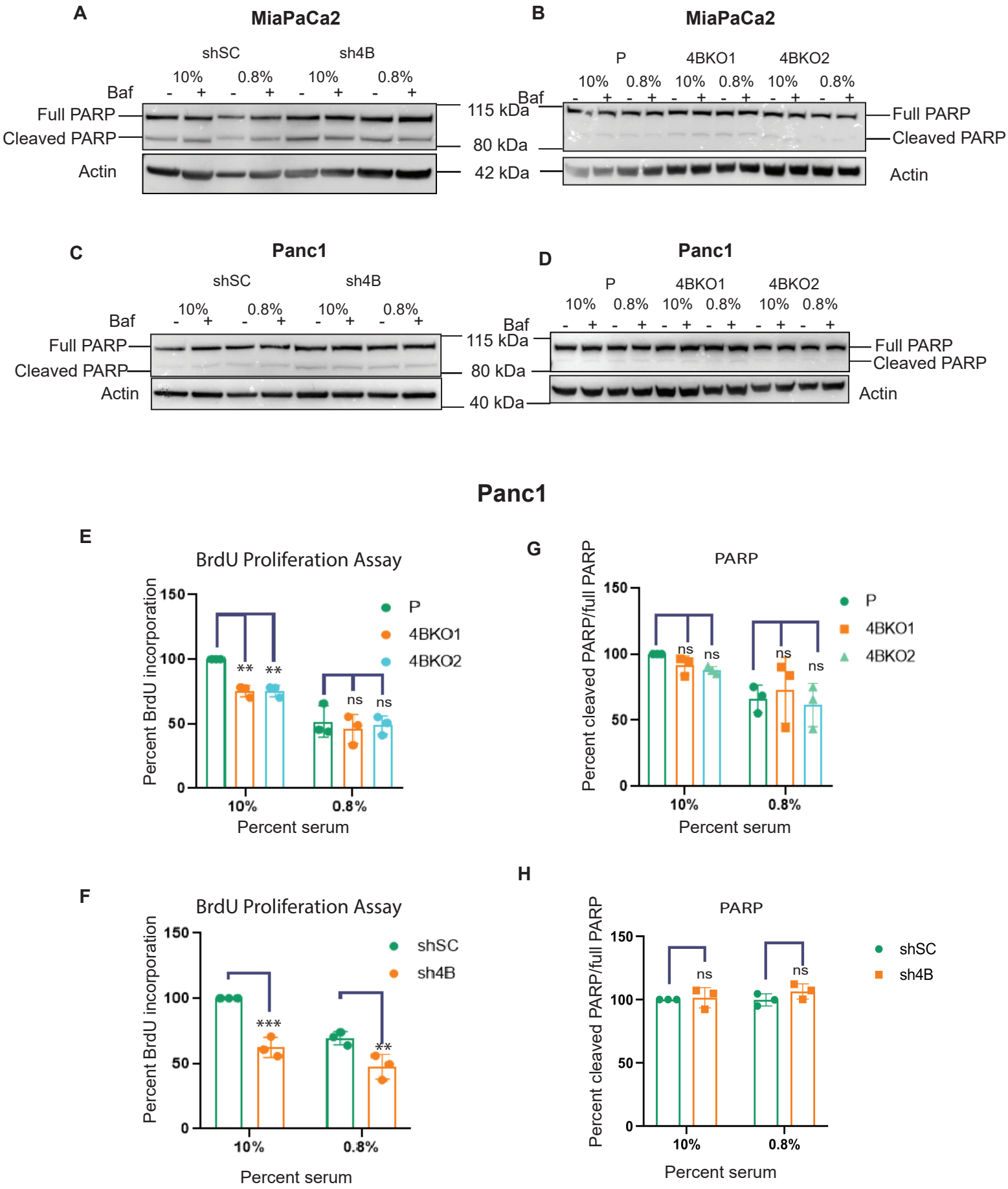


Fig. S1. BrDU Proliferation and PARP cleavage cell death assay in PDAC cell lines. **(A-D)** Representative immunoblots of PARP (full length and cleaved) and actin (loading control) in the sh4B and ATG4B KO lines of **(A and B)** MiaPaCa2 and **(C and D)** Panc1 PDAC cell lines (n=3) in both 10% and 0.8% serum condition with and without Baf treatment. **(E-F)** Quantification of BrdU incorporation assay in Panc1 ATG4B KO **(E)** and sh4B **(F)** cell lines in 10% and 0.8% serum conditions. **(G-H)** Quantification of PARP cleavage assay (cleaved PARP/full-length PARP) performed in ATG4B KO **(G)** and sh4B **(H)** Panc1 lines in 10% and 0.8% culture conditions. Each cell line in **(E)**, **(F)**, **(G)** and **(H)** was normalized against scramble control (shSC) or parental (P) line under 10% serum condition. Ordinary one-way ANOVA with Dunnett's multiple tests was used to determine statistical significance between parental and ATG4A KO lines. Error bars indicate SE; n=3. **p<0.01. *** p<0.001.

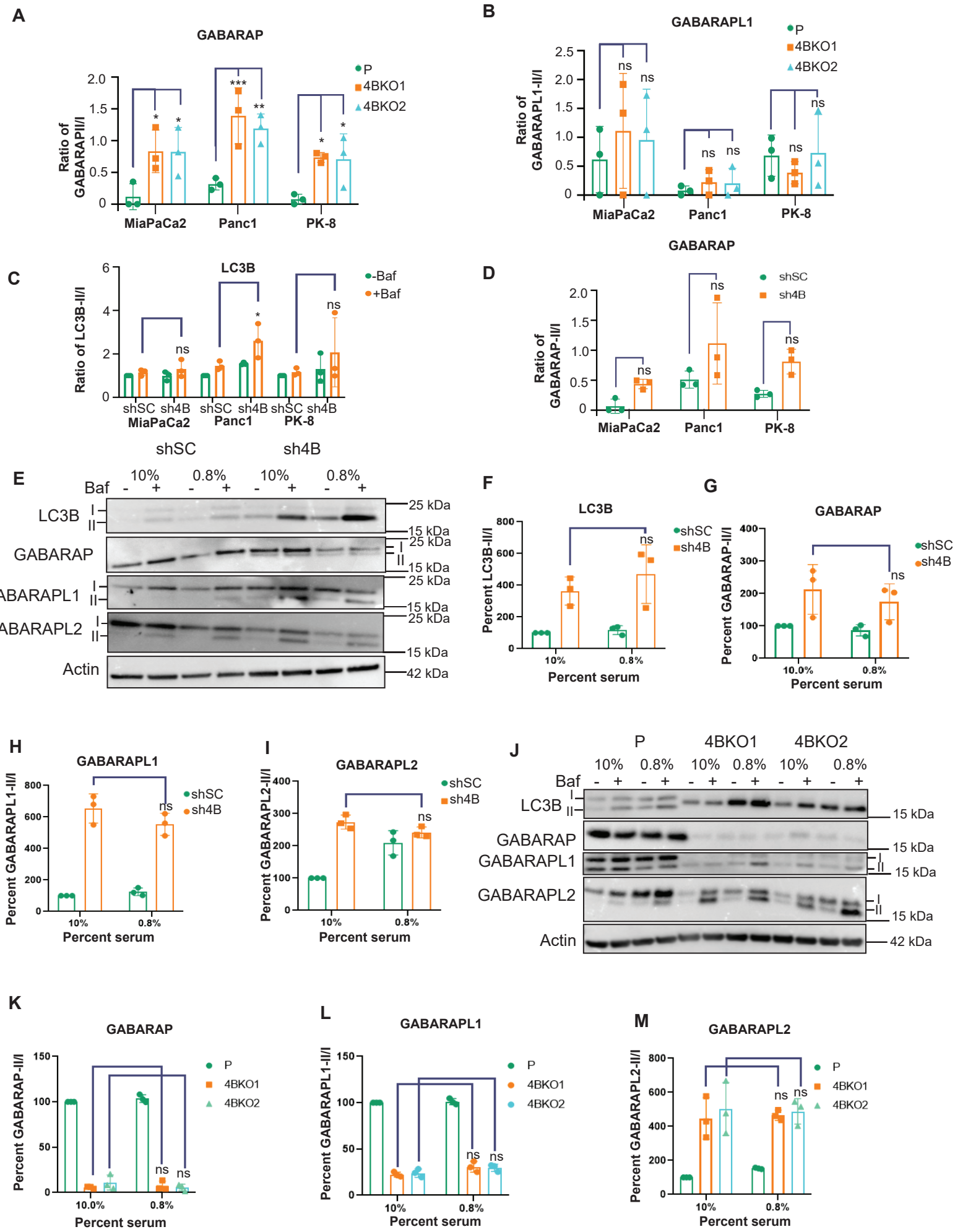
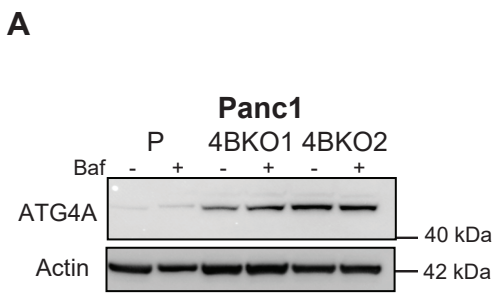
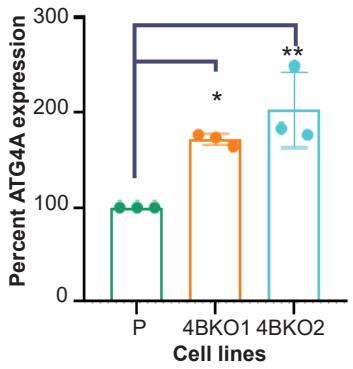
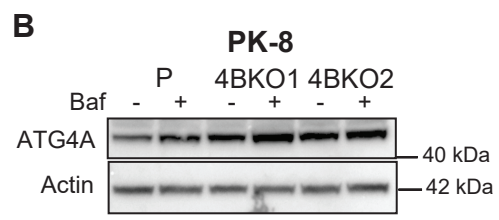
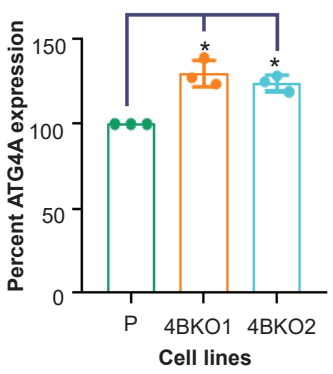


