Supplementary Figure 1

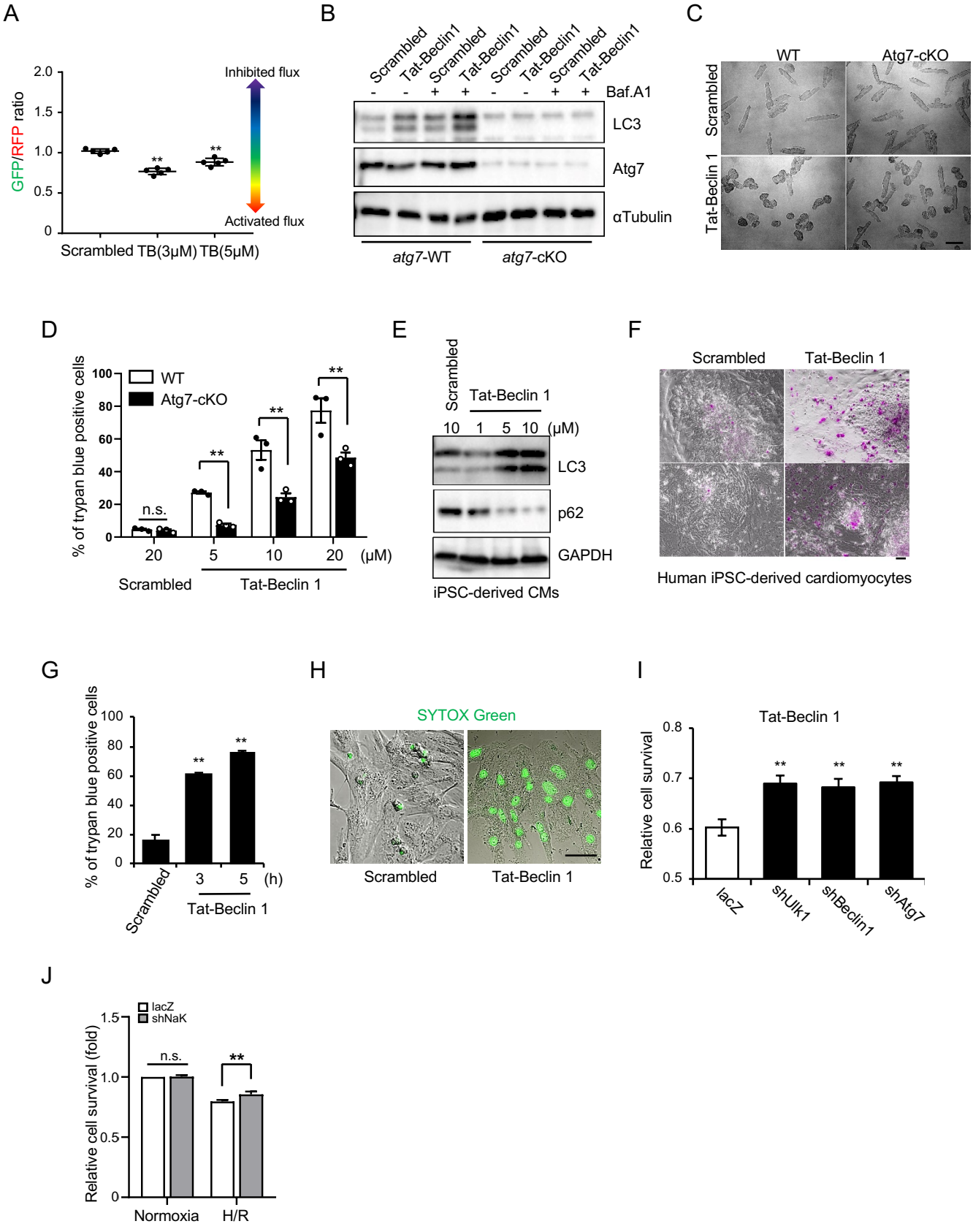
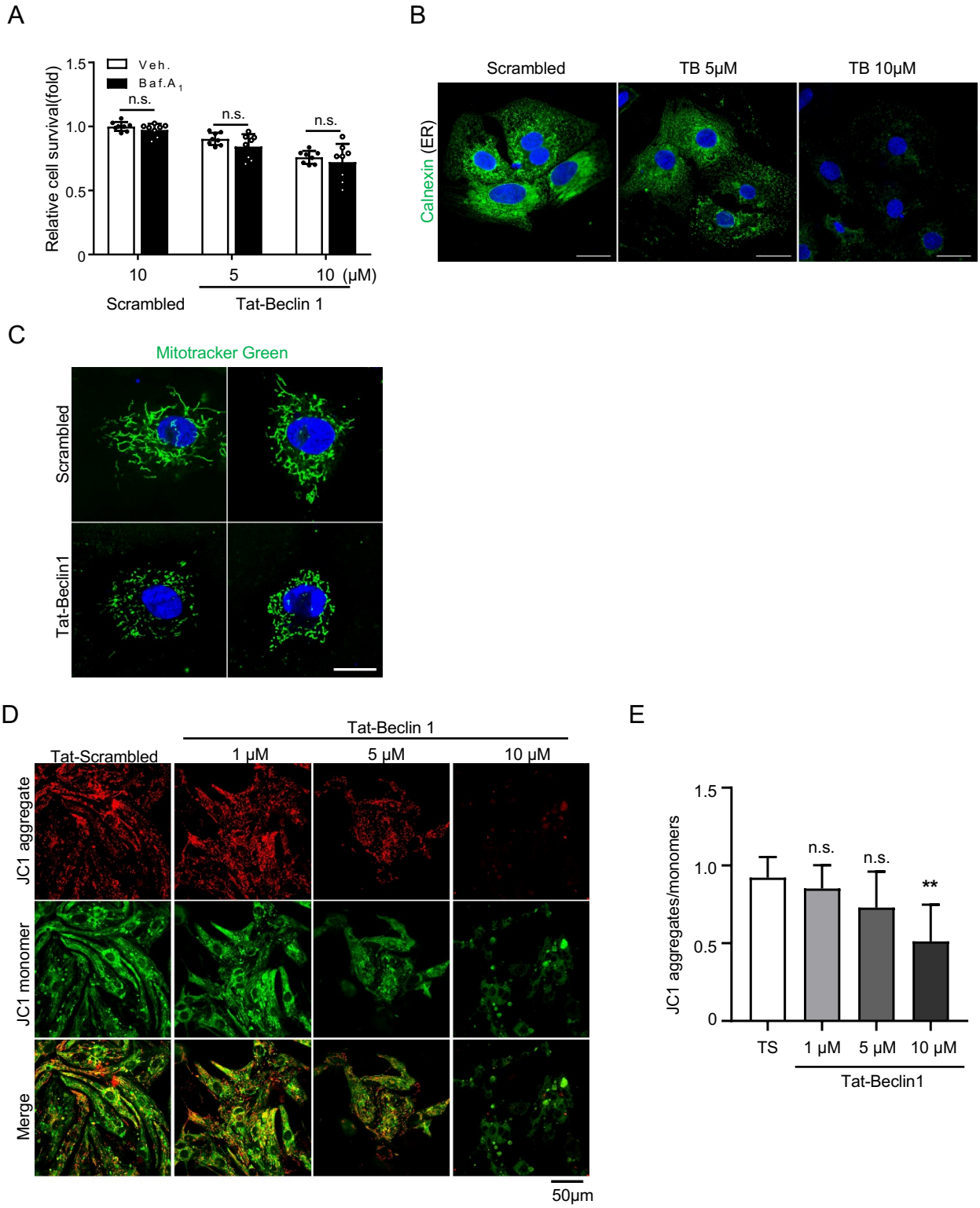


Figure S1. Tat-Beclin 1 induces autosis in CMs. A. Quantification of the fluorescence intensity ratio for GFP/RFP in NRCMs treated with Scrambled or 3 μM or 5 μM Tat-Beclin 1 (mean values ± S.D., n=5; $^{**}p < 0.01$) (one-way ANOVA with Dunnett's post hoc). B - D. Adult mouse CMs isolated from $Atg7^{flox/flox}$ -Cre negative (WT) or -Cre positive (Atg7-cKO) mice were treated with Scrambled or 5 μM of Tat-Beclin 1 for 2 hours and analyzed by Western blotting using anti-Atg7, anti-LC3 and anti-αTubulin antibodies (B). Adult mouse CMs were treated with 20 μM Scrambled or 5, 10 or 20 μM Tat-Beclin 1 for 3 hours. Cells were observed under a microscope (C) and cell death was quantified by trypan blue assays (mean value ±S.E., n=3; $^{**}p < 0.01$) (scale bar = 100 μm) (2-way ANOVA) (D). E and F. Human iPSC-derived CMs were treated with Scrambled or Tat-Beclin 1 at indicated concentrations for 2 hours and analyzed by Western blot analyses (E). Cell death was evaluated with SYTOX Green assays (scale bar = 20 μm) (F). G and H. NRCMs were treated with 20 μM Scrambled or Tat-Beclin 1 for 3 or 5 hours, and then cell death was quantified by trypan blue assay (G) and SYTOX Green assay (H) (mean values ±S.D., n=4; $^{**}p < 0.01$) (scale bar = 20 μm) (one-way ANOVA with Dunnett's post hoc). I. NRCMs were treated with Ad-shULK1, Ad-shBeclin1, or Ad-shAtg7 for 48 hours and then CMs were treated with 5 μM Tat-Beclin 1 for 3 hours. Cell death was quantified by CellTiter-Blue assays (mean values ±S.D., n=3; values were measured from more than 8 wells per experiments, $^{**}p < 0.01$ vs lacZ) (one-way ANOVA with Dunnett's post hoc). J. NRCMs were treated with Ad-shNa^+,K^+-ATPase α1 virus or control virus for 48 hours. Cells were subjected to 3 hours of hypoxia and 3 hours of reoxygenation. Cell death was quantified by CellTiter-Blue assays (mean values ±S.D., n=3; values were measured from more than 8 wells per experiments, $^{**}p < 0.01$ vs lacZ) (2-way ANOVA).

Supplementary Figure 2



