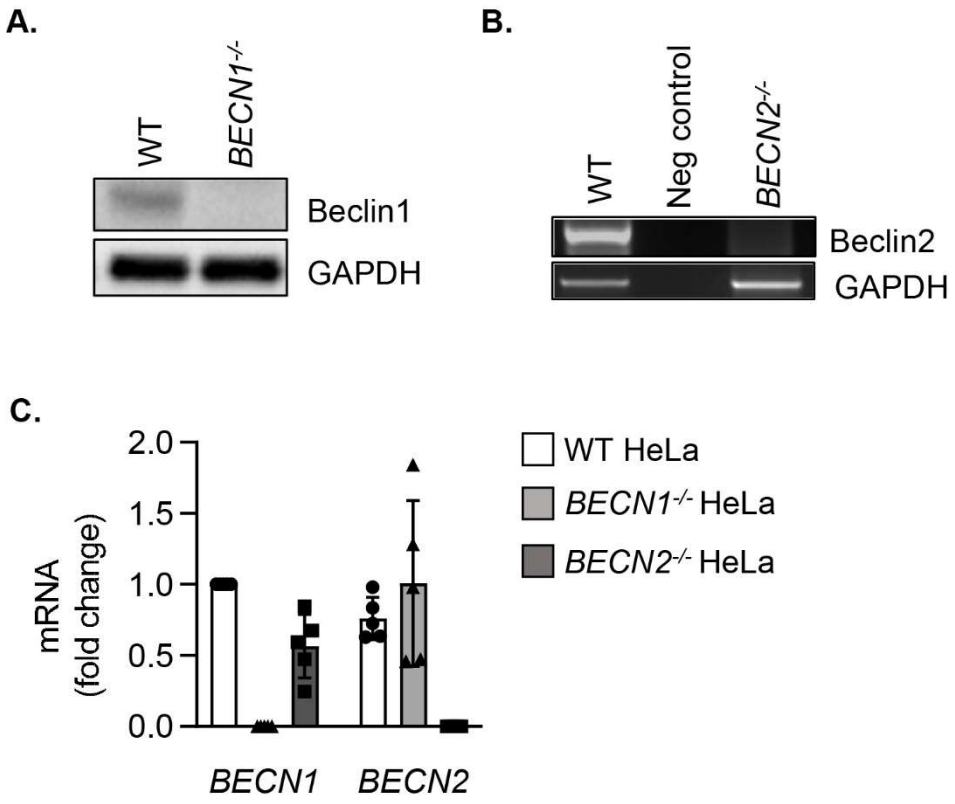

Figure S1. Generation of *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells using CRISPR-Cas-9. **A.** Representative Western blot for Beclin1 in WT and *BECN1*$^{-/-}$ HeLa cells. **B.** Ethidium bromide-stained agarose gel showing PCR-amplified *BECN2* transcripts in WT, negative control, and *BECN2*$^{-/-}$ HeLa cells. **C.** Quantitative PCR analysis of *BECN1* and *BECN2* transcript levels in WT, *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells (n=5 biological replicates per group).

