
SLC3A2	Rabbit monoclonal	CST	47213S	WB
SLC7A11	Rabbit monoclonal	CST	12691S	WB
GPX4	Rabbit monoclonal	Abcam	ab125066	WB, IF, and IHC
4-Hydroxynonenal	Rabbit polyclonal	Abcam	ab46545	IHC
NCOA4	Rabbit monoclonal	CST	66849S	WB
FSP1 (AMID)	Mouse monoclonal	Santa Cruz	sc-376987	WB
FTH1	Rabbit monoclonal	CST	4393S	WB
DMT1	Rabbit monoclonal	CST	15083S	WB
GAPDH	Mouse monoclonal	Proteintech	60004-1-Ig	WB
GAPDH	Rabbit polyclonal	Proteintech	10494-1-AP	WB
FLAG	Mouse monoclonal	Sigma Aldrich	F1804	WB
HA	Rabbit monoclonal	Abclonal	AE105	WB

TAF1: TATA-box binding protein-associated factor 1; IREB2: iron response element binding protein 2; ACSL4: acyl-CoA synthetase long chain family member 4; TP53: tumor protein p53; FTL: ferritin light chain; SLC3A2: solute carrier family 3 member 2; SLC7A11: solute carrier family 7 member 11; GPX4: glutathione peroxidase 4; NCOA4: nuclear receptor coactivator 4; FSP1 (AMID): ferroptosis suppressor protein 1; FTH1: ferritin heavy chain 1; DMT1: divalent metal transporter 1; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; HA: hemagglutinin; WB: western blotting; IF: immunofluorescence; IHC: immunohistochemical; Abcam (MA, USA); Abclonal (Wuhan, China); Cell signaling technology (CST; MA, USA); Proteintech (Wuhan, China); Santa Cruz (CA, USA); Sigma Aldrich (MO, USA).

RNA-seq and enrichment analysis

The polyA-RNA of SKOV3-Mock and SKOV3-KO cells treated with dimethyl sulfoxide (DMSO), (1S,3R)-RAS-selective lethal 3 (RSL3) and RSL3+ferrostatn-1 was extracted, sequenced, and analyzed by Bioacme (Wuhan, China). Three biological replicates were used for each condition. The individual RNA sequencing (RNA-seq) libraries were pooled based on their respective sample specific-6 bp adaptors and sequenced at 150 bp/sequence pair-read using an Illumina NovaSeq system. Reads were mapped into the hg19 reference genome by STAR (Dobin et al., 2013) and quantified by relative standard error of the mean (RSEM) (Li and Dewey, 2011). Gene differential expression analysis was accomplished by the DESeq2 (Love et al., 2014) package in R. The Benjamini-Hochberg false discovery rate method was applied to correct for multiple hypothesis testing. The genes with adjust P of <0.05 and fold change of >1 or <-1 were defined as differentially expressed genes (DEGs) and candidates for further analysis. Gene expression heatmap was accomplished with the R package heatmap. Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed using the online tools (Database for Annotation,

Visualization, and Integrated Discovery, DAVID) (https://davidbioinformatics.nih.gov). The results were visualized by the R package ggplot2 in R software.

Immunofluorescence assay

Cells were gently washed three times with phosphate-buffered saline (PBS) at 37 °C before fixation in 4% (volume fraction) paraformaldehyde for 20 min. After permeabilization with 0.5% (volume fraction) Triton X-100 for 10 min, cells were blocked with 10% (volume fraction) fetal bovine serum for 1 h at room temperature, and then incubated with the primary antibody overnight at 4 °C in a humidified chamber. The following day, the cells were incubated with an Alexa Fluor 546-conjugated anti-rabbit secondary antibody (A-11010, Invitrogen, CA, USA) for 1 h at room temperature in the dark. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (C1002, Beyotime, Shanghai, China) for 20 min in PBS. Fluorescence signals were captured using high-resolution confocal microscopy (IX83-FV3000-OSR, Olympus, Tokyo, Japan). Representative images depicting the subcellular localization of each molecule are shown. All confocal analyses were performed in triplicate.

