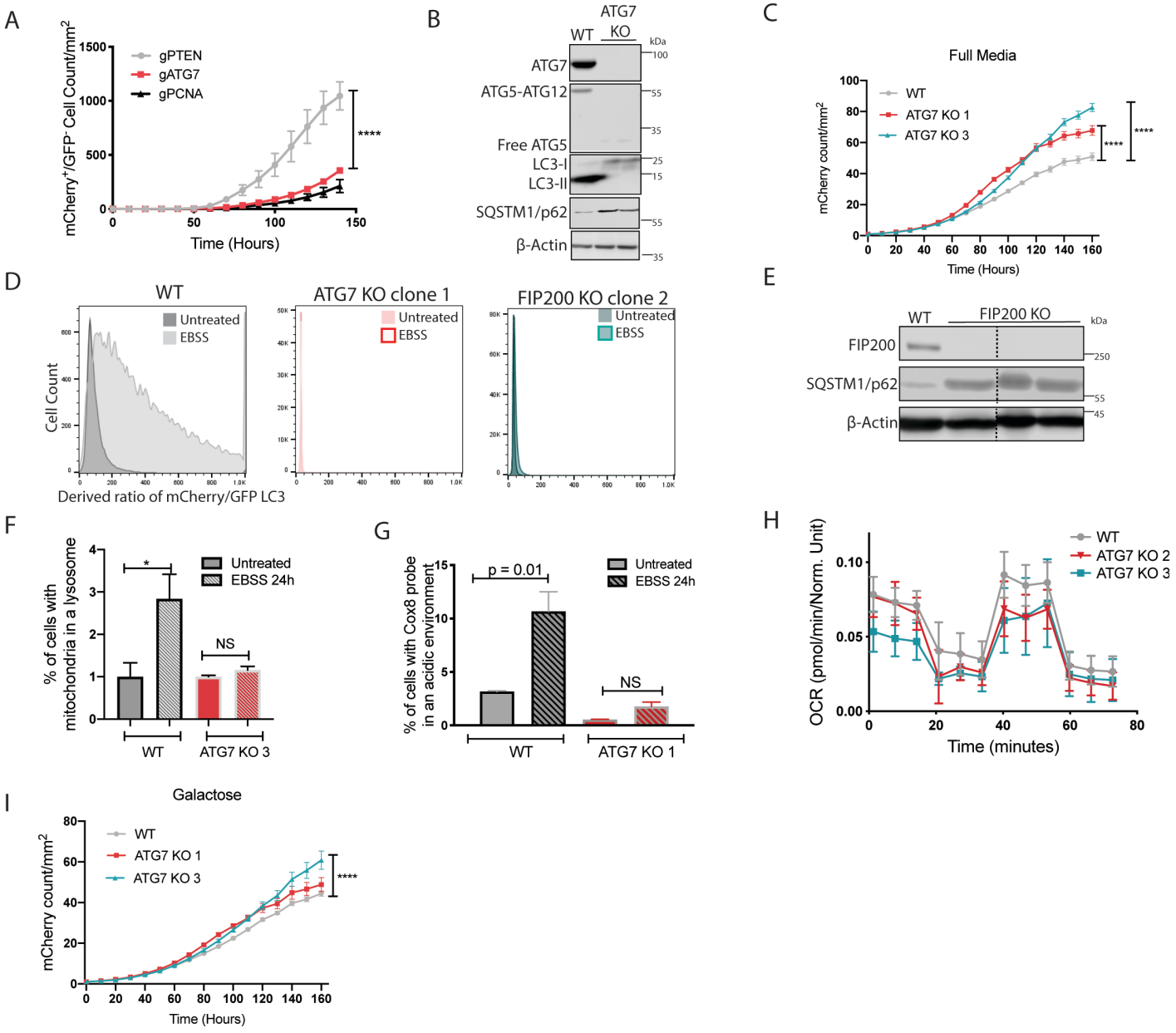


Figure S1. Rare clones derived from multiple autophagy-dependent cell lines adapt to maintain mostly functional mitochondria, Related to Figure 1. (A) In H292 cells, incucyte live cell imaging of the mCherry+/GFP- cell count/mm^2 immediately after delivery of gRNAs targeting the indicated genes along with gRNAs targeting GFP in cells with stable expression of GFP-NLS and mCherry-NLS. Data are represented as mean ± SEM for technical replicates (N of 3). The graphs are representative of 3 experiments. Statistical analysis: two-way ANOVA and the significance at the last time point is shown. (B) Western blot analysis of rare clones derived from H292 cells that survived loss of ATG7. Blots are representative of 3 experiments. (C) In H292 cells WT and ATG7 KO clones, incucyte live cell imaging of mCherry+ cell count/mm^2 normalized to time point 0. Data are represented as mean ± SEM for technical replicates (N of 6). The graphs are representative of 3 experiments. Statistical analysis: two-way ANOVA and the significance at the last time point is shown. (D) In BT549 WT, ATG7, and FIP200 KO cells with stable expression of mCh-GFP-LC3: representative histograms of ratiometric flow cytometry analysis after 24hours of starvation in EBSS medium. Graphs are representative of 3 experiments. (E) Western blot analysis of rare clones derived from BT549 cells that survived loss of FIP200. Blots are representative of 3 experiments. Unnecessary lanes were removed from the blot indicated by a dotted line. (F) Quantitative analysis of ratiometric flow cytometry (mCherry/GFP) in H292 WT and ATG7 KO clones with stable expression of mCh-GFP-Fis1 