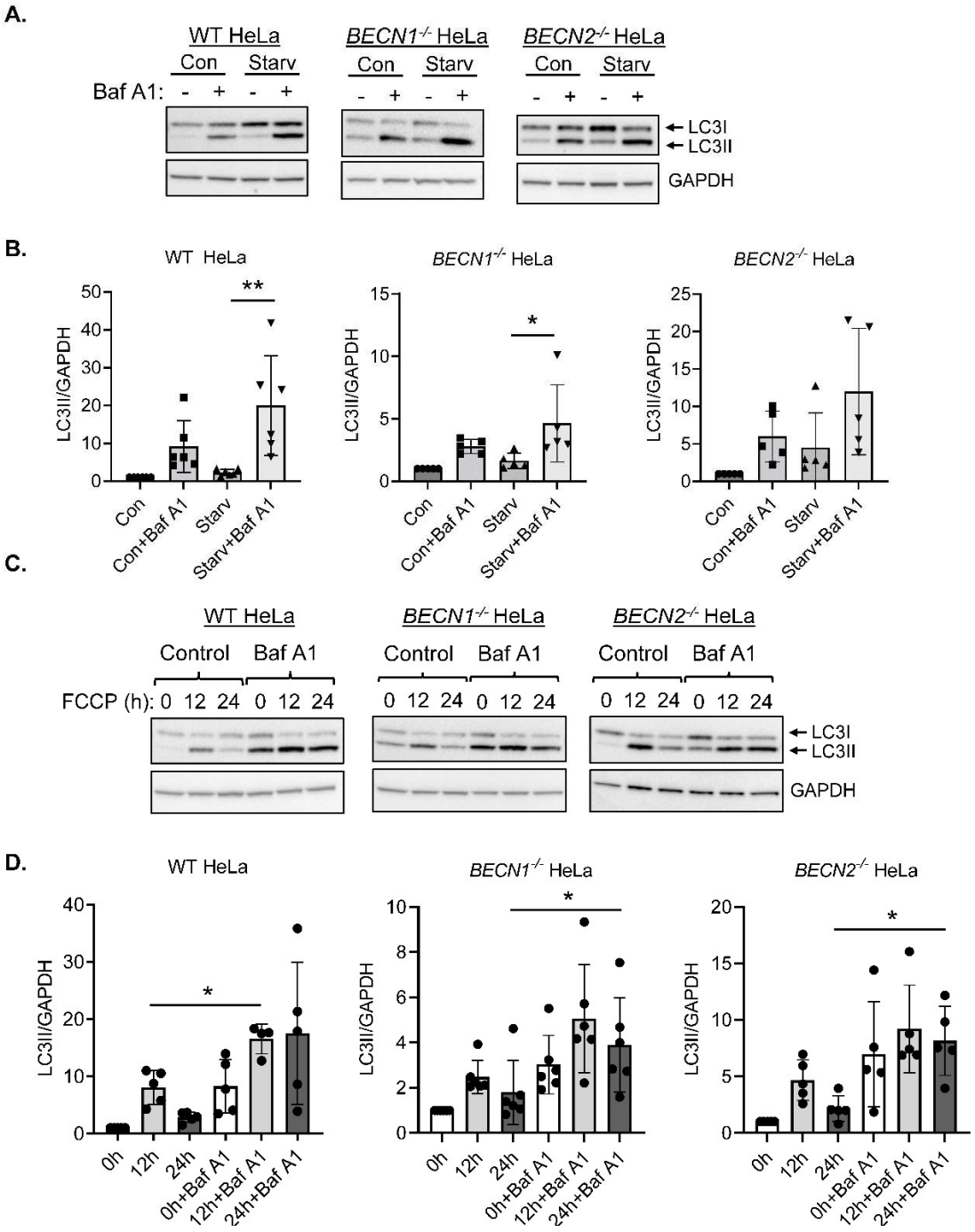

Figure S2. Evaluation of autophagic flux in WT, *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells. **A.** Representative Western blots of cells treated or not with Bafilomycin A1 (Baf A1, 3h). **B.** Quantification of LC3II protein levels in WT, *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells. **C.** Representative Western blots of cells treated with 10 μM FCCP in the absence or presence of Baf A1. **D.** Quantification of LC3II protein levels in WT, *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells (n=5 biological replicates per group). *p<0.05, **p<0.01 by one-way ANOVA followed by Tukey's multiple comparison test.

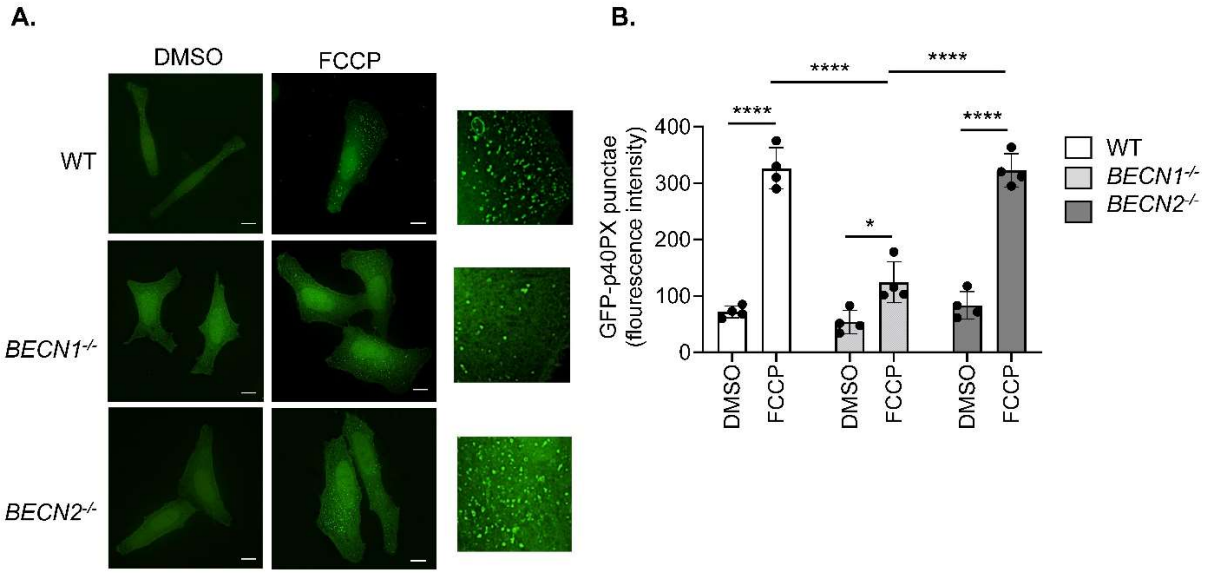

Figure S3. Vps34 activity in WT, *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells. **A.** Representative images of WT, *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells overexpressing the GFP-p40PX reporter before and after treatment with FCCP (10 μM) for 6 h. **B.** Quantification of GFP-p40PX positive puncta in cells (n=120 cells/group from 4 independent experiments). *p<0.05 and ****p<0.0001 by two-way ANOVA followed by Tukey's multiple comparison test.