Cellular reactive oxygen species assay

In order to assess intracellular reactive oxygen species (ROS) levels, harvested cells were washed three times with serum-free culture media and then incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (S0033S, Beyotime, Shanghai, China) at 37 °C with 5% CO_2 in the dark for 20 min. Following incubation, cells were washed three additional times with serum-free culture media. The cell suspension was filtered through a 40-μm nylon mesh cell strainer and analyzed using a DxFLEX Flow Cytometer (Beckman, CA, USA).

To evaluate the effects of ferroptosis and other regulated cell death (RCD) inhibitors on ROS levels, cells were co-treated with RSL3 (S8155, Selleck, TX, USA) in combination with ferrostatin-1 (S7243, Selleck, TX, USA), benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (Z-VAD-FMK) (S7023, Selleck, TX, USA), necrostatin-1 (S8037, Selleck, TX, USA), or chloroquine (HY-17589A, MCE, NJ, USA) for the indicated duration before ROS measurement.

Cell viability assay

Equal numbers of cells were seeded into 96-well plates and incubated with increasing concentrations of RSL3 for 48 h. Cell viability was assessed using the cell counting kit-8 (CCK8; AR1160, Boster, Wuhan, China) according to the manufacturer's instructions. Absorbance at 450 nm was measured using a microplate reader.

Lipid peroxidation assay

Lipid peroxidation was assessed using Boron-Dipyrromethene (BODIPY) 581/591 C11 staining (S0043S, Beyotime, Shanghai, China). Cells were washed three times with PBS and incubated with 2 μmol/L BODIPY 581/591 C11 in PBS at 37 ℃ for 30 min in a 5% CO_2 incubator. After staining, cells were washed three times with PBS and fluorescence signals were visualized using a confocal microscope (IX83-FV3000-OSR, Olympus, Tokyo, Japan). The shift in mean fluorescence intensity from the non-oxidized (red) to the oxidized (green) channel was quantified using ImageJ software.

Cellular Fe^{2+} assay

In order to assess the intracellular Fe^{2+} levels via fluorescence microscopy, cells were pre-seeded in glass-bottom cell culture dishes (801001, Nest, Wuxi, China) and incubated overnight at 37 °C in a 5% CO_2 incubator. The following day, the supernatant was carefully removed and cells were washed three times with Hank's balanced salt solution (HBSS) (24020117, Gibco, NY, USA). A working solution of FerroOrange (1 μmol/L; F374, Dojindo, Tabaru, Japan) was then added and cells were incubated for 30 min at 37 °C in a 5% CO_2 incubator. Fluorescent signals were visualized using a high-resolution confocal microscope

(IX83-FV3000-OSR, Olympus, Tokyo, Japan).

Mouse models

All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of Zhejiang University (Ethics Committee No. ZJU20240161).

To evaluate whether TAF1 deletion affects the susceptibility of p53-mutant cells to ferroptosis in vivo, 4×10^6 SW620 TAF1-Mock or SW620 TAF1-KO cells were subcutaneously injected into nude mice (male, 5 weeks old). When the tumor volumes reached 50 mm^3, mice were administered intraperitoneal injections of either 250 μL vehicle (5% DMSO, 40% polyethylene glycol 300 (PEG300), 5% Tween-80, and 50% ddH_2O, volume fraction) or RSL3 (30 mg/kg in the same vehicle; S8155, Selleck, TX, USA) every other day. Tumor diameters were measured every other day and tumor volumes were calculated using the formula of length×width×width×0.5.