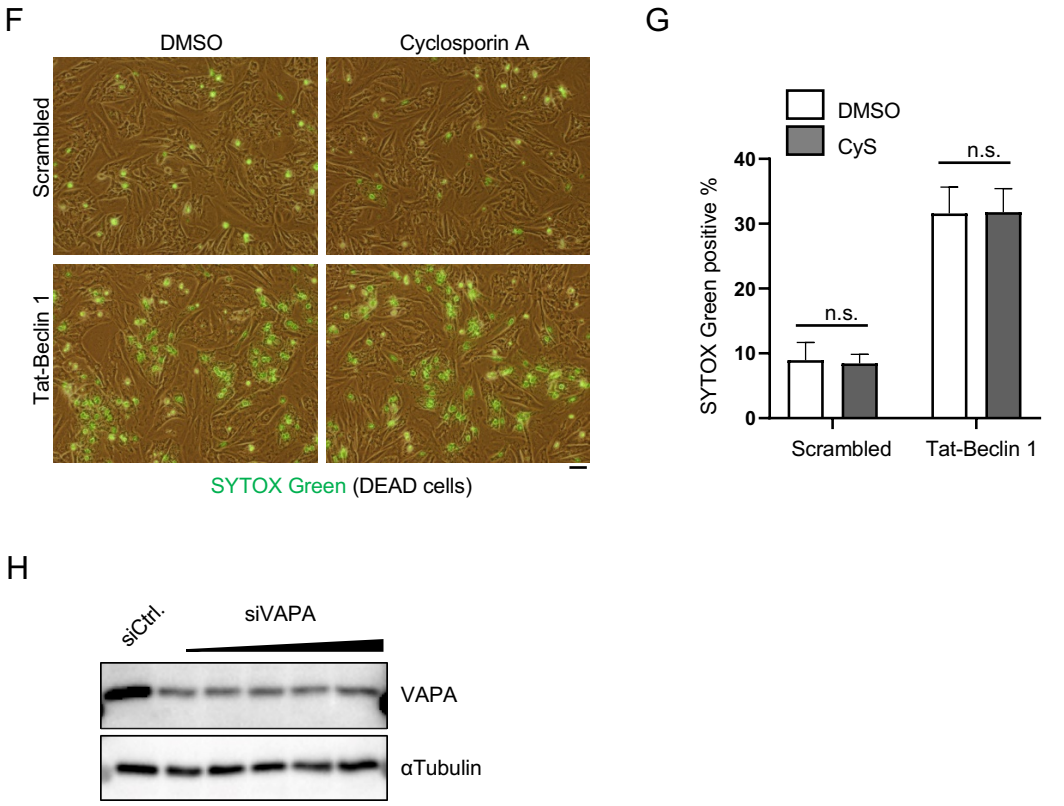


Figure S2. Tat-Beclin 1-treated CMs show abnormal ER and mitochondrial morphology. A. NRCMs were pre-treated with bafilomycin A1 for 12 hours and then treated with Scrambled or Tat-Beclin 1 at the indicated doses for 3 hours. Relative cell death was quantified by CellTiter-Blue assays (mean values ± S.D., n=8; not significant (n.s.)) (2-way ANOVA). B. NRCMs were treated with 5 μM and 10 μM of Tat-Beclin 1 for 3 hours and then fixed with 4% paraformaldehyde, stained using anti-calnexin antibody and observed under a confocal microscope. Scale bar = 20 μm. C. NRCMs were treated with 5 μM of Tat-Beclin 1 for 3 hours and then treated with Mitotracker dye for 30 minutes and observed under a confocal microscope. Scale bar = 20 μm. D and E. NRCMs were treated with indicated dose of Tat-Beclin 1 for 3 hours and then loaded with JC-1 dye for 20 minutes, which selectively enters mitochondria and reversibly changes color from JC-1 red to JC-1 green as the membrane potential decreases. Representative images