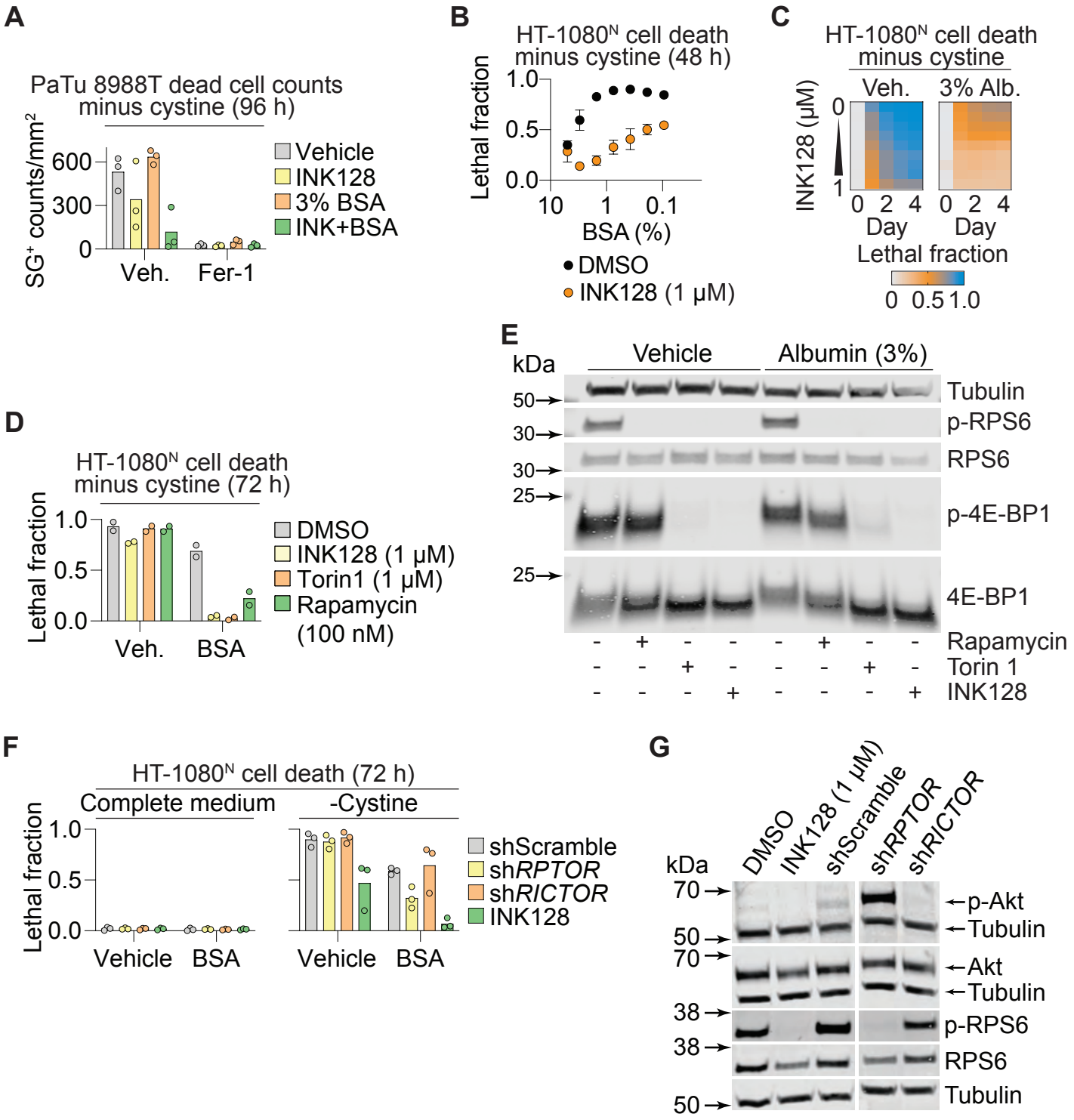

Figure S1. Ferroptosis suppression by extracellular albumin, Related to Figure 1. (A) SYTOX Green-positive (SG^+) dead cell counts in PaTu 8988T cells. Bovine serum albumin (BSA) was used at 3% w/v, ferrostatin-1 (Fer-1) and INK128 (INK) at 1 μM. (B) Cell death at various concentrations of BSA expressed in % w/v. Data are mean ± SD from three independent experiments. (C) Heat map summary of cell death. INK128 was used at 1000 nM, then 125 nM, then two-fold dilutions down to 2 nM, then DMSO vehicle only. Data are mean values from three independent experiments. (D) Cell death in the indicated conditions. BSA was used at 3% w/v. (E) mTORC1 activity after 8 h treatment by western blotting in HT-1080 cells. p-RPS6 is phosphorylated at Ser235/236 and p-4E-BP1 at Thr37/46. Rapamycin, 50 nM. Torin 1, 1 μM. INK128, 1 μM. (F) Cell death in HT-1080^N cells. shRNA transduction was for 72 h and positive control INK128 (1 μM) treatment was for 24 h before the start of cystine deprivation (or control) treatment. BSA (3% w/v). (G) mTORC1/2 pathway activity in HT-1080 cells transduced (72 h) or treated with INK128 (24 h) as in (F). p-RPS6 is phosphorylated at Ser235/236, p-Akt at Ser473. RPS6 and Akt were run on different gels in parallel from the same lysates. All samples are from the same blot with irrelevant lanes eliminated. Individual datapoints from three independent experiments are shown in (A), (D) and (F). Blots in (E) and (G) are representative of three independent experiments.