Histological analysis

Subcutaneous tumors in mice were fixed in 4% (volume fraction) paraformaldehyde for over 48 h and then paraffin-embedded. Tissue sections were cut to a thickness of 4 μm and subjected to hematoxylin and eosin (H&E) staining and immunohistochemistry. The sections were first dewaxed and rehydrated in xylene and graded ethanol, followed by incubation in 3% (volume fraction) hydrogen peroxide methanol to block endogenous peroxidase activity. Antigen retrieval was performed using citrate buffer (0.01 mol/L, pH 6.0), and tissues were blocked with 10% (volume fraction) normal goat serum for 30 min at room temperature. Sections were then incubated overnight with rabbit anti-TAF1 (1:600; 20260-1-AP, Proteintech, Wuhan, China), anti-GPX4 (1:100; ab125066, Abcam, MA, USA), or anti-4-hydroxynonenal (1:100; ab46545, Abcam, MA, USA) at 4 °C in a humidified chamber. Afterwards, tissues were incubated with a secondary antibody for 30 min at room temperature. The tissues were developed using 3,3-diaminobenzidine (Zhongshan Golden Bridge Biotechnology, Beijing, China), counterstained with hematoxylin, and then dehydrated in graded alcohols. Finally, sections were mounted with coverslips and observed under a light microscope.

Quantifications and statistical analysis

All data are presented as mean±standard deviation (SD). Statistical comparisons between two groups were performed using Student's *t*-test for paired or independent samples. One-way analysis of variance (ANOVA) was used for comparisons among multiple groups. Kaplan-Meier survival analysis was conducted using IBM SPSS Statistics 20, with significance assessed by the log-rank test. A *P*-value of <0.05 was considered statistically significant. Significance levels are indicated as follows: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, and ns (not significant).

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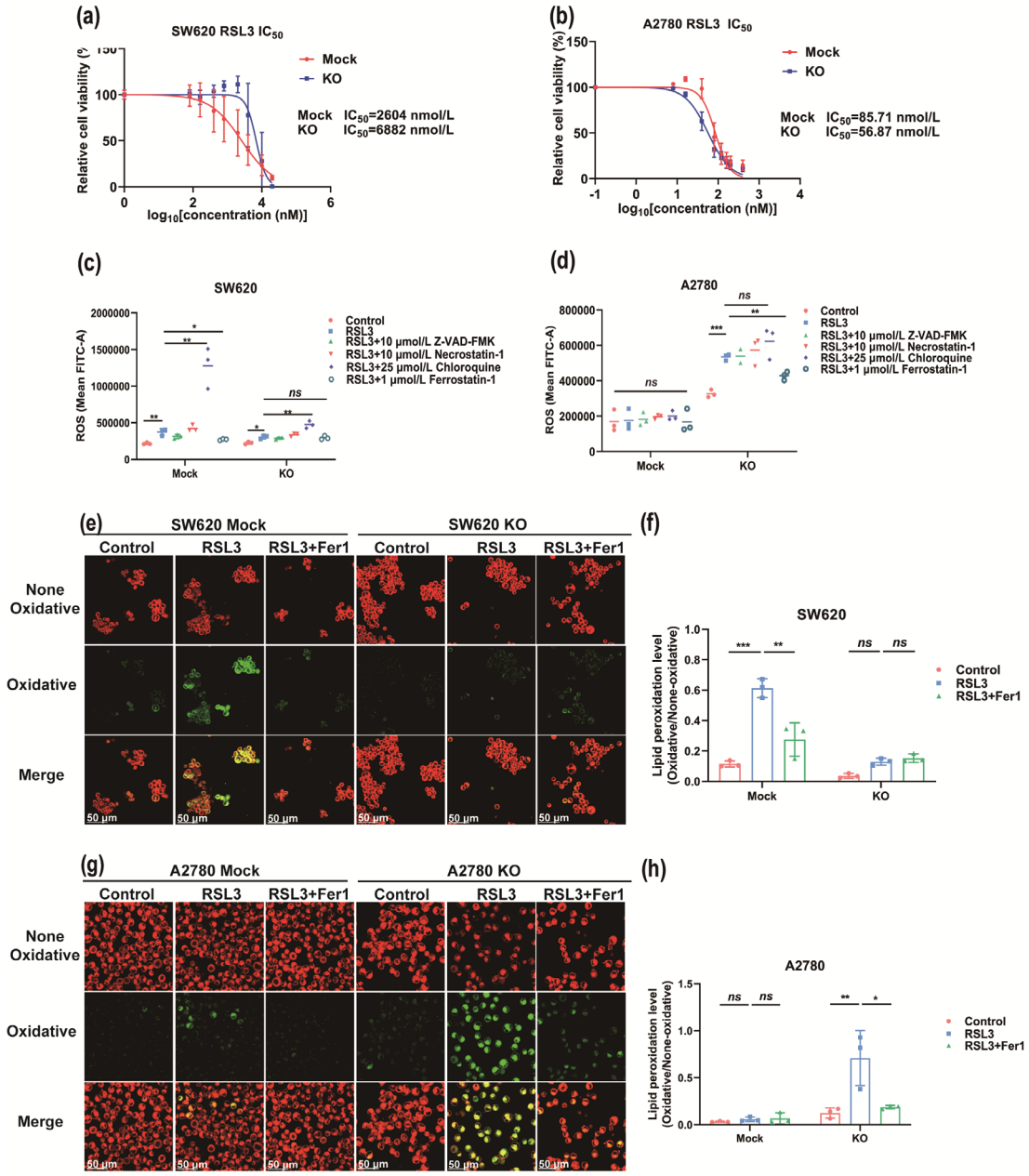