were shown (D). The ratio between red (JC1 aggregates) and green (JC1 monomer) signal were quantified (mean values ± S.D., n=3, values were measured from more than 4 different parts per each experiment; *p < 0.05, **p < 0.01 vs Scrambled, not significant (n.s.)) (one-way ANOVA with Dunnett's post hoc) (E). F and G. NRCMs were pretreated with 0.2 μM of cyclosporine A (CyS) for 1 hour and then cells were treated with 10 μM Scrambled or Tat-Beclin 1 for 3 hours. Cell death was quantified by SYTOX Green assay. Representative images shown in F and SYTOX Green positive cells were quantified (G) (mean values ±S.D., n=5 with different pictures; not significant (n.s.)) (2-way ANOVA) (scale bar = 20 μm). H. NRCMs were transfected with siControl (siCtrl.) or siVAPA and siVAPB dose dependently. Cell lysates were subjected to Western blotting using anti-VAPA antibody and anti-αTubulin antibody.

Supplementary Figure 3

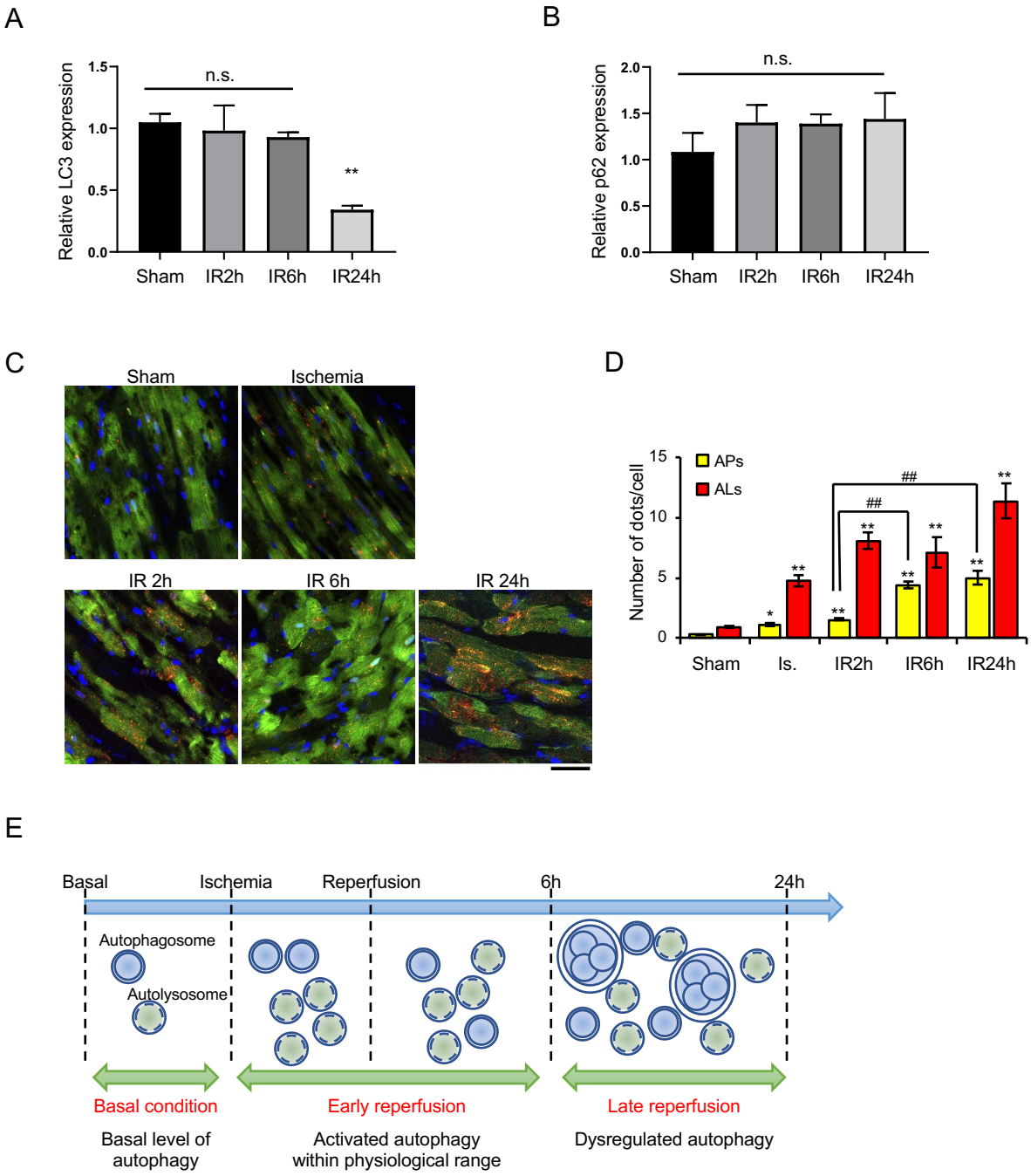


Figure S3. Autophagic flux is activated during early reperfusion but is inhibited during late reperfusion in the mouse heart. A and B. Mice were subjected to 30 minutes of ischemia and reperfusion for the indicated durations. Some mice were subjected to sham operation. Mouse heart samples were subjected to qPCR analyses using LC3 and p62 primer sets (mean values ±S.E., n=4/each group; $^{**}p < 0.01$ versus Sham, not significant (n.s.)) (one-way ANOVA with Tukey's post hoc). C and D. Three-month-old transgenic mice expressing cardiac-specific-tandem-fluorescent RFP-GFP-LC3 (tf-LC3) were subjected to 30 minutes of ischemia with reperfusion for the indicated durations, and the infarction border area was analyzed by confocal microscopy to assess autophagic flux (scale bar = 50 μm) (C). The quantification of autophagosomes (yellow puncta) and autolysosomes (red puncta) is shown (mean values ±S.E., n=3; $^{*}p < 0.05$, $^{**}p < 0.01$ versus Sham; $\#\#p < 0.01$ versus IR2h, values were measured from 20 different areas per mouse) (2-way ANOVA) (D). E. A schematic model of autophagy regulation during ischemia/reperfusion in the mouse heart.