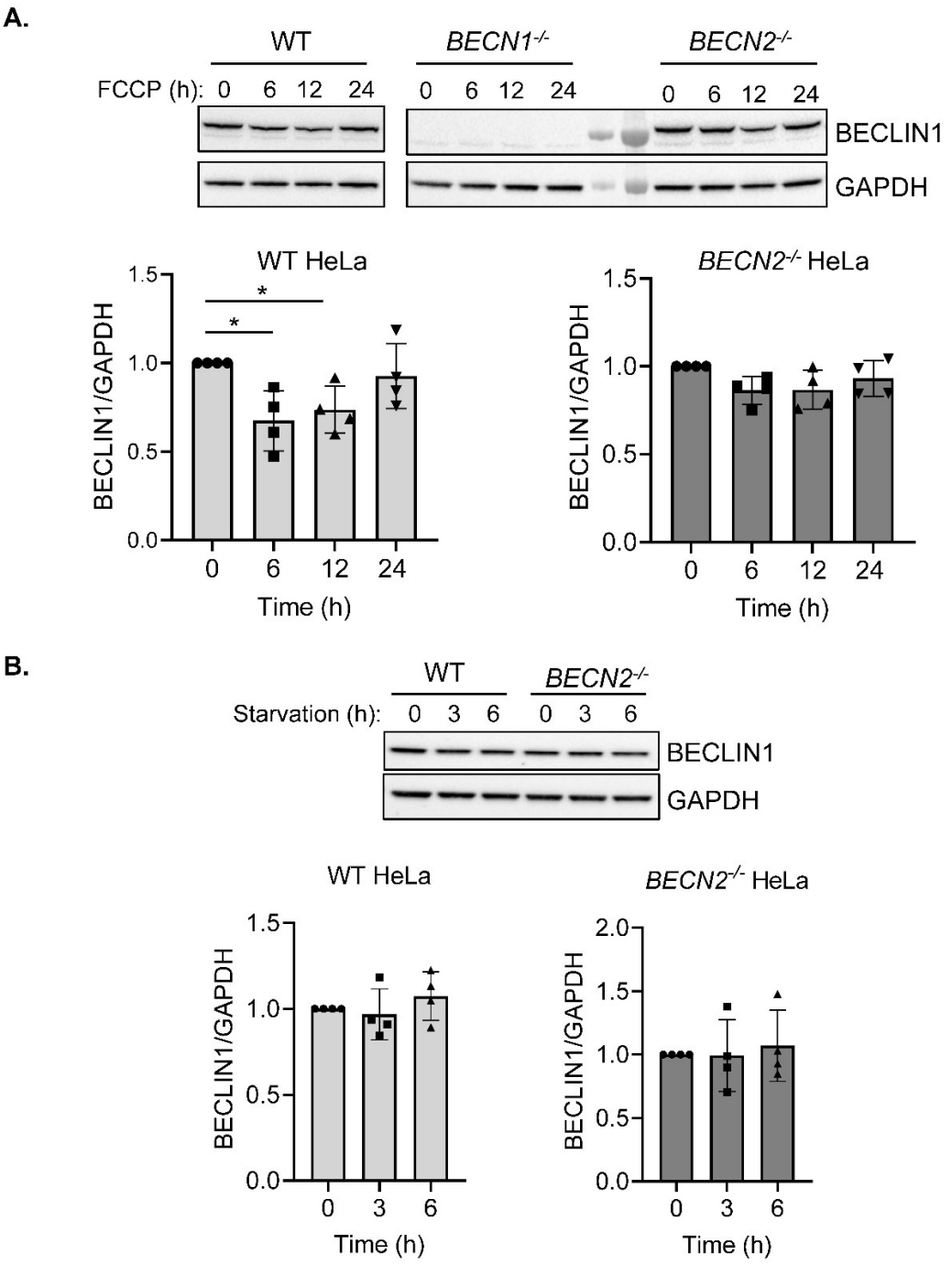

Figure S4. Beclin1 protein levels in HeLa cells after challenge. **A.** Representative Western blots and quantification of BECLIN1 protein levels in WT and *BECN2*$^{-/-}$ HeLa cells after exposure to 10 μM FCCP (n=4 independent experiments). **B.** Representative Western blots and quantification of BECLIN1 protein levels in WT and *BECN2*$^{-/-}$ HeLa cells after starvation (n=4 independent experiments). *p<0.05 by one-way ANOVA followed by Tukey's multiple comparison test.

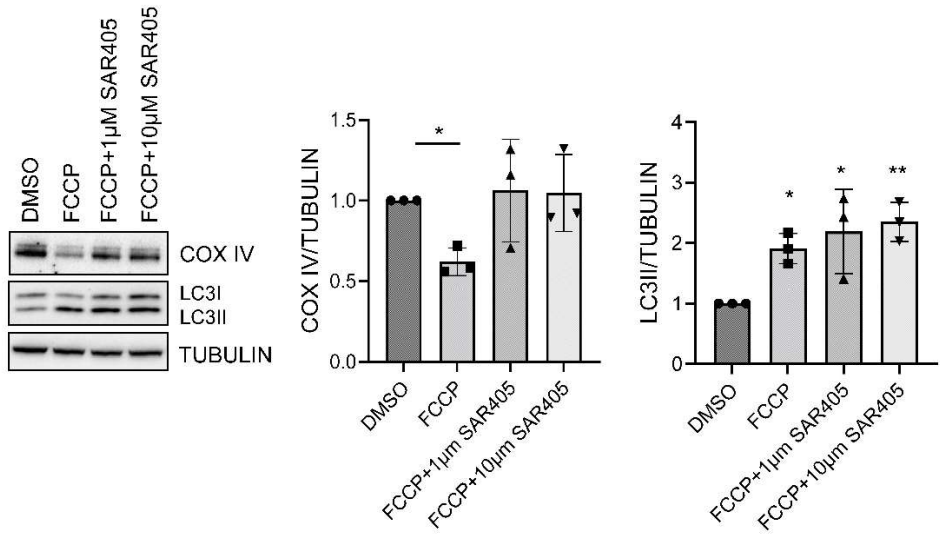

Figure S5. Vps34 inhibition and mitophagy in WT HeLa cells. Representative Western blot and quantification of COX IV and LC3II in WT HeLa cells treated with FCCP (10 µM) in the presence or absence of SAR405 for 12h (n=3 independent experiments). *p<0.05 and **p<0.01 compared to DMSO by one-way ANOVA followed by Tukey's multiple comparison test. FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