Fig. S1 Validation of ferroptosis in A2780 and SW620 cells. (a) Cell viability of TATA-box binding protein-associated factor 1 (TAF1)-Mock and TAF1-knockout (KO) cells of SW620 (a) and A2780 (b) treated with (1S,3R)-RAS-selective lethal 3 (RSL3) for 48 h at the indicated concentrations. (c) Intracellular reactive oxygen species (ROS) levels in SW620 TAF1-Mock and TAF1-KO cells treated with 2.5 μmol/L RSL3 for 48 h in the absence or presence of benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (Z-VAD-FMK), necrostatin-1, chloroquine, or ferrostatin-1. (d) Intracellular ROS levels in A2780 TAF1-Mock and TAF1-KO cells treated with 40 nmol/L RSL3 for 24 h in the absence or presence of Z-VAD-FMK, necrostatin-1, chloroquine, or ferrostatin-1. (e, f) Representative images and quantitative analysis of lipid peroxidation (LPO) levels in SW620 TAF1-Mock and TAF1-KO cells treated with 2.5 μmol/L RSL3 for 48 h, with or without ferrostatin-1. (g, h) Representative images and quantitative analysis of LPO levels in A2780 TAF1-Mock and TAF1-KO cells treated with 40 nmol/L RSL3 for 24 h, with or without ferrostatin-1. All experiments were performed in triplicate. The data were presented as the mean±standard deviation (SD) (a, b, f, h) or as the mean (c, d). * P<0.05; ** P<0.01; *** P<0.001; not statistically significant (ns), P>0.05; paired Student's t-tests (c, d), one-way analysis of variance (ANOVA) (f, h). IC_{50}: half maximal inhibitory concentration; FITC-A: fluorescein isothiocyanate-area; Fer1: ferrostatin-1.