after 24hours of starvation in EBSS medium. Graphs are represented as mean ± SEM for biological replicates (N of 3). Statistical analysis: one-way ANOVA. (G) Quantitative analysis of ratiometric flow cytometry (561 channel/488 channel) in BT549 WT and ATG7 KO clones with stable expression of cox8-Keima before and after 24hours of starvation in EBSS medium. Graphs are represented as mean ± SEM for biological replicates (N of 3). Statistical analysis: one-way ANOVA. (H) Oxygen consumption rates measured via a Seahorse mitochondrial stress test in H292 WT and ATG7 KO clones. Data are represented as the mean ± SD for technical replicates (N = 8) and are representative of 3 individual experiments. (I) In H292 WT and ATG7 KO clones, incucyte live cell imaging of mCherry+ cell count/mm^2 normalized to time point 0 of cells grown in media where the glucose was substituted with 100mM galactose. Data are represented as mean ± SEM for technical replicates (N of 6). The graphs are representative of 3 experiments. Statistical analysis: two-way ANOVA and the significance at the last time point is shown. *p≤0.05, **** p≤0.001

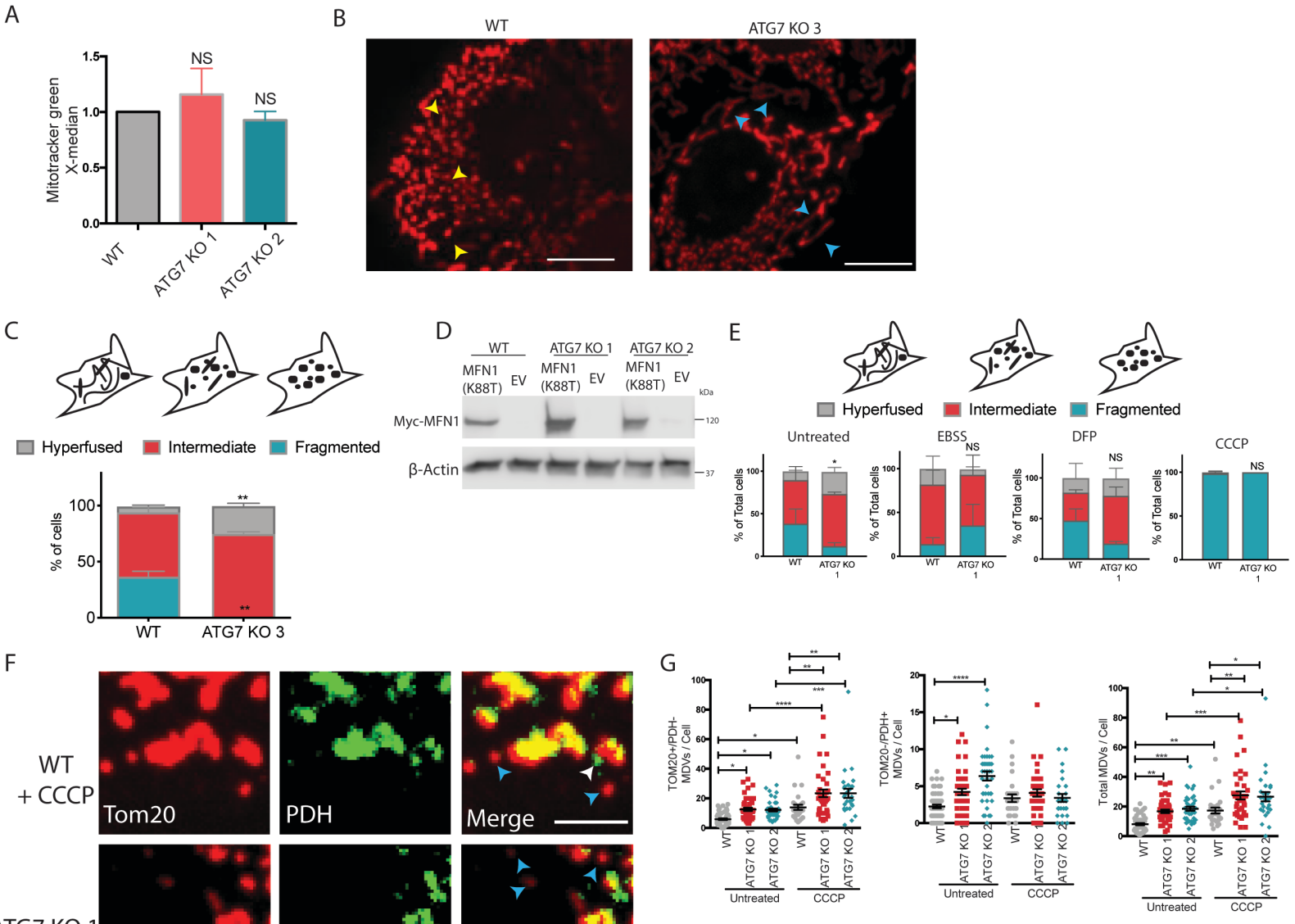
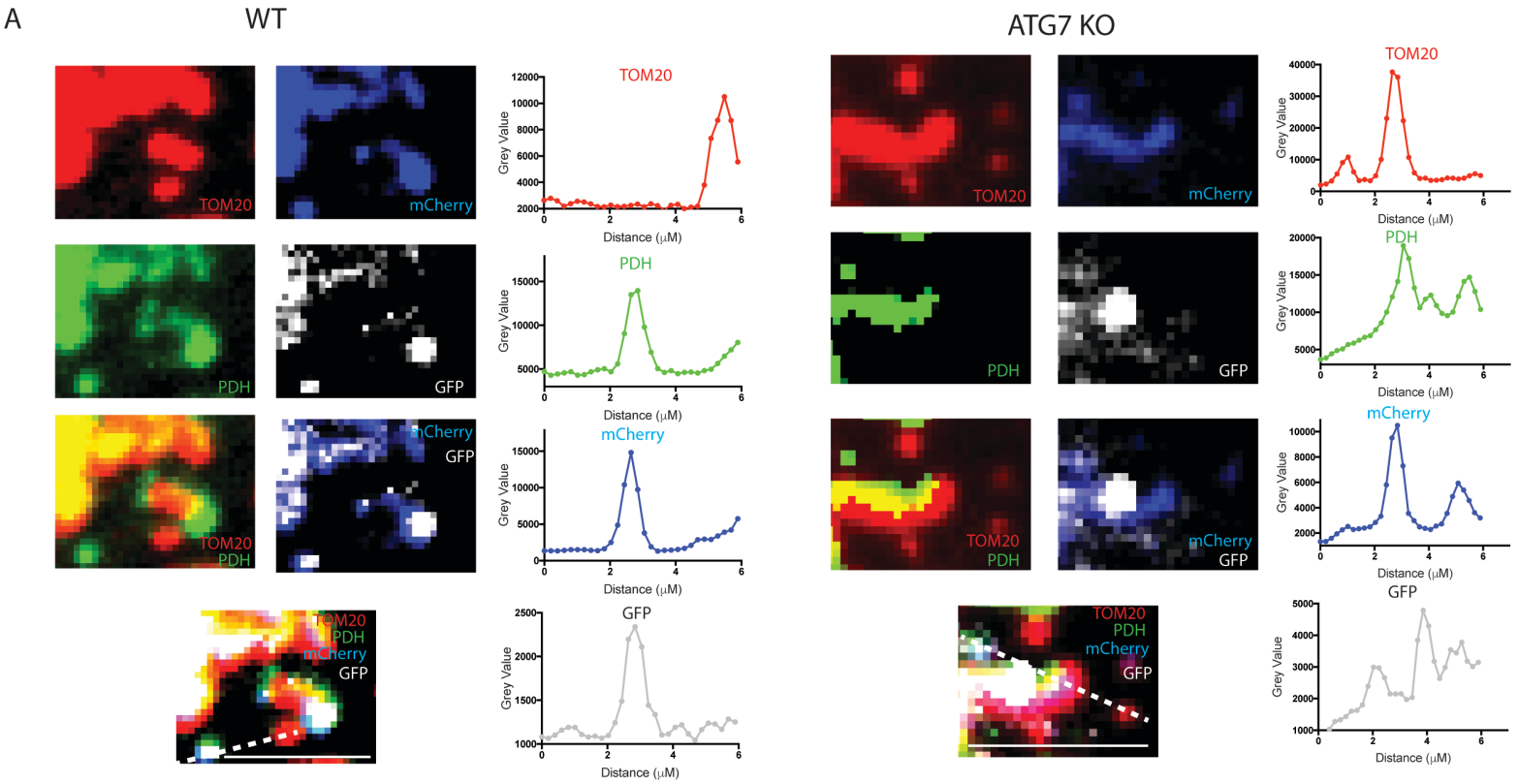
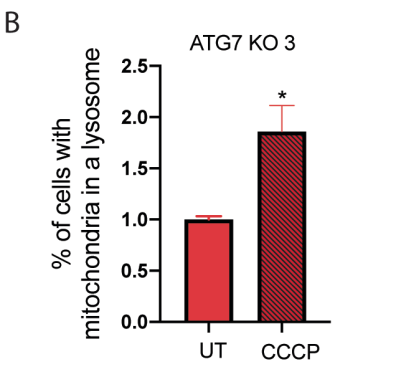
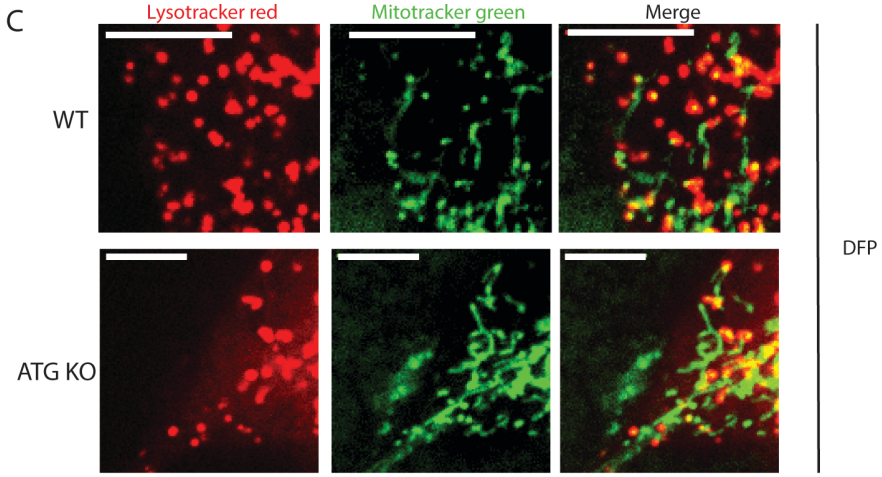


Figure S2. Autophagy deficient clones have increased mitochondrial fusion and increased mitochondrial derived vesicles, Related to Figures 2 and 3. (A) In BT549 WT and ATG7 KO clones, flow cytometry analysis to determine the relative amount of mitochondrial mass based on mitotracker green staining where the fold change in the X-median compared to WT is shown and the mean ± SEM for biological