

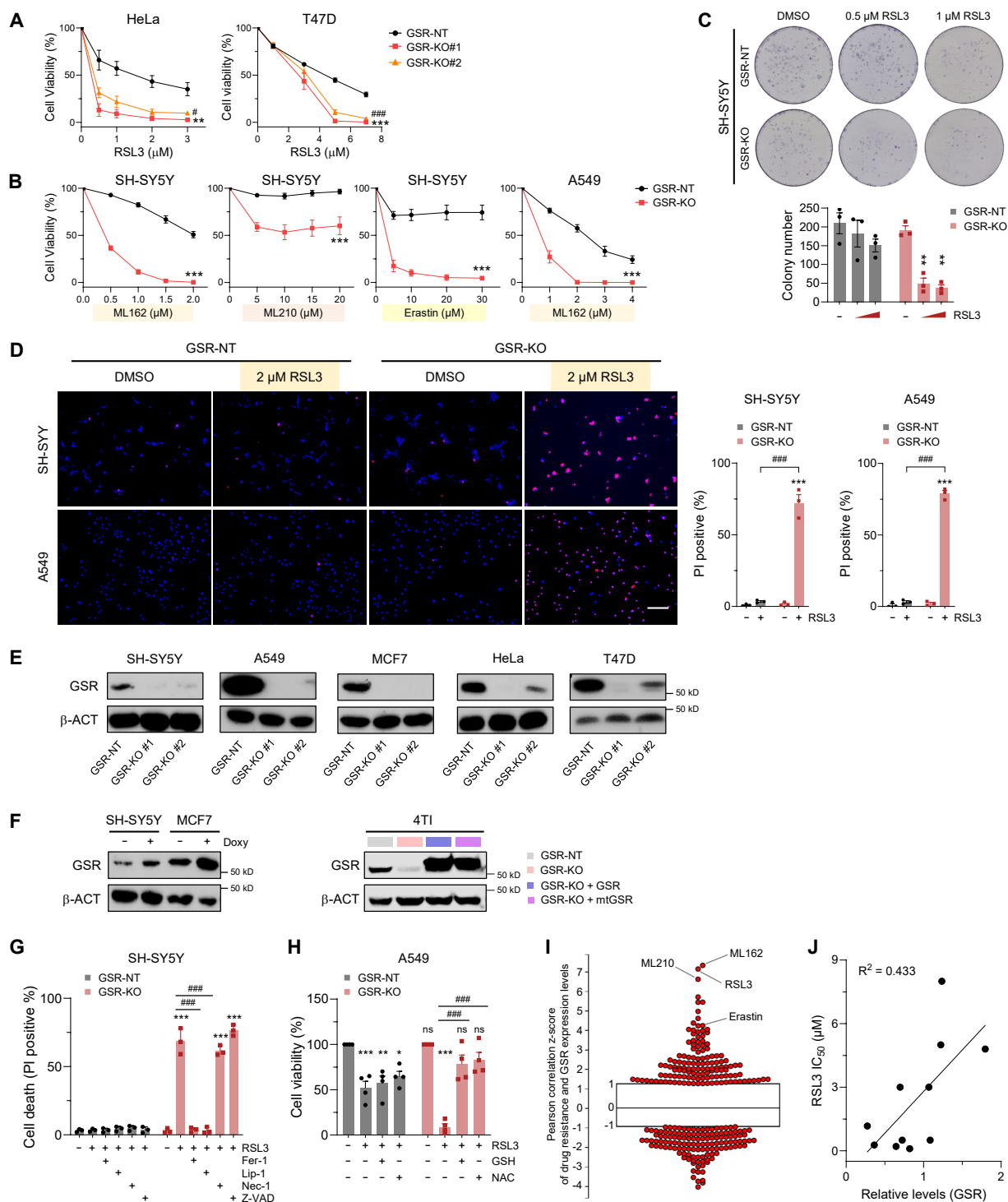
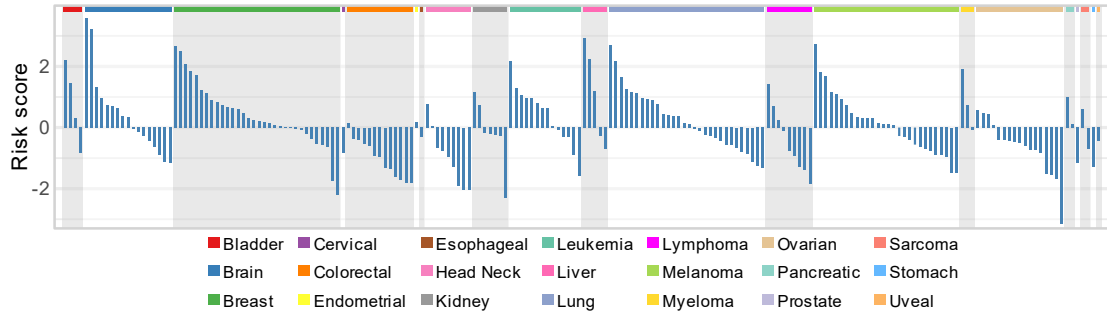


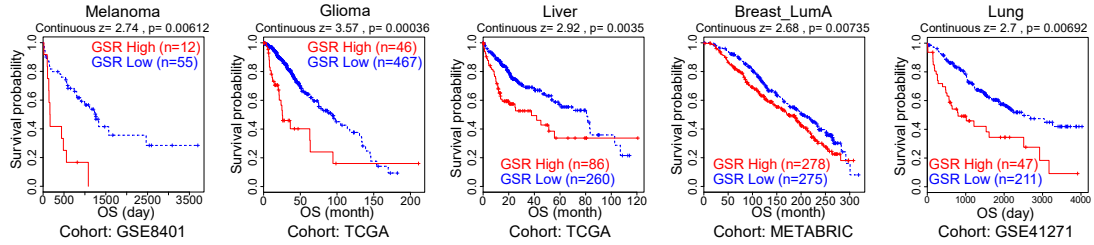
Fig. S1. Genetic deficiency of GSR enhances sensitivity to ferroptosis.

(A) Cell viability in GSR-KO cell lines treated with the ferroptosis inducer RSL3 for 48 h, compared to non-targeting (NT) controls. (B) Cell viability in GSR-KO and NT control cells treated with ferroptosis inducers ML162, ML210, or erastin for 48 h. (C) Representative images and quantification of colony formation in GSR-KO and NT control cells treated with RSL3. (D) Representative images and quantification of cell death rate in GSR-KO and NT control cells treated with 2 μ M RSL3 for 24 h, measured by propidium iodide (PI) staining (Scale bars: 200 μ m). (E, F) Western blotting analysis of GSR levels in GSR-KO (E) and GSR-overexpressing (F) cell lines. (G, H) Cell viability in GSR-KO cells treated with 2 μ M RSL3 for 24 h, compared to NT controls, showing no rescue by apoptosis inhibitor Z-VAD-FMK (10 μ M) or necroptosis inhibitor necrostatin-1 (Nec-1, 2 μ M), but full rescue by ferroptosis inhibitors ferrostatin-1 (Fer-1, 10 μ M) and liproxstatin-1 (Lip-1, 10 μ M) (G) or thiol-containing antioxidants GSH (1 mM) and N-acetylcysteine (NAC, 3 mM) (H). (I) Correlation analysis showing the Pearson z-scores between GSR expression and drug resistance profiles for 481 compounds in the CTRP dataset. (J) Correlation analysis showing a positive relationship between GSR protein levels and RSL3 resistance (IC₅₀) across a panel of 11 cancer cell lines. Statistics were analyzed by unpaired two-tailed Student's *t*-test (B) or one-way ANOVA with Tukey's correction (A, C, D, G, H). Asterisks indicate comparison between NT and KO#1, and hash symbols denote comparison between NT and KO#2 (A).

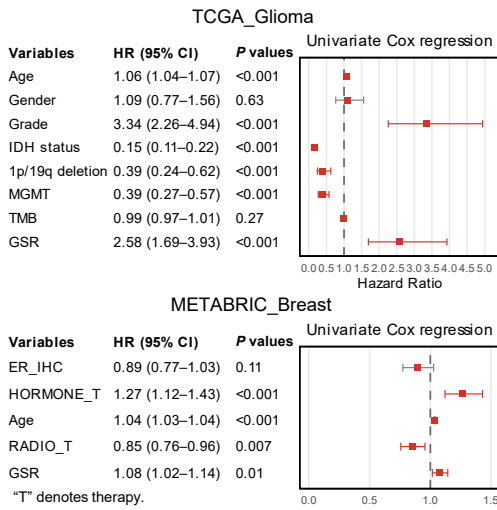
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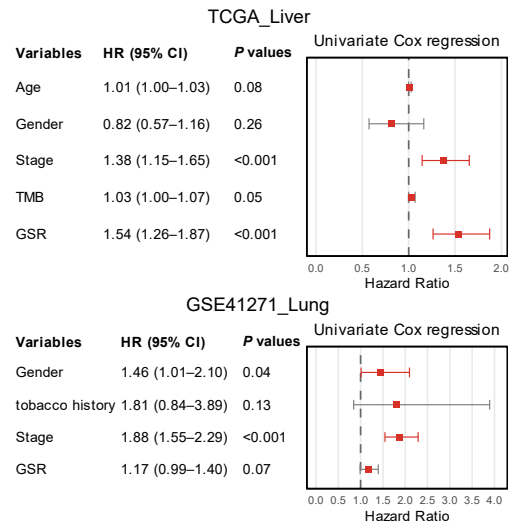
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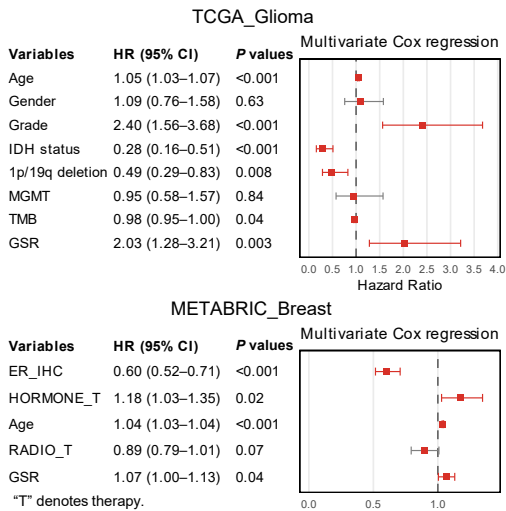
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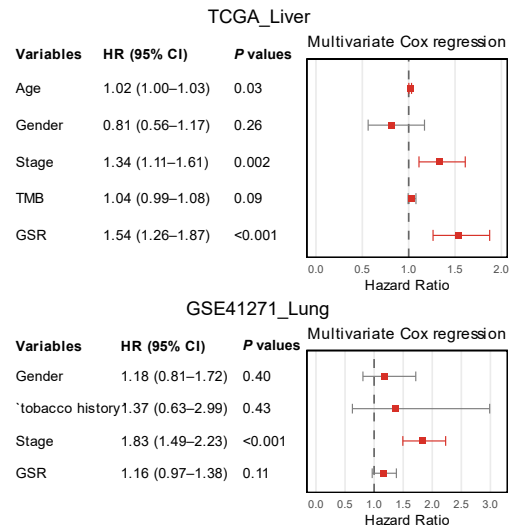


Fig. S2. GSR expression negatively correlates with clinical outcomes.