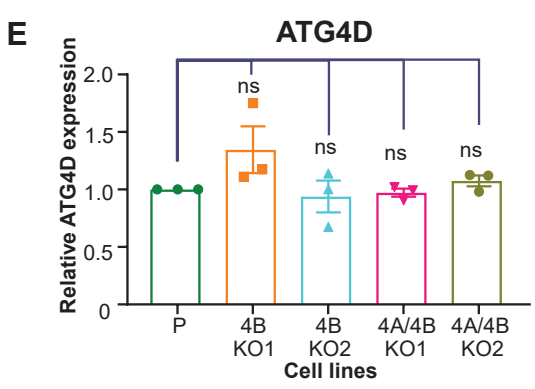
Fig. S2. Expression and lipidation of LC3/GABARAP families in ATG4B KO and KD cell lines. **(A)** Ratio of lipidated GABARAP (GABARAP-II) to un-lipidated GABARAP (GABARAP-I or pro-GABARAP) in Baf-treated samples in ATG4B KO lines and their respective parental lines (n=3). **(B)** Ratio of lipidated GABARAPL1 (GABARAPL1-II) to un-lipidated GABARAPL1 (GABARAPL1-I) in Baf-treated samples in ATG4B KO lines and their respective parental lines (n=3). **(C):** The ratio of LC3B-II to LC3B-I in sh4B and their respective scramble control (shSC) from MiaPaCa2, Panc1 and PK-8 with (+; orange) and without (-; green) BafA1. Values were normalized to the shSC controls without Baf (-Baf) for each respective cell line (n=3). **(D)** Ratio of lipidated GABARAP (GABARAP-II) to un-lipidated GABARAP (GABARAP-I or pro-GABARAP) in Baf-treated samples in sh4B lines and their respective shSC control lines (n=3). **(E)** Representative immunoblot of LC3B, GABARAP, GABARAPL1 and GABARAPL2 and actin (loading control) in MiaPaCa2 shSC and sh4B cell lines under 10% and 0.8% serum conditions with and without Baf treatment (n=3). Graphical representation of the percent ratio of **(F)** LC3B-II/LC3B-I, **(G)** GABARAP-II/GABARAP-I, **(H)** GABARAPL1-II/GABARAPL1-I and **(I)** GABARAPL2-II/GABARAPL2-I in sh4B and scramble control (shSC) of MiaPaCa2 Baf-treated samples under 10% and 0.8% serum conditions. Values were normalized to the shSC control in 10% serum condition (n=3). **(J)** Representative immunoblots of LC3B, GABARAP, GABARAPL1, GABARAPL2 and actin in parental (P) and ATG4B knockout (4BKO1 or 4BKO2) MiaPaCa2 cell lines under 10% and 0.8% serum conditions with and without Baf treatment (n=3). Quantification of **(K)** total GABARAP (GABARAP-I and GABARAP-II) or **(L)** total GABARAPL1 (GABARAPL1-I and GABARAPL1-II) in the presence of Baf from 3 biological replicates in 10% and 0.8% serum conditions. **(M)** Ratio of lipidated GABARAPL2 (GABARAPL2-II) to un-lipidated GABARAPL2 (GABARAPL2-I) in Baf-treated samples in MiaPaCa2 ATG4B KO and parental lines under 10% and 0.8% serum conditions Each ATG4B KO cell line was normalized to its respective parental line under 10% serum condition (n=3). All error bars represent SE; Two-way ANOVA with Dunnett's multiple tests was used to compare the statistical significance of each ATG4B KO line under 10% and 0.8% serum conditions. Two-way ANOVA with Sidak's multiple comparisons test was used for sh4B lines. ns=not significant.

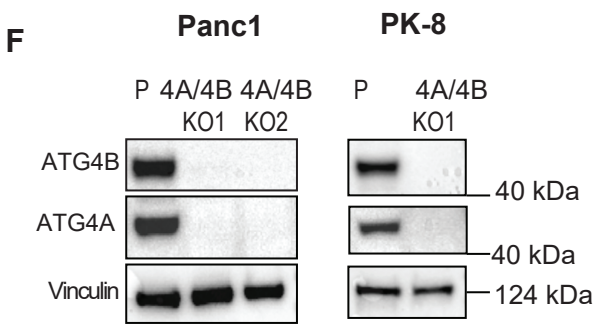


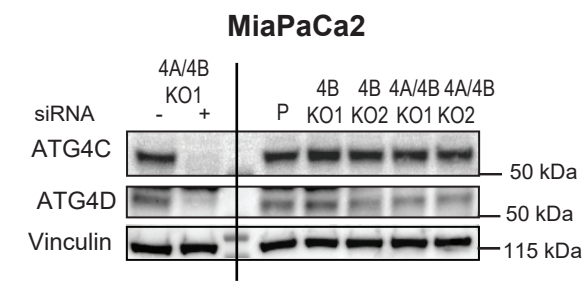


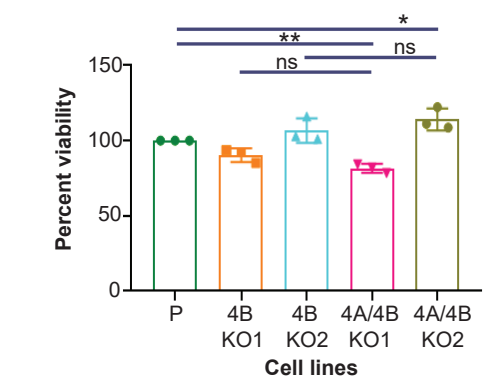


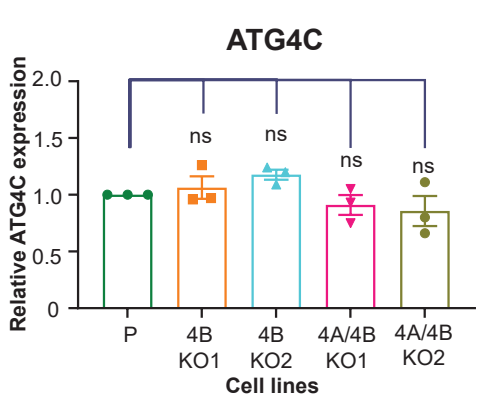












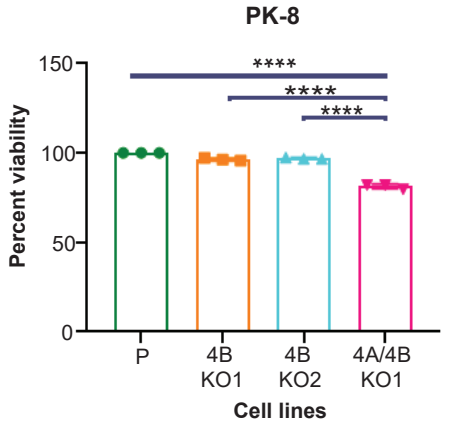
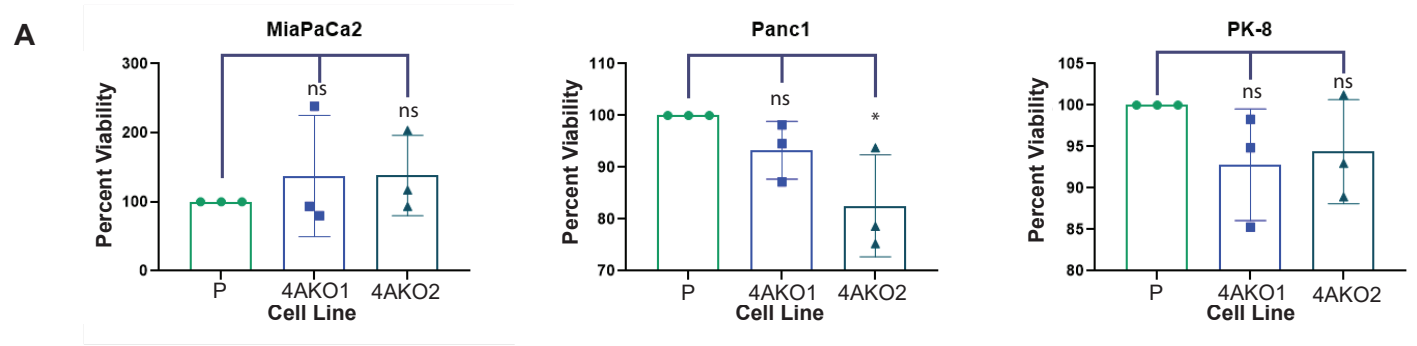
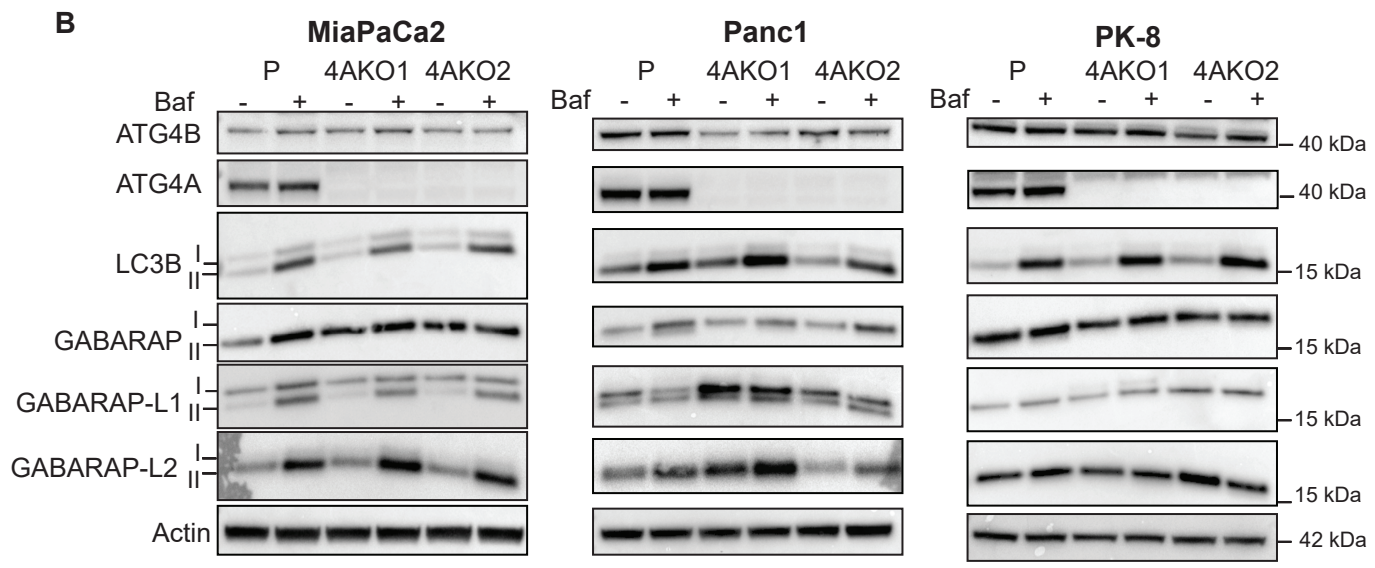
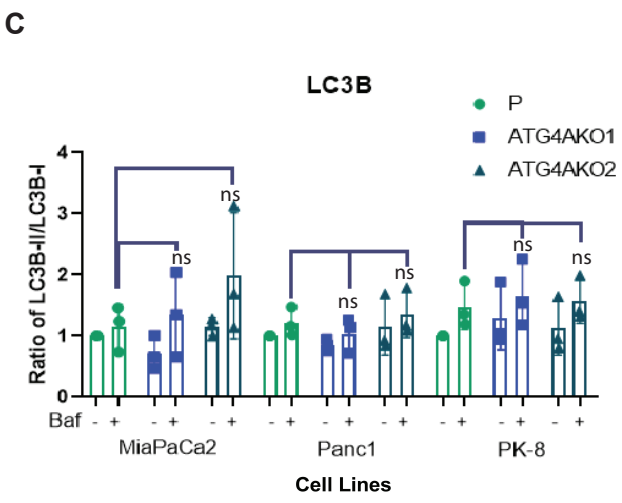
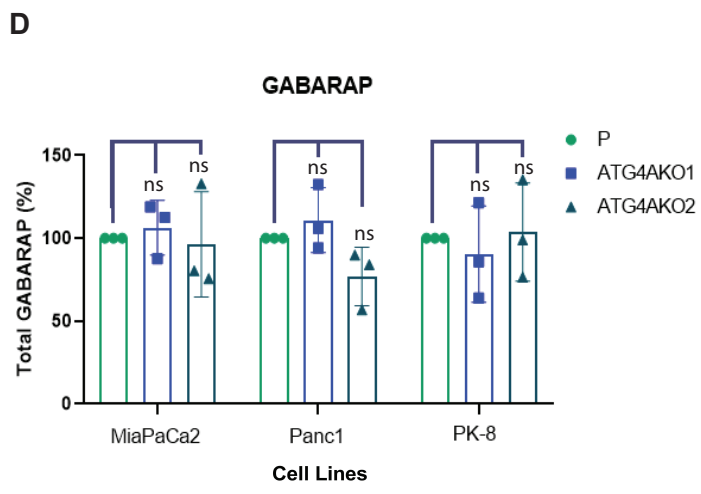