replicates (N of 4). (B-C) In H292 WT and ATG7 KO cells, (B) representative confocal images of mitotracker-red labeled mitochondria. Yellow arrow heads: fractured mitochondria, red arrow heads: elongated/hyperfused mitochondria. Scale bars represent 10μm (C) Top: Cartoon of how cells were categorized. Bottom: Quantification of categorized cells. Images from 2 individual experiments were combined and the data are represented as the mean±SD and an unpaired T-test was performed to determine statistical significance. (D) In BT549 WT and ATG7 KO cells, representative western blot of exogenous Myc expression after transient transfection with 3X-MYC-MFN1(K88T). Blot are representative of 2 experiments. (E-H) In BT549 WT and ATG7 KO clones, (E) Top: Cartoon of how cells were categorized. Bottom: Quantification of categorized cells. Images from 2-3 individual experiments were combined and the data are represented as the mean± SEM and an unpaired T-test was performed to determine statistical significance. (F) Representative confocal images from IF labeled cells with antibodies against TOM20 and PDH with or without treatment with CCCP (20μM) for 24hours. Scale bar represents 5μm. Blue and white arrow heads point to examples of TOM20+/PDH- and TOM20-/PDH+, respectively. (G) Quantification of the number of MDVs identified per cell across multiple images collected from 2 individual experiments. The data is shown as the mean ± SEM and each dot represents a cell. The total MDV count was determined by adding the TOM20+/PDH- count and the TOM20-/PDH+ count and a one-way ANOVA was performed to determine statistical analysis. (H) Representative western blot of PARKIN expression in mitochondrial fractions and probed for COXIV to verify purity of the fractionation. The blots are representative of 3 experiments. *p≤ 0.05, **p≤0.01, *** p≤0.01, **** p≤0.001