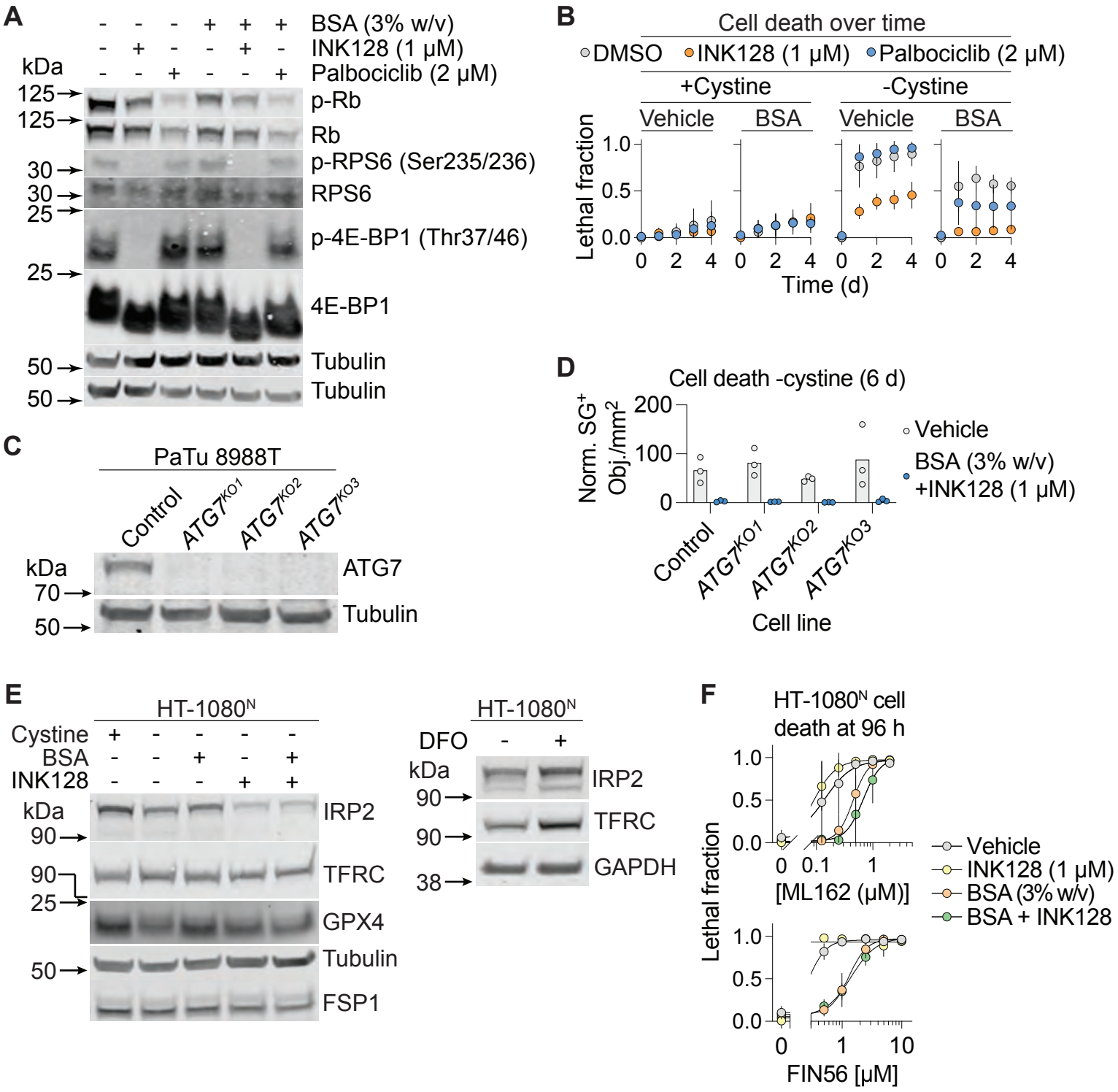

Figure S2. Effects of mTOR-independent cytostasis on albumin-mediated protection, Related to Figure 1. (A) mTORC1 activity and Rb phosphorylation assessed after 8 h treatment by western blotting in HT-1080 cells. Phospho- and total blots were made in parallel from the same lysates. Upper tubulin is from the phospho-blot and lower tubulin from the total blot. (B) Cell death in HT-1080^{N} cells ± cystine ± INK128 ± palbociclib over time. (C) ATG7 protein levels assessed in non-targetting Control and *ATG7* gene disrupted ("KO") cell lines by western blotting. (D) SYTOX Green positive (SG^{+}) dead cell counts, normalized to initial cell confluence, in Control and *ATG7* gene disrupted cell lines. Individual datapoints from three independent experiments. (E) Protein levels assessed by western blotting after 10 h treatment (left panel), or 72 h treatment (right panel) in HT-1080^{N} cells. BSA was 3% w/v, and INK128 used at 1 μM. Deferoxamine (DFO, 50 μM) was used as a postive control for iron deprivation. The 10 h timepoint was used in the left panel as this is the timepoint after which cells deprived of cystine will die. (F) Cell death examined in cells treated with various doses of ML162 and FIN56 ± cystine ± INK128 ± BSA. Data in (B) and (F) are mean ± SD from three independent experiments. All western blots are representative of three independent experiments.