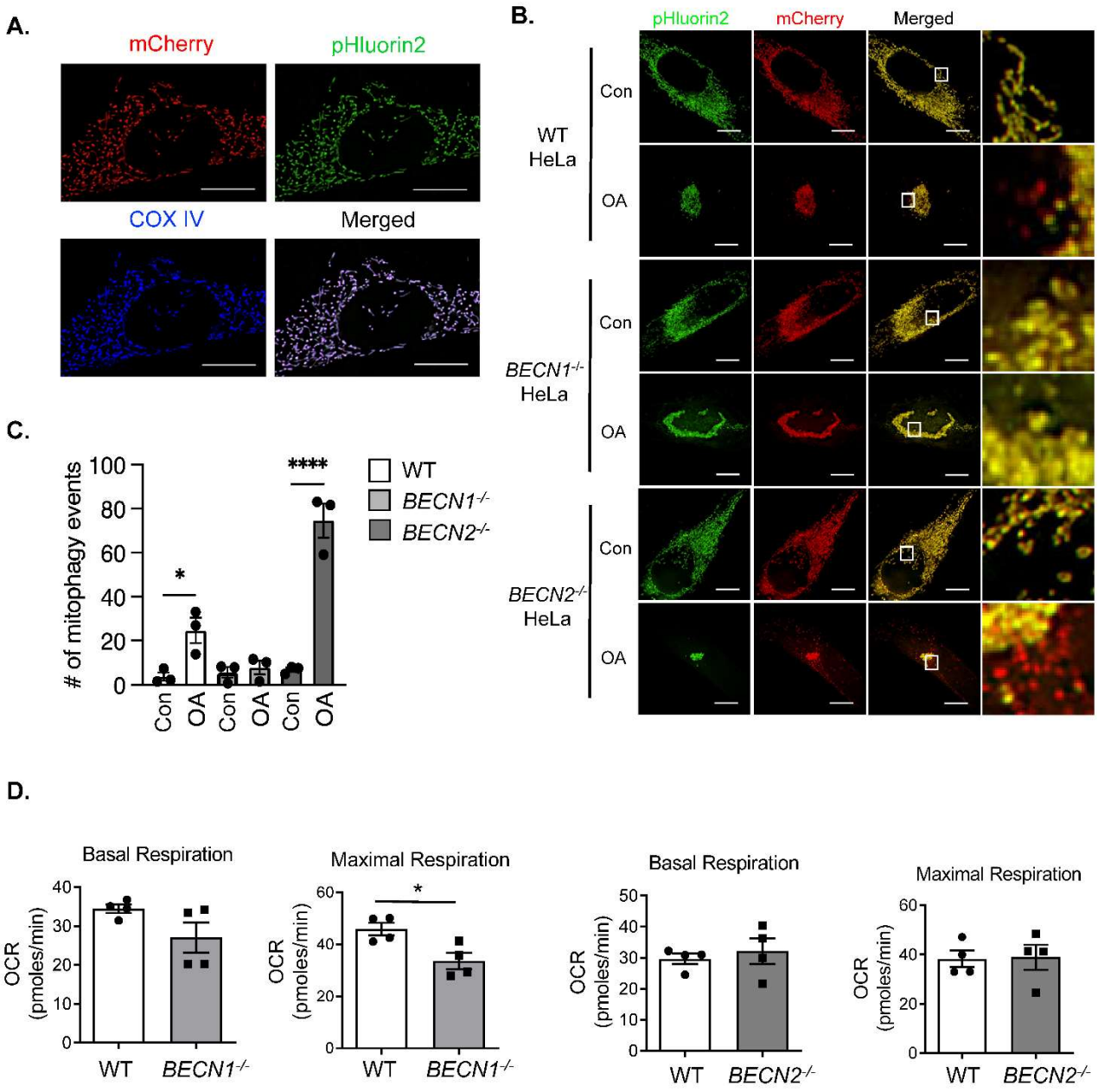

Figure S6. Mitophagy and mitochondrial respiration in WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells. **A.** Representative fluorescent image of WT MEFs overexpressing the MTS-mCherry-pHleurin2 mitophagy reporter. Cells are stained with anti-COX IV to label mitochondria. **B.** Representative images of WT, *BECN1*$^{-/-}$ and *BECN2*$^{-/-}$ HeLa cells overexpressing the mitophagy reporter before and after treatment with Oligomycin (4 μM) plus Antimycin A (4 μM) for 6h. **C.** Quantification of mitophagy events/cell (n=90 cells/group from 3 independent experiments). **D.** Mitochondrial respiration was measured in WT, *BECN1*$^{-/-}$, and *BECN2*$^{-/-}$ HeLa cells using a Seahorse XFp analyzer. Basal and maximal oxygen consumption rates (OCR) were normalized to cell number (n=4 independent experiments). *p<0.05 and ****p<0.0001 by one-way ANOVA followed by Tukey's multiple comparison test or Student's t-test. Scale bar = 10 μm

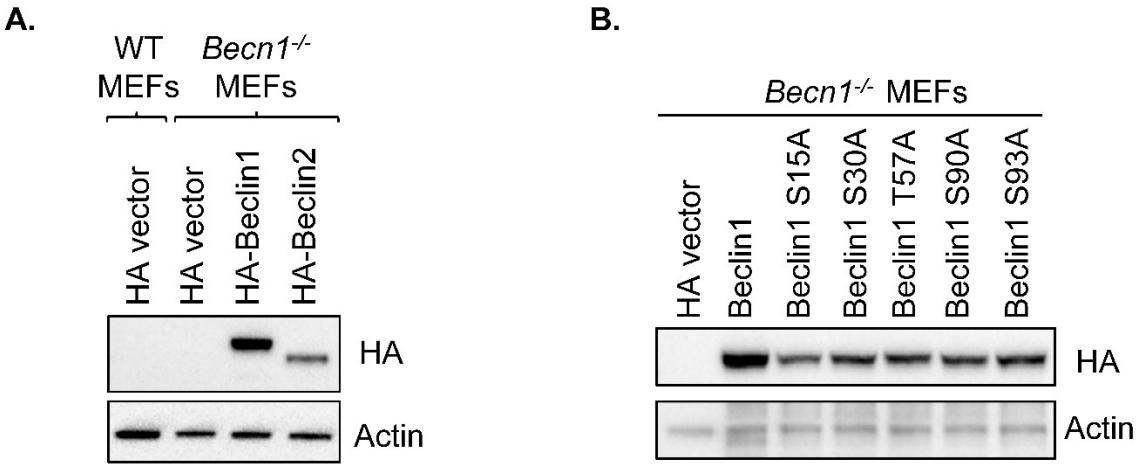

Figure S7. Expression of Beclin1 and mutant constructs in MEFs. **A.** Western blot confirming overexpression of HA-Beclin1 and HA-Beclin2 in transfected MEFs. **B.** Western blot confirming overexpression of the various HA-tagged mutants in *Becn1*$^{-/-}$ MEFs. Membranes were blotted with anti-HA. Actin was used as a loading control.