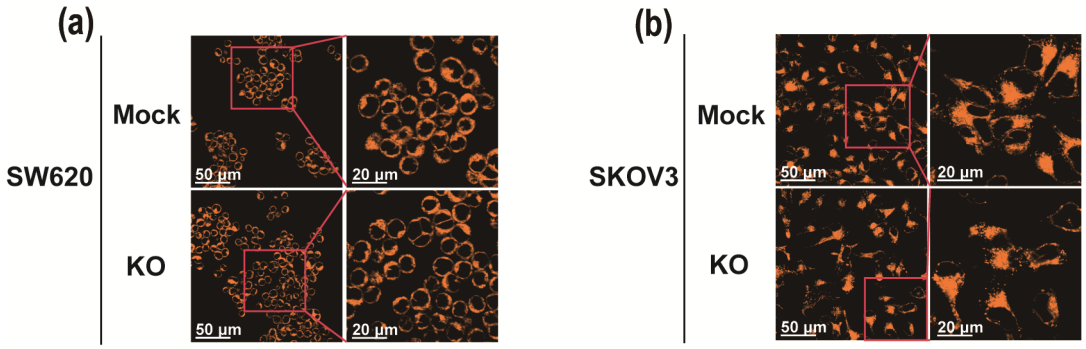

Fig. S2 Intracellular Fe^{2+} in tumor protein p53 (*TP53*)-mut cells. (a) Immunofluorescence detection of intracellular Fe^{2+} in SW620 TATA-box binding protein-associated factor 1 (TAF1)-Mock and TAF1-knockout (KO) cells. (b) Immunofluorescence detection of intracellular Fe^{2+} in SKOV3 TAF1-Mock and TAF1-KO cells. All experiments were performed in triplicate.