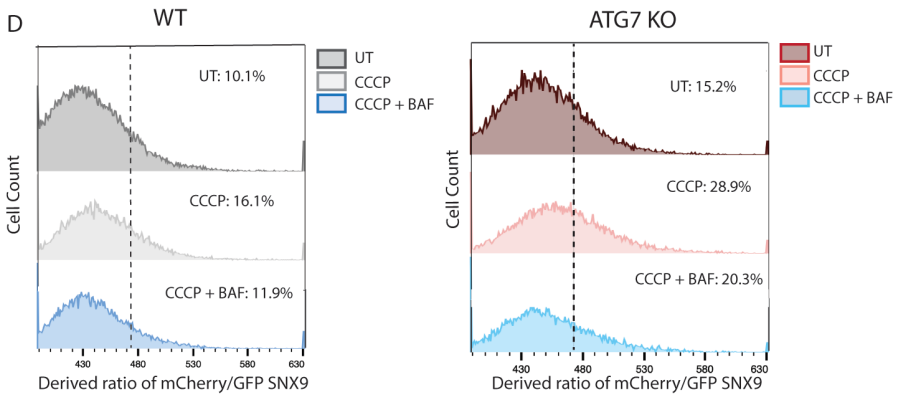


Figure S3. Mitochondrial derived vesicles can be labeled with pH sensitive mitochondrial probes, Related to Figure 4.
(A) In BT549 WT and ATG7 KO cells, representative confocal images from IF labeled cells with antibodies against TOM20 and PDH. Scale bar represents 5μm. The Keima-561 is pseudo-colored blue and the Keima-488 is pseudo-colored white. Blue and white arrow heads point to examples of TOM20+/PDH- and TOM20-/PDH+, respectively. The merged image shows the ROI quantified for intensity in the graphs to the right where the left side of the ROI line corresponds to the graph origin. (B) In H292 ATG7 KO cells with stable expression of mCh-GFP-Fis1, quantitative analysis of ratiometric flow cytometry (mCherry/GFP) 24hr after treatment with CCCP (20μM). Graphs are represented as mean ± SD for biological replicates (N of 2) and an unpaired T-test was performed for statistical analysis *p≤0.05. (C) Live cell imaging of mitotracker green (50nM) and lysotracker red (50nM) in WT and ATG7 KO cells treated with DFP(1mM) to induce mitochondrial turn over. Images are representative of multiple cells across 10-12 fields imaged over multiple experiments. Scale bars are 10μM. (D) Quantitative ratiometric flow cytometry in BT549 WT and ATG7 KO cells with stable expression of mCh-GFP-Fis1 and treated with CCCP (50μM) with or without Bafilomycin-A1 (20nM) for 24hrs. The histograms show the derived ratio of mCherry/GFP and are representative of 3 experiments.