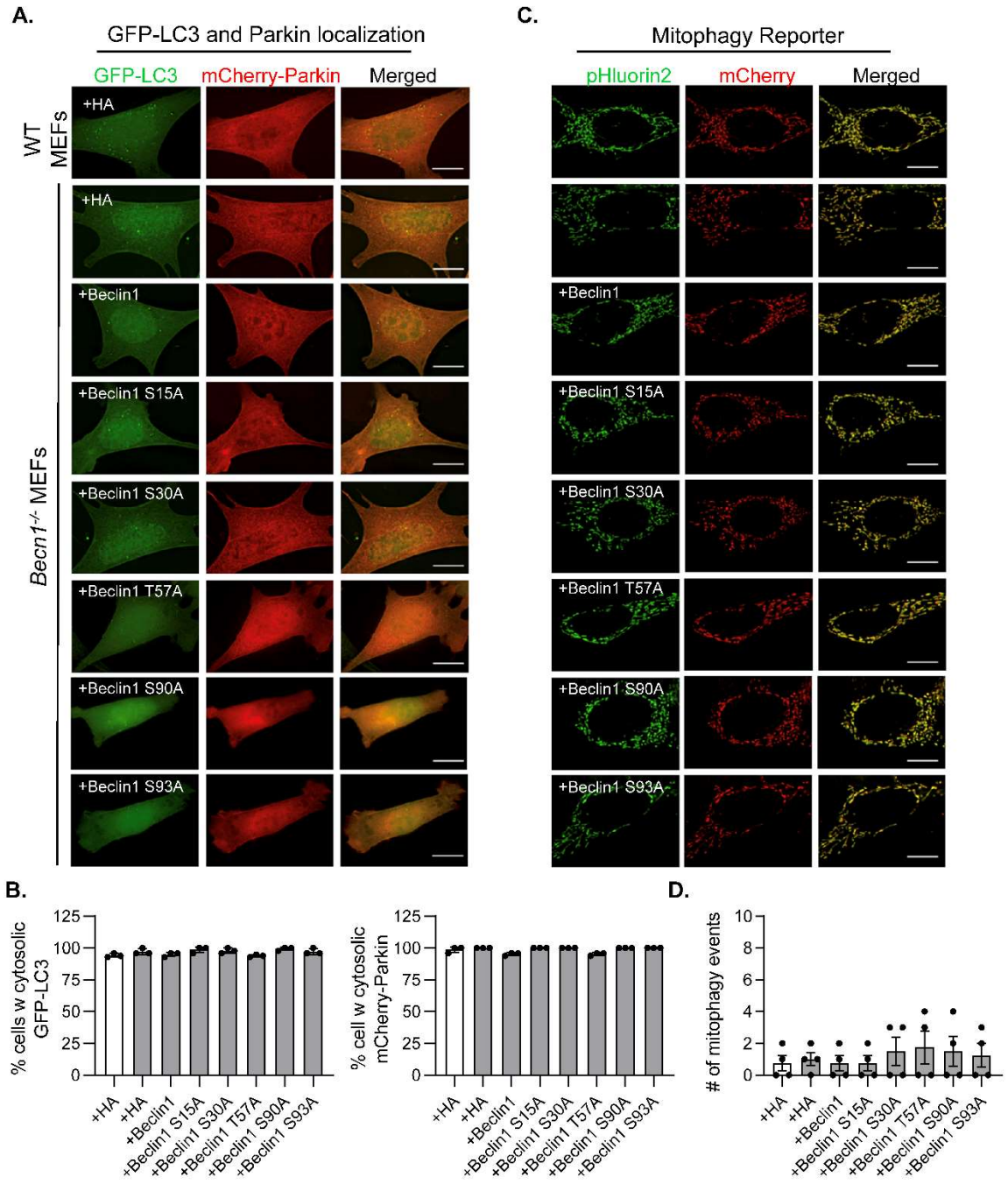

Figure S8. Cellular localization of GFP-LC3, mCherry-Parkin and mitophagy reporter in WT and *Becn1*$^{-/-}$ MEFs under baseline conditions. **A.** Representative images of WT and *Becn1*$^{-/-}$ MEFs overexpressing Beclin1 or Beclin1 phosphorylation resistant mutants plus GFP-LC3 and mCherry-Parkin under baseline conditions. **B.** Quantification of cells with cytosolic distribution (n=90 cells/group from 3 independent experiments). **C**. Representative images of WT and *Becn1*$^{-/-}$ MEFs overexpressing MTS-mCherry-pHluorin2 plus myc-Parkin, and Beclin1 or Beclin1 mutants at baseline. **D.** Quantification of mitophagy events at baseline (n=120 cells/group from 4 independent experiments). One-way ANOVA followed by Tukey's multiple comparison test. Scale bar = 10 μm