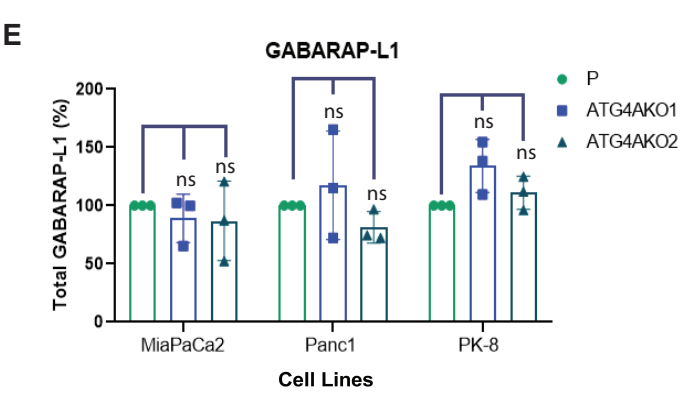
Fig. S3. ATG4B KO lines have elevated levels of ATG4A but not ATG4C or ATG4D. (A) Representative immunoblot of ATG4A and actin (loading control) in Panc1 parental (P) and ATG4B KO lines with (+) and without (-) Baf treatment. n= 3. Bar graph on the right indicates ATG4A levels (% relative to P) in Panc1 parental (P) and ATG4B KO lines in the Baf-treated samples (n=3). **(B)** Representative immunoblot of ATG4A and actin (loading control) in PK-8 parental (P) and ATG4B KO lines with (+) and without (-) Baf treatment. n= 3. Bar graph on the right indicates ATG4A levels (% relative to P) in PK-8 parental (P) and ATG4B KO lines in the Baf-treated samples (n=3). **(C)** Representative immunoblot of ATG4C, ATG4D and vinculin (loading control) in MiaPaCa2 parental (P), ATG4B KO and ATG4A/4B KO lines. The ATG4A/4B KO1 line was treated with ATG4C-siRNA or ATGD-siRNA to validate the antibodies (left). **(D)** Levels of ATG4C in MiaPaCa2 ATG4B KO and ATG4A/4B KO lines relative to the parental (P) line. n=3. **(E)** Levels of ATG4D in MiaPaCa2 ATG4B KO and ATG4A/4B KO lines relative to the parental (P) line. n=3. **(F)** Immunoblot representation of ATG4B, ATG4A and vinculin in parental (P) and ATG4A/4B KO lines of Panc1 and PK-8 (n=3). **(G)** Graphical representation of cell viability (%), determined using the crystal violet assay, of Panc1 parental (P), ATG4B KO and ATG4A/4B KO lines grown in 10% serum conditions for 72 hours. **(H)** Graphical representation of cell viability (%), determined using the crystal violet assay, of PK-8 parental (P), ATG4B KO and ATG4A/4B KO lines grown in 10% serum conditions for 72 hours. Each sample was normalized to the parental line but statistical significance was determined by comparing each ATG4A/4B KO line to the ATG4B KO line it was derived from (e.g. ATG4B KO1 vs ATG4A/4B KO1) or by comparing parental and ATG4A/4B KO lines. All error bars represent SE. Ordinary one-way ANOVA with Dunnett's multiple tests was used to determine statistical significance between ATG4B KO and ATG4A/4B KO lines or between parental and ATG4A/4B KO lines. ****p-value<0.0001; **p-value<0.01, *p-value<0.05; ns = not significant.











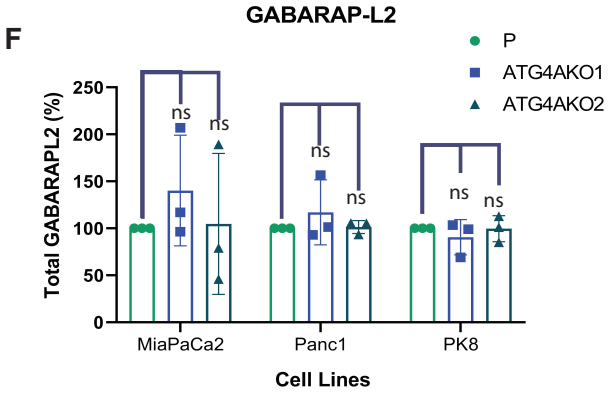


Fig. S4. ATG4A KO does not lead to consistent patterns of substrate alterations across multiple PDAC cell lines. (A) Graphical representation of cell viability (%), determined using the crystal violet assay, of MiaPaCa2 (left), Panc1 (middle) and PK-8 (right) parental (P) and ATG4A KO (4AKO1 and 4AKO2) lines grown in 10% serum conditions. Each sample was normalized to the parental line (n=3). **(B)** Representative immunoblots for ATG4A, LC3B, GABARAP, GABARAPL1 and GABARAPL2 in MiaPaCa2 (left), Panc1 (middle) and PK-8 (right) parental and ATG4A KO lines under 10% serum condition with and without Baf treatment. **(C)**The ratio of LC3B-II to LC3B-I in ATG4A KO lines and their respective parental lines (MiaPaCa2, Panc1 and PK-8) with and without Baf. Values were normalized to the respective parental line without Baf (-Baf) (n=3). Quantification of **(D)** total GABARAP (GABARAP-I and GABARAP-II), **(E)** total GABARAPL1 (GABARAPL1-I and GABARAPL1-II) and **(F)** total GABARAPL2 in the presence of Baf from 3 biological replicates. Each ATG4A KO cell line was normalized to its respective parental line (MiaPaCa2, Panc1 and PK-8). Error bars indicate SE; *p<0.05; ns=not significant.