(A) TCGA pan-cancer analysis showing that elevated GSR expression is associated with higher risk scores across multiple cancer types. (B) Kaplan-Meier survival curves stratified by tumor GSR expression levels across multiple cancer cohorts. (C-F) univariate and multivariate Cox regression analyses across four independent cohorts confirmed GSR as an independent prognostic factor after adjusting for clinical variables.

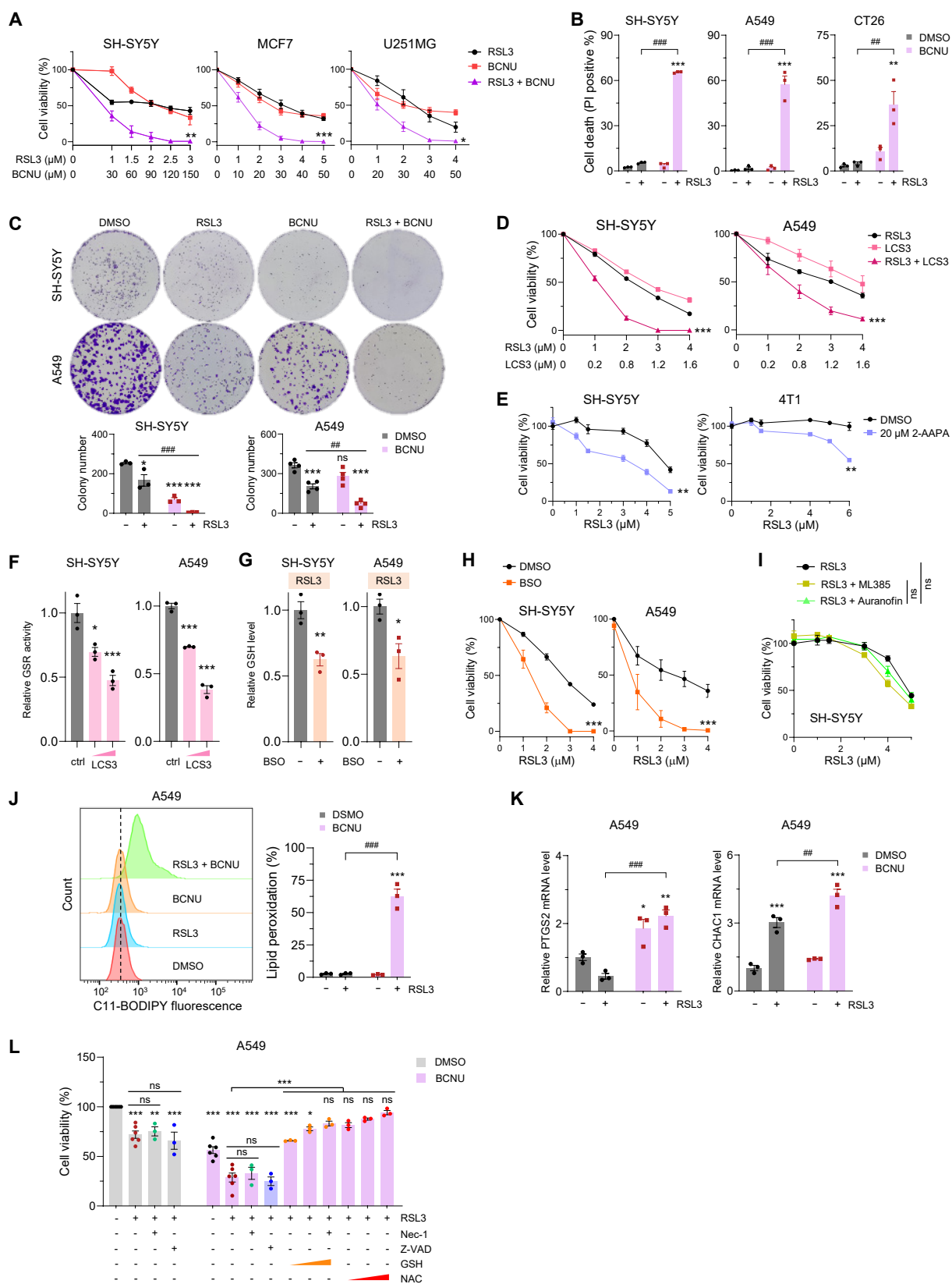


Fig. S3. Pharmacological GSR inhibition enhances ferroptosis sensitivity.

(A-C) Co-treatment with the ferroptosis inducer RSL3 and the GSR inhibitor BCNU for 48 h decreases cell viability (A) and colony formation (C) while increases cell death (B) compared to single treatments. (D, E) Co-treatment with RSL3 and either GSR inhibitor LCS3 (D) or 2-AAPA (E) similarly reduces cell viability compared to individual treatments. (F) GSR enzymatic activity is decreased upon treatment with GSR inhibitors LCS3 (0.8 or 1.6 μ M for 12 h). (G) Cellular GSH levels are reduced after BSO (3 μ M for 24 h) treatment. (H) Co-treatment with RSL3 and the glutamate-cysteine ligase inhibitor BSO reduces cell viability. (I) Co-treatment with RSL3 and inhibitors of NRF2 (ML385) or thioredoxin reductase (auranofin) has minimal effects on cell viability. (J, K) Co-treatment with 3 μ M RSL3 and 90 μ M BCNU for 6 h enhances lipid peroxidation (J) and induces PTGS2 and CHAC1 expression (K). (L) The cytotoxicity induced by RSL3 plus BCNU is not rescued by 10 μ M Z-VAD-FMK or 2 μ M Nec-1, but is fully rescued by 1 mM GSH or 3 mM NAC. Statistics were analyzed by unpaired two-tailed Student's *t*-test (E, G, H) or one-way ANOVA with Tukey's correction (A-D, F, I-L). Asterisks and ns (not significant) without connecting bars indicate comparisons versus the DMSO control (B, C, F, J-L) or RSL3 alone (A, D).

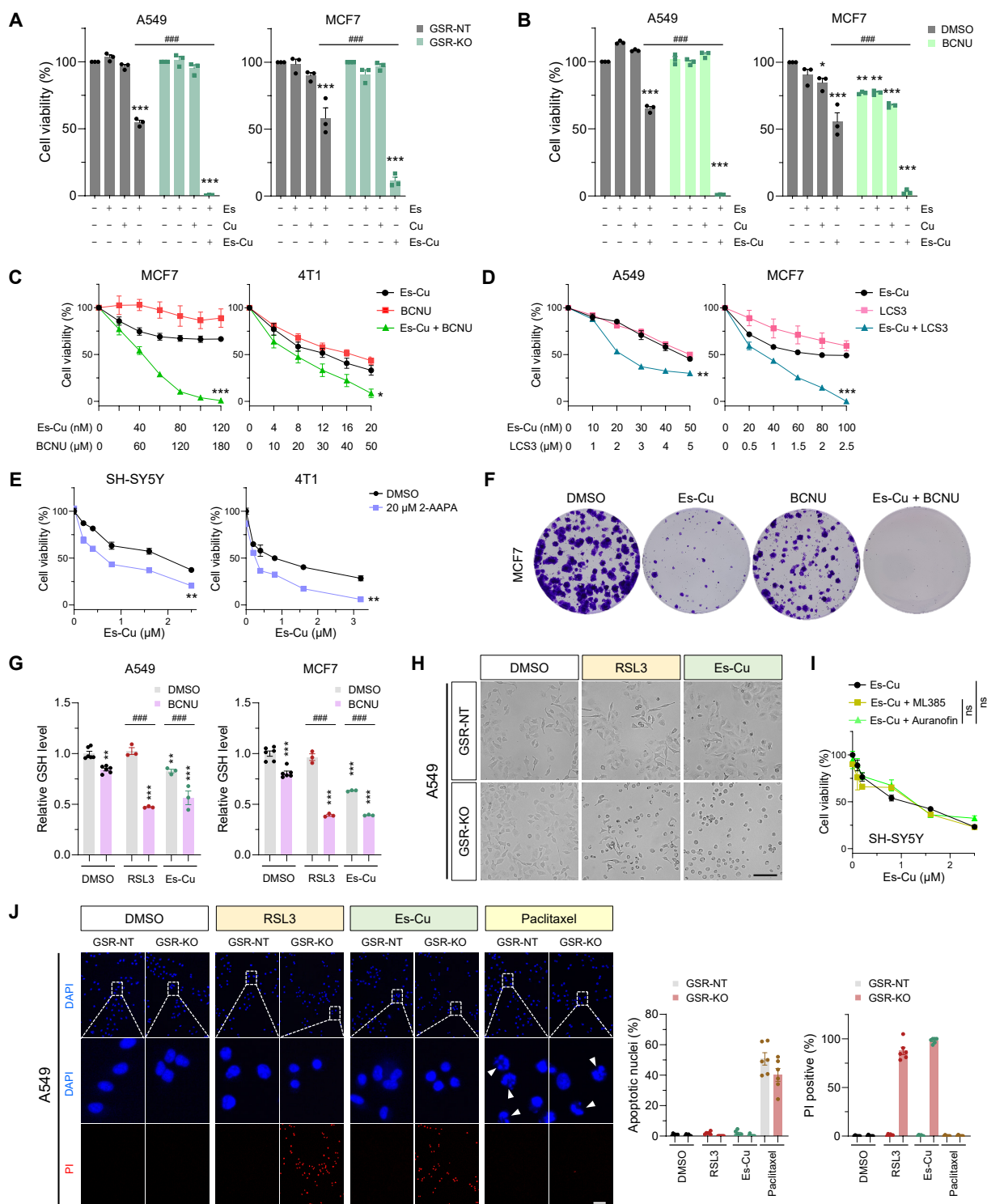


Fig. S4. Shared features of GSR-dependent sensitization to ferroptosis and cuproptosis.

(A, B) Cell viability decreases only upon co-treatment with the 80 nM copper ionophore elesclomol (Es) and 80 nM CuCl₂ (Cu) for 24 h, with further sensitization under genetic (A) or pharmacological (B) GSR inhibition (80 μM BCNU). (C-E) Synergistic reduction in cell viability upon co-treatment with Es-Cu and the GSR inhibitors BCNU (C), LCS3 (D), or 2-AAPA (E) for 24 h. (F) Representative images of colony formation in cancer cells treated with 15 μM BCNU and Es-Cu each of 10 nM, individually or combined. (G) Cellular GSH levels are markedly reduced in cells treated with BCNU plus RSL3 or Es-Cu compared with single treatments for 12 h. (H) Representative images of RSL3- or Es-Cu-induced cell death morphology in NT control and GSR-KO cells (Scale bars: 200 μm). (I) Co-treatment with Es-Cu and inhibitors of NRF2 (ML385) or thioredoxin reductase (auranofin) has minimal effects on cell viability. (J) Nuclear and PI staining in NT or GSR-KO cells treated with 5 μM RSL3, Es-Cu each of 100 nM, or 200 nM paclitaxel for 12 h. RSL3 and Es-Cu cause cell death without nuclear fragmentation, unlike paclitaxel (Scale bar: 100 μm). White arrowheads indicate apoptotic nuclear damage. Statistics were analyzed by unpaired two-tailed Student's *t*-test (E) or one-way ANOVA with Tukey's correction (A-D, G, I, J). Asterisks and ns (not significant) without connecting bars indicate comparisons versus the DMSO control (A, B, G) or Es-Cu alone (C, D, I).