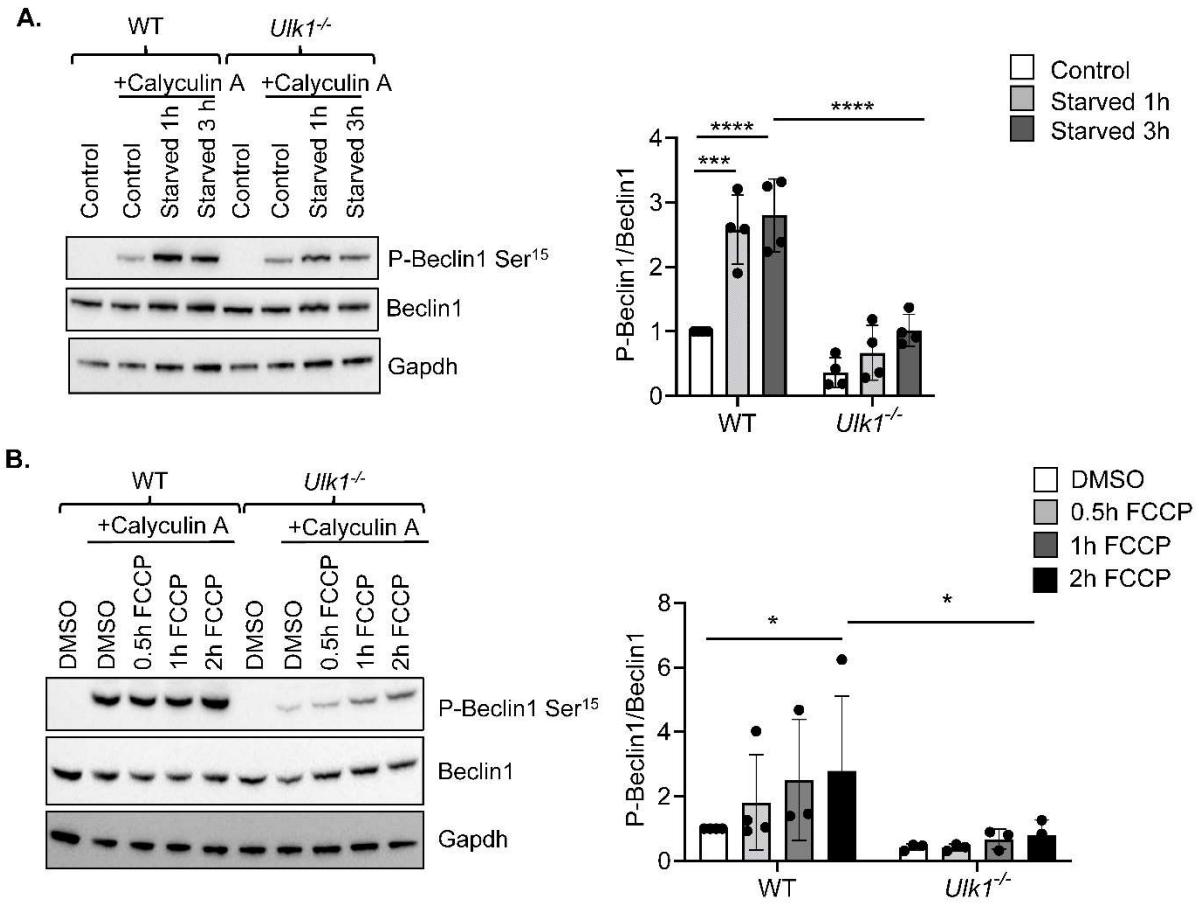

Figure S9. Phosphorylation of Beclin1 at Ser^{15} in WT and *Ulk1*$^{-/-}$ MEFs. **A.** Representative Western blot of P-Beclin1 Ser^{15}, Beclin1 and Gapdh in WT and *Becn1*$^{-/-}$ MEFs. Quantification of P-Beclin1/Beclin1 protein levels in the presence of 100 nM Calyculin A to inhibit dephosphorylation (n=4 independent experiments). **B.** Representative Western blot for P-Beclin1 Ser^{15}, Beclin1 and Gapdh in WT and *Becn1*$^{-/-}$ MEFs after treatment with 10 µM FCCP. Quantification of P-Beclin1/Beclin1 protein levels in the presence of 100 nM Calyculin A (n=3-4 independent experiments). ***p<0.001, ****p<0.0001 by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

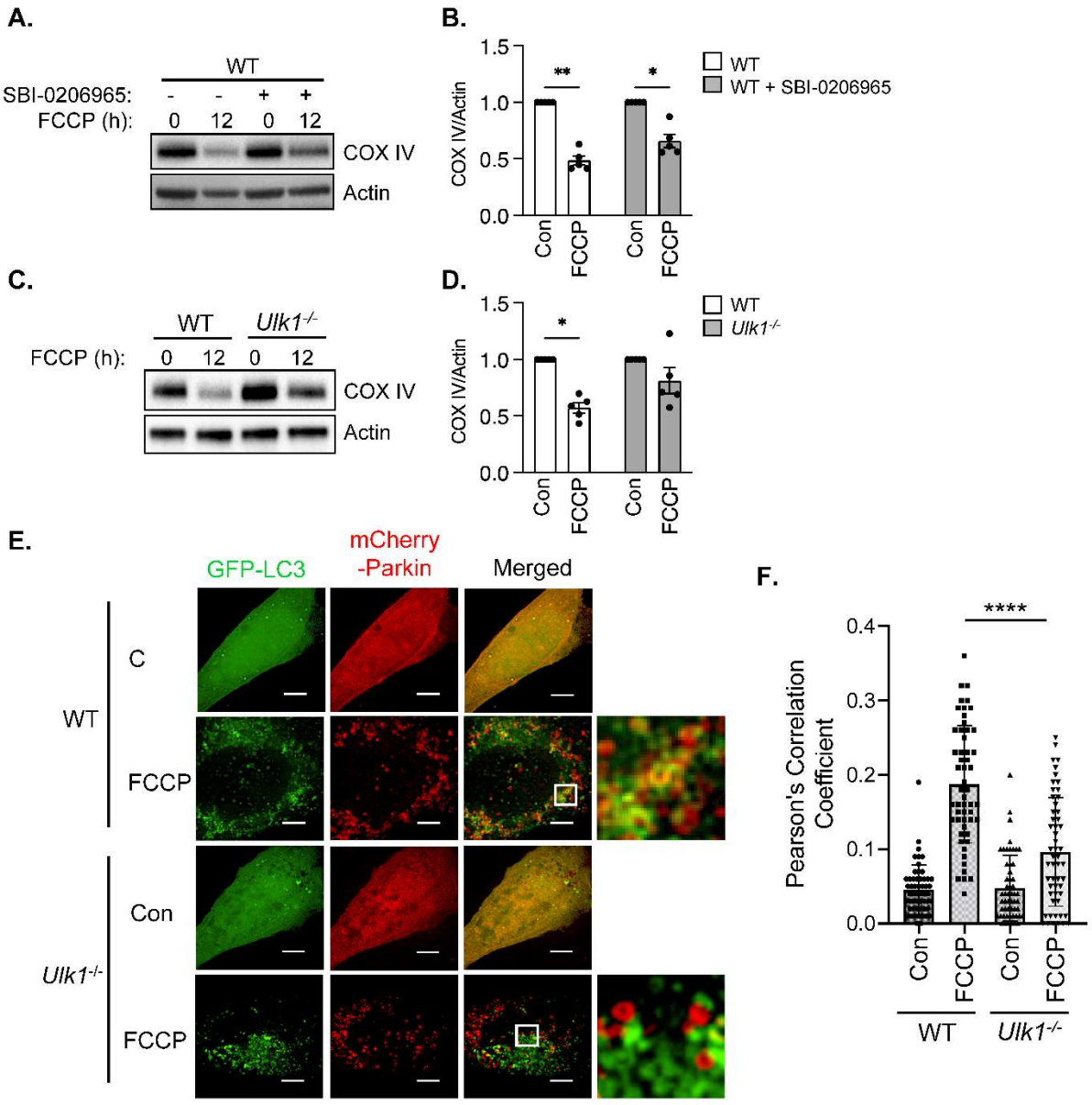

Figure S10. Effect of pharmacologic inhibition or genetic deletion of Ulk1 on mitophagy in MEFs. **A.** Representative Western blot for COX IV in WT MEFs. Cells were treated with the Ulk inhibitor SBI-0206965 (20 μM) plus FCCP (10 μM) for 12 hours. **B.** Quantification of COX IV protein levels (n=5 independent experiments). **C.** Representative Western blot for COX IV in WT and *Ulk1-/-* MEFs after treatment with 25 μM FCCP (12h). **D.** Quantification of COX IV protein levels (n=5 independent experiments). **E.** Representative images of WT and *Ulk1*$^{-/-}$ MEFs overexpressing GFP-LC3 and mCherry-Parkin treated with 10 μM FCCP for 6 hours. **F.** Quantification of co-localization between mCherry-Parkin and GFP-LC3 by Pearson's correlation coefficient (n=56 cells/group from 3 independent experiments). *p<0.05, **p<0.01, and ****p<0.0001 by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Scale bar = 10 μm