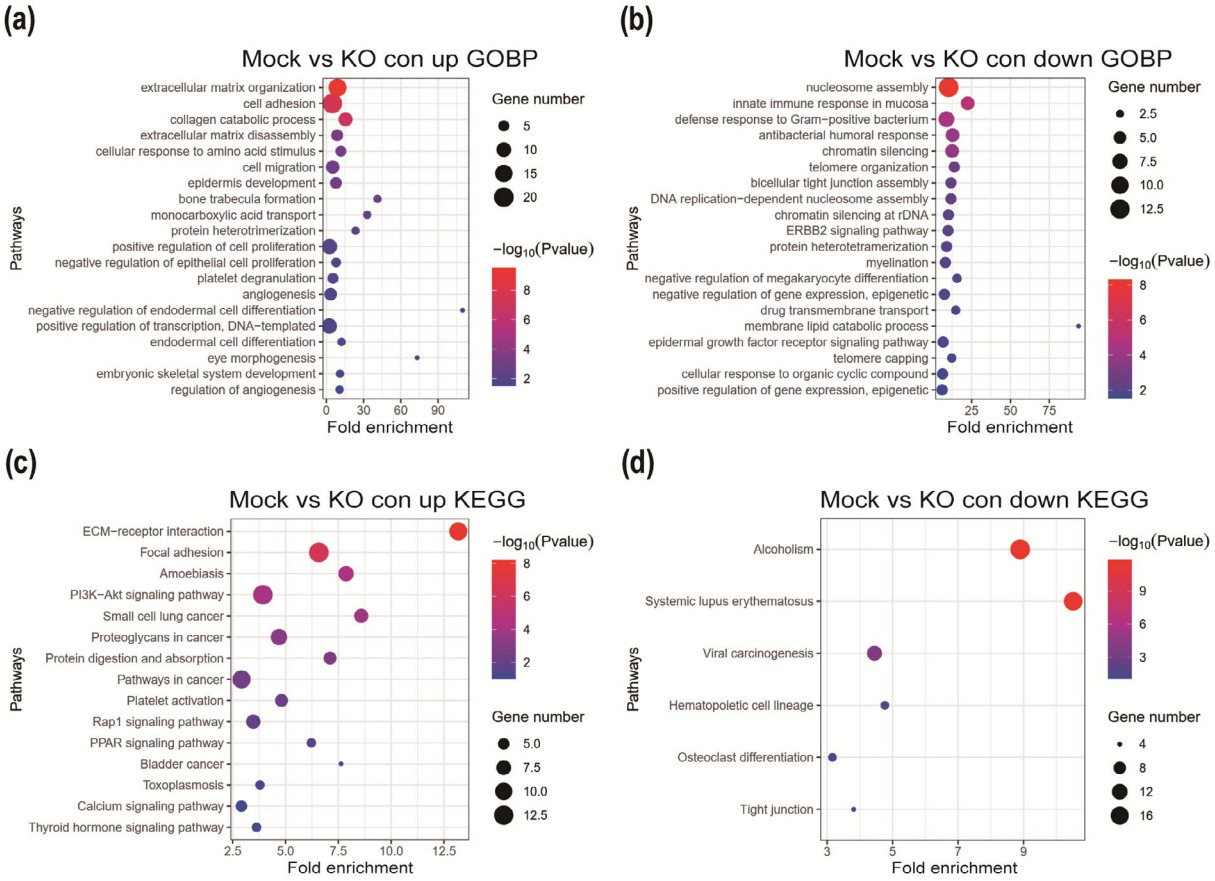

Fig. S3 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. (a) Top 20 upregulated GO pathways in SKOV3 TATA-box binding protein-associated factor 1 (TAF1)-Mock cells compared with TAF1-knockout (KO) cells. (b) Top 20 downregulated GO pathways in SKOV3 TAF1-Mock cells compared with TAF1-KO cells. (c) Top 15 upregulated KEGG pathways in SKOV3 TAF1-Mock cells compared with TAF1-KO cells. (d) Top downregulated KEGG pathways in SKOV3 TAF1-Mock cells compared with TAF1-KO cells.