Supplementary Figure 4

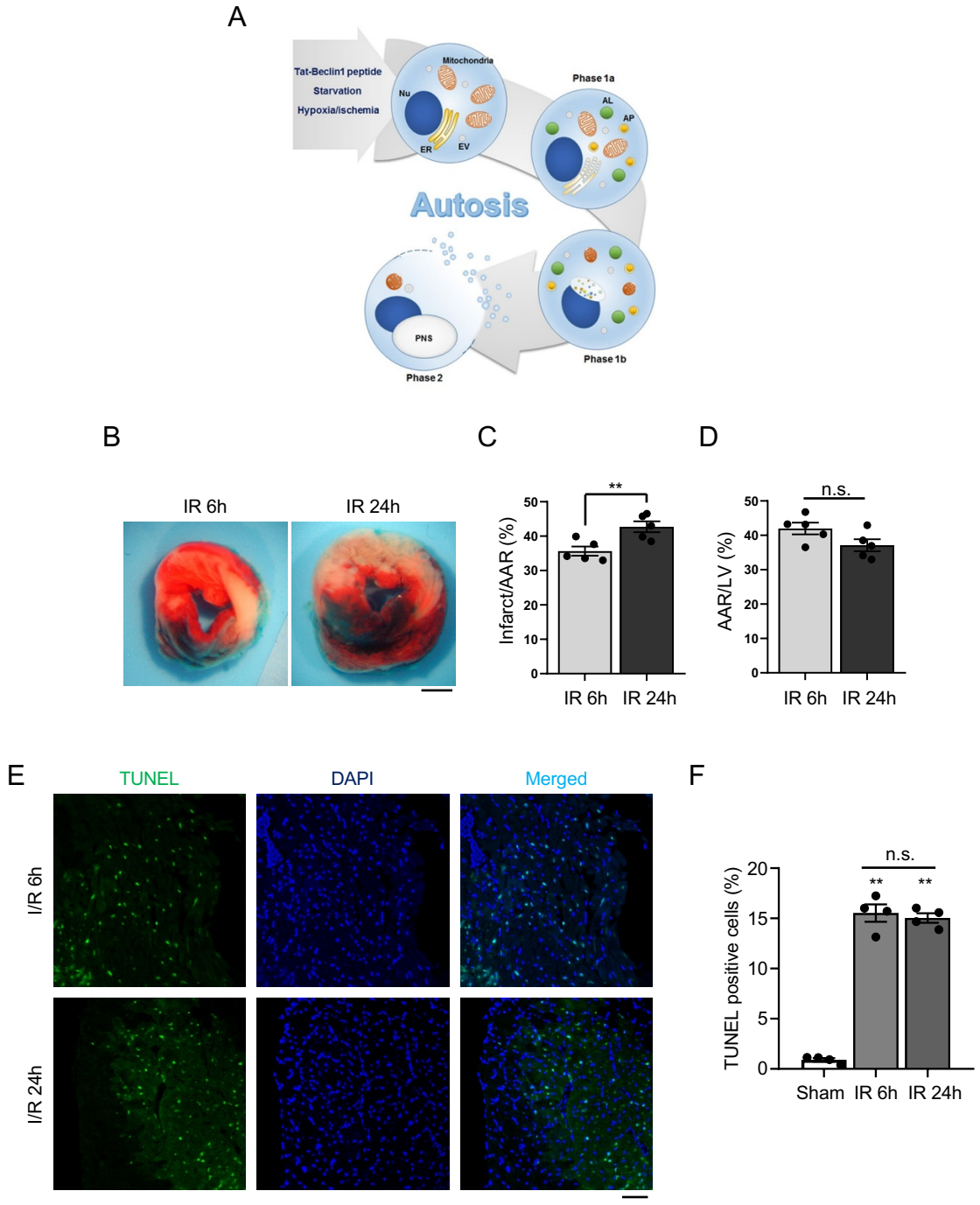


Figure S4. Autosis is induced in CMs during late reperfusion. A. A schematic model of the morphological features of autosis. B - F. Three-month-old C57BL/6J mice were subjected to 30 minutes of ischemia and 6 or 24 hours of reperfusion. Representative images of LV myocardial sections after Alcian blue and triphenyltetrazolium chloride (TTC) staining (scale bar = 1 mm) (B). The ratio of infarction area to area at risk (AAR) (C) and AAR to LV (D) were compared between 6 and 24 hours after reperfusion in the mouse heart (mean value ±S.E., n=5; **p<0.01) (unpaired t test). The heart samples were subjected to TUNEL assays. Representative immunofluorescent DAPI (blue) and TUNEL (green) staining of sections from the infarction area in the hearts of mice (E). The apoptotic index was determined by calculating the percentage of TUNEL positive cells (measured in 3 different view fields per section, 3 sections per mouse with 4 mice per group, mean value ±S.E., **p<0.01 vs sham; not significant (n.s.)) (scale bar = 50 μm) (one-way ANOVA with Tukey's post hoc) (F).