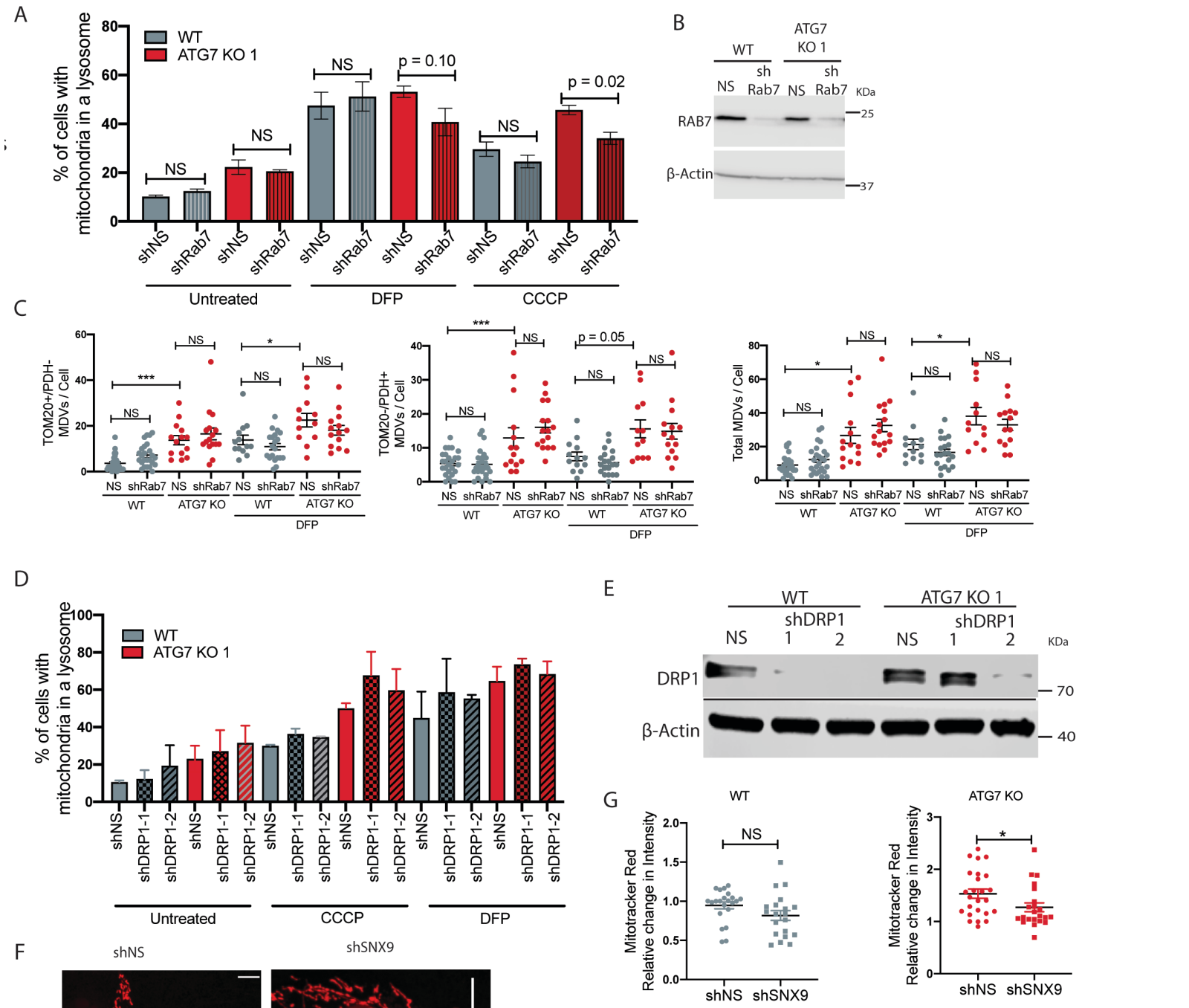


Figure S4. Mitochondrial derived vesicles can be labeled with pH sensitive mitochondrial probes. Related to Figure 5. (A-C) In BT549 WT and ATG7 KO cells with transient shRNAs targeting RAB7A and (A-B) stable expression of mCh-GFP-Fis1, quantitative analysis of ratiometric flow cytometry (mCherry/GFP) 24hr after treatment with CCCP (20μM) or DFP (1mM). Graphs are represented as mean ± SEM for biological replicates (N of 4) and a student's T-test was performed for statistical analysis comparing shNS to shRAB7A. (B) Western blot to assess protein knock down. Blots are representative of 4 experiments. (C) Quantification of the number of MDVs identified per cell across multiple images combined from 2 independent experiments. The data is shown as the mean ± SEM and each dot represents a cell. The total MDV count was determined by adding the TOM20+/PDH- count and the TOM20-/PDH+ count and a one-way ANOVA was performed to determine statistical analysis. (D-E) In BT549 WT and ATG7 KO cells with stable expression of mCh-GFP-Fis1 and transient shRNAs targeting DRP1, quantitative analysis of ratiometric flow cytometry (mCherry/GFP) 24hr after treatment with CCCP (20μM) or DFP (1mM). Graphs are represented as mean ± SD for biological replicates (N of 2) and a One-way ANOVA was performed for statistical analysis. (E) Western blot to assess protein knock down. Blots are representative of 2 experiments. (F-G) Live cell imaging of WT and ATG7 KO cells with stable mCherry-NLS expression 4 days after treatment with SNX9 targeting shRNAs and stained with the membrane potential-sensitive mitotracker red dye. To quantify the mitotracker red signal in G, a region of interest was drawn in each cell that excluded the mCherry+ nucleus and focused on the mitochondria near the periphery of the cell to avoid any nuclear signal. The data are represented as the relative change in intensity where each dot represents a cell and 2 individual experiments are combined. Scale bar is 10μM. *p≤0.05, *** P≤0.001