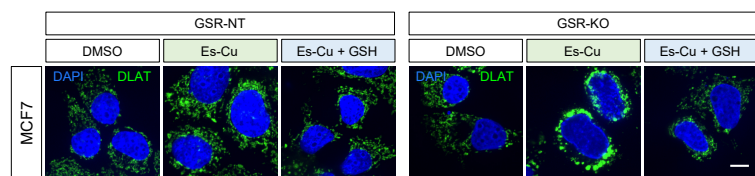
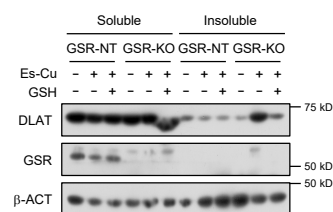
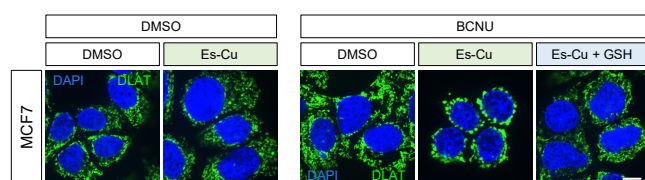
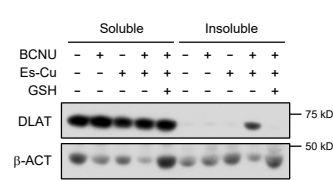
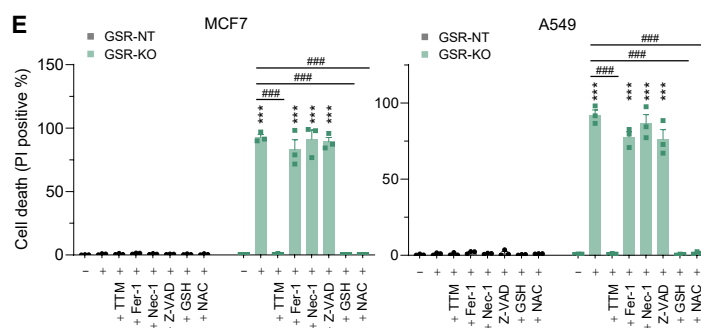
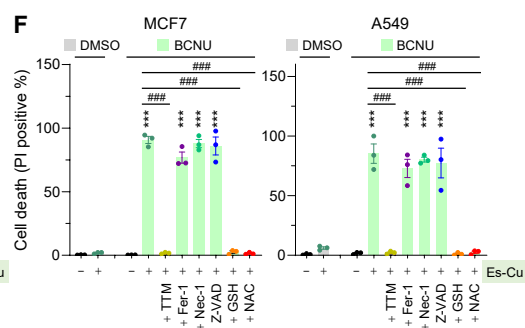
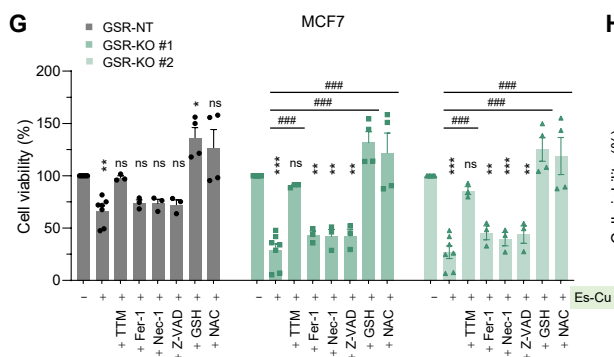
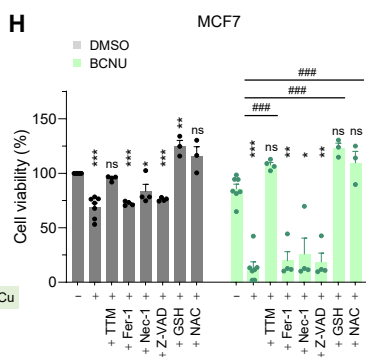
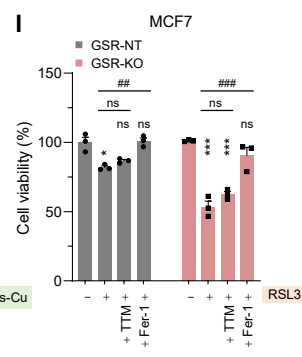
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Fig. S5. GSR deficiency enhances cuproptosis sensitivity.

(A-D) Increased aggregation of dihydrolipoamide S-acetyltransferase (DLAT) in MCF7 cancer cells treated with Es-Cu under genetic (A, B) or pharmacological (C, D) GSR inhibition (Scale bars: 10 μ m). (E, F) Increased cell death induced by Es-Cu under genetic (E) or pharmacological (F) GSR inhibition is rescued by 10 μ M TTM, 1 mM GSH, or 3 mM NAC, but not by 10 μ M Z-VAD-FMK or 2 μ M Nec-1, assessed by resazurin assay. (G, H) Reduced cell viability induced by Es-Cu with GSR inhibition is rescued by TTM, GSH, or NAC, but not by Z-VAD-FMK or Nec-1, assessed by PI staining. (I) RSL3-induced reduction in cell viability in GSR-KO SH-SY5Y cancer cells is rescued by 5 μ M Fer-1, but not by 10 μ M TTM, as assessed by resazurin assay. All statistics were analyzed by one-way ANOVA with Tukey's correction. Asterisks and ns (not significant) without connecting bars indicate comparisons versus the DMSO control.

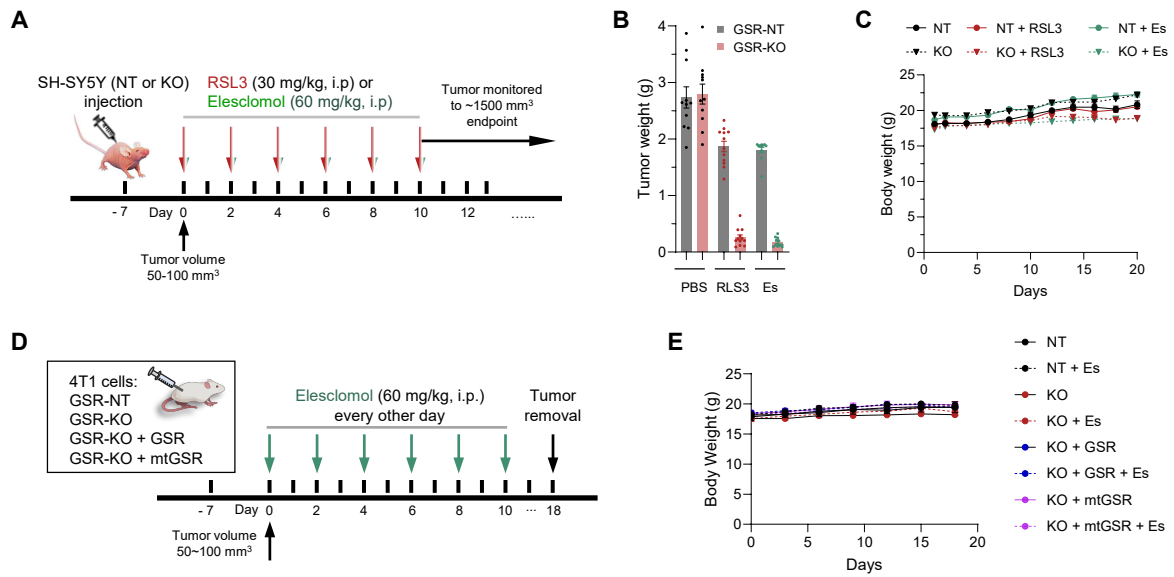


Fig. S6. Xenograft models.

(A) Schematic of the SH-SY5Y neuroblastoma xenograft model and treatment regimen. (B) Tumor weights at the study endpoint. (C) Mouse body weights measured throughout the treatment course. (D) Schematic of the 4T1 xenograft model using GSR-NT, GSR-KO, and GSR-KO cells with genetic reconstitution (GSR or mtGSR), along with the treatment regimen. (E) Mouse body weights measured throughout the treatment course.

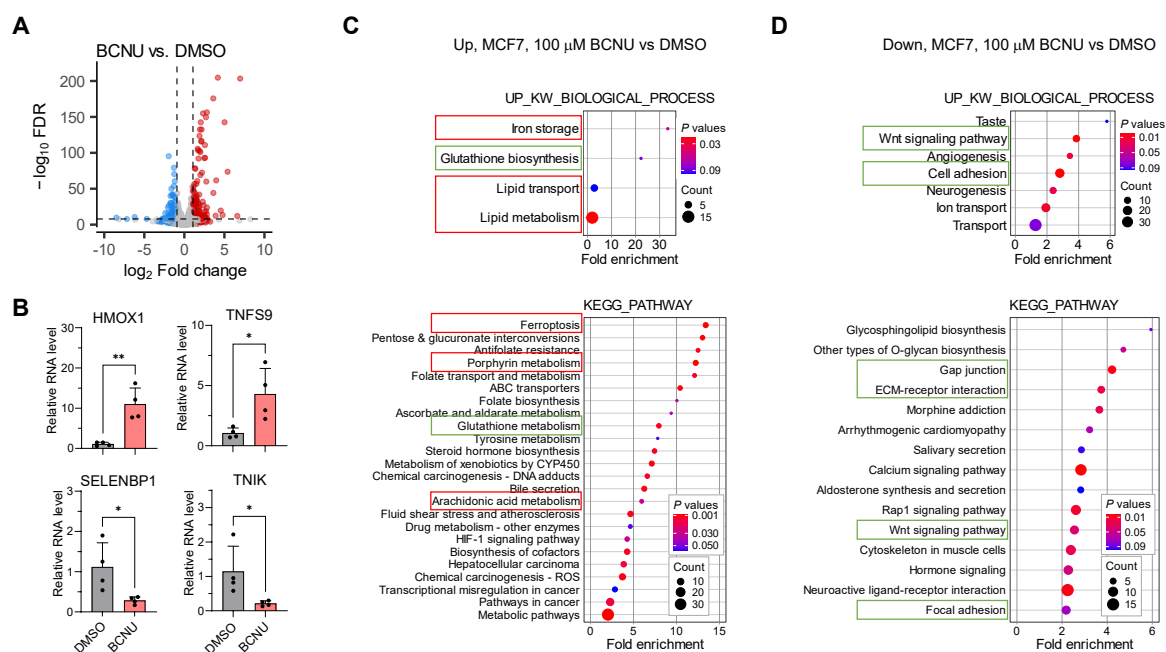


Fig. S7. KEGG pathway analysis of BCNU-treated cells.