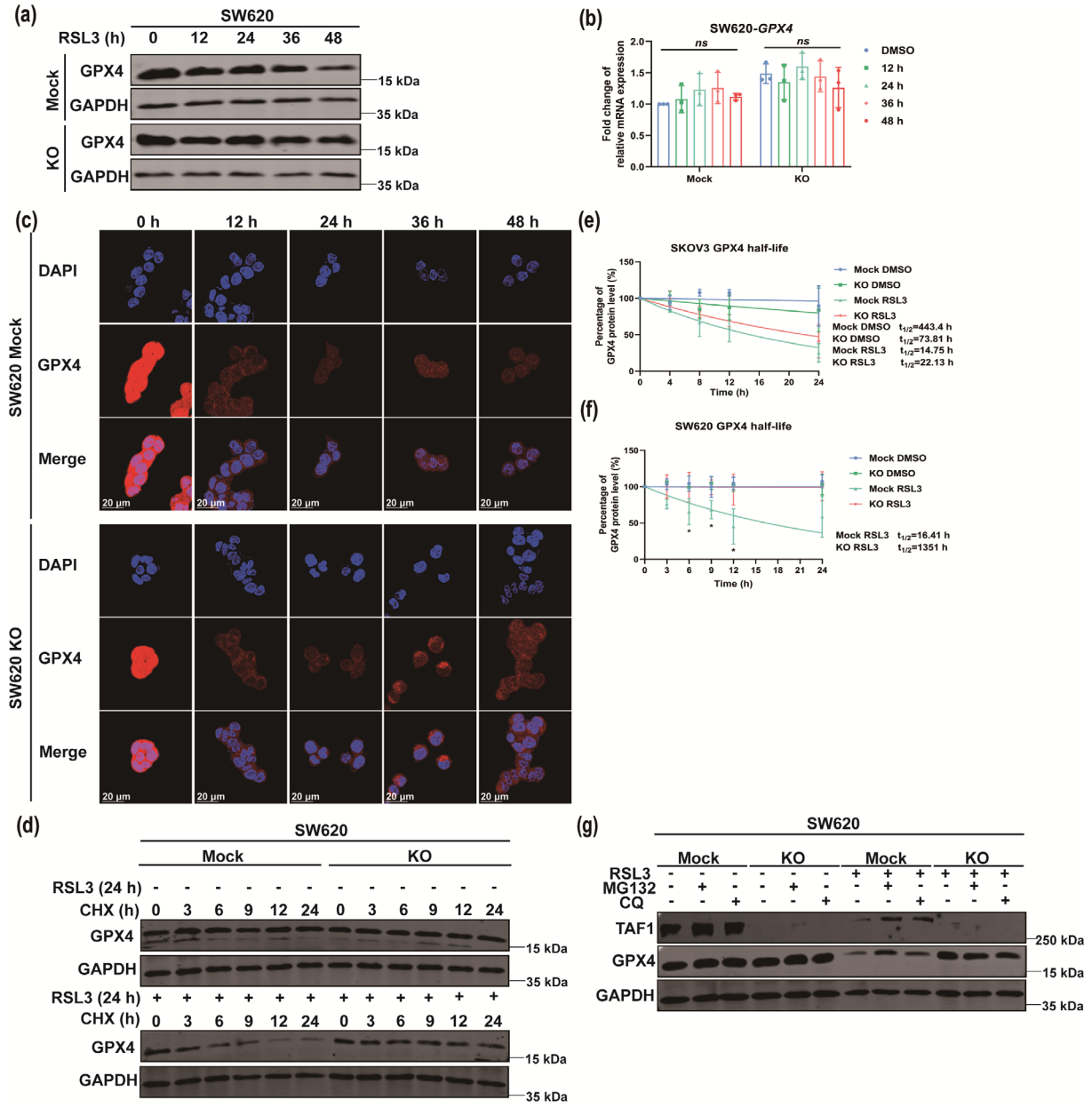

Fig. S4 TATA-box binding protein-associated factor 1 (TAF1)-mediated degradation of nuclear glutathione peroxidase 4 (nGPX4) in SW620 cells. (a) Western blotting (WB) analysis of GPX4 in SW620 TAF1-Mock and TAF1-knockout (KO) cells treated with 2.5 μmol/L (1S,3R)-RAS-selective lethal 3 (RSL3) for the indicated times. (b) Fold changes in relative *GPX4* messenger RNA (mRNA) levels in SW620 TAF1-Mock and TAF1-KO cells treated with 2.5 μmol/L RSL3 for the indicated times. (c) Immunofluorescence staining of GPX4 in SW620 TAF1-Mock and TAF1-KO cells treated with 2.5 μmol/L RSL3 for the indicated times. (d) WB analysis of GPX4 in SW620 TAF1-Mock and TAF1-KO cells treated with 2.5 μmol/L RSL3 for 24 h, followed by incubation with 50 μg/mL cycloheximide (CHX) for the indicated durations. Cells were collected at the indicated time points for protein analysis. (e) CHX chase assay of GPX4 in SKOV3 TAF1-Mock and TAF1-KO cells. Relative protein levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Related to Fig. 4e (f) CHX chase assay of GPX4 in SW620 TAF1-Mock and TAF1-KO cells. Relative protein levels were normalized to GAPDH. Related to Fig. S4d (g) WB analysis of GPX4 in SW620 TAF1-Mock and TAF1-KO cells treated with 2.5 μmol/L RSL3 for 48 h, followed by 10 μmol/L MG132 or 25 μmol/L chloroquine (CQ) for 6 h. All experiments were performed in triplicate, and the data are presented as mean±standard deviation (SD). * *P*<0.05; ns, *P*>0.05; one-way analysis of variance (ANOVA) (b, e, f). DMSO: dimethyl sulfoxide; DAPI: 4',6-diamidino-2-phenylindole.