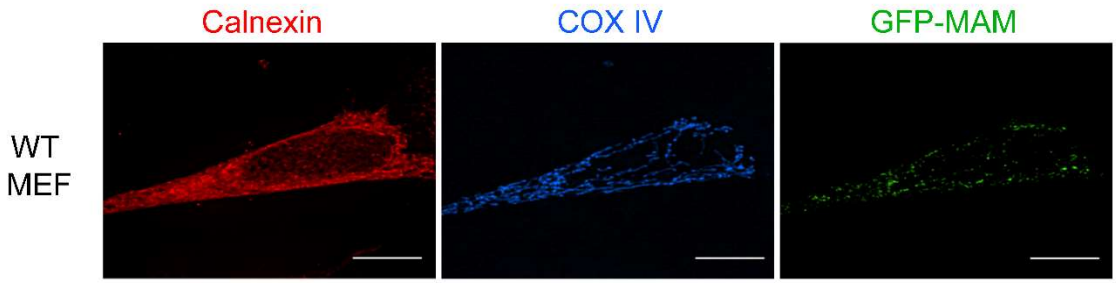

Figure S11. Expression of MAM reporter in WT MEFs. Representative fluorescent image of a WT MEF overexpressing the split GFP-MAM reporter (green). Cells were fixed and stained with anti-calnexin to label the endoplasmic reticulum (red) and anti-COX IV (blue) to label mitochondria. Scale bar = 10 μm.