(A) Volcano plot of differentially expressed genes following BCNU treatment. (B) Among the genes differentially expressed upon BCNU treatment identified by RNA-seq, the expression changes of HMOX1, TNFSF9, SELENBP1, and TNIK were validated by qPCR. (C, D) KEGG pathway analysis categorizing differentially expressed genes into biological processes. Green boxes highlight pathways promoting cell proliferation and survival; red boxes indicate ferroptosis-related pathways.

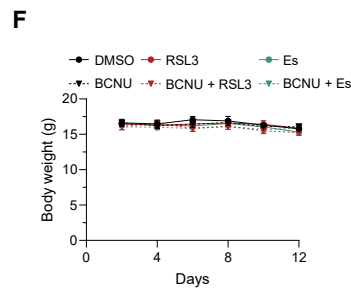
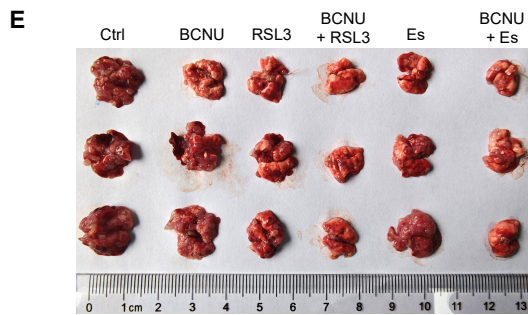
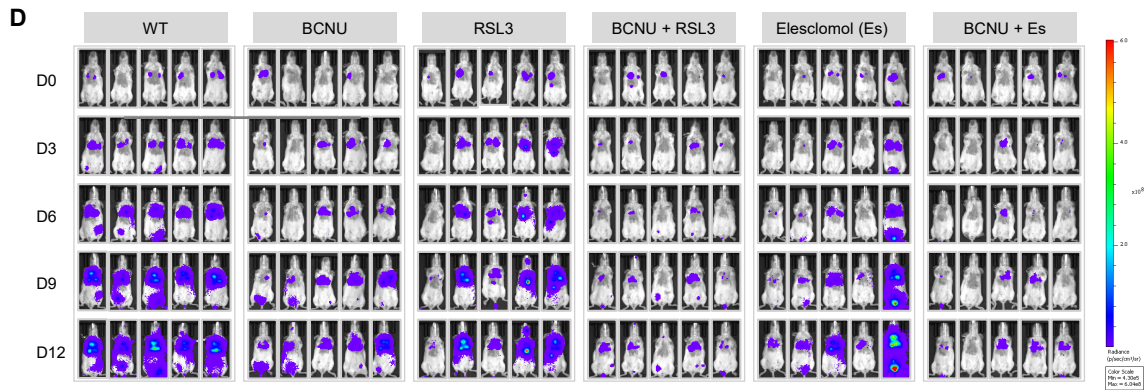
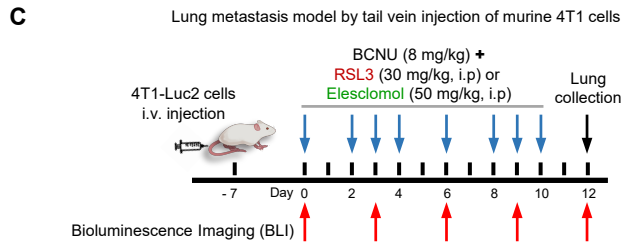
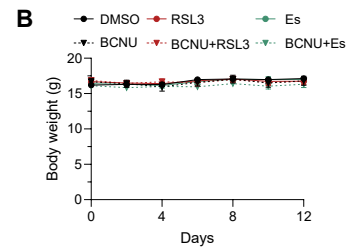
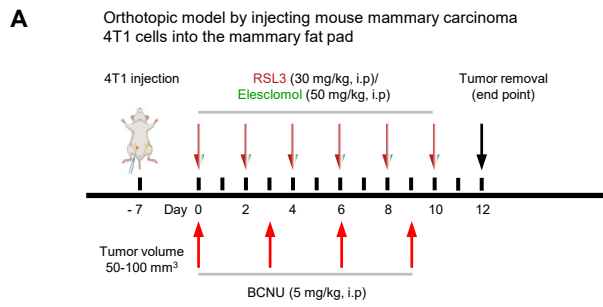


Fig. S8. Syngeneic orthotopic and experimental lung metastasis models of breast cancer.

(A) Schematic of 4T1 tumor inoculation into the mammary fat pad and treatment regimen. (B) Mouse body weights measured throughout the treatment course. (C) Schematic of 4T1-luc intravenous tumor inoculation and treatment regimen. (D) Bioluminescent images of 4T1-luc tumor-bearing mice throughout the treatment course under indicated treatments. (E) Representative images of lungs at the study endpoint. (F) Mouse body weights measured throughout the treatment course.

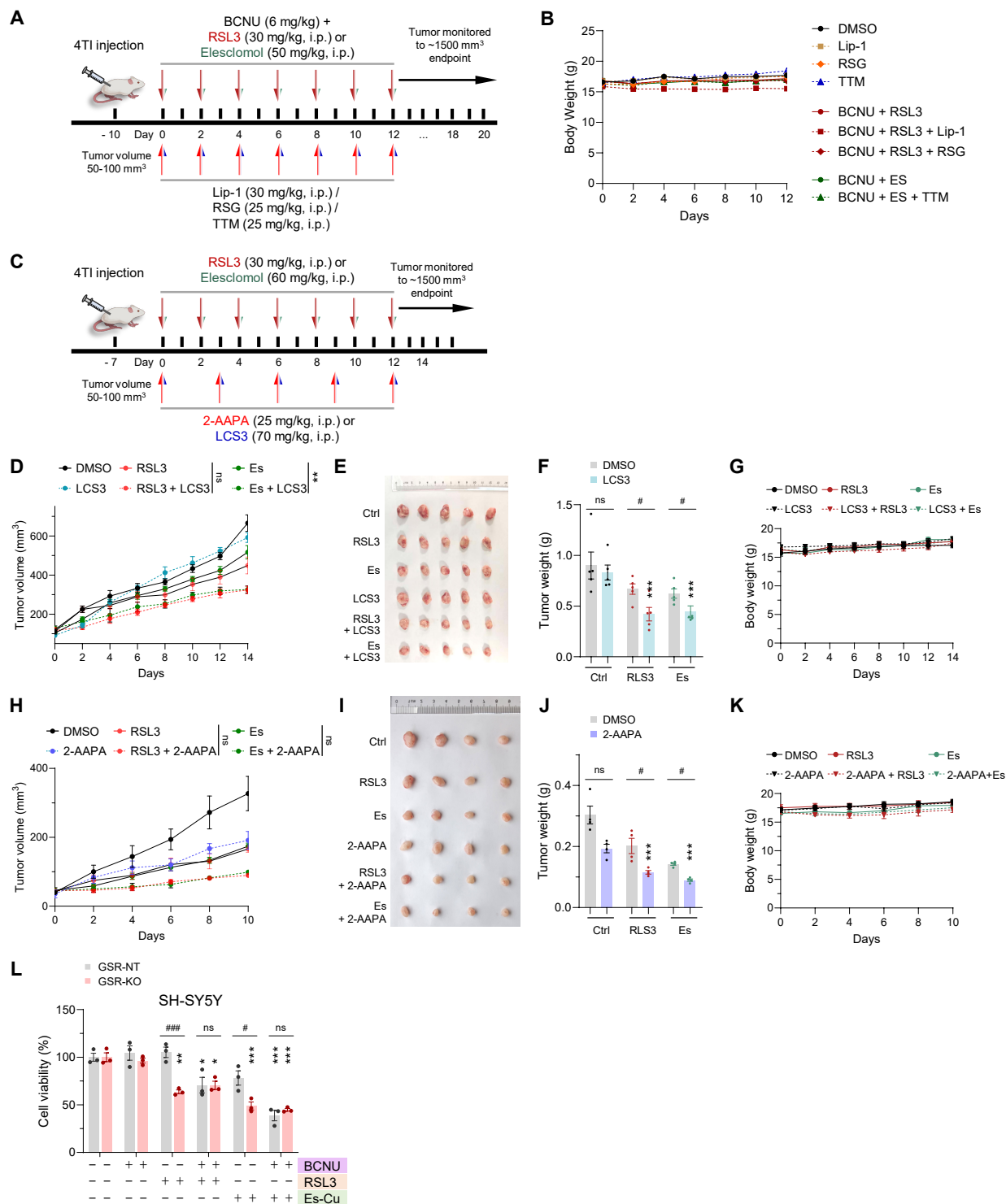


Fig. S9. Enhanced antitumor efficacy upon combinational treatments.

(A) Schematic of 4T1 xenograft model and treatment regimen using cell death-specific inhibitors. (B) Mouse body weights measured throughout the treatment course. (C) Schematic of 4T1 xenograft model and treatment regimen using different GSR inhibitors. (D-G) Tumor growth curves, final tumor weights, and measurements mouse body weights of 4T1 tumors treated with RSL3 or Es in combination with GSR inhibitor LCS3. (H-K) Tumor growth curves, final tumor weights, and measurements mouse body weights of 4T1 tumors treated with RSL3 or Es in combination with GSR inhibitor 2-AAPA. (L) Cell viability in GSR-KO and NT control SH-SY5Y cells treated with RLS3, Es-Cu, or BCNU alone, or with BCNU in combination with RSL3 or Es-Cu, for 24 h. All statistics were analyzed by one-way ANOVA with Tukey's correction. Asterisks indicate comparison between the control and the indicated drug treatment (F, J, L).

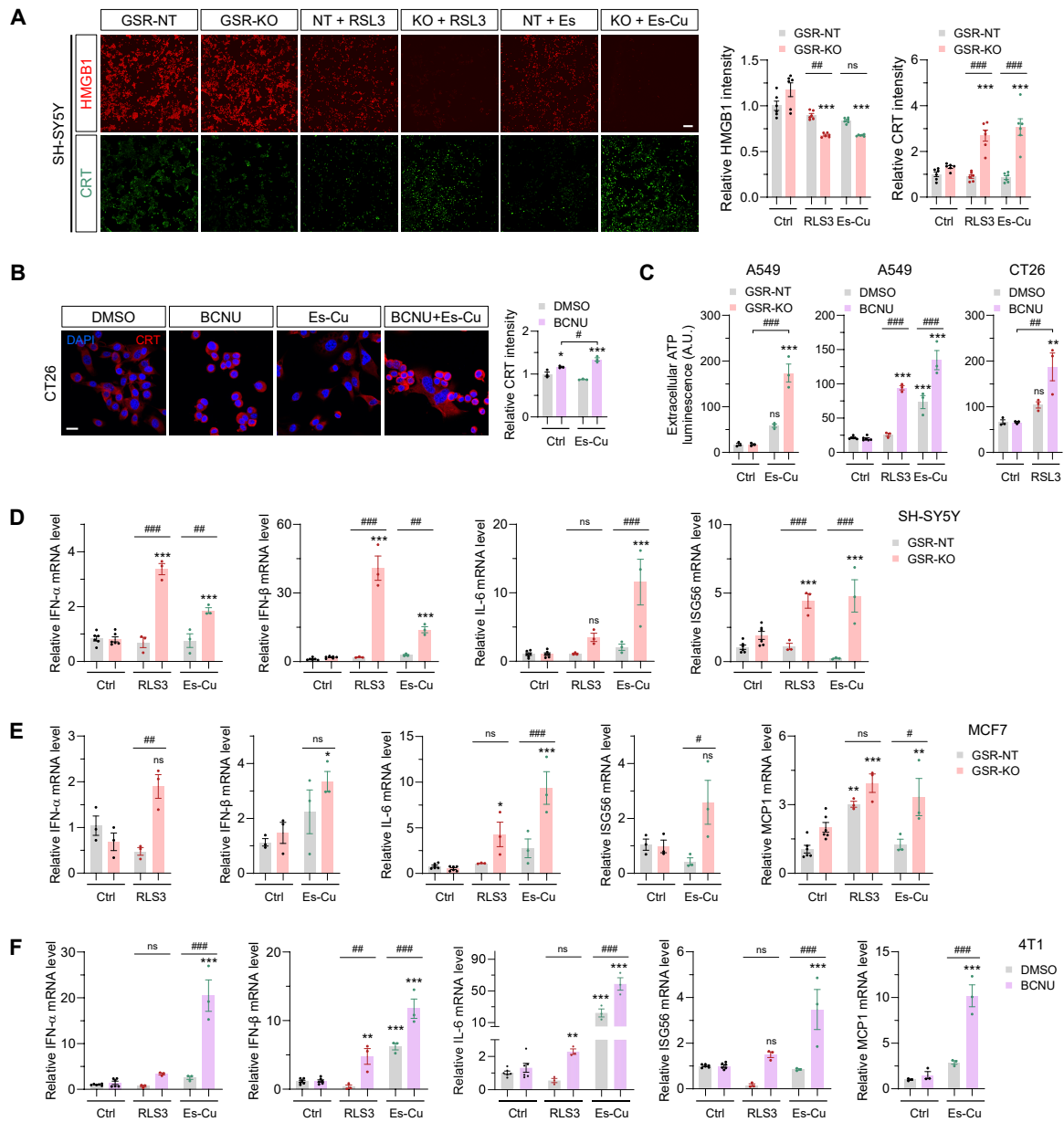


Fig. S10. Enhanced DAMP signals and inflammatory gene expression upon combinational treatments in vitro.

(A) Representative images and quantification of HMGB1 and CRT staining in SH-SY5Y GSR-KO or NT control cells treated with RSL3 or Es-Cu (Scale bars: 200 μ m).

(B) Representative images and quantification of HMGB1 and CRT staining in CT26 cells treated with RSL3 or Es-Cu, alone or combined with BCNU. Scale bars: 25 μ m.

(C-F) Increased extracellular ATP levels (C) and inflammatory gene expression (IFN- α , IFN- β , IL-6, ISG56, MCP1) (D-F) in multiple cancer cell lines treated with RSL3 or Es-Cu under genetic or pharmacological GSR deficiency. All statistics were analyzed by one-way ANOVA with Tukey's correction. Asterisks or ns (not significant) without connecting bars indicate comparisons with the DMSO control.

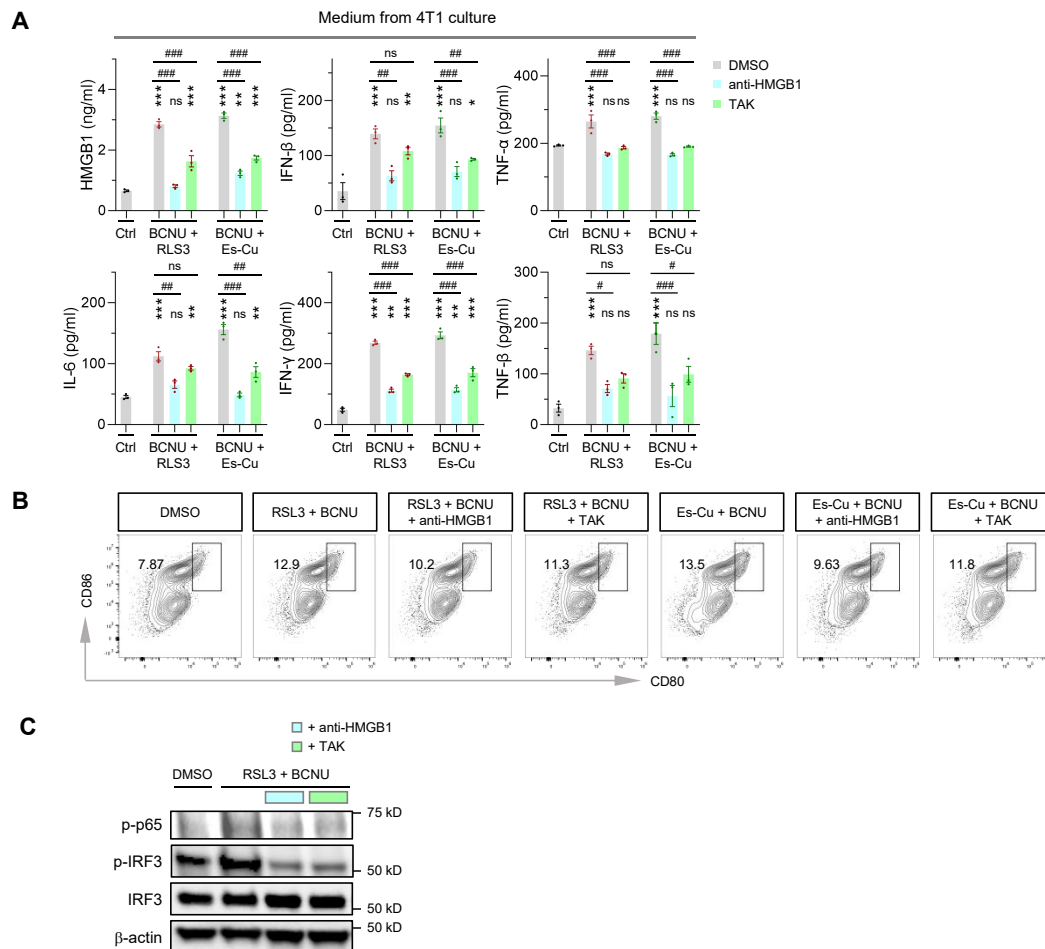


Fig. S11. Enhanced immunogenicity induced by combinational treatments in vitro.

(A) In 4T1 breast cancer cells treated with RSL3 or Es-Cu plus BCNU, HMGB1 neutralization or TLR4 inhibition with TAK-242 (0.2 μ M; TAK) significantly reduced the production of immunogenic cytokines. All statistics were analyzed by one-way ANOVA with Tukey's correction. Asterisks or ns (not significant) indicate comparisons with the DMSO control. (B) In co-culture experiments, HMGB1 neutralization or TLR4 inhibition with 0.2 μ M TAK-242 markedly decreased dendritic cell (DC) activation ($CD86^{+}$), as measured by flow cytometry. (C) In 4T1 cells, HMGB1 neutralization or TLR4 inhibition with 0.2 μ M TAK-242 substantially attenuated phosphorylation of NF- κ B p65 and IRF3.

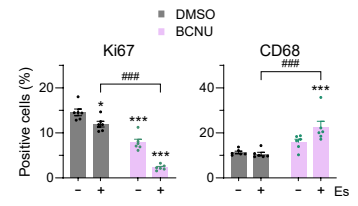
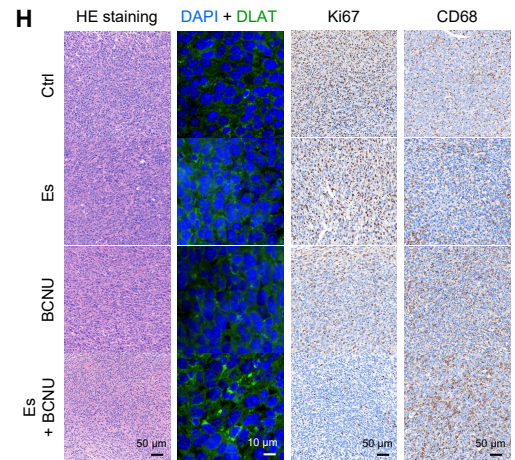
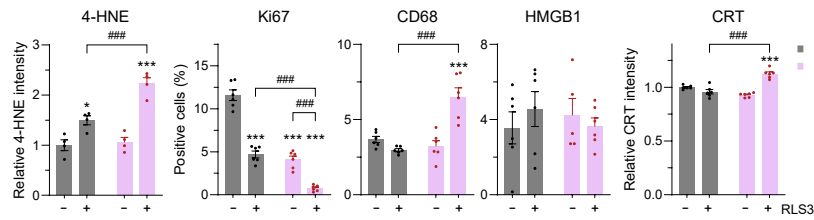
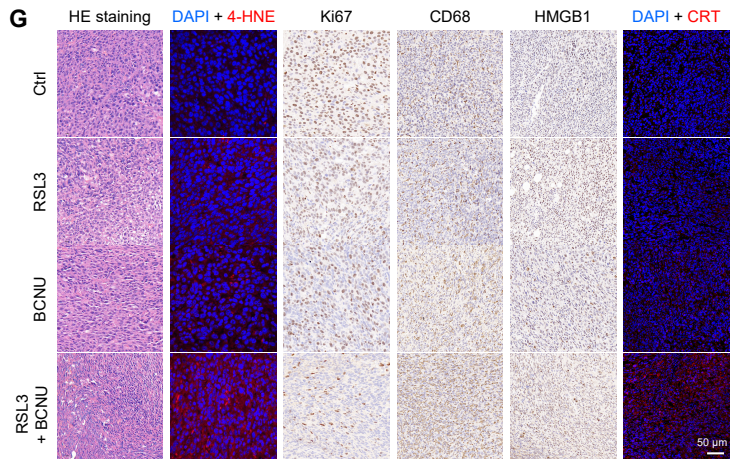
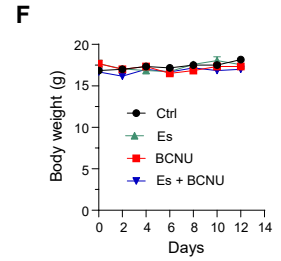
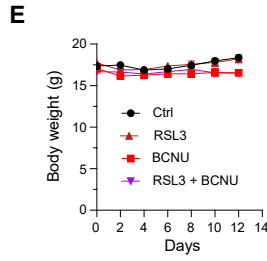
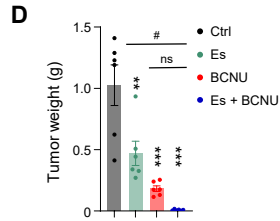
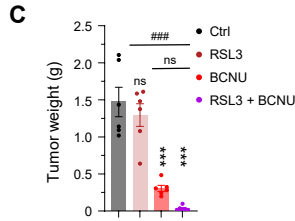
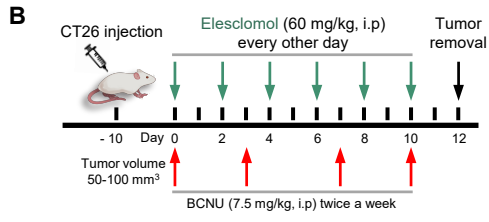
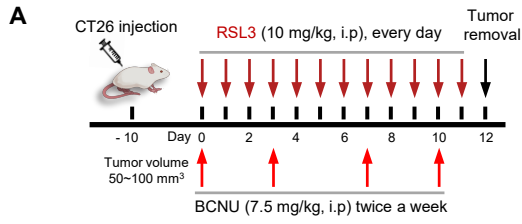


Fig. S12. Syngeneic immunocompetent tumor models.