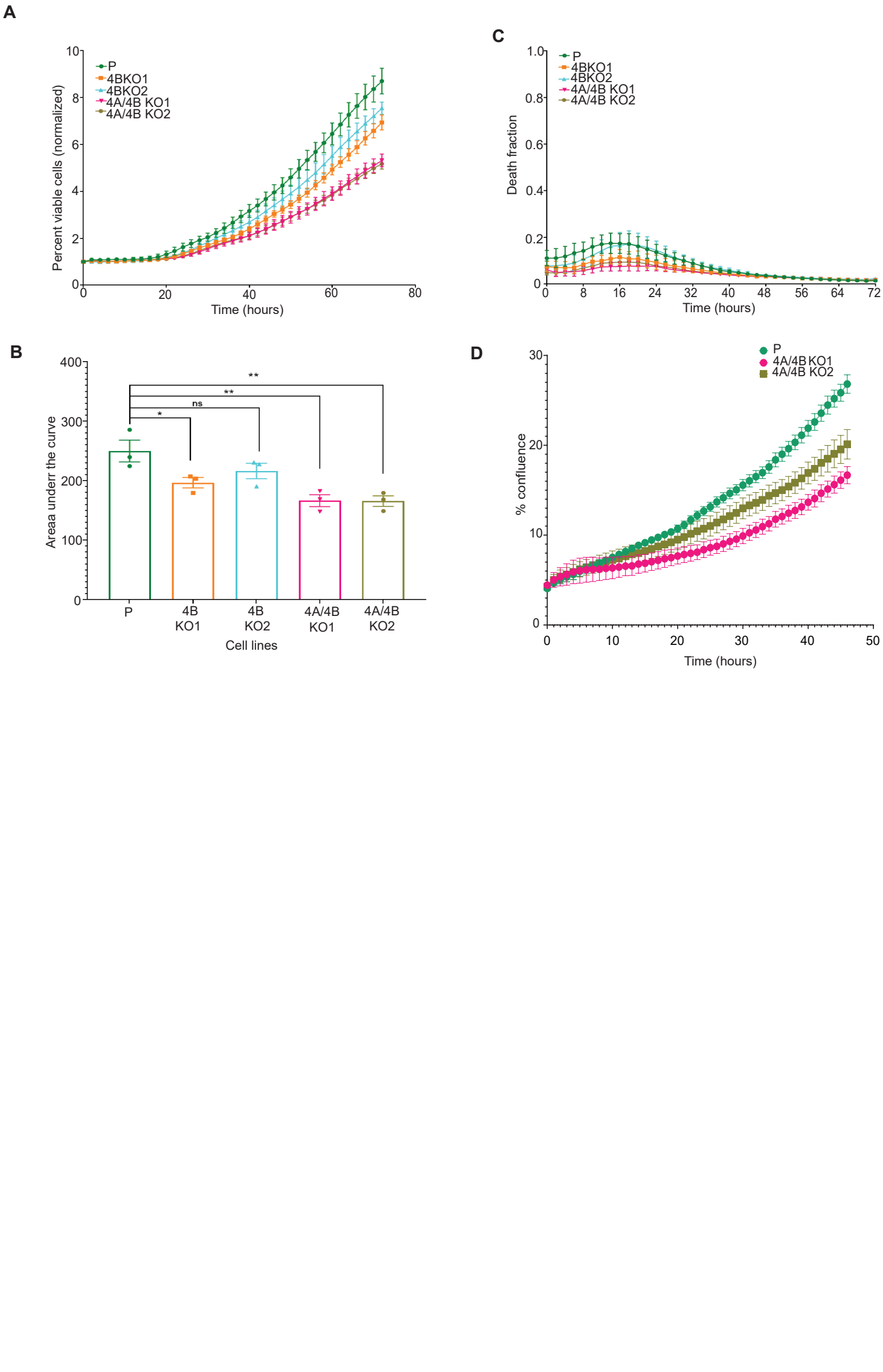
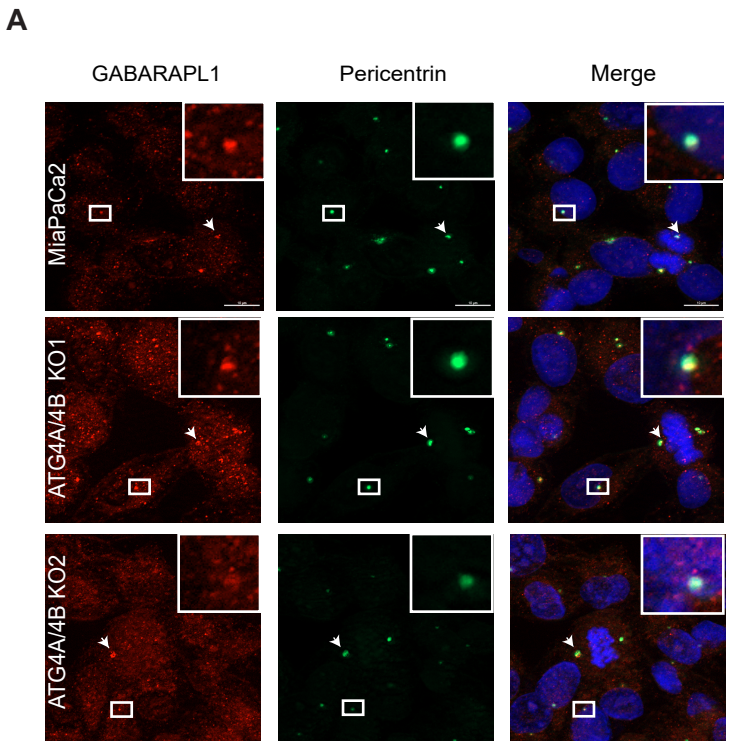
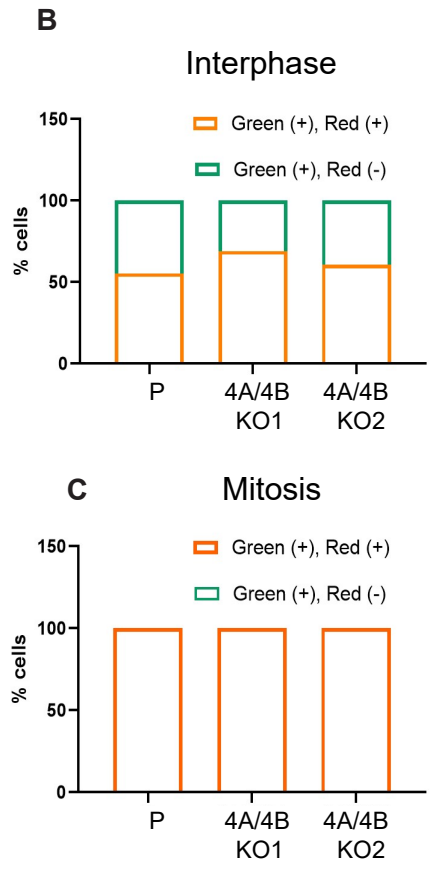
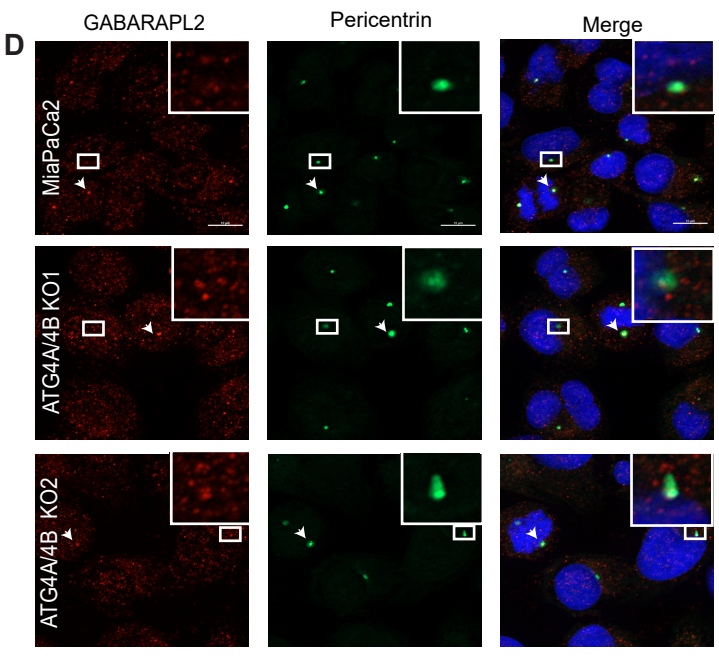


Fig. S5. ATG4A/4B KO lines display reduced proliferation. ((A) Viability curves of MiaPaCa2 parental (P) and ATG4A/4B KO (ATG4A/4B KO1 and 2) lines generated using the live-cell sensor Nuclight Red dye and Incucyte. **(B)** Graphical representation of area under the curve from (A) of MiaPaCa2 parental (P), ATG4B KO and ATG4A/4B KO lines. **(C)** Cell death fraction, detected using Nuclight Cytotox dye and Incucyte, in MiaPaCa2 parental (P), ATG4B KO and ATG4A/4B KO lines. **(D)** Confluence-based viability curves for MiaPaCa2 parental (P) and ATG4A/4B KO lines were generated using Incucyte analysis. Curves in (A), (C) and (D) were generated over the span of 72 hours. Error bars indicate mean ±SEM of 5 replicates.







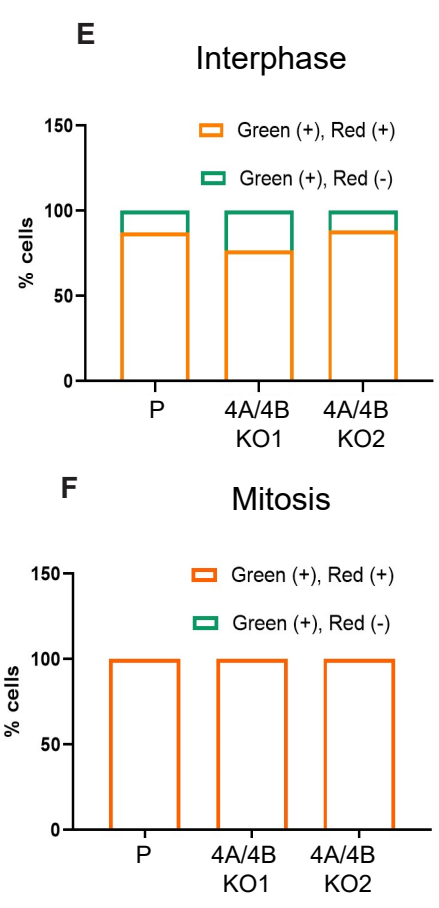


Fig. S6. GABARAPL1 and GABARAPL2 localize to centrosomes during both interphase and mitosis. **(A)** Representative images showing GABARAPL1 (red) and pericentrin (green) in MiaPaCa2 parental and ATG4A/4B KO cells. Insets show zoomed images of boxed regions indicating PCNT and GABARAPL1 co-localization. Arrows indicate mitotic cells displaying PCNT and GABARAPL1 colocalization to centrosomes. The scale bars represent 10 μm. Quantification of PCNT (green) and GABARAPL2 (red) co-localization (green+red) at both **(B)** interphase (parental, n = 56 cells; ATG4A/4B KO1, n= 55; ATG4A/4B KO2, n=38 from 1 experiment) and **(C)** mitosis (parental, n = 10 cells; ATG4A/4B KO1, n= 7; ATG4A/4B KO2, n=8 from 1 experiment) are shown as bar graphs on the right. **(D)** Representative images showing GABARAPL2 (red) and PCNT (green) in MiaPaCa2 parental and ATG4A/4B KO cells. Insets show zoomed images of boxed regions indicating PCNT and GABARAPL2 co-localization. Arrows indicate mitotic cells with PCNT and GABARAPL2 colocalization to centrosomes. The scale bars represent 10 μm. Quantification of PCNT (green) and GABARAPL2 (red) co-localization (green+red) at both **(E)** interphase (parental, n=94 cells; ATG4A/4B KO1, n=69; ATG4A/4B KO2, n=61 from 1 experiment) and **(F)** mitosis ((parental, n=4 cells; ATG4A/4B KO1, n=6; ATG4A/4B KO2, n=10 from 1 experiment) is shown as bar graphs on the right.

Supplementary Figure 7

Figure 2A

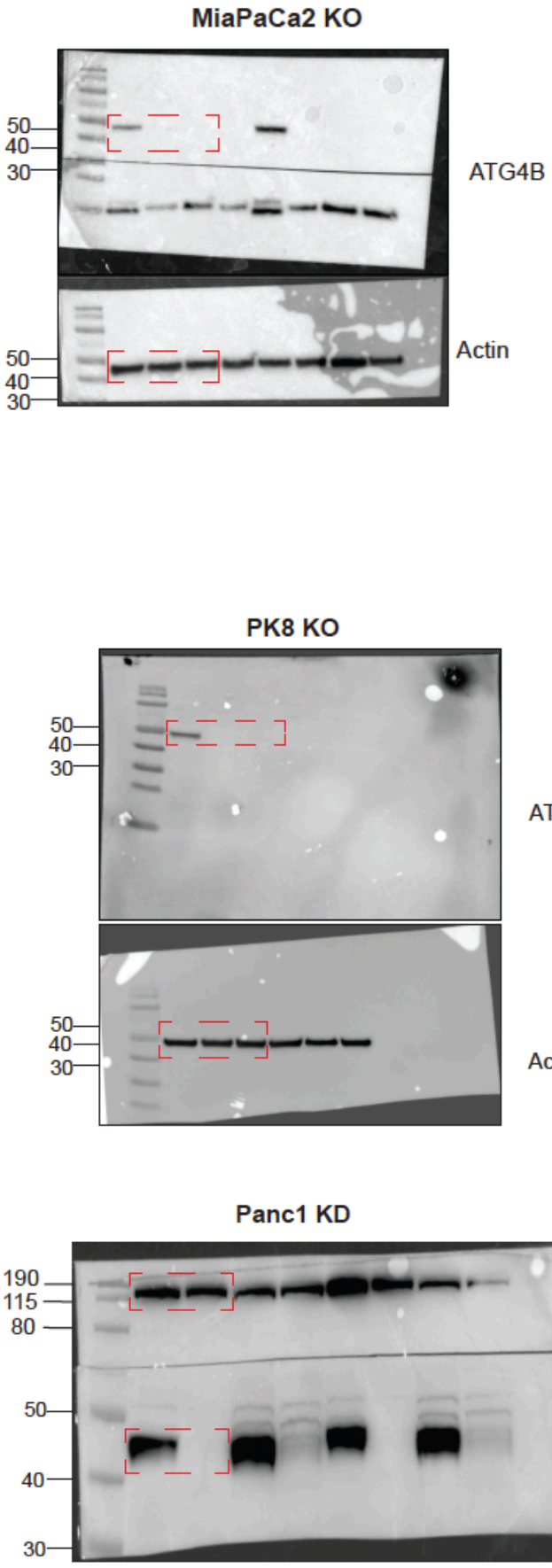


Figure 3

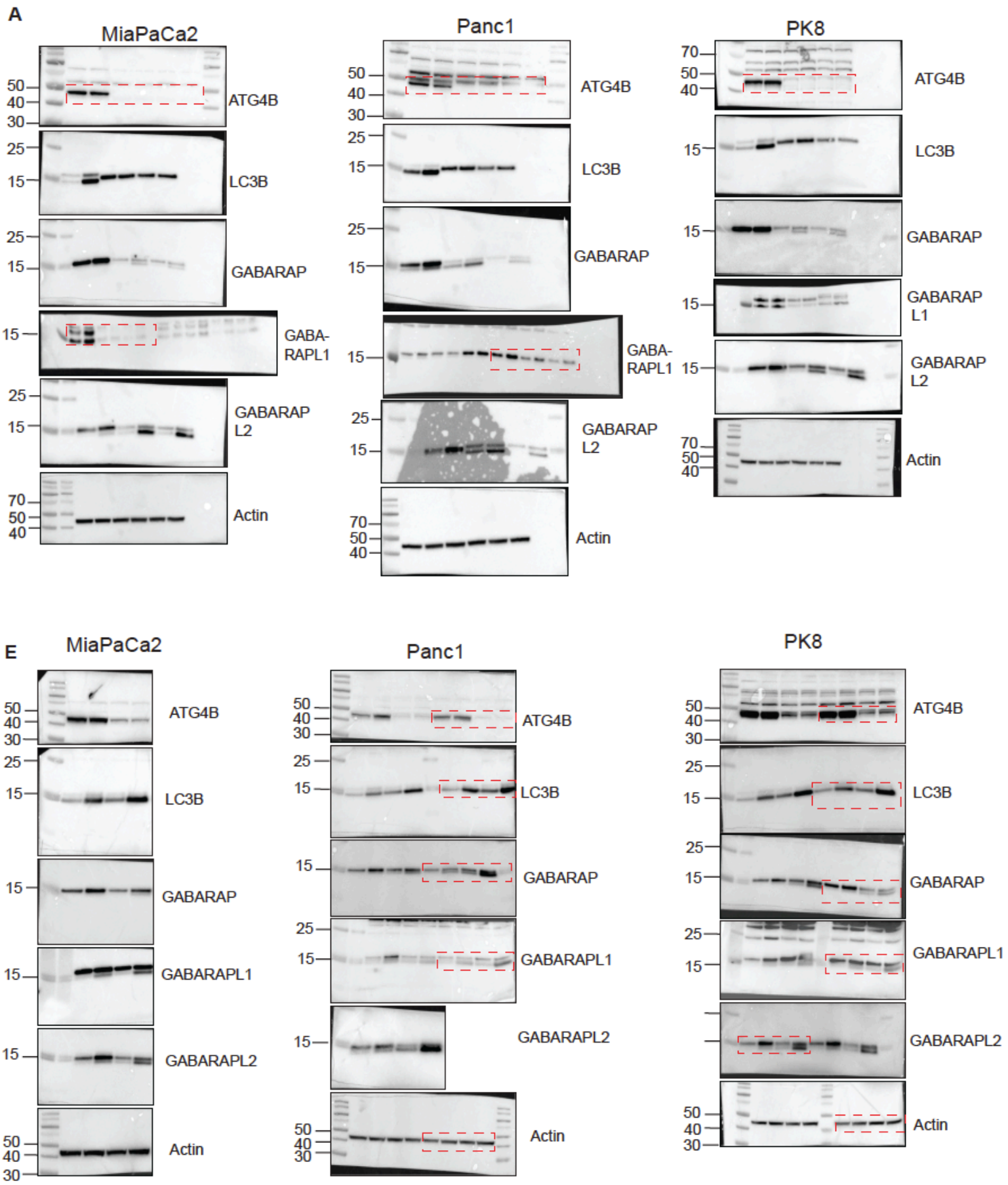


Figure 4A

MiaPaCa2

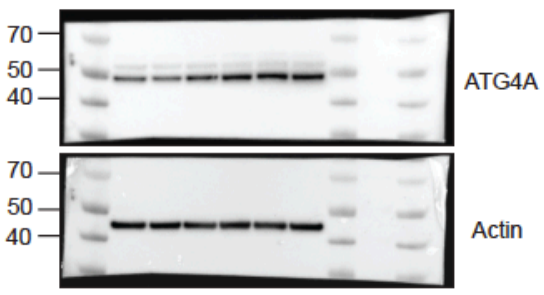


Figure 4E

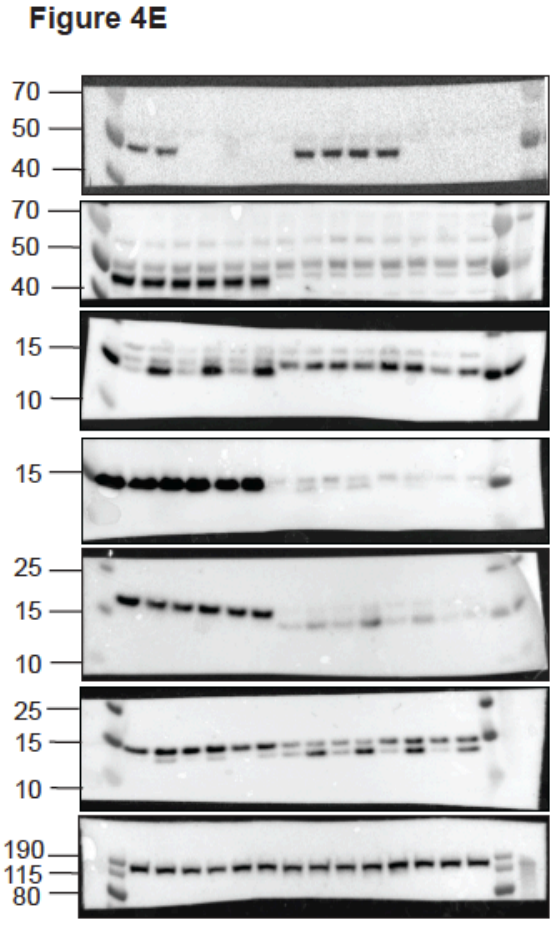


Figure 7E

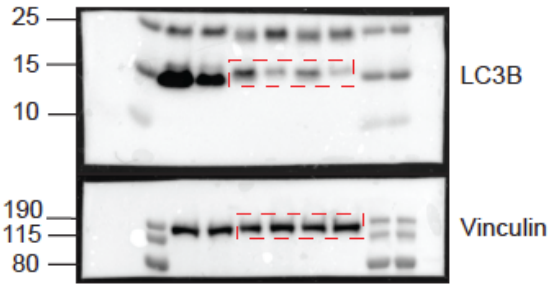


Figure 4C

MiaPaCa2

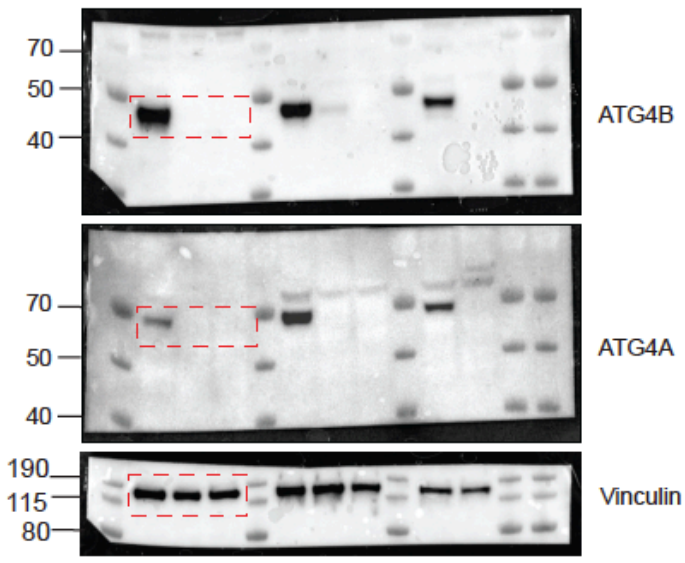


Figure 6C

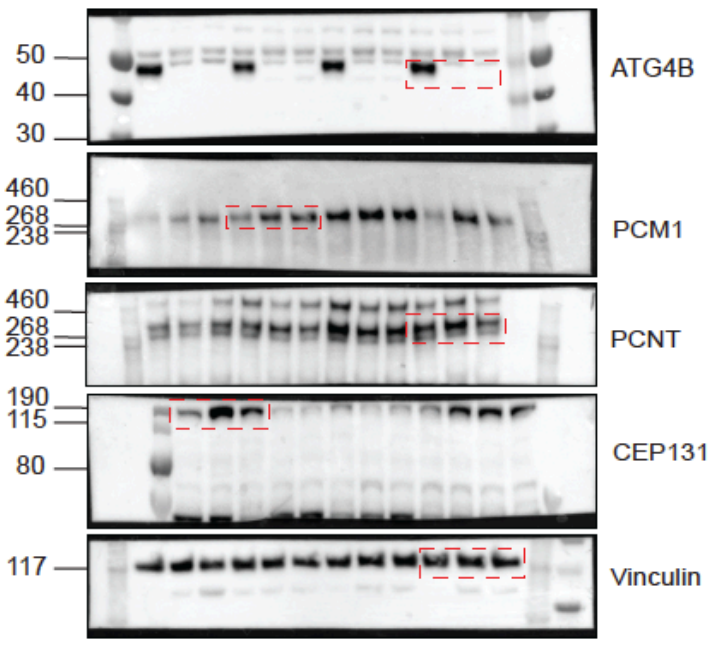


Figure 8A

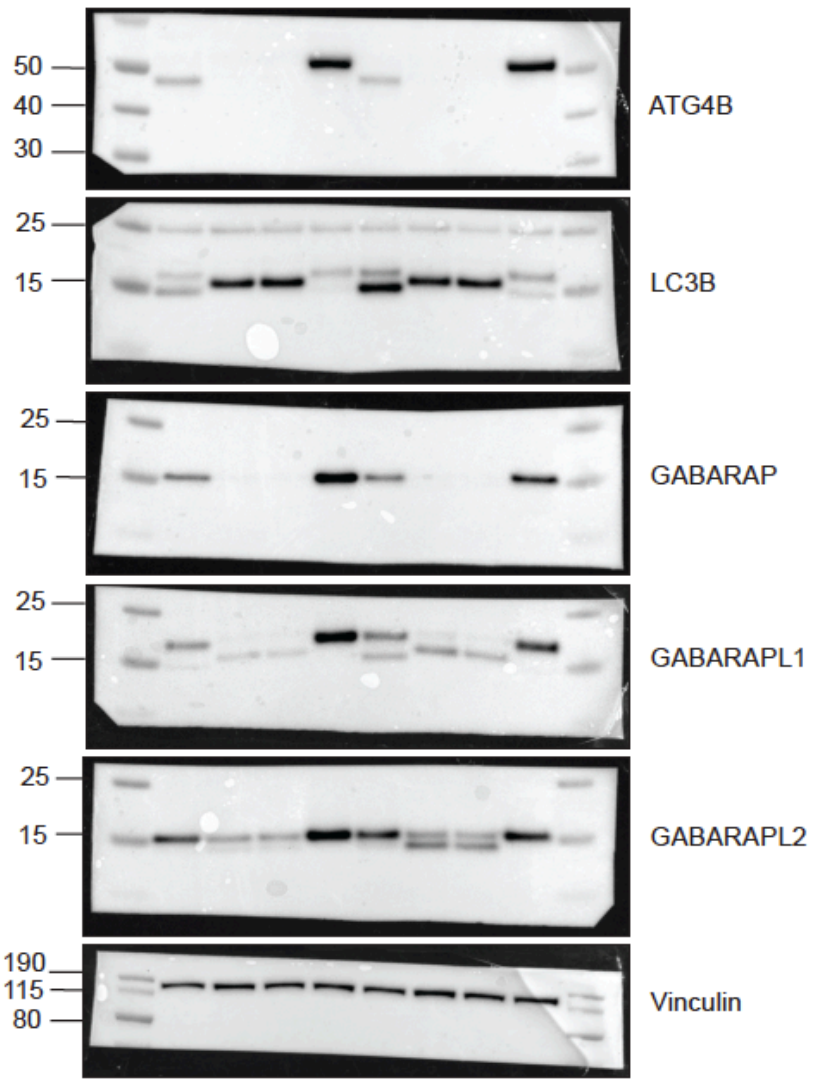
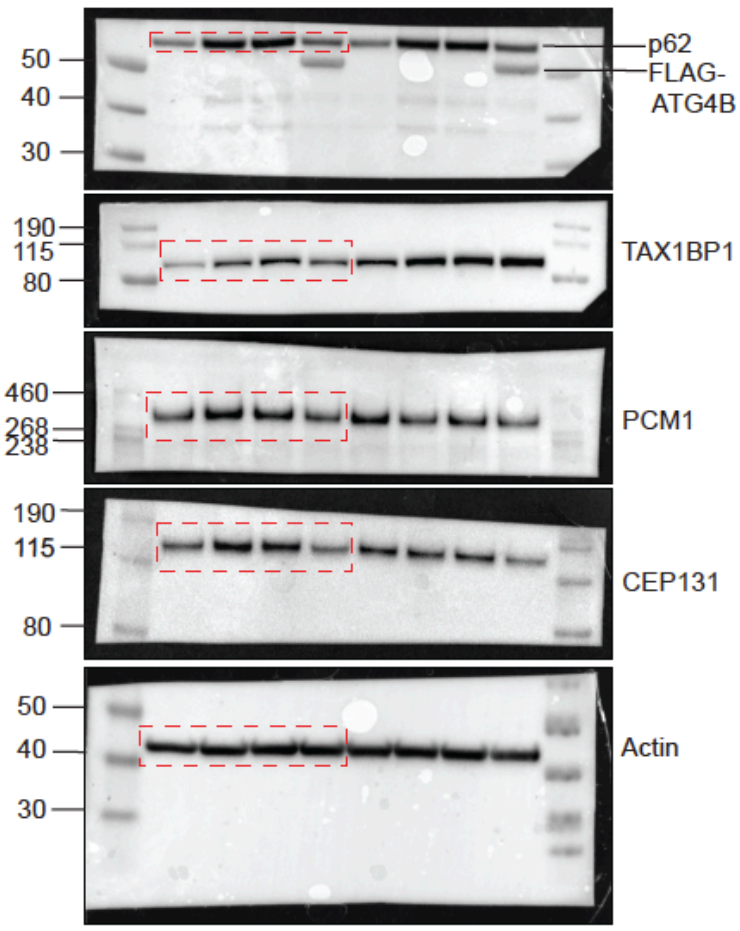
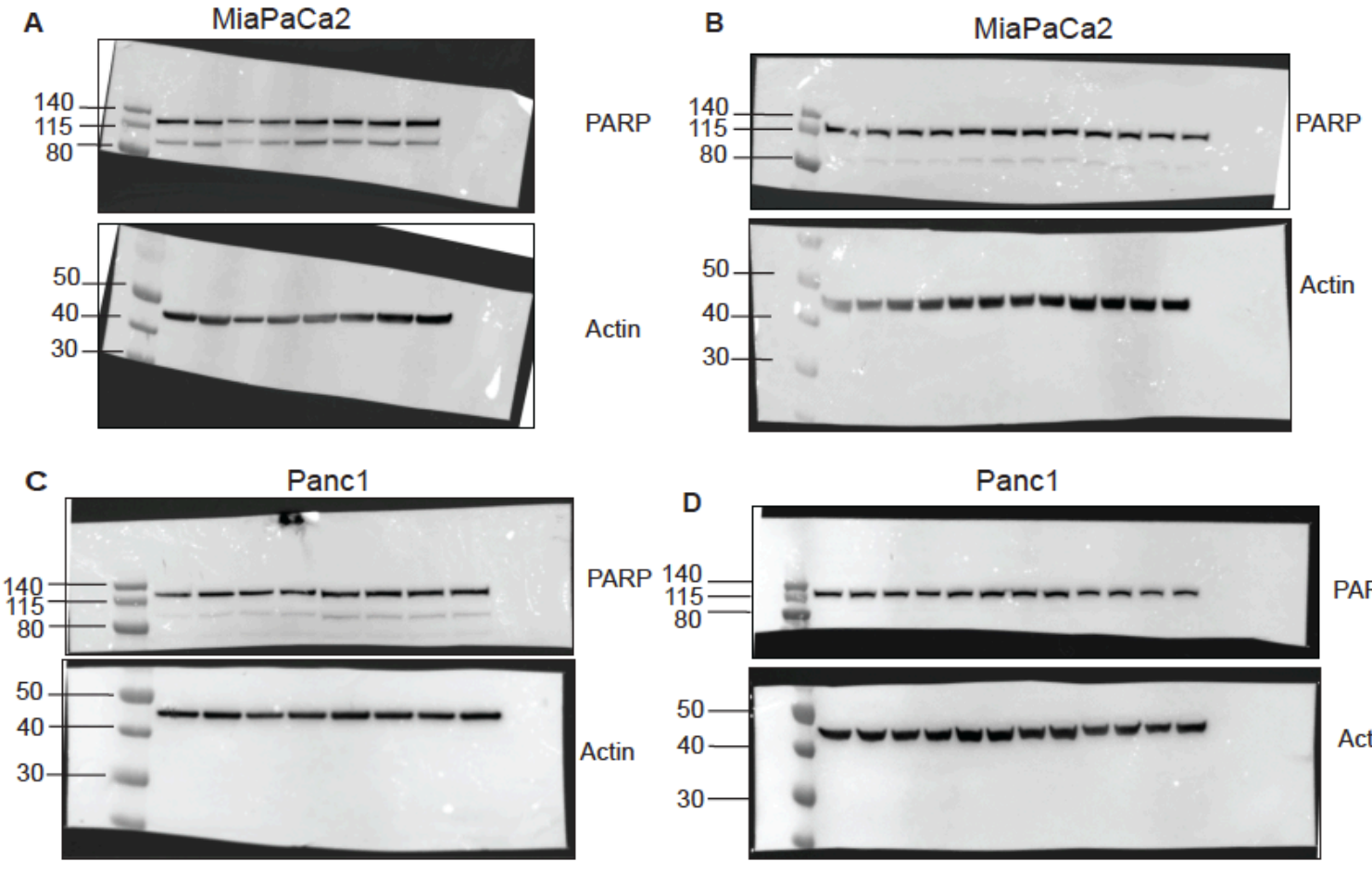


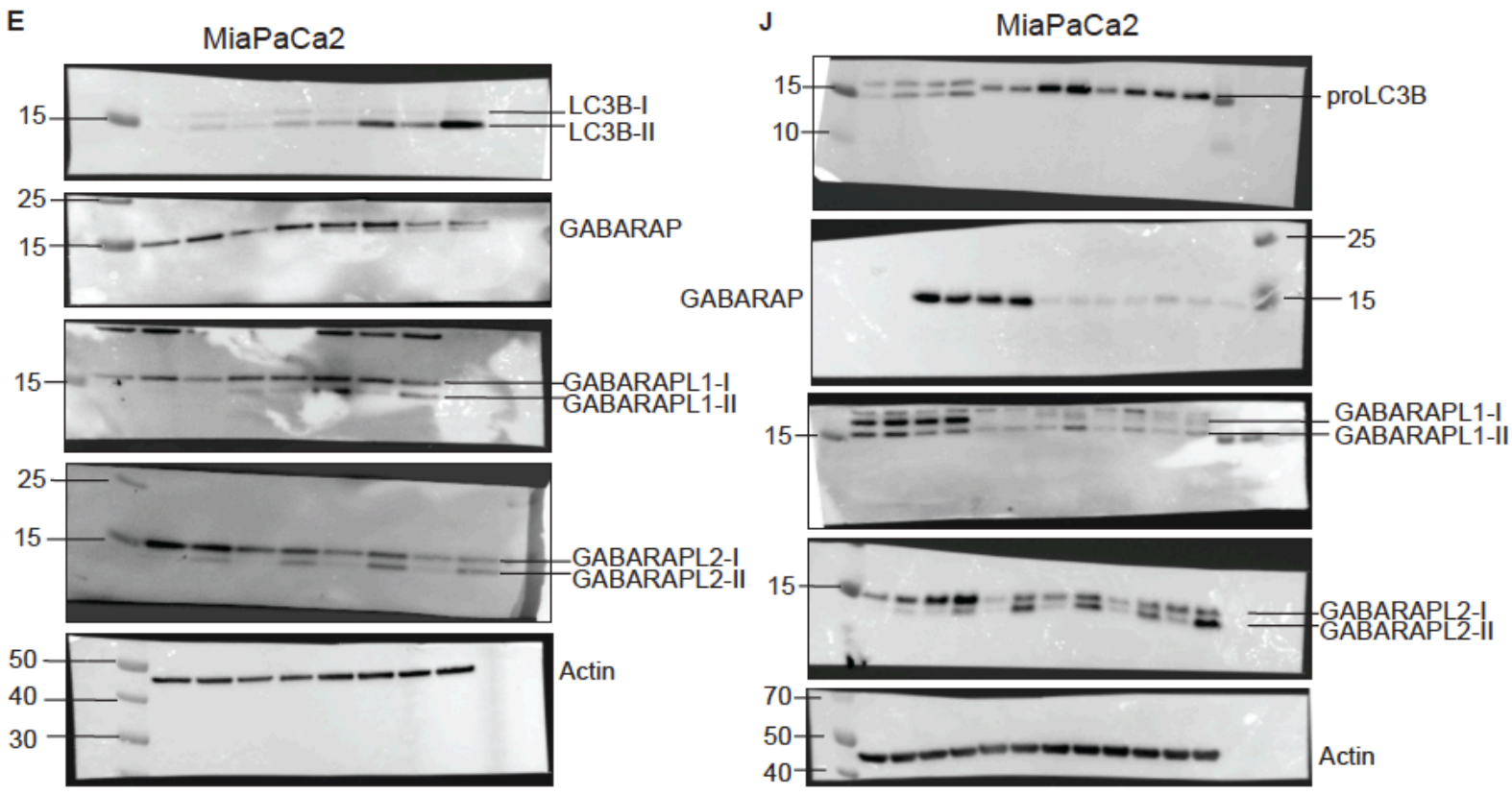
Figure 8F



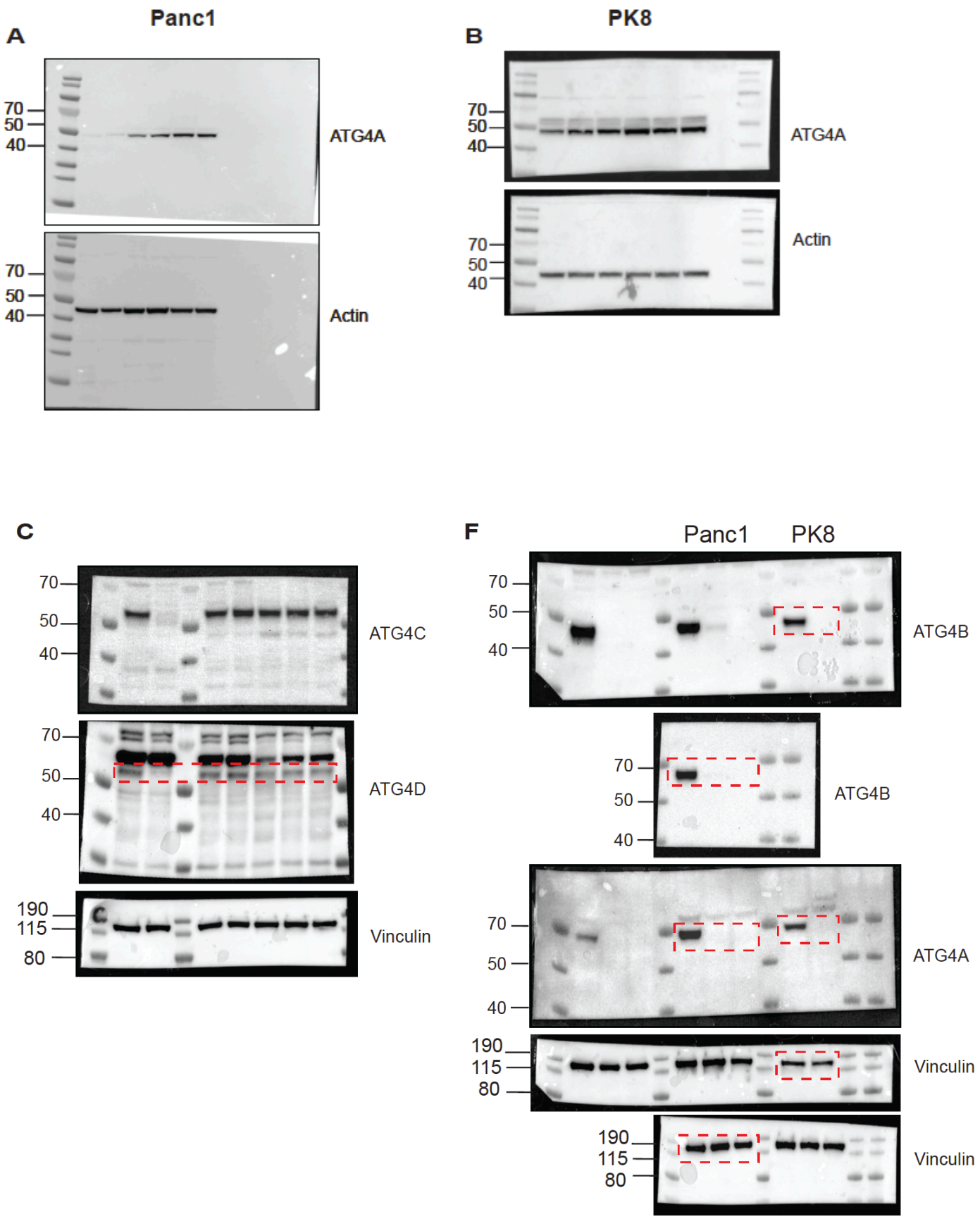
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4

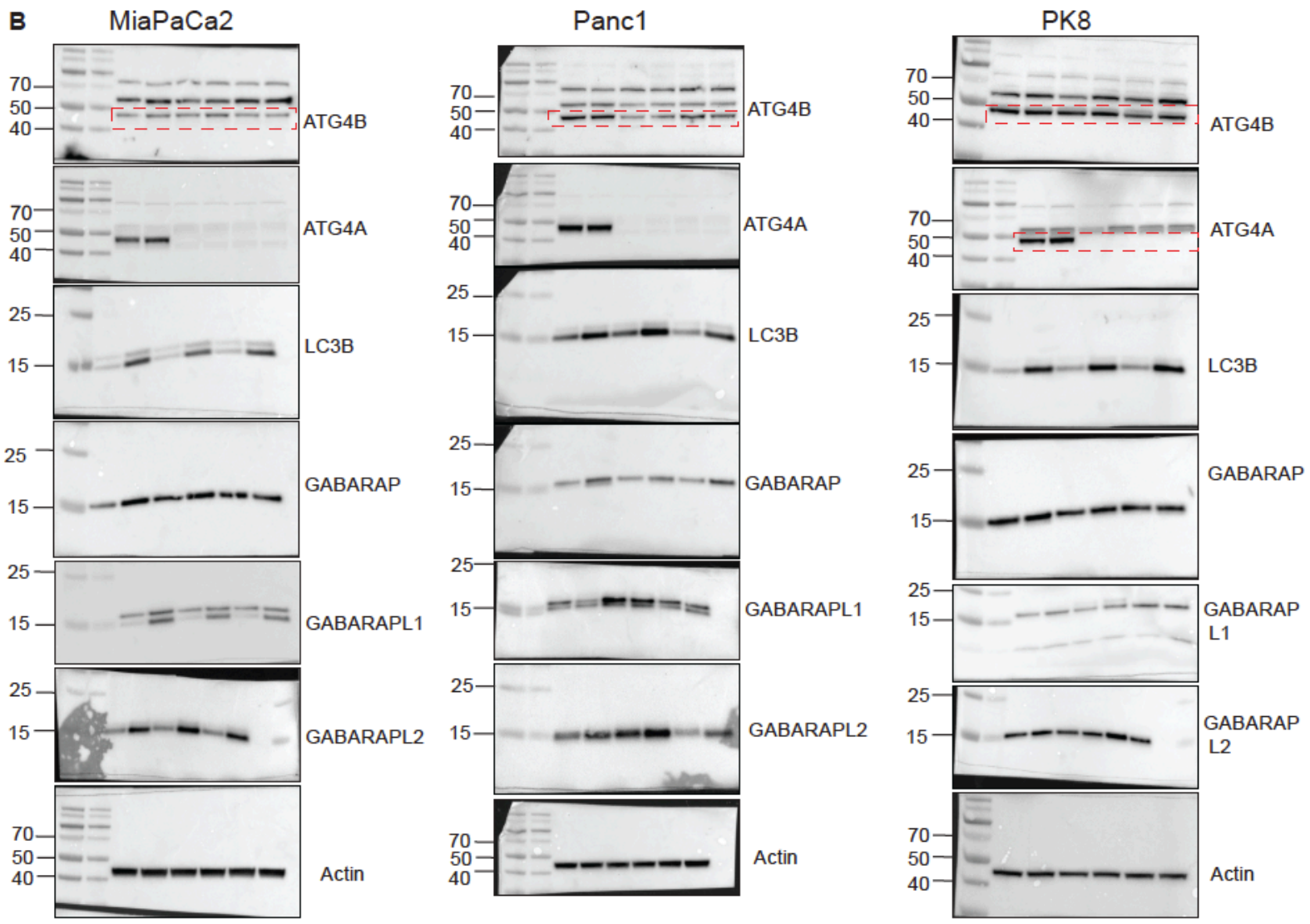
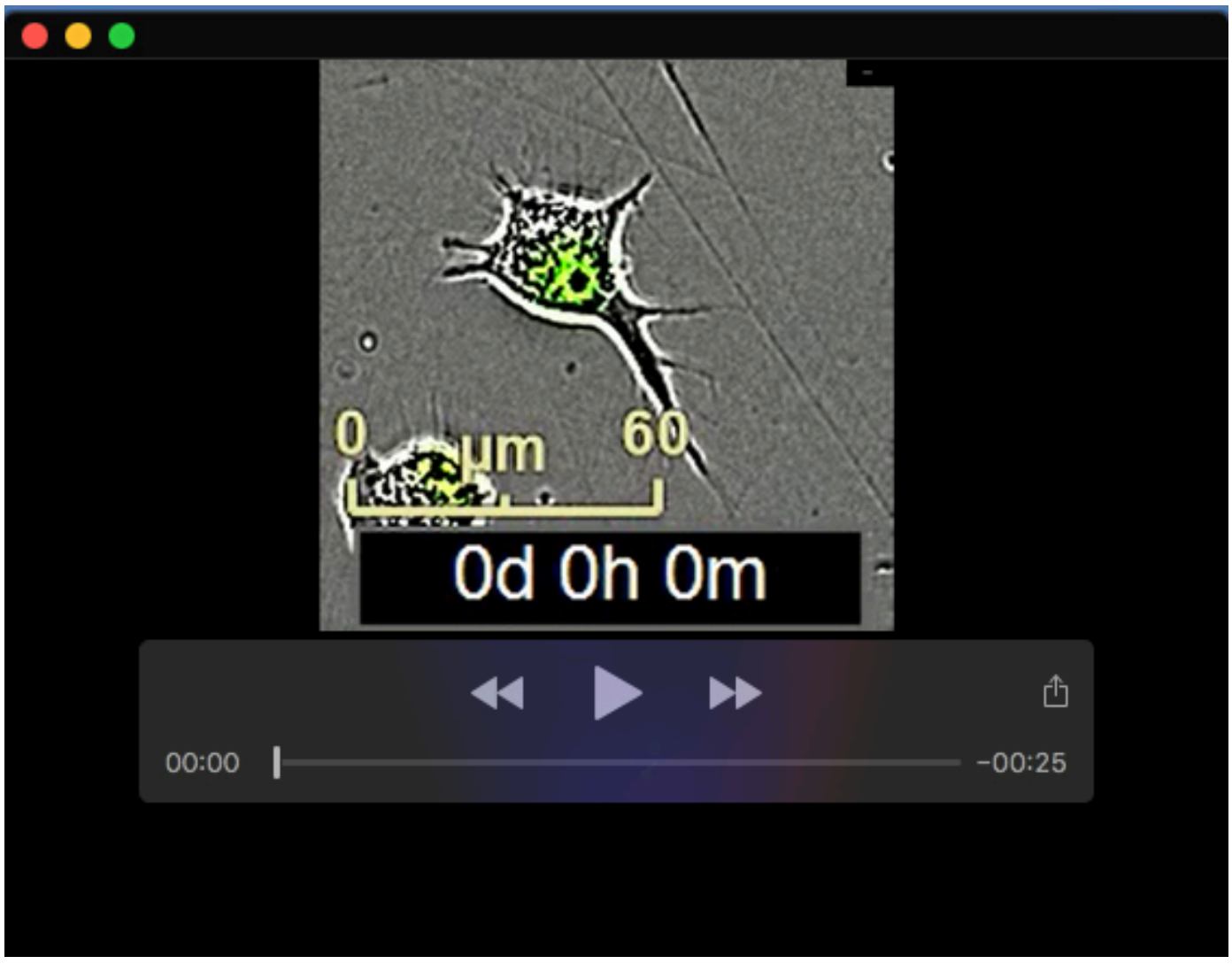


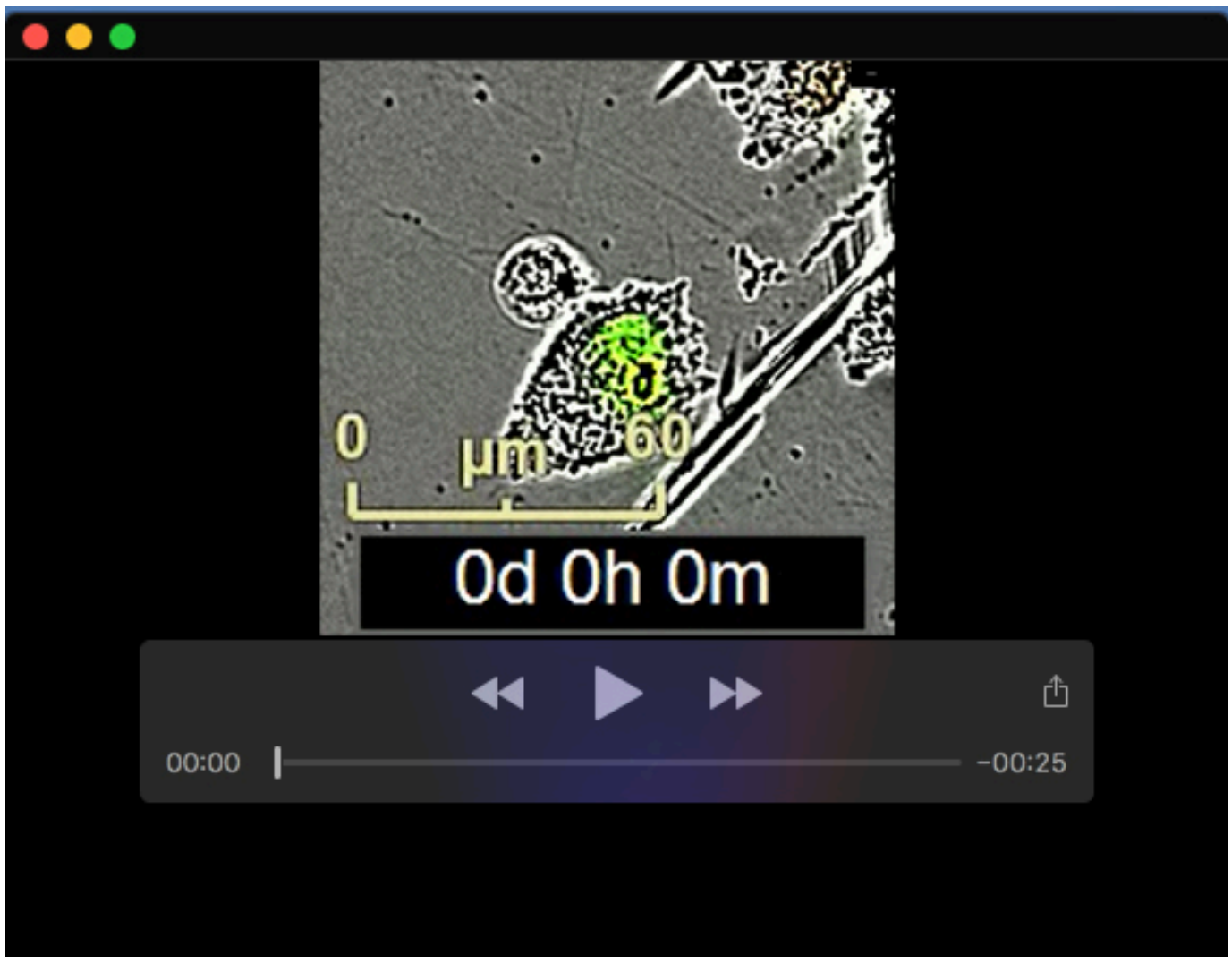
Fig. S7. Blot Transparency. Images of the full, uncropped blots with molecular weight markers (in kDa) are shown for all corresponding figures and supplementary figures.

Table S1. Analysis of ATG4B H-Score by clinicopathological features.

Factor	Count	Mean	Std Error	Lower 95%	Upper 95%	p-value
Histological grade						**0.0001**
Differentiation:						
G1: Well	2	240.000	67.338	107.30	372.70	
G2: Moderate	162	184.691	7.482	169.95	199.44	
G3: Poor	63	124.603	11.998	100.96	148.25	
Sex						**0.8407**
Female	106	168.255	9.6688	149.20	187.31	
Male	124	165.605	8.9396	147.99	183.22	
Adjuvant Chemotherapy						**0.0474**
No	164	158.598	7.707	143.41	173.78	
Yes	66	187.273	12.149	163.33	211.21	
Regional Lymph Nodes Metastasis Status						**0.5812**
pN0: No	53	180.472	13.603	153.67	207.28	
pN1: Yes	170	164.971	7.595	150.00	179.94	
pNX: Cannot be assessed	3	186.667	57.175	73.99	299.34	
Resection Status						**0.0899**
R0	169	169.941	7.594	155.0	184.90	
R1	57	165.088	13.076	139.3	190.85	
N/A	4	60.000	49.361	-37.3	157.26	
Lymphovascular Invasion						**0.2275**
No	96	177.760	10.072	157.91	197.61	
Yes	131	161.718	8.622	144.73	178.71	
Perineural Invasion						**0.1534**
No	18	200.833	23.240	155.04	246.63	
Yes	208	166.130	6.837	152.66	179.60	



Movie 1. Mitosis in MiaPaCa2 parental cells stably expressing *FUCCI.* Green indicates cells in S, G2 and G2-M transition; colorless indicates cells in mitosis; red indicates cells in G1; yellow indicates cells in G1-S transition. The images were captured using an IncuCyte live-imaging system with frames collected every 10 minutes. The display rate is 1 frame/second. Related to Figure 6A and 6B.



Movie 2. Mitosis in ATG4A/4B KO1 cells stably expressing FUCCI. Green indicates cells in S, G2 and G2-M transition; colorless indicates cells in mitosis; red indicates cells in G1; yellow indicates cells in G1-S transition. The images were captured using the IncuCyte live-imaging system with frames collected every 10 minutes. The display rate is 1 frame/second. Related to Figure 6A and 6B.