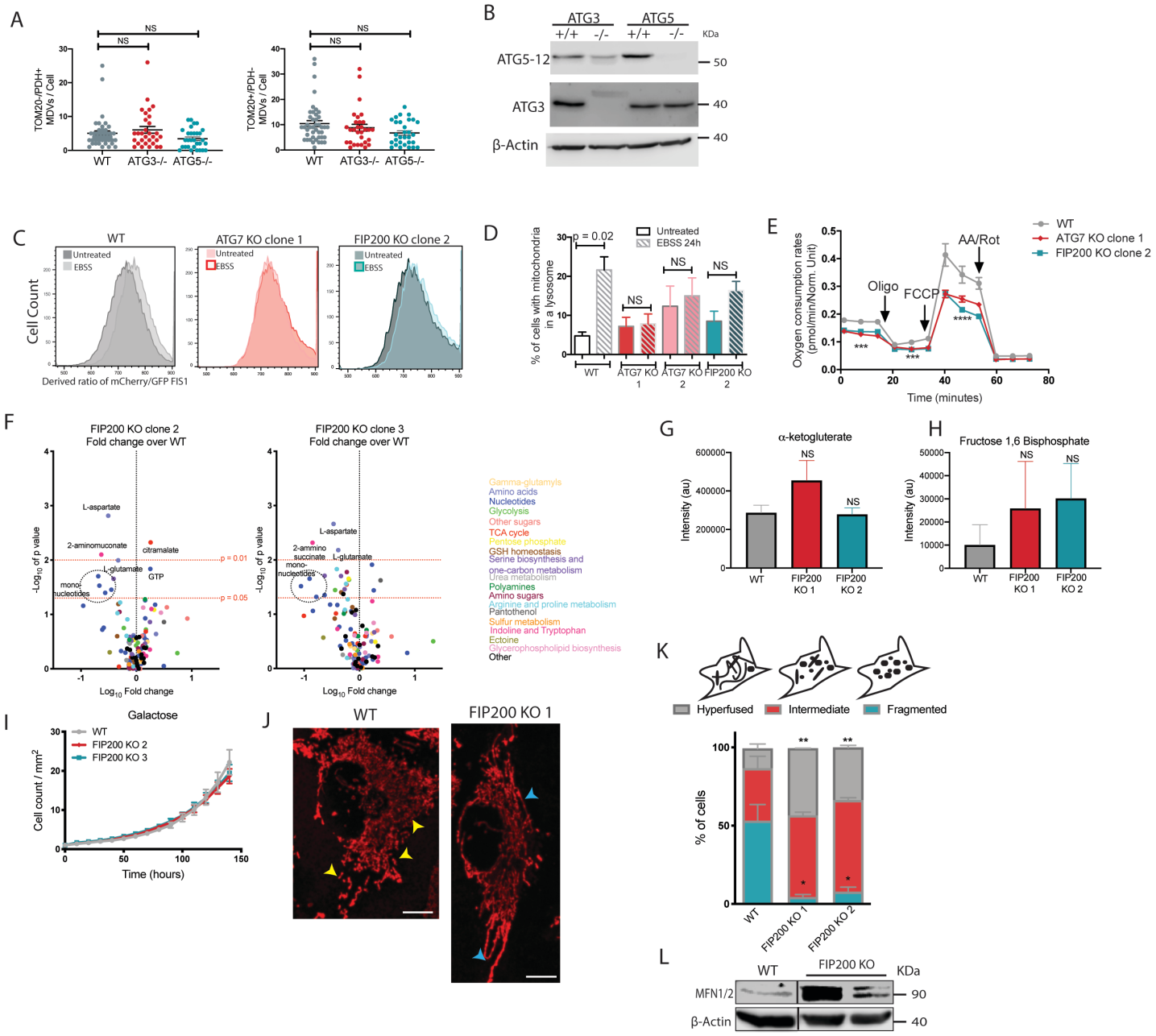


Figure S5: Rare FIP200 KO clones derived from autophagy dependent cells maintain functional mitochondria and have increased mitochondrial fusion. Related to Figures 1 and 2. (A) In murine embryonic fibroblasts (MEFs) derived from ATG5 and ATG3-/- animals, IF for Tom20 and PDH was performed and the graphs show the quantification of the number of MDVs identified per cell across multiple images combined from 2 independent experiments. The data is shown as the mean ± SEM and each dot represents a cell. A one-way ANOVA was performed to determine statistical analysis. (B) Western blot for ATG3 and ATG5 expression in KO MEFs. (C-D) In BT549 WT, ATG7, and FIP200 KO cells with stable expression of mCh-GFP-Fis1 (C) representative histograms of ratiometric flow cytometry analysis before and after 24hours of starvation in EBSS medium. Graphs are representative of 3 experiments. (D) Quantitative analysis of ratiometric flow cytometry (mCherry/GFP). Graphs are represented as mean ± SEM for biological replicates (N of 3-4). Statistical analysis: one-way ANOVA and note that the WT and ATG7 KO data is the same as shown in Figure 1F and displayed here as a control for comparison. (E) In BT549 WT, ATG7, and FIP200 KO clones, oxygen consumption rates measured via a Seahorse mitochondrial stress test. Data are represented as the mean ± SD for technical replicates (N = 3) and are representative of 3 individual experiments. (F-H) In BT549 WT and FIP200 KO clones, quantification of relative intensity of metabolite peaks determined by mass spectrometry and displayed as a waterfall plot of the Log10 fold change of each clone compared to WT cells graphed relative to the p-value. Levels