Supplementary Figure 5

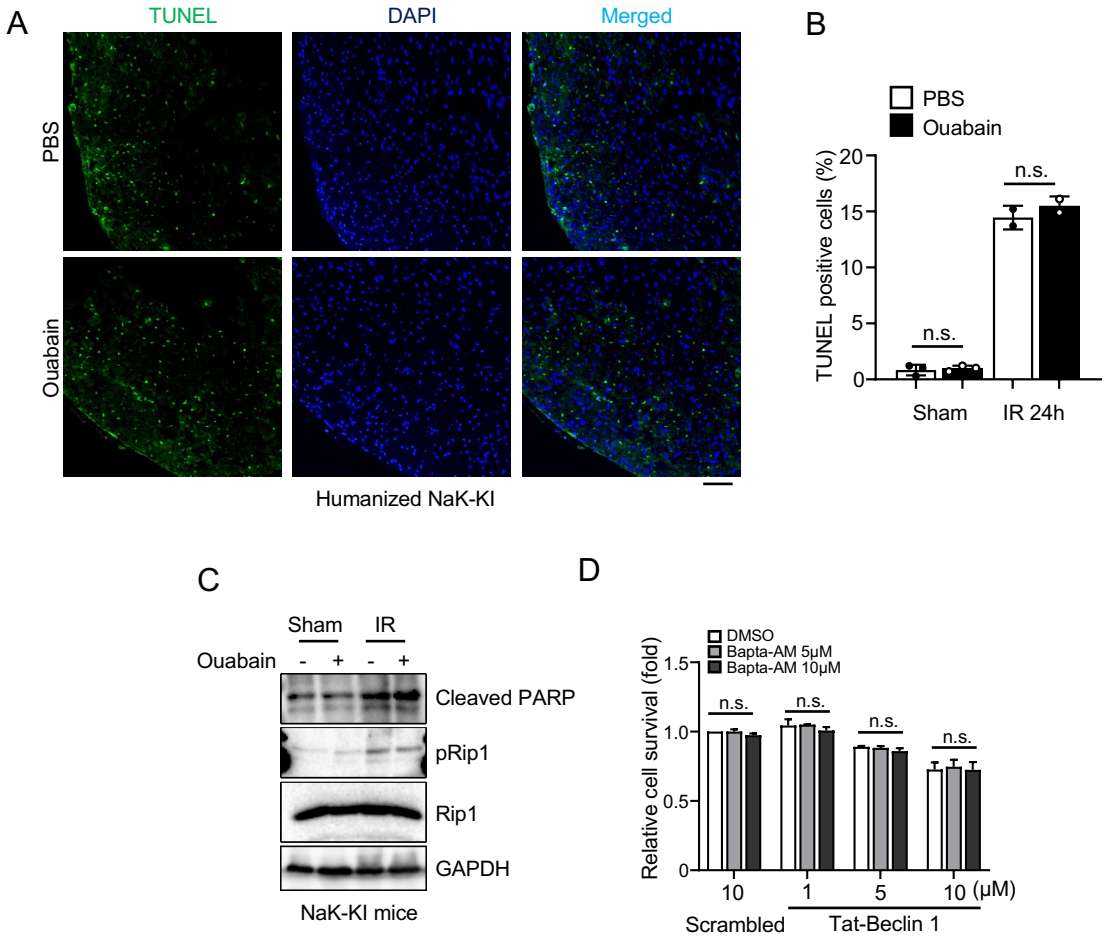
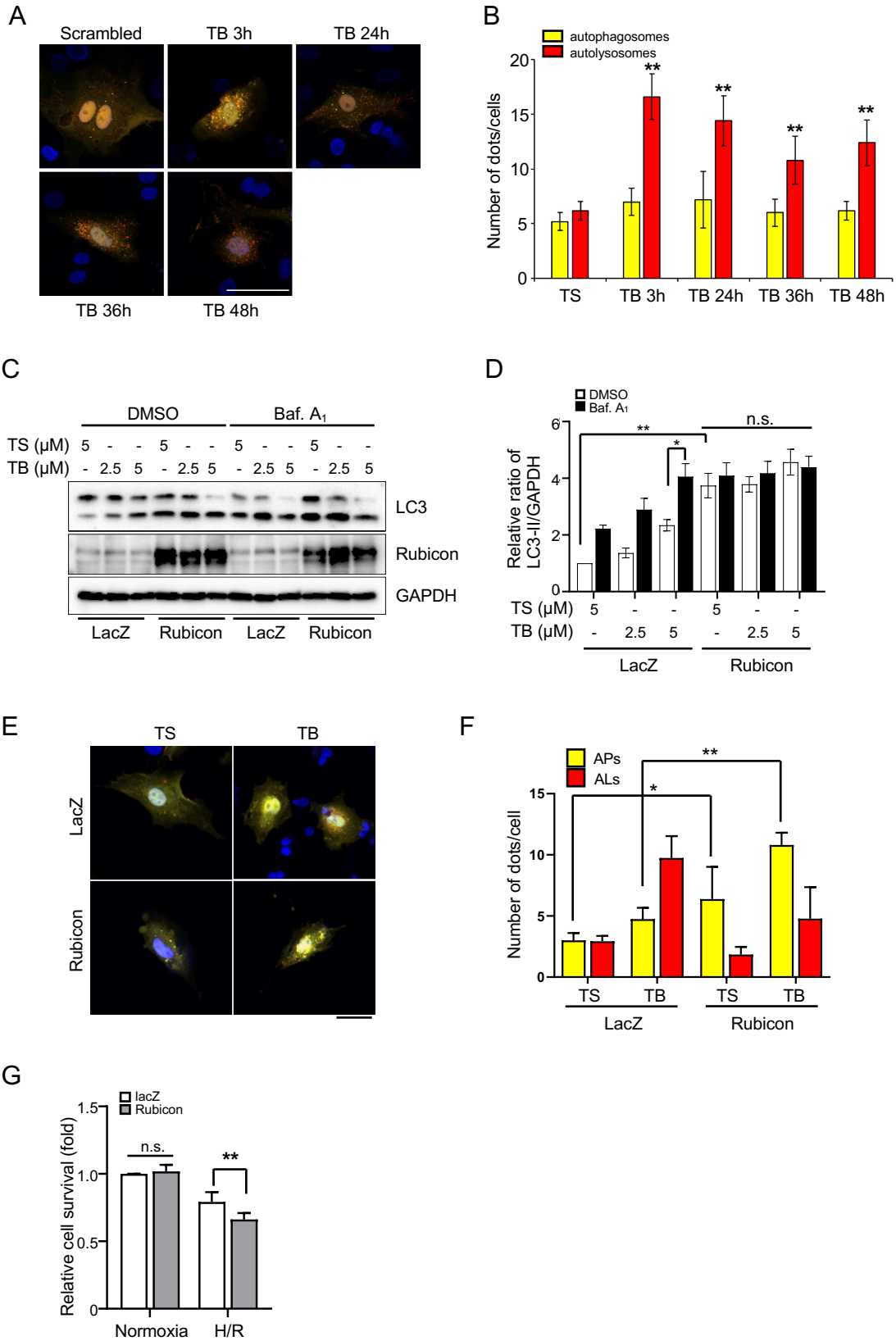


Figure S5. Three-month-old homozygous Na+,K+-ATPase humanized knock-in mice were subjected to 30 minutes of ischemia and 24 hours of reperfusion. Either PBS or ouabain was injected intraperitoneally, as indicated in Figure 5B. Heart samples were subjected to TUNEL assays. Representative immunofluorescent DAPI (blue) and TUNEL (green) staining of heart sections around the infarction area (A). The apoptotic index was determined by calculating the percentage of TUNEL positive cells (measured in 3 different view fields per section, 3 sections per mouse with 3 mice per group, not significant (n.s.)) (scale bar = 50 μm) (2-way ANOVA) (B). Heart lysates were analyzed by immunoblotting with anