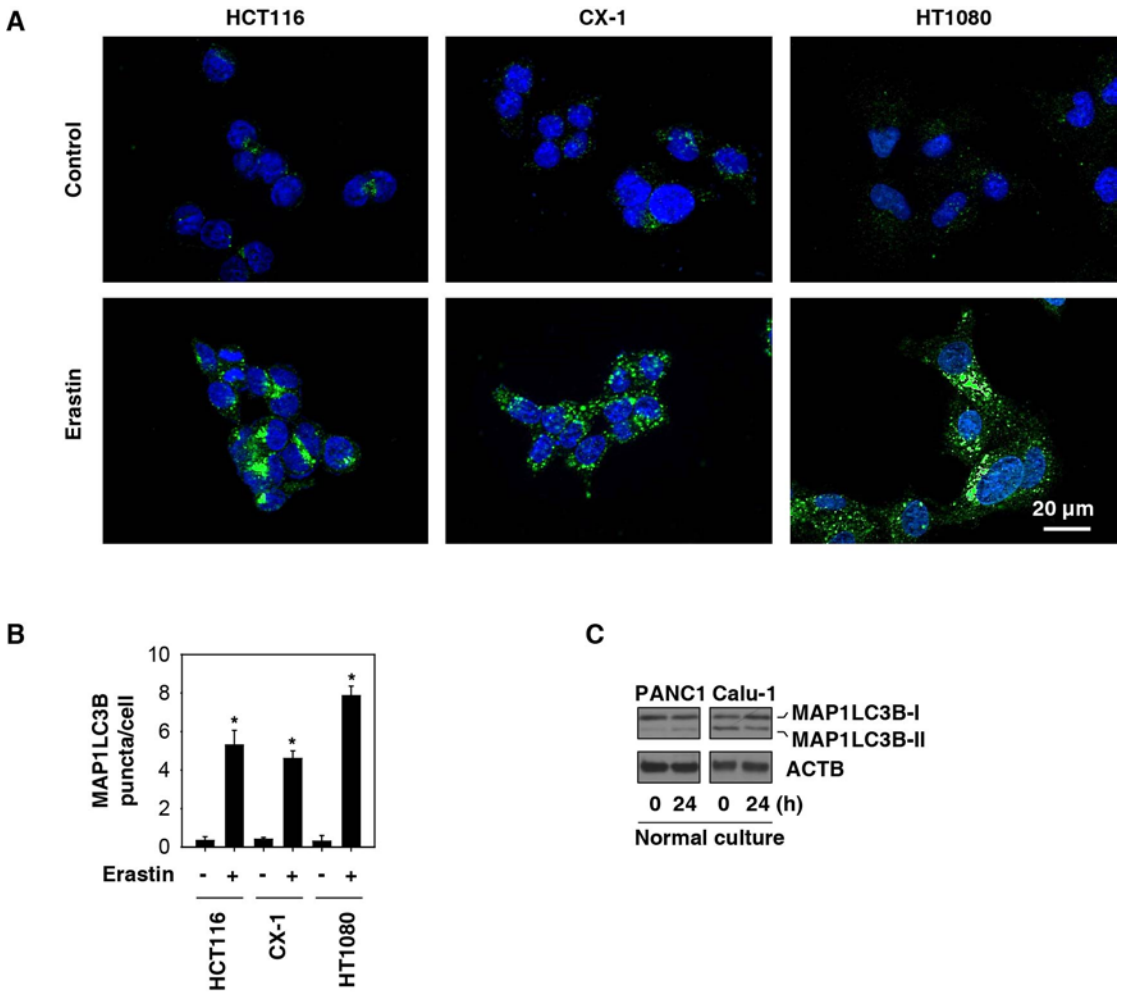

Figure S1. Erastin induces MAP1LC3B puncta formation, Related to Figure 1. Analysis of MAP1LC3B puncta formation in HCT116 (20 μM, 6 h), CX-1 (20 μM, 6 h), and HT1080 (10 μM, 6 h) cells by image analysis. Images were acquired digitally from a randomly selected pool of 10 fields under each condition. (**A**) Representative images (green, MAPLC3B; blue, nucleus). (**B**) Quantitative analysis of MAP1LC3B puncta per cell (*, $P<0.05$ versus control group, *t* test). **(C)** Western blot analysis of MAP1LC3B protein expression in PANC1 and Calu-1 cells under normal cell culture condition for 24 hours.

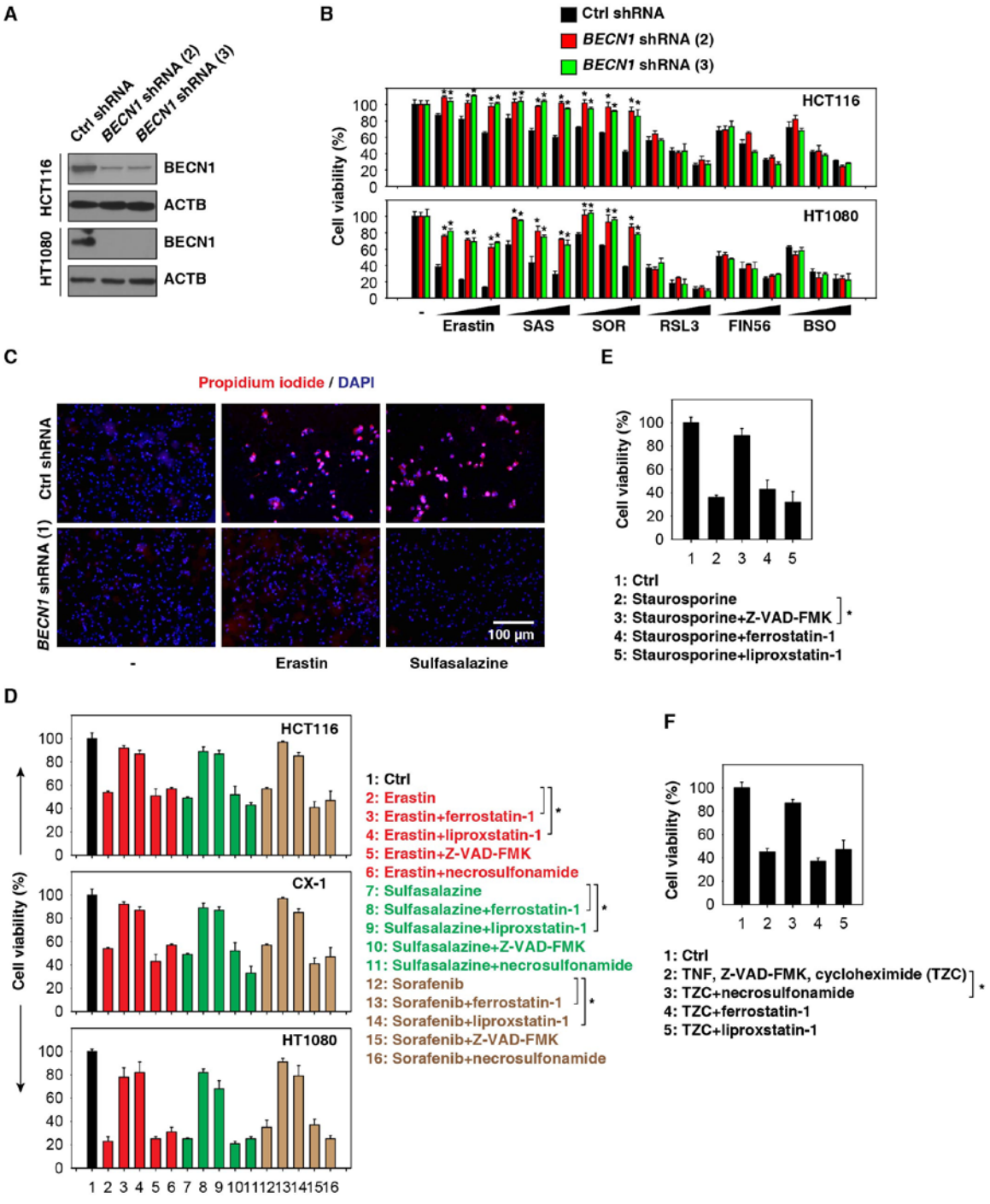

Figure S2. BECN1 is required for system X_c^- inhibitor-induced ferroptosis, Related to Figure 1. (**A**) Western blot analysis of BECN1 expression in BECN1-knockdown cells. (**B**) Knockdown of BECN1 inhibited erastin (20 μM)-, sulfasalazine (SAS, 1 mM)-, and sorafenib (SOR, 10 μM)-induced cell death, but not RSL3 (1 μM)-, FIN56 (5 μM)- and buthionine sulfoximine (BSO, 100 μM)-induced cell death at 24, 48, and 72 h (n=3, *,

P<0.05 versus control group, *t* test). **(C)** Analysis of cell death by propidium iodide staining (red) in indicated HT1080 cells with or without erastin (5 μM) and sulfasalazine (1 mM) for 24 hours. (**C**) The indicated BECN1-overexpressing cells were treated with erastin (20 μM for HCT116 and CX-1 cells; 5 μM for HT1080 cells) in the absence or presence of Z-VAD-FMK (20 μM), ferrostatin-1 (500 nM), liproxstatin-1 (200 nM), and necrosulfonamide (1 μM) for 24 h. Cell viability was assayed (n=3, *p < 0.05, t-test). (**D**) HCT116 cells were treated with staurosporine (1 μM) in the absence or presence of Z-VAD-FMK (20 μM), ferrostatin-1 (500 nM), and liproxstatin-1 for 24 h. Cell viability was assayed (n=3, *p < 0.05, t-test). (**E**) HCT116 cells were treated with TZC (TNF [50 nM], Z-VAD-FMK [20 μM], cycloheximide [10 μg/ml]) in the absence or presence of necrosulfonamide (1 μM), ferrostatin-1 (500 nM), and liproxstatin-1 for 24 h. Cell viability was assayed (n=3, *p < 0.05, t-test).

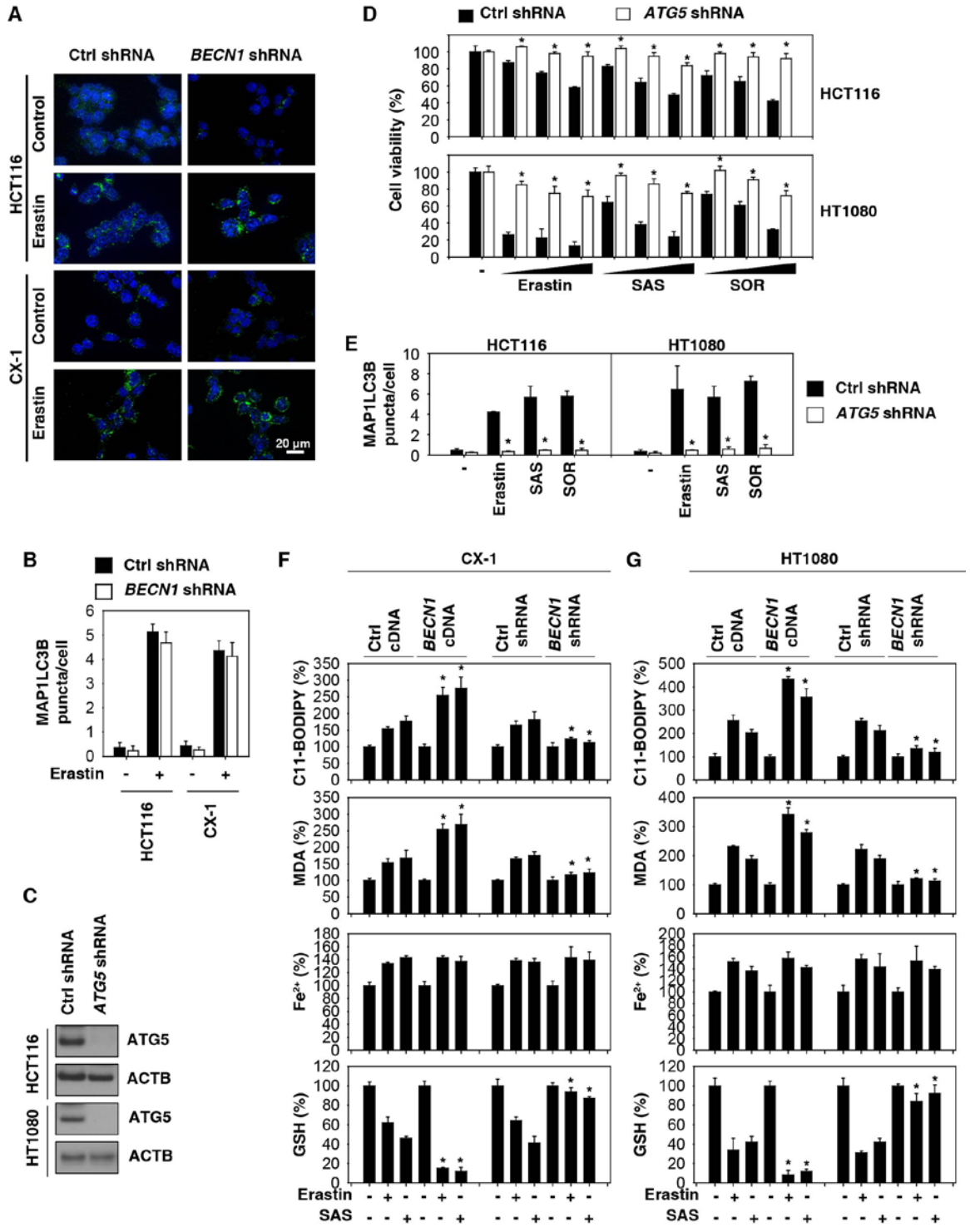

Figure S3. Effects of BECN1 and ATG5 on ferroptosis and autophagy, Related to Figure 2. (**A**) Representative images (green, MAPLC3B; blue, nucleus) of erastin (20

μM, 6 h)-induced MAP1LC3B puncta formation in HCT116 and CX-1 cells by image analysis. (**B**) Quantitative analysis of MAP1LC3B puncta per cell in panel A. Images were acquired digitally from a randomly selected pool of 10 fields under each condition. (**C**) Western blot analysis of ATG5 expression in ATG5-knockdown cells. (**D**) Knockdown of ATG5 inhibited erastin (20 μM for HCT116 cells; 5 μM for HT1080 cells)-, sulfasalazine (SAS, 1 mM)-, and sorafenib (SOR, 10 μM)-induced cell death at 24, 48, and 72 h (n=3, *, $P<0.05$ versus control shRNA group, *t* test). (**E**) Knockdown of ATG5 inhibited erastin (20 μM for HCT116 cells; 5 μM for HT1080 cells)-, sulfasalazine (1 mM)-, and sorafenib (10 μM)-induced MAP1LC3B puncta formation (*, $P<0.05$ versus control shRNA group, *t* test). (**F**) The indicated CX-1 cells were treated with erastin (20 μM) or sulfasalazine (SAS, 1 mM) for 24 h. The relative levels of C11-BODIPY, MDA, Fe^{2+}, and GSH were assayed (n=3, *, $P<0.05$ versus control group, *t* test). (**G**) The indicated HT1080 cells were treated with erastin (5 μM) or sulfasalazine (1 mM) for 24 h. The relative levels of C11-BODIPY, MDA, Fe^{2+}, and GSH were assayed (n=3, *, $P<0.05$ versus control group, *t* test).

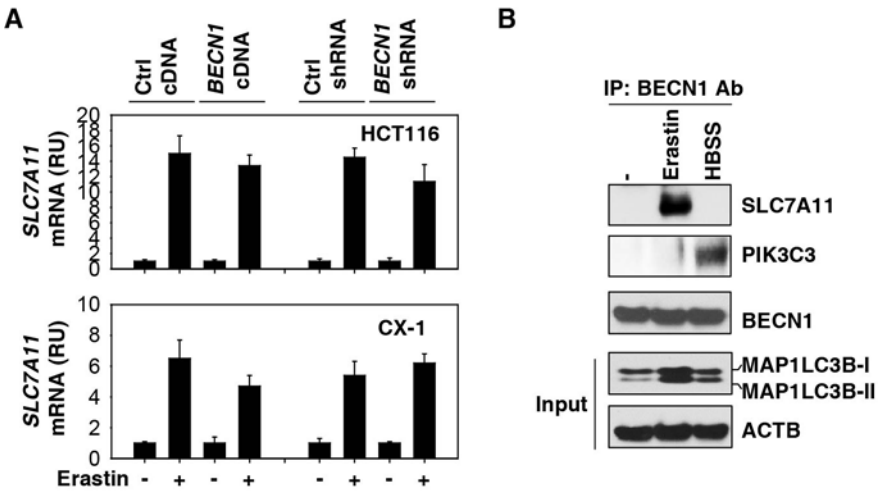

Figure S4. BECN1 protein complex in ferroptosis and autophagy, Related to Figure 3. (A) The indicated HCT116 and CX-1 cells were treated with erastin (20 μM) for 24 h. The relative mRNA levels of *SLC7A11* were assayed (n=3). RU, relative units. **(B)** IP analysis of BECN1-SLC7A11 and BECN1-PIK3C3 formation in HCT116 cells following erastin (20 μM, 24 h) or HBSS (6 h) treatment.

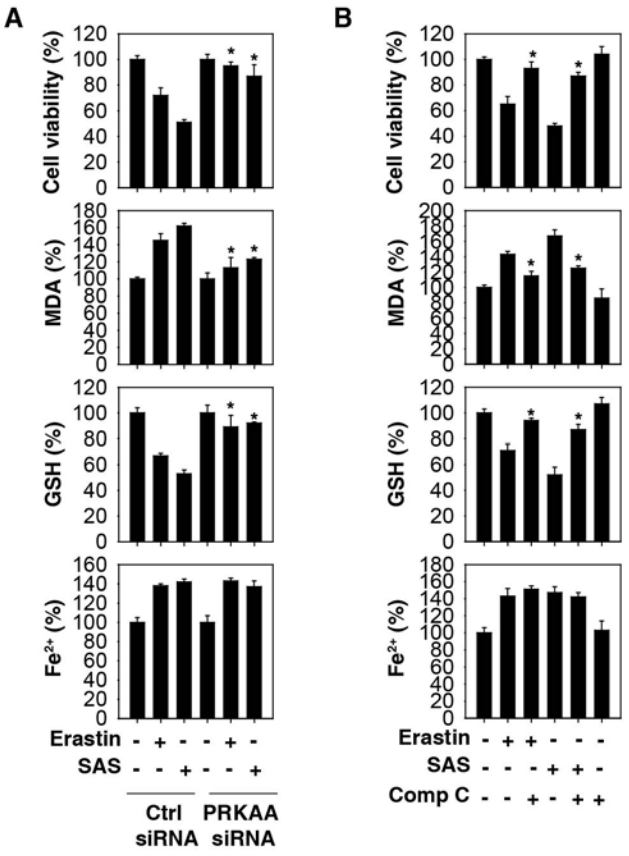

Figure S5. AMPK is required for System X_c^- inhibitor-induced ferroptosis, Related to Figure 5. (**A**) Analysis of the levels of cell viability, MDA, GSH, and Fe^{2+} in the indicated CX-1 cells following erastin (20 μM) or sulfasalazine (SAS, 1 mM) treatment for 24 h (n=3, *, P<0.05 versus control group, *t* test). (**B**) Analysis of the levels of cell viability, MDA, GSH, and Fe^{2+} in CX-1 cells following erastin (20 μM) or sulfasalazine (1 mM) treatment with or without compound C (Comp C, 1 μM) for 24 h (n=3, *, P<0.05 versus erastin or SAS group, *t* test).