of significance are indicated with dotted red lines and metabolites are color coded according to pathway. The data is represented as the mean of 3 individual experiments. (G-H) Relative intensity (au) of the peaks corresponding to the indicated metabolites. The data is represented as the mean ± SEM of 3 individual experiments. Statistical analysis was performed with a one-way ANOVA and note that the data corresponding to the WT cells is the same as shown in Figure 1J and 1K and displayed here as a reference control. (I) In BT549 WT and FIP200 KO cells, incucyte live cell imaging of mCherry+ cell count/mm^2 normalized to time point 0 of cells grown in media where the glucose was substituted with 100mM galactose. Data are represented as mean ± SEM for technical replicates (N of 3). The graphs are representative of 3 experiments. Statistical analysis: two-way ANOVA and the significance at the last time point is shown. (J) Representative confocal images of mitotracker-red labeled mitochondria. Yellow arrow heads: fractured mitochondria, red arrow heads: elongated/hyperfused mitochondria. Scale bars represent 10μm (K) Top: Cartoon of how cells were categorized. Bottom: Quantification of categorized cells. Images from 4 individual experiments were combined and the data are represented as the mean±SEM and one-way ANOVA was performed to determine statistical significance. Note the WT data is the same as that shown in Figure 2G and is shown here as a reference control. (L) Western blot in BT549 WT and FIP200 KO clones showing increased protein expression of MFN1. The black line indicates where unnecessary lanes were removed. The blots are representative of 3 experiments. *p≤0.05, **p≤0.01, *** p≤0.01, **** p≤0.001

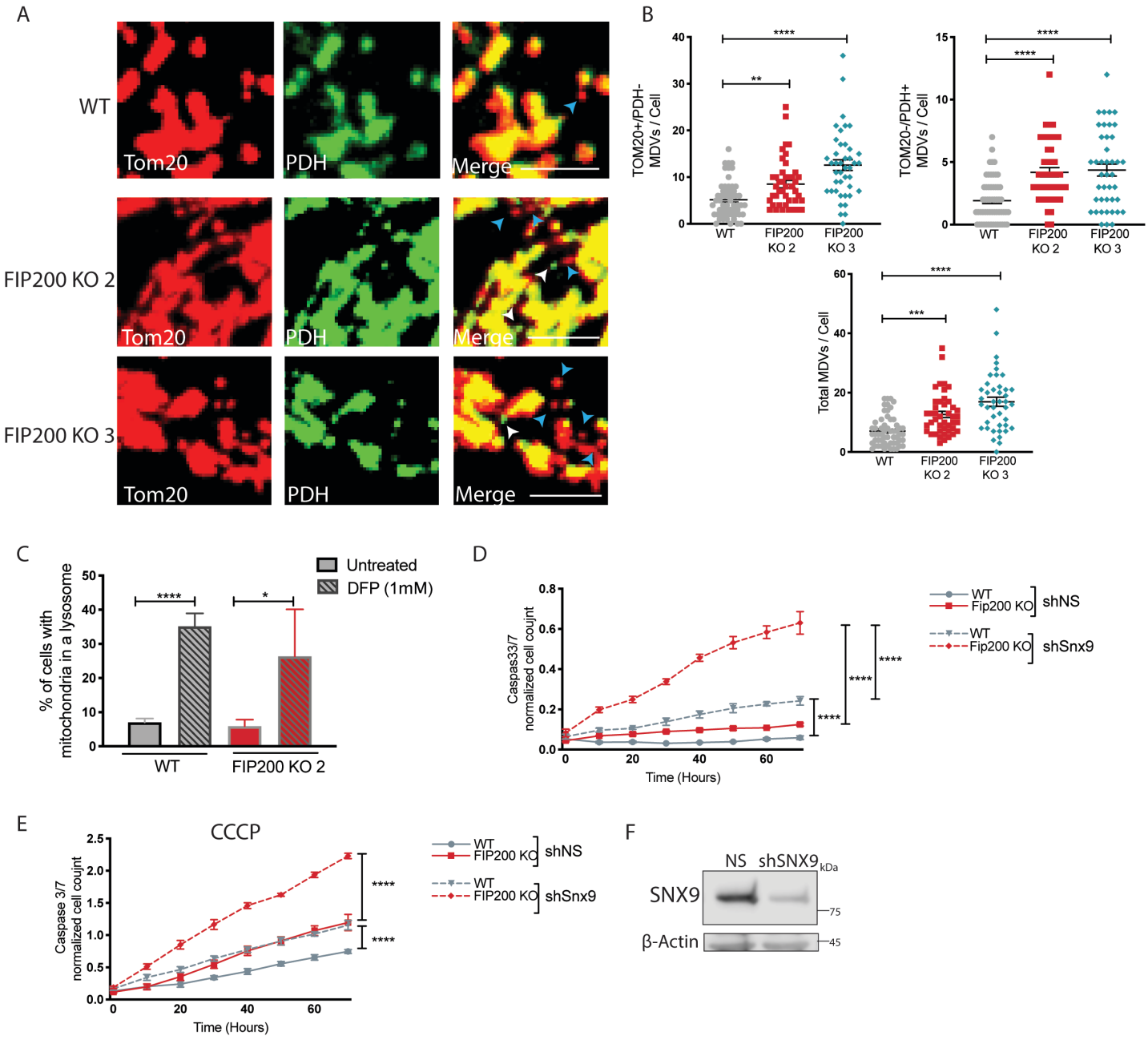


Figure S6: FIP200 KO cells have an acquired dependency on SNX9-mediated MDVs. Related to Figures 3, 4, and 6.