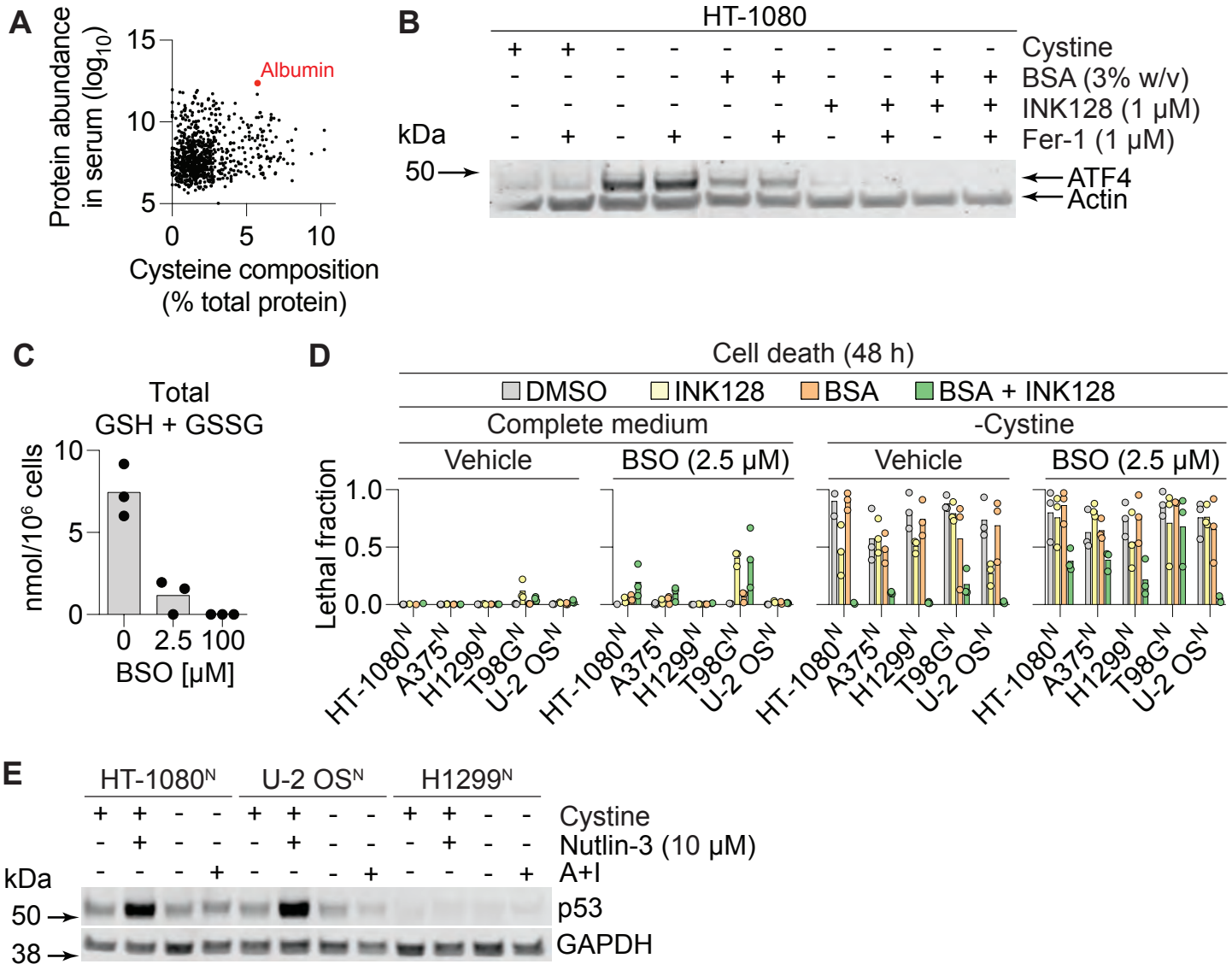

Figure S3. Glutathione synthesis is required for ferroptosis suppression, Related to Figure 2. (A) Abundance of 858 proteins in blood assessed by label-free quantification of mass spectrometry (Geyer, et al. (2016), Cell Systems), plotted against the percent cysteine residues in each protein. Each dot represents a single protein. Albumin is highlighted in red. (B) ATF4 protein levels in response to the indicated conditions of treatment (10 h). Fer-1: ferrostatin-1. (C) Total glutathione (GSH + GSSG) in HT-1080^{N} cells determined using Ellman's reagent. Treatment was for 24 h. BSO: buthionine sulfoximine. Glutathione levels are normalized to mKate2 cell counts. (D) Cell death in different cell lines and treatment conditions. BSA: 3% w/v, INK128: 1 μM, BSO: 2.5 μM. (E) p53 protein expression in different conditions. Cystine deprivation and cystine deprivation with A+I (bovine serum albumin [3% w/v] + INK128 [1 μM]) treatments were for 10 h. The positive control treatment (nutlin-3) was for 48 h. H1299 cells do not express p53 and are a negative control. Blots in (B) and (E) are representative of three independent experiments. Results in (C) and (D) depict individual datapoints from independent experiments.