(A) Schematic of CT26 murine colon carcinoma subcutaneous graft model and ferroptosis treatment regimen. (B) Schematic of CT26 subcutaneous graft model and cuproptosis treatment regimen. (C, D) Tumor weights at the study endpoint of ferroptosis (C) and cuproptosis (D) treatment regimens. (E, F) Mouse body weights monitored throughout the treatment course of ferroptosis (E) and cuproptosis (F) regimens. (G) Representative images and quantification of tumor tissue stained with hematoxylin and eosin (H&E) or immunolabeled for 4-HNE, Ki67, CD68, HMGB1, and CRT. (H) Representative H&E and IHC images with quantification for DLAT, Ki67, and CD68 in tumor tissue. All statistics were analyzed by one-way ANOVA with Tukey's correction. Asterisks or ns (not significant) without connecting bars indicate comparisons with the DMSO control (C, D, G, H).

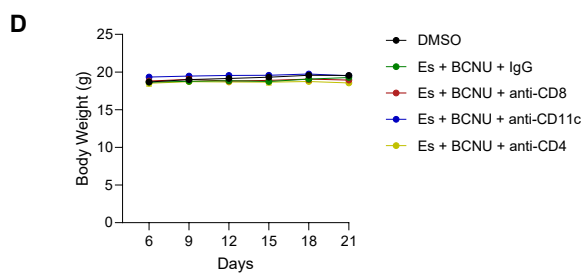
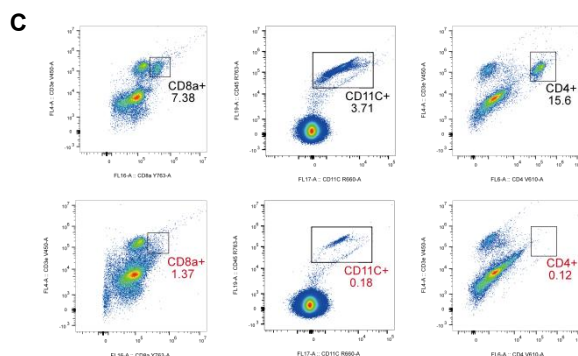
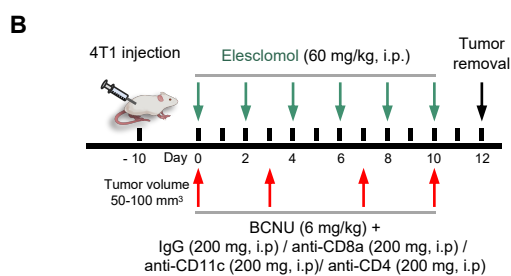
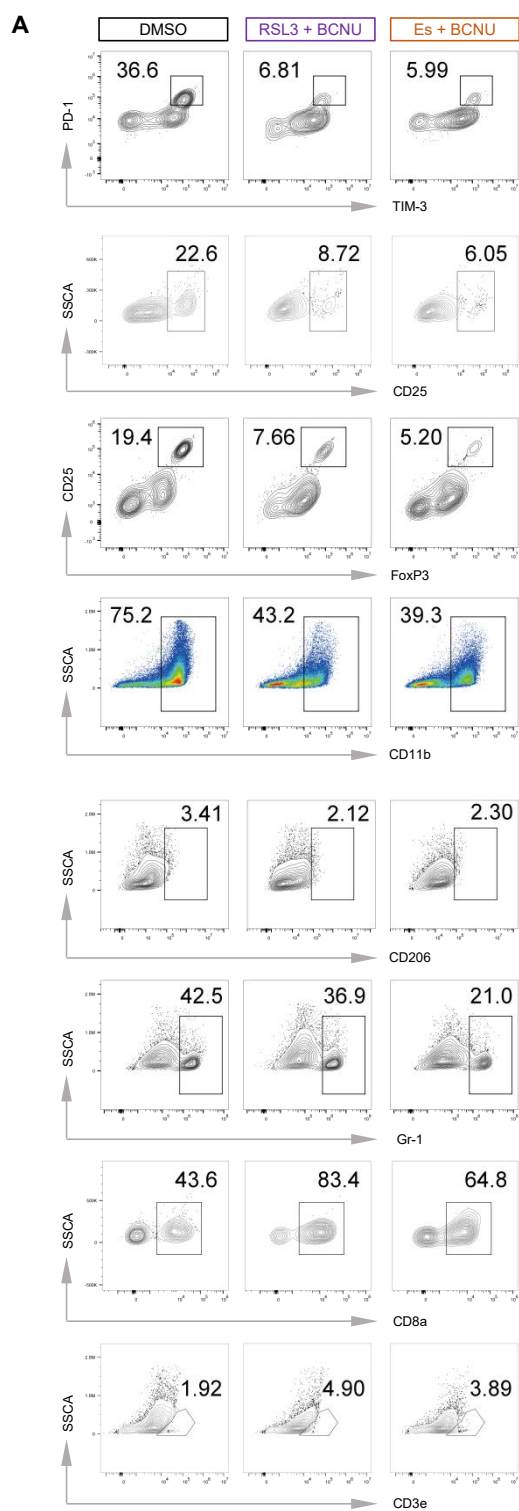


Fig. S13. Immune infiltration in ferroptosis and cuproptosis treatment regimens.

(A) Flow cytometric profiling of 4T1 tumors subjected to treatment of RSL3 or Es combined with BCNU. Related to Fig. 6G. (B) Schematic of in vivo immune cell depletion experiment using 4T1 xenograft model treated with immune cell-specific antibodies. (C) Depletion efficiency was confirmed by flow cytometry. (D) Mouse body weights measured throughout the treatment course. B-C are related to Fig. 6I-K.

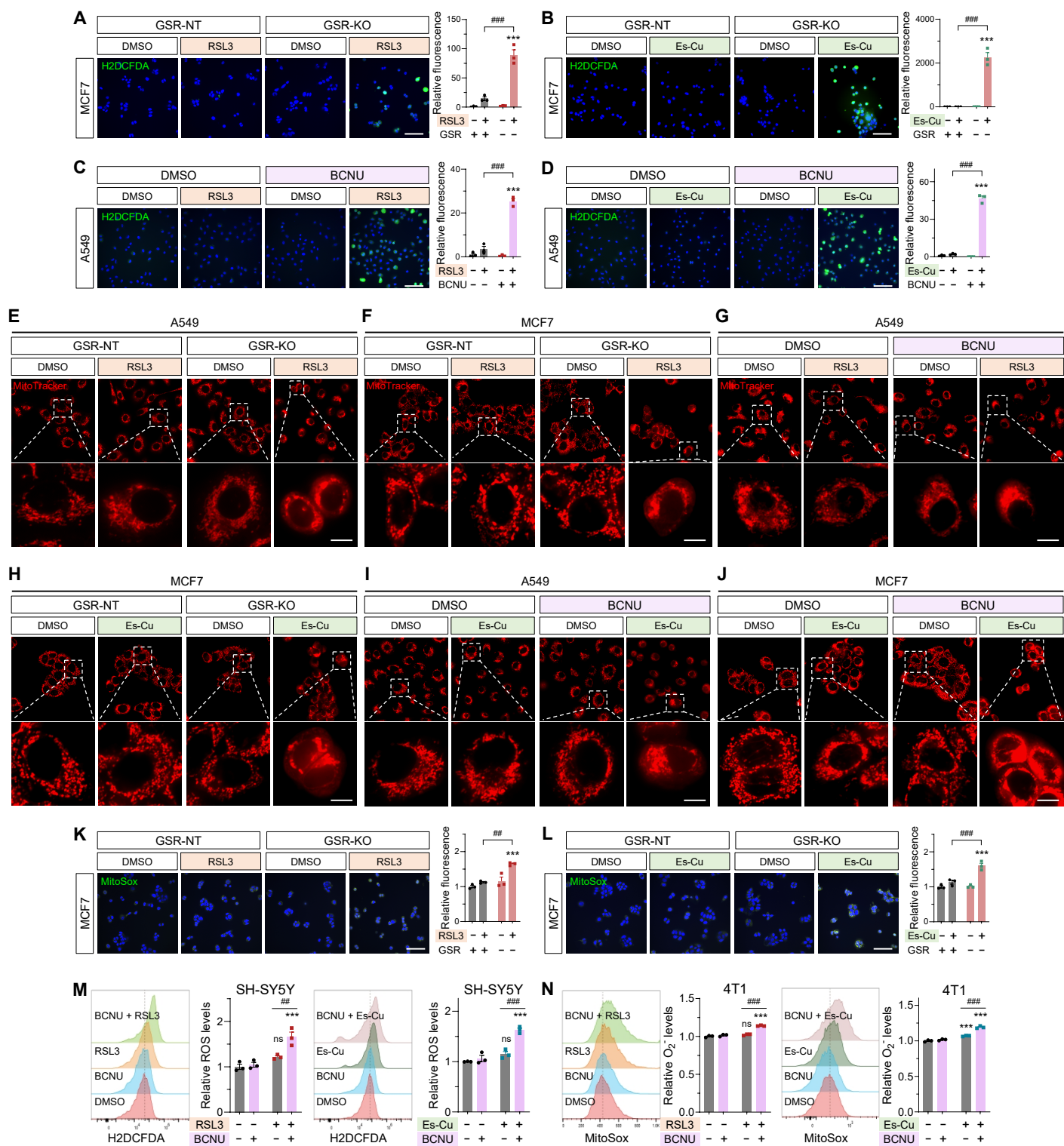


Fig. S14. GSR deficiency enhances cellular ROS levels and causes mitochondrial dysfunction.

(A-L) Genetic or pharmacological GSR inhibition, combined with ferroptosis or cuproptosis inducers, elevated cellular ROS levels (A-D) as measured by H2DCFDA staining (Scale bars: 100 μ m), promotes mitochondrial aggregation (E-J) as visualized by MitoTracker staining (Scale bars: 10 μ m), and elevated mitochondrial superoxide levels (K, L) as indicated by MitoSox staining (Scale bars: 100 μ m). (M, N) Flow cytometry analysis of intracellular ROS (M) using H2DCFDA and mitochondrial superoxide (N) using MitoSox following the indicated treatments, with representative histograms shown on the left and quantification presented as bar plots on the right for each experiment. All statistics were analyzed by one-way ANOVA with Tukey's correction. Asterisks and ns (not significant) without connecting lines indicate comparisons with the DMSO control.

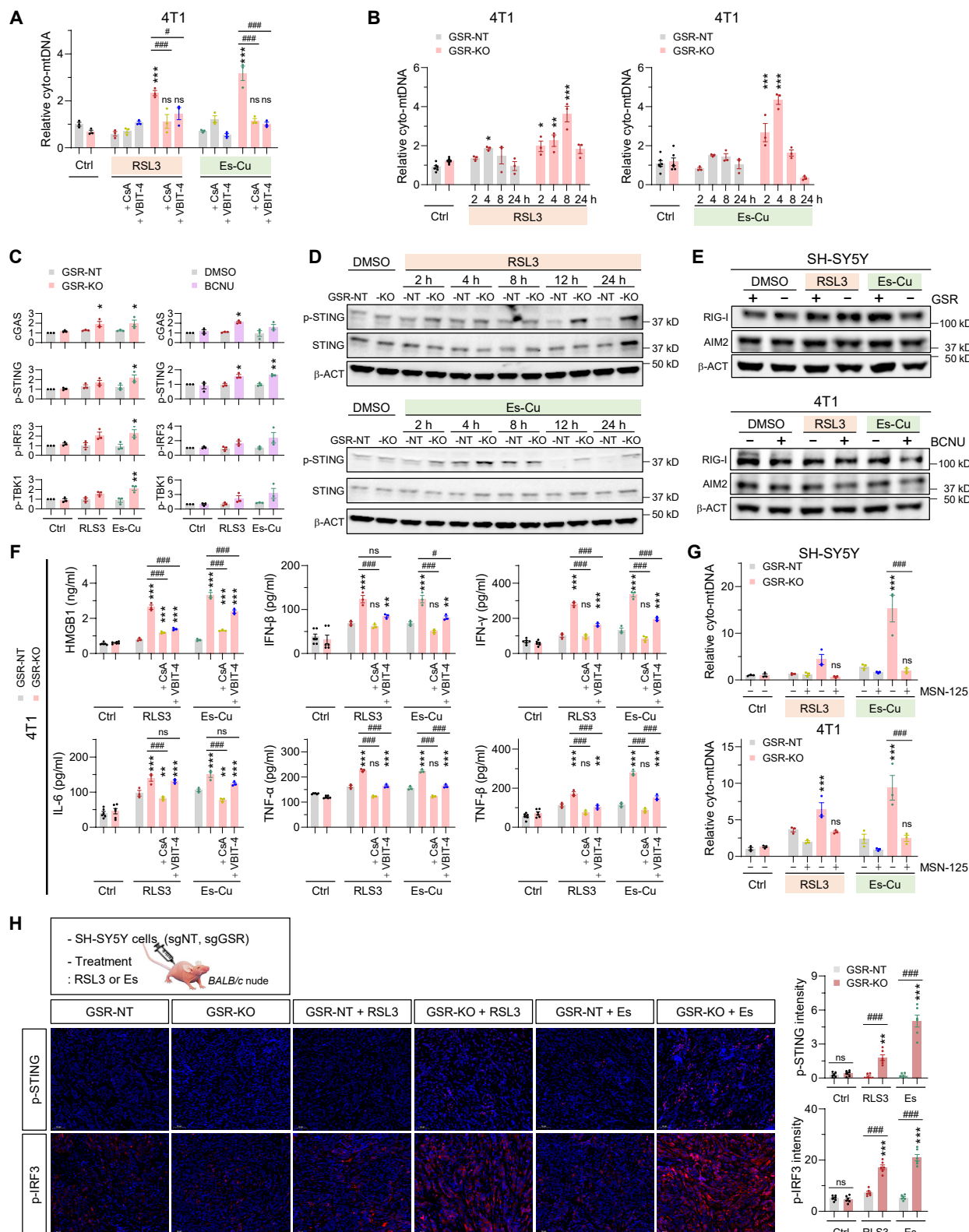


Fig. S15. Increased immunogenic cell death is mediated through mtDNA release and activation of the downstream STING pathway.

(A) Increased cytosolic mitochondrial DNA (mtDNA) release was blocked by 0.2 μ M of cyclosporine A (CsA, mPTP inhibitor) or 2 μ M of VBIT-4 (VDAC inhibitor). (B) Time course of cytosolic mitochondrial DNA release following the indicated treatments. (C) Quantification of cGAS, p-STING, p-IRF3, and p-TBK1 levels in GSR-KO (left) or BCNU-treated (right) cancer cells treated with RSL3 or Es-Cu, as determined by western blotting. (D) Time course of STING activation following the indicated treatments. (E) RIG-I and AIM2 were not significantly altered by the indicated treatments. (F) Increased release of inflammatory mediators, including HMGB1, IFN- β , IFN- γ , IL-6, TNF- α , and TNF- β , following the indicated treatments. (G) Increased cytosolic mtDNA release was abolished by 5 μ M of MSN-125, a selective inhibitor of BAX/BAK oligomerization that blocks BAX/BAK-mediated mitochondrial outer membrane permeabilization (MOMP). (H) Representative immunofluorescence images and quantification of p-STING and p-IRF3 staining, showing elevated levels in GSR-KO SH-SY5H tumor tissues during ferroptosis and cuproptosis compared with NT control tumors. All statistics were analyzed by one-way ANOVA with Tukey's correction. Asterisks and ns (not significant) without connecting bars indicate comparisons versus the DMSO control (A-C, F-H).

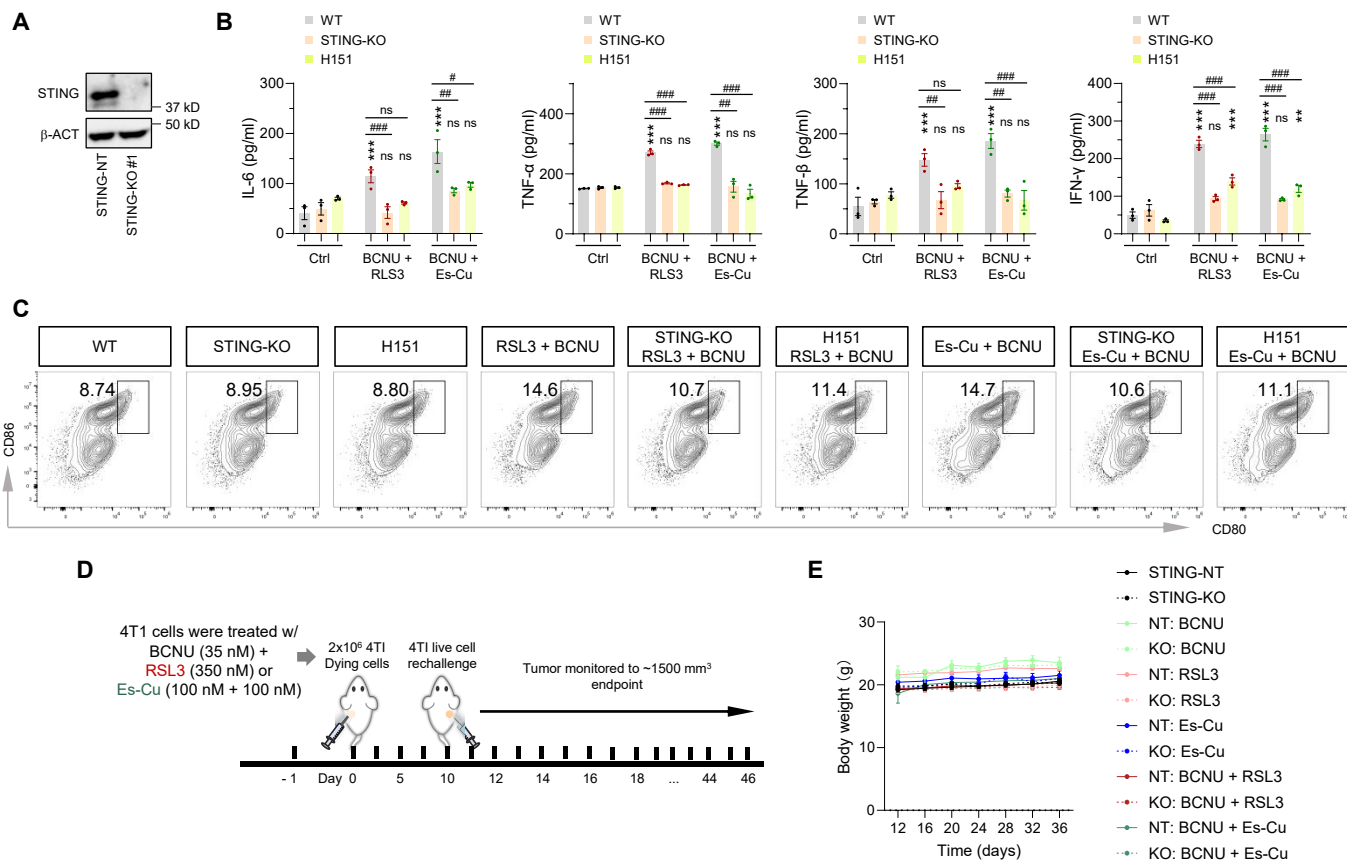


Fig. S16. STING pathway mediates enhanced immunogenicity.

(A) Western blot confirming STING knockout (KO). (B, C) STING-KO cells or pharmacological inhibition of STING with 1 μ M of H151 failed to induce the release of inflammatory cytokines upon BCNU combination treatment (B) and markedly reduced the ability to stimulate dendritic cell activation (C) in vitro. (D) Schematic of the tumor rechallenge assay using ex vivo-treated 4T1 STING-NT or STING-KO cell following monotherapy or combination treatments to induce ferroptosis or cuproptosis for evaluation of their “vaccination” effects. (E) Mouse body weights measured throughout the treatment course. All statistics were analyzed by one-way ANOVA with Tukey’s correction (B). Asterisks or ns (not significant) without connecting lines indicate comparisons with the DMSO control (B).

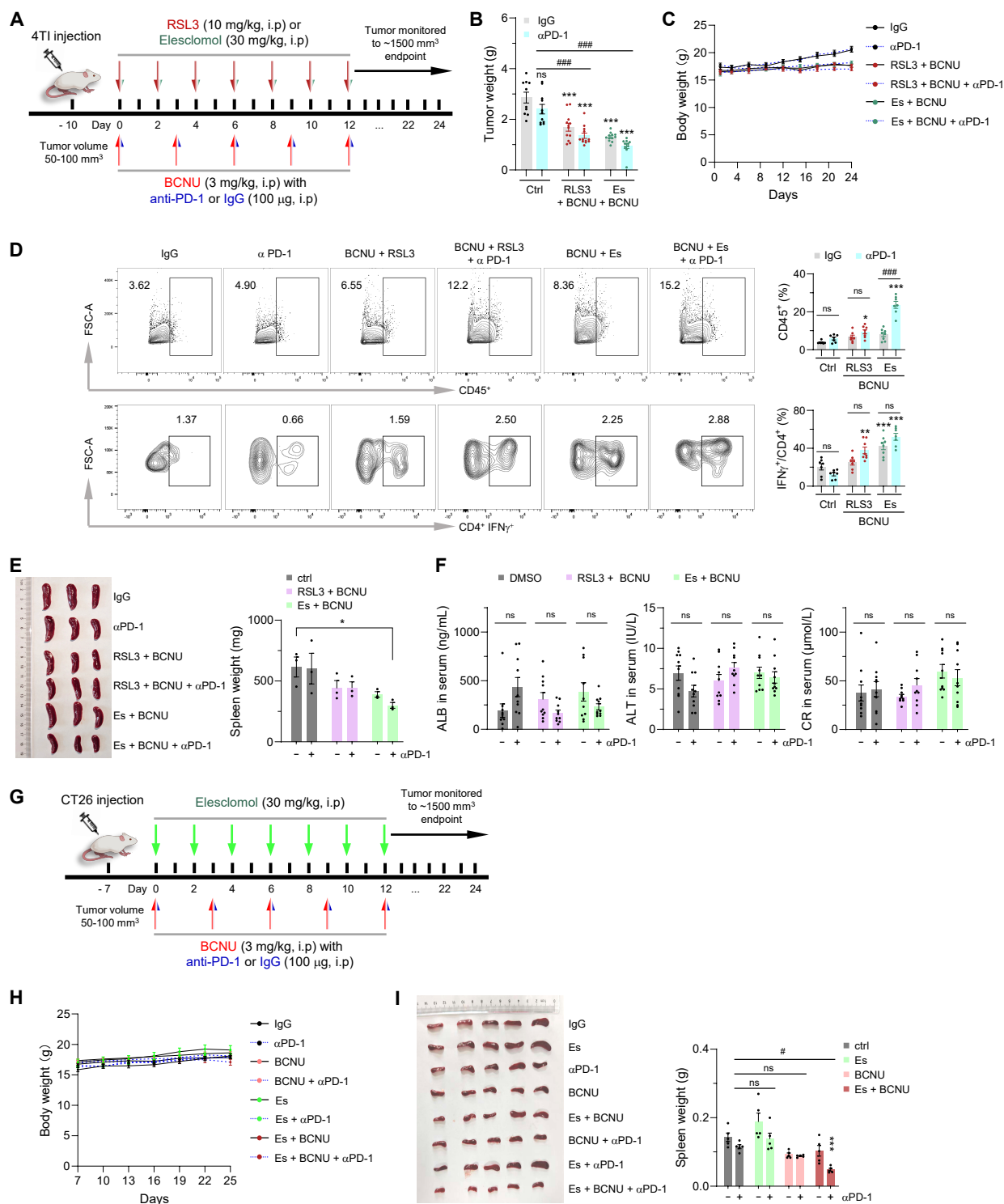


Fig. S17. GSR deficiency synergizes with immune checkpoint inhibitor (ICI) therapy.

(A) Schematic of the 4T1 tumor graft model and treatment regimen with immune checkpoint inhibitor (ICI) therapy, BCNU, and ferroptosis or cuproptosis inducers. (B) Tumor weight at the study endpoint. (C) Mouse body weights measured throughout the treatment course. (D) Flow cytometry analysis of intratumoral CD45⁺ leukocytes, and CD4⁺ T cells expressing IFN γ in 4T1-derived tumors with indicated treatment. (E) Spleen images and quantified weights at the study endpoint under indicated treatments. (F) Serum levels of albumin (ALB), alanine aminotransferase (ALT), and creatinine (CR) in treated mice. (G) Schematic of the CT26 tumor graft model and treatment regimen combining ICI therapy, BCNU, and ferroptosis or cuproptosis inducers. (H) Mouse body weights measured throughout the treatment course. (I) Spleen images and quantified weights at the study endpoint under indicated treatments. All statistics were analyzed by one-way ANOVA with Tukey's correction. Asterisks or ns (not significant) without connecting bars indicate comparisons with the control (B, D, I).

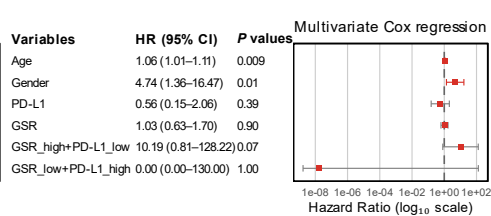
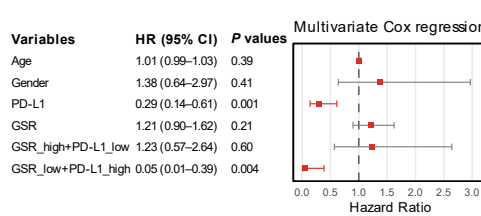
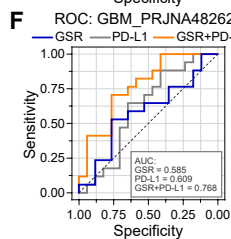
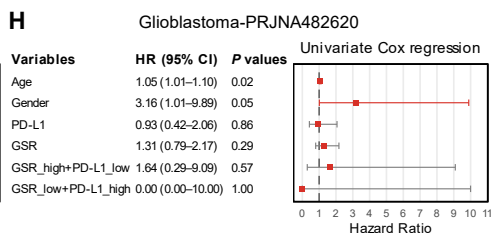
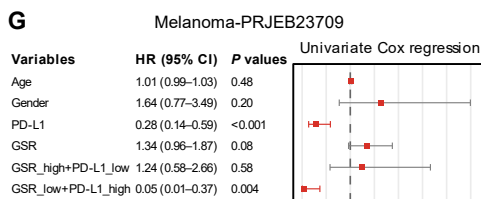
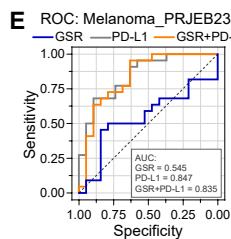
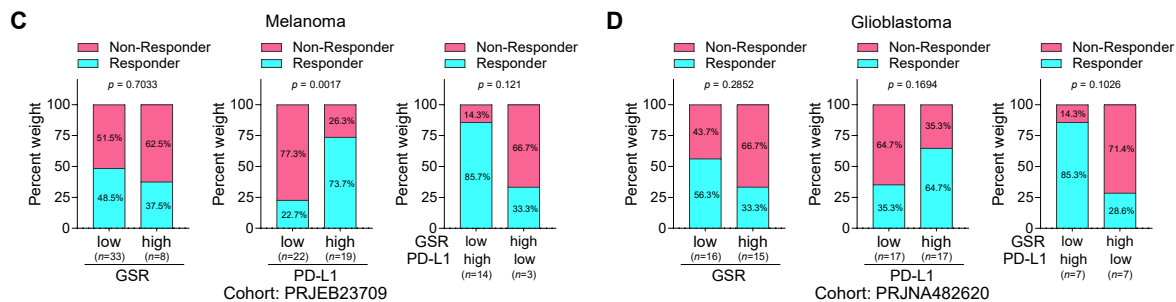
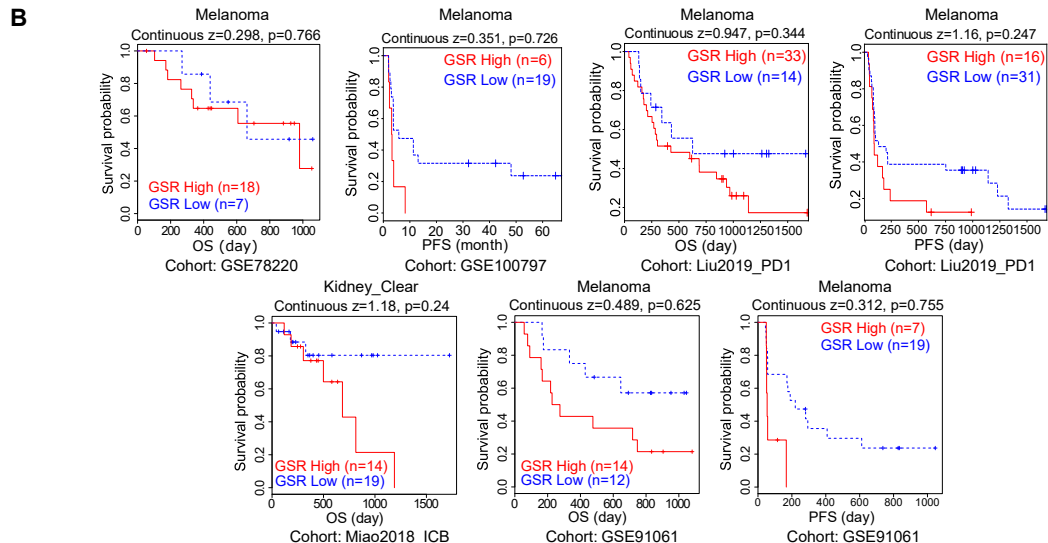
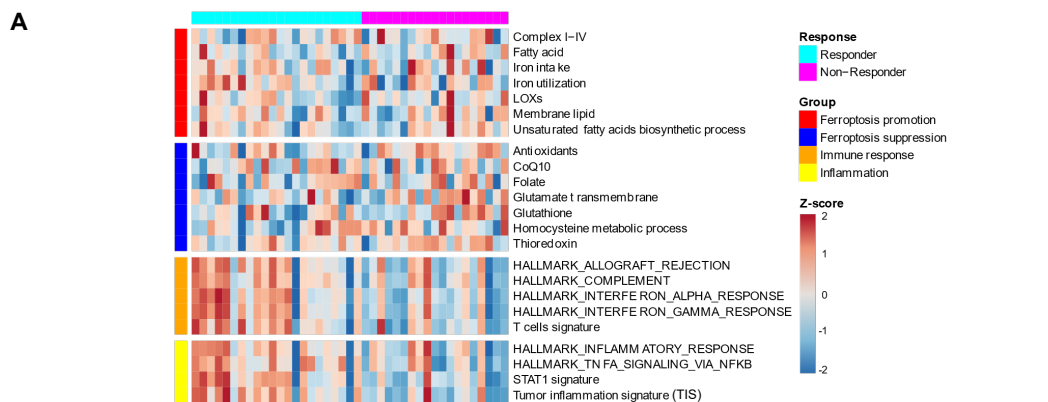


Fig. S18. High GSR expression predicts poor prognosis and immunotherapy outcomes.

(A) Heatmap of single-sample gene set enrichment analysis (ssGSEA) showing enrichment of ferroptosis-related and immune-related pathways in anti-PD-1 immunotherapy responders versus non-responders in the Gide2019_PD1 melanoma cohort. (B) Kaplan-Meier survival curves from multiple clinical cohorts receiving anti-PD-1 therapy, stratified by tumor high vs. low tumor GSR expression levels. (C, D) predictive value of GSR expression for immunotherapy response in comparison with PD-L1 and GSR-PD-L1 combined. (E-H) ROC analysis and univariate and multivariate Cox regression of the GSR-PD-L1 signature.