(A-C) In BT549 WT and FIPF200 KO cells, (A) representative confocal images from IF labeled cells with antibodies against TOM20 and PDH. Scale bar represents 5 µM. Blue and white arrow heads point to examples of TOM20+/PDH- and TOM20-/PDH+, respectively. (B) Quantification of the number of MDVs identified per cell across multiple images combined from 2 independent experiments. The data is shown as the mean ± SEM and each dot represents a cell. The total MDV count was determined by adding the TOM20+/PDH- count and the TOM20-/PDH+ count and a one-way ANOVA was performed to determine statistical analysis. (C) Quantitative analysis of ratiometric flow cytometry (mCherry/GFP) 24hr after treatment with DFP (1mM). Note the WT data is the same as that shown in Figure 4D and is shown here as a comparative control. Graphs are represented as mean ± SEM for biological replicates (N of 2-6). Statistical analysis: one-way ANOVA. (D-E) In BT549 WT and FIP200 KO cells 4 days after transduction with shRNAs targeting SNX9 or shNS, Incucyte live cell imaging of mCherry+ cells and CellEvent caspase 3/7 green either (D) untreated or (E) treated with CCCP (20µM). Data for the WT cells is also shown in Figure 5E and 5H, respectively, and shown again here as a reference. The data is shown as the Caspase3/7 green count normalized to the mCherry+ red count over time and represented as the mean ± SD for technical replicates (N of 3). The graphs are representative of 2 experiments. Statistical analysis: two-way ANOVA and the significance at the last time point is shown. (F) In BT549 FIP200 KO cells, western blot showing knock down. Blots are representative of 2 experiments. *p≤0.05, **p≤0.01, *** p≤0.01, **** p≤0.001

Table S2: Oligonucleotide sequences used. Related to STAR Methods.

Oligonucleotide name	**Oligonucleotide sequence**
sh-SNX9-1	CCGGGATGGAATGTAATCACGAGTACTCGAGTACTCGTGATTACATTCCATCTTTTTTG
sh-SNX9-2	CCGGCAAGCTGAGATGAATCACTTTCTCGAGAAAGTGATTCATCTCAGCTTGTTTTTTG
sh-DNML1-1 (DRP1)	CCGGGCTACTTTACTCCAACTTATTCTCGAGAATAAGTTGGAGTAAAGTAGCTTTTTG
sh-DNML1-2 (DRP1)	CCGGCGGTGGTGCTAGAATTTGTTACTCGAGTAACAAATTCTAGCACCACCGTTTTTG
sh-Rab7a	GTACCGGACCAGTATGTGAATAAGAAATCTCGAGATTTCTTATTCACATACTGGTTTTTTTG
gRNA ATG7 1 F	TAATACGACTCACTATAGGTATGATGAGAACATG
gRNA ATG7 1 R	TTCTAGCTCTAAAACGCACCATGTTCTCATCATA
gRNA ATG7 2 F	TAATACGACTCACTATAGGAAGCTGAACGAGTAT
gRNA ATG7 2 R	TTCTAGCTCTAAAACGCCGATACTCGTTCAGCTT
gRNA PTEN 1 F	TAATACGACTCACTATAGGCAGCAATTCACTGTA
gRNA PTEN 1 R	TTCTAGCTCTAAAACGCTTTACAGTGAATTGCTG
gRNA PTEN 2 F	TAATACGACTCACTATAGGAGTAACTATTCCCAG
gRNA PTEN 2 R	TTCTAGCTCTAAAACCTGACTGGGAATAGTTACT
gRNA FIP200 1 F	TAATACGACTCACTATAGGTGTACCTACAGTGCT
gRNA FIP200 1 R	TTCTAGCTCTAAAACTCCCAGCACTGTAGGTACA
gRNA FIP200 2 F	TAATACGACTCACTATAGGATGAACATCTTCAAC
gRNA FIP200 2 R	TTCTAGCTCTAAAACTGGTGTTGAAGATGTTCAT
gRNA PCNA 1 F	TAATACGACTCACTATAGGCAAGGTATCCGCGTTATCT
gRNA PCNA 1 R	TTCTAGCTCTAAAACAAGATAACGCGGATACCTT
gRNA PCNA 2 F	TAATACGACTCACTATAGGCAGGTTGCGGTCGCAGCGG
gRNA PCNA 2 R	TTCTAGCTCTAAAACACCGCTGCGACCGCAACCT
gRNA GFP 1 F	TAATACGACTCACTATAG GTGAACCGCATCGAGC
gRNA GFP 1 R	TTCTAGCTCTAAAACTTCAGCTCGATGCGGTTCA
gRNA GFP 2 F	TAATACGACTCACTATAG GGAGCGCACCATCTTC
gRNA GFP 2 R	TTCTAGCTCTAAAACTGAAGAAGATGGTGCGCTC