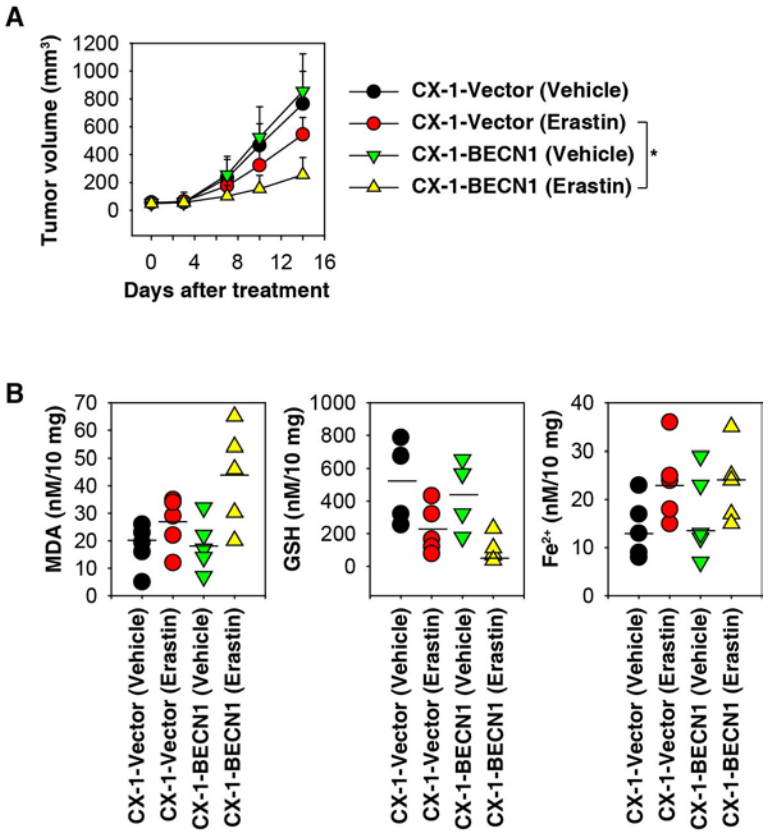

Figure S6. BECN1 contributes to the anticancer activity of erastin *in vivo*, Related to Figure 6. (**A**) Athymic nude mice were injected subcutaneously with the indicated CX-1 cells and treated with erastin (40 mg/kg/intraperitoneal injection, once every day) at day 7 for two weeks. Tumor volume was calculated weekly (n=5 mice/group, * $p < 0.05$, ANOVA *LSD* test). (**B**) In parallel, MDA, GSH, and Fe^{2+} levels in the isolated tumors at day 14 after treatment were assayed (n=5 mice/group).

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Human SLC7A11 Q-PCR primers: 5’-TCCTGCTTTGGCTCCATGAACG-3’ and 5’-AGAGGAGTGTGCTTGCGGACAT-3’	Sigma-Aldrich	This paper
Human RNA18S Q-PCR primers: 5’-CTACCACATCCAAGGAAGCA-3’ and 5’-TTTTTCGTCACTACCTCCCCG-3’	Sigma-Aldrich	This paper
Human FTH1 Q-PCR primers: 5’-TGAAGCTGCAGAACCAACGAGG-3’ and 5’-GCACACTCCATTGCATTCAGCC-3’	Sigma-Aldrich	This paper
Human FTL Q-PCR primers: 5’-TACGAGCGTCTCCTGAAGATGC-3’ and 5’-GGTTCAGCTTTTTCTCCAGGGC-3’	Sigma-Aldrich	This paper
Human TFRC Q-PCR primers: 5’-ATCGGTTGGTGCCACTGAATGG-3’ and 5’-ACAACAGTGGGCTGGCAGAAAC-3’	Sigma-Aldrich	This paper
Human SLC11A2 Q-PCR primers: 5’-AGCTCCACCATGACAGGAACCT-3’ and 5’-TGGCAATAGAGCGAGTCAGAACC-3’	Sigma-Aldrich	This paper
Human SLC40A1 Q-PCR primers: 5’-GAGACAAGTCCTGAATCTGTGCC-3’ and 5’-TTCTTGCAGCAACTGTGTCACAG-3’	Sigma-Aldrich	This paper
pcDNA4-Becn1 S15A mutant primers: S15A-F, GCACCATGCAGGTGGCCTTCGTGTGCCAGC; S15A-R, GCTGGCACACGAAGGCCACCTGCATGGTGC	Sigma-Aldrich	This paper

pcDNA4-Becn1 S30A mutant primers: S30A-F, CCCTGAAACTGGACACGGCTTTCAAGATCCTGGACC; S30A-R, GGTCCAGGATCTTGAAAGCCGTGTCCAGTTTCAGGG	Sigma-Aldrich	This paper
pcDNA4-Becn1 S90-93-96A mutant primers: S90-93-96A-F, CCAGCCAGGATGATGGCCACAGAAGCTGCCAACGCCTTCACTCTGATTGG; S90-93-96A-R, CCAATCAGAGTGAAGGCGTTGGCAGCTTCTGTGGCCATCATCCTGGCTGG	Sigma-Aldrich	This paper
pcDNA4-Becn1 T108A mutant primers: T108A-F, CATCTGATGGCGGCGCCATGGAGAACCTC; T108A-R, GAGGTTCTCCATGGCGCCGCCATCAGATG	Sigma-Aldrich	This paper
pcDNA4-Becn1 T119A mutant primers: T119A-F, CCGAAGACTGAAGGTCGCTGGGGACCTTTTTGA; T119A-R, TCAAAAAGGTCCCCAGCGACCTTCAGTCTTCGG	Sigma-Aldrich	This paper

pcDNA4-Becn1 S113A mutant primers: 113Aa- reverse, CATGGAGAACCTCAGCCAGACAGATGTGGATC; 129Aa- forward, GATCCACATCTGTCTGGCTGAGGTTCTCCATG; 1Aa-forward (BamH1), AGCTCGGATCCATGGAAGGGTCTAAGACGTCC; 450Aa-reverse (Not 1) GTAGTCGCGGCCGCTTTGTTATAAAATTGTGAGGAC	Sigma-Aldrich	This paper

Table S1. Primers used for mutant and qPCR, related to STAR Methods