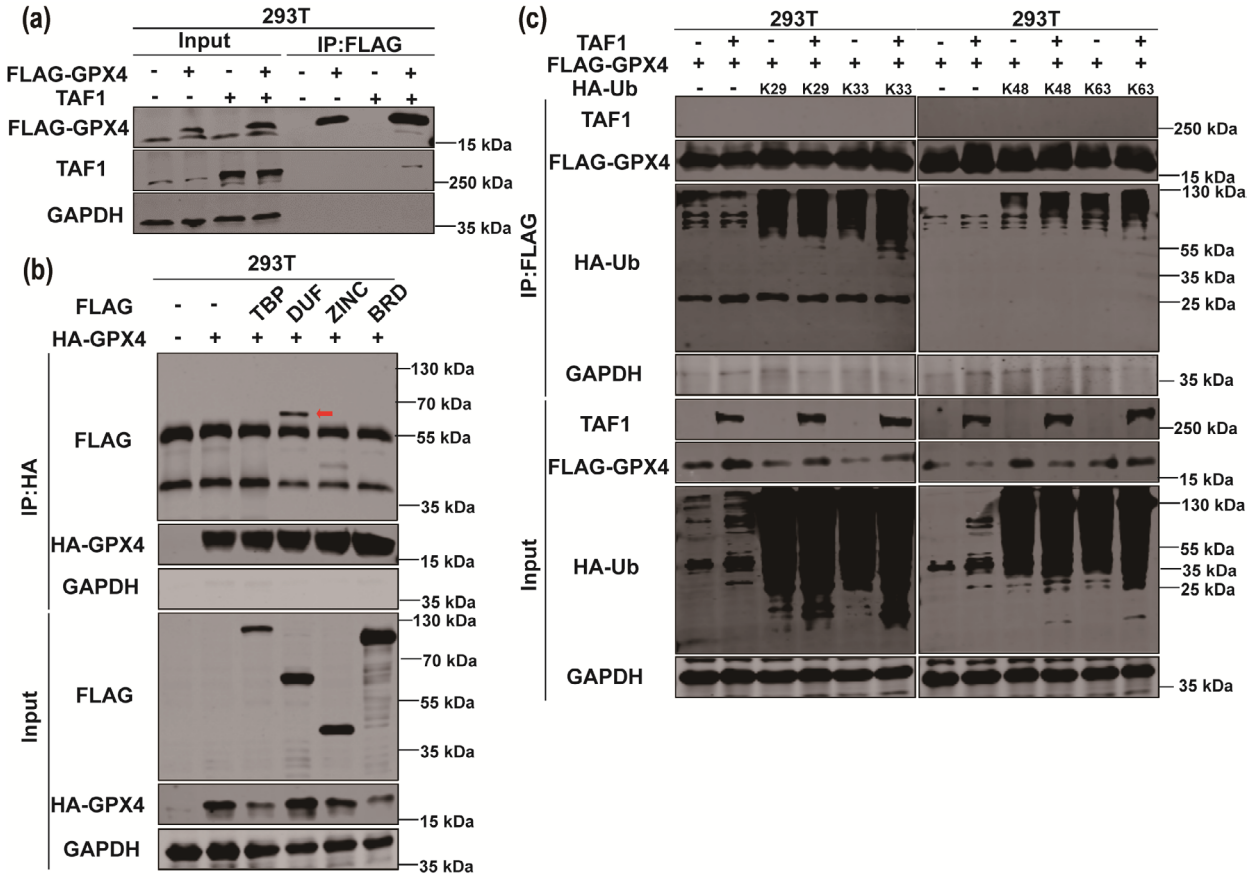

Fig. S5 Interaction between glutathione peroxidase 4 (GPX4) and TATA-box binding protein-associated factor 1 (TAF1). (a) Western blotting (WB) analysis of immunoprecipitated exogenous FLAG-tagged GPX4 and green fluorescent protein (GFP)-tagged TAF1 in HEK293T cells. (b)WB analysis of immunoprecipitated hemagglutinin (HA)-tagged GPX4 and exogenous FLAG-tagged TAF1 truncations in HEK293T cells. (c) HEK293T cells were co-transfected with GFP-TAF1 and FLAG-GPX4 together with different ubiquitin mutants. After treatment with MG132 (10 μmol/L) for 4 h, the cells were collected and subjected to immunoprecipitation and WB analysis. All experiments were performed in triplicate. TBP: TATA-box binding protein; DUF: domain of unknown function; ZINC: zinc finger domain; BRD: bromodomain; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Ub: ubiquitin.