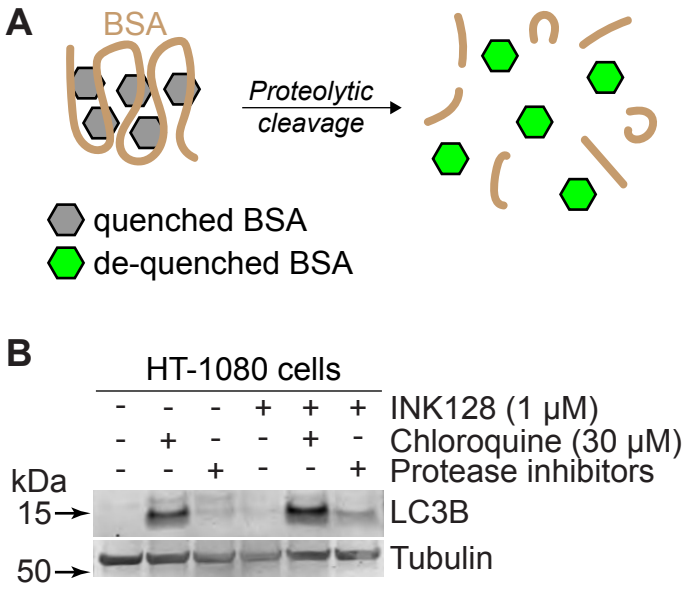

Figure S4. Effect on lysosome protease inhibition on LC3B accumulation, Related to Figure 3. (A) Schematic of the Dye Quenched-Bovine Serum Albumin (DQ-BSA) assay. (B) Protein levels in HT-1080 cells. Treatments were for 24 h. Blot is representative of three independent experiments.