Figure S12 – Uncropped Western blots

Figure 1B

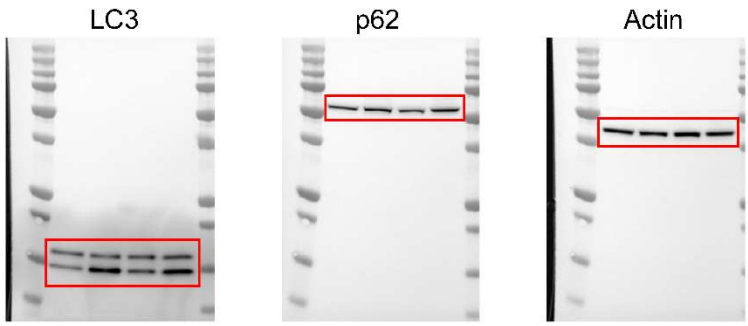

Figure 1E

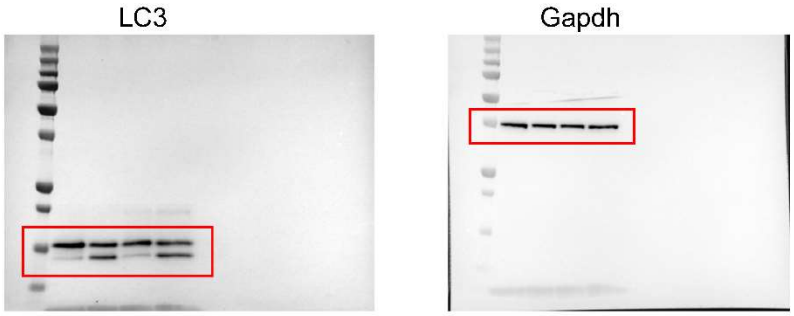

Figure 1H

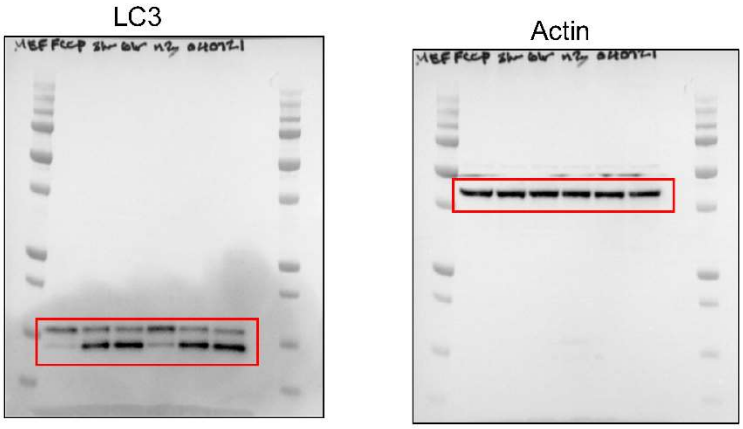

Figure 2B

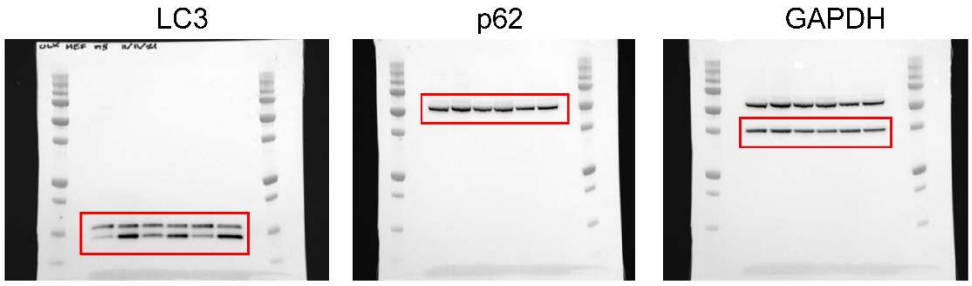

Figure 2E

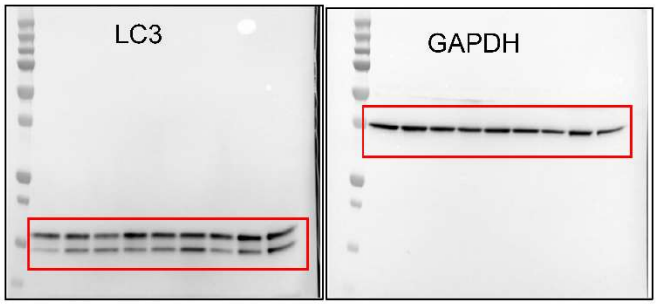

Figure 2H

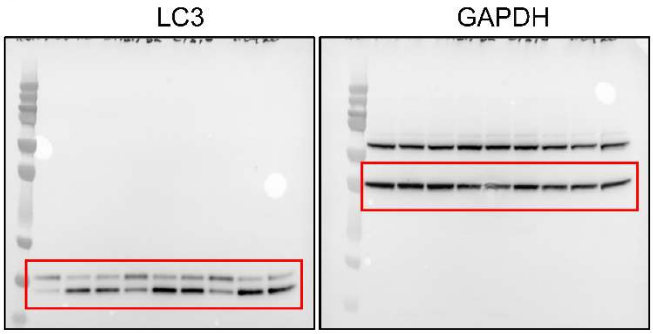

Figure 3C

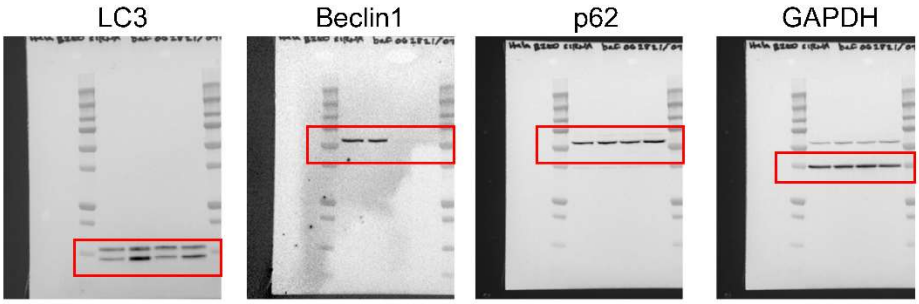

Figure 3E

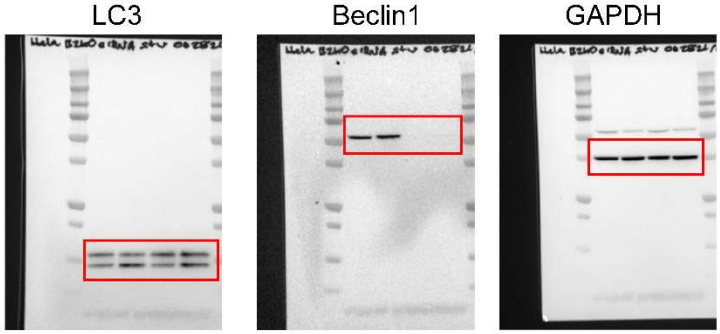

Figure 3G

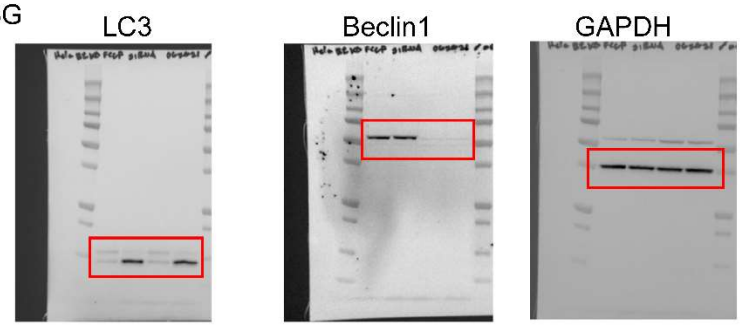

Figure 4D

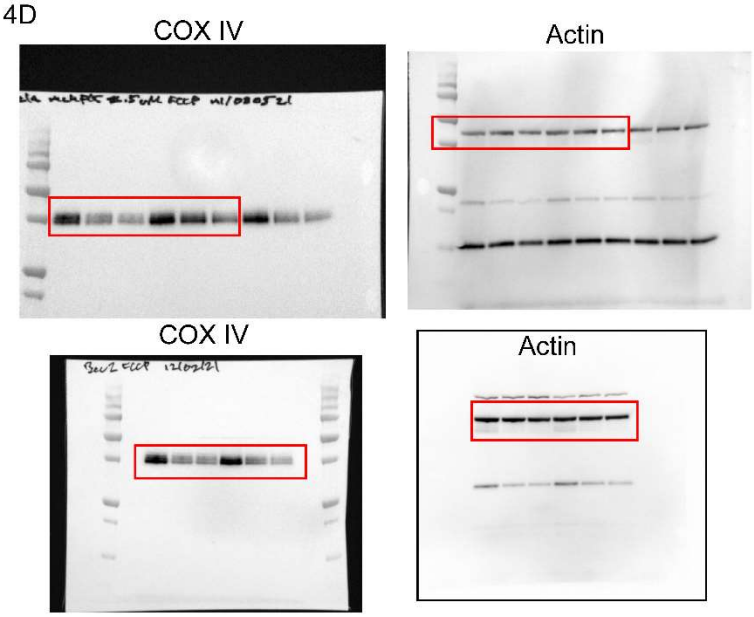

Figure 4G

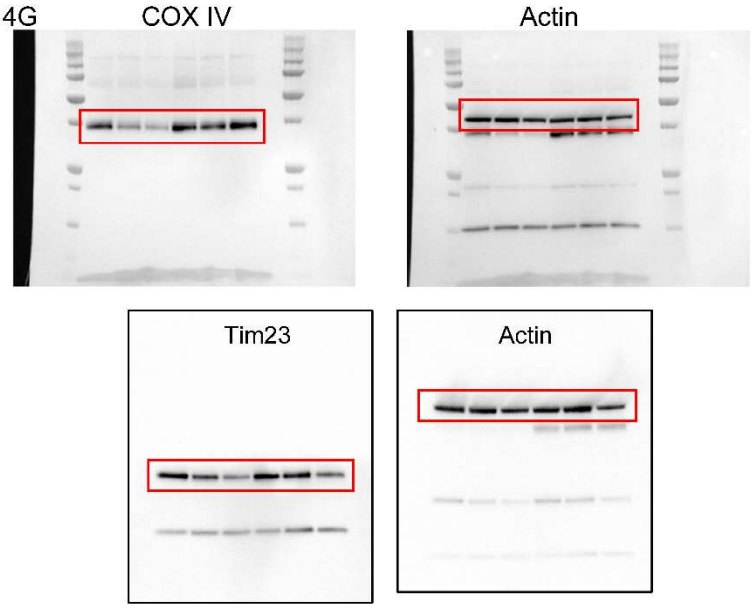

Figure 5C