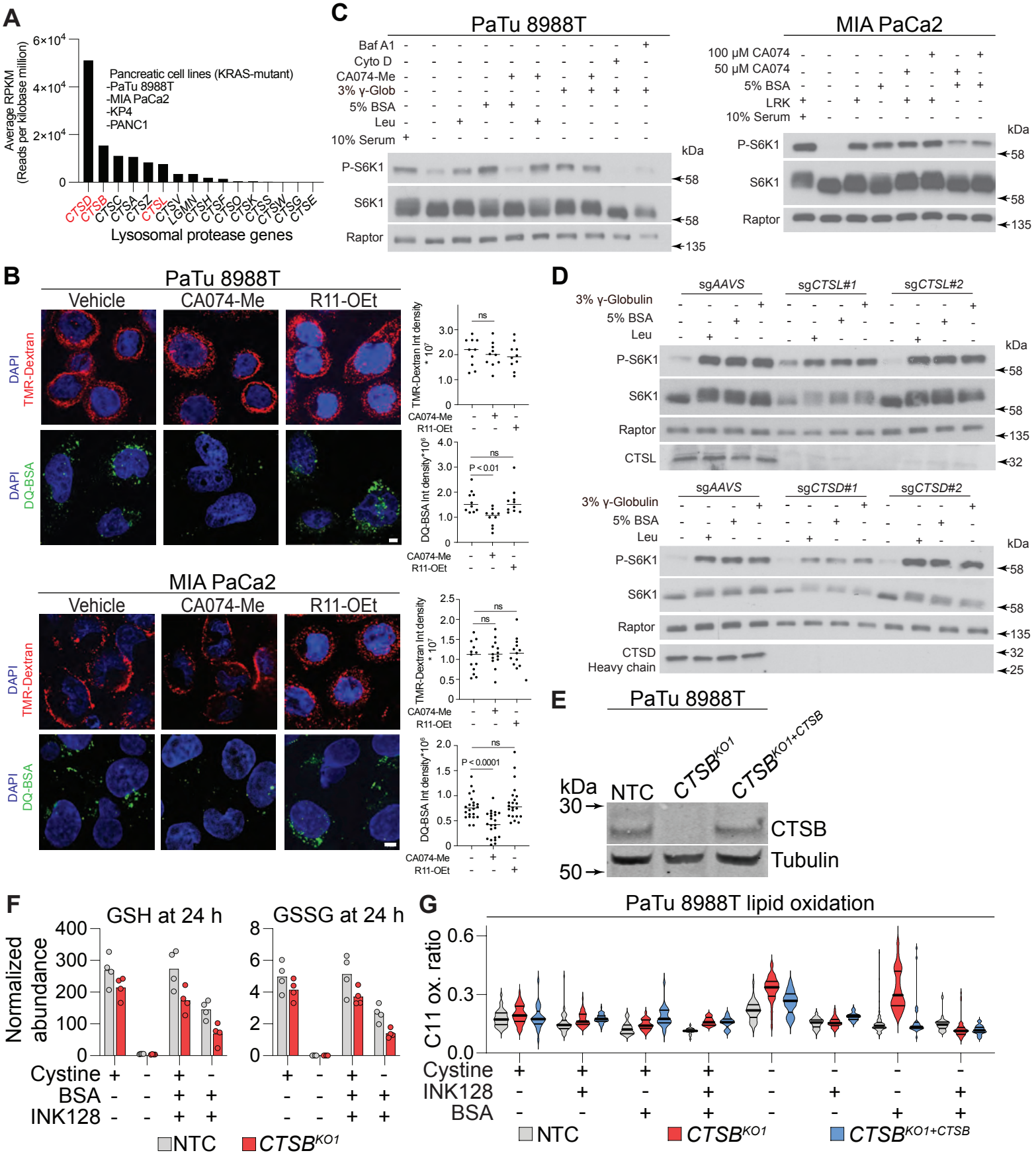
A
Average RPKM (Reads per kilobase million)
6×10^4
4×10^4
2×10^4
0
Pancreatic cell lines (KRAS-mutant)
-PaTu 8988T
-MIA PaCa2
-KP4
-PANC1
CTSD CTSB CTSC CTSA CTSZ CTSL CTSV LGMN CTSH CTSF CTSO CTSK CTSS CTSW CTSG CTSE
Lysosomal protease genes
C
PaTu 8988T
Baf A1
Cyto D
CA074-Me
3% γ-Glob
5% BSA
Leu
10% Serum
kDa
P-S6K1
S6K1
Raptor
58
58
135
MIA PaCa2
100 μM CA074
50 μM CA074
5% BSA
LRK
10% Serum
kDa
P-S6K1
S6K1
Raptor
58
58
135
B
PaTu 8988T
Vehicle
CA074-Me
R11-OEt
DAPI
TMR-Dextran
DAPI
DQ-BSA
TMR-Dextran Int density * 10^7
DQ-BSA Int density*10^6
ns
P < 0.01
CA074-Me
R11-OEt
MIA PaCa2
Vehicle
CA074-Me
R11-OEt
DAPI
TMR-Dextran
DAPI
DQ-BSA
TMR-Dextran Int density * 10^7
DQ-BSA Int density*10^6
ns
P < 0.0001
CA074-Me
R11-OEt
D
sgAAVS
sgCTSL#1
sgCTSL#2
3% γ-Globulin
5% BSA
Leu
kDa
P-S6K1
S6K1
Raptor
CTSL
58
58
135
32
sgAAVS
sgCTSD#1
sgCTSD#2
3% γ-Globulin
5% BSA
Leu
kDa
P-S6K1
S6K1
Raptor
CTSD Heavy chain
58
58
135
32
25
E
PaTu 8988T
kDa
NTC
CTSB^KO1
CTSB^KO1+CTSB
30
50
CTSB
Tubulin
F
GSH at 24 h
GSSG at 24 h
Normalized abundance
400
300
200
100
0
8
6
4
2
0
Cystine
BSA
INK128
NTC
CTSB^KO1
G
PaTu 8988T lipid oxidation
C11 ox. ratio
0.6
0.3
0.0
Cystine
INK128
BSA
NTC
CTSB^KO1
CTSB^KO1+CTSB

Figure S5. Cathepsin B promotes lysosomal albumin catabolism, Related to Figure 4. (A) Averaged reads per kilobase per million mapped reads (RPKM) across four KRAS mutated pancreatic cell lines. Data were obtained from CCLE, accessed 22 Feb 2022. (B) Assessment and quantification of DQ-BSA and TMR-Dextran. Cells starved of leucine and treated with vehicle, CA-074 Me (50 μM) or R11-OEt (100 nM) for 2 h, then incubated with TMR-Dextran or DQ-BSA for 4 h. Representative images are shown. Scale bar = 5 μm. Quantification of TMR-dextran and DQ-BSA fluorescence in cells is shown to the right. Data are represented as mean ± SD (n ≥ 3 fields of view with ≥ 10 cells in total). (C) Assessment of mTORC1 signaling. (Left) PaTu 8988T cells were starved of leucine for 2 h, then treated with vehicle, CA-074 Me (50 μM), cytochalasin D (Cyto D, 10 μM), or bafilomycin A1 (Baf A1, 250 nM) for 2 h, then incubated with bovine serum albumin (BSA) or γ-globulin (γ-Glob) for 4 h. (Right) MIA PaCa2 cells were starved of leucine, arginine, and lysine (LRK) and treated with CA074-Me (CA074) for 2 h, then incubated with BSA. (D) Activity of the mTORC1 signaling pathway in PaTu 8988T cells starved of leucine for 2 h, then restimulated with medium containing leucine (Leu), BSA, or γ-globulin for 4 h. Raptor was used as loading control. (E) Protein levels assessed by western blot. NTC: non-targeting control. Representative of three independent blots. (F) Reduced (GSH) and oxidized (GSSG) glutathione measured by liquid chromatography coupled to mass spectrometry. Integrated peak intensity is normalized to mg protein in each sample. INK128: 1 μM, BSA: 3% w/v. (G) C11 BODIPY 581/591 (C11) oxidation in PaTu 8988T cells assessed by microscopy after 30 h treatment. Violin plots are from 5-146 individual cells/condition from one experiment. Data are representative of three independent experiments. Blots in (C) and (D) show phosphorylation of S6K1 at Thr389.

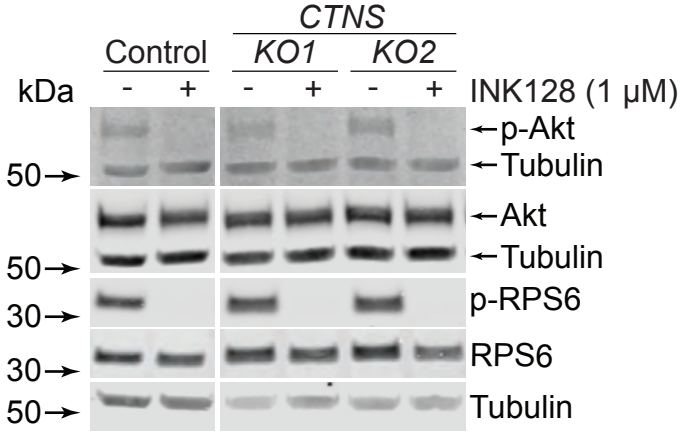

Figure S6. *CTNS* gene disruption does not affect mTOR pathway function, Related to Figure 5. mTOR pathway activity assessed by western blot in HT-1080 Control and two independent *CTNS* gene-disrupted ("KO1/2") clonal cell lines. INK128 treatment was for 8 h. The sites of phosphorylation detected are p-RPS6: Ser235/236, and p-Akt: Ser473. RPS6 and Akt were run on different gels in parallel from the same lysates. The vertical cut represents a crop of two parts of the same blots. Blot is representative of three independent experiments.

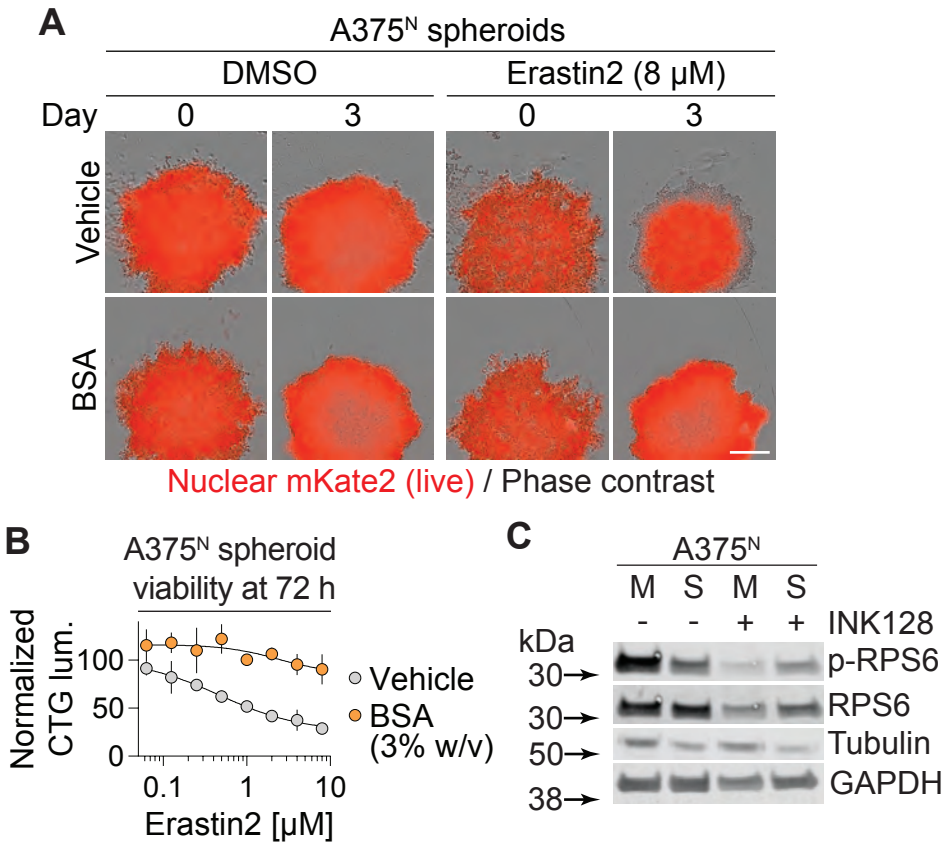

Figure S7. Spheroid viability promoted by albumin and mTOR inhibition, Related to Figure 6. (A) Images of A375^{N} spheroids over time. BSA: bovine serum albumin (3% w/v). Scale bar = 300 μm. Images are representative of three independent experiments. (B) Spheroid viability determined by CellTiter-Glo assay. Data are mean ± SD from three independent experiments. Lum., luminescence. (C) mTOR pathway activity assessed by western blot. INK128: 1 μM, 24 h. M: cells grown in monolayer, S: cells grown as spheroids. p-RPS6 is phosphorylated at Ser235/236. Blots are representative of three independent experiments.

Table S1, related to Figures S2, 4, S5 and S6. Oligonucleotides used in this study.

OLIGO NAME and SEQUENCE	SOURCE	Identifier
ATG7 KO1 (S): caccgTGGGAAAAGAACCAGAAAGG	This paper	N/A
ATG7 KO1 (AS): aaacCCTTTCTGGTTCTTTTCCCAc	This paper	N/A
ATG7 KO2 (S): caccgGAAGCTGAACGAGTATCGGC	This paper	N/A
ATG7 KO2 (AS): aaacGCCGATACTCGTTCAGCTTCc	This paper	N/A
CTSB KO1 (S): caccgGTGGTGCTCACAGGGAGGGA	This paper	N/A
CTSB KO1 (AS): aaacTCCCTCCCTGTGAGCACCACc	This paper	N/A
CTSB KO2 (S): caccgGTCACCGGAGAGATGATGGG	This paper	N/A
CTSB KO2 (AS): aaacCCCATCATCTCTCCGGTGACc	This paper	N/A
CTSD KO1 (S): caccgGGAGAGGCAGGTCTTTGGGG	This paper	N/A
CTSD KO1 (AS): aaacCCCCAAAGACCTGCCTCTCCc	This paper	N/A
CTSD KO2 (S): caccgCGTCTCAAAGTACTCCCAGG	This paper	N/A
CTSD KO2 (AS): aaacCCTGGGAGTACTTTGAGACGc	This paper	N/A
CTSL KO1 (S): caccgGCACAACAGATTATACGGCA	This paper	N/A
CTSL KO1 (AS): aaacTGCCGTATAATCTGTTGTGCc	This paper	N/A
CTSL KO2 (S): caccgAAGGCAGCAAGGATGAGTGT	This paper	N/A
CTSL KO2 (AS): aaacACACTCATCCTTGCTGCCTTc	This paper	N/A
CTSN KO (S): CACCGCCAGCCTACCCGGTCTGAT	This paper	N/A
CTSN KO (AS): AAACATCAGACCGGGTAGGCTGGC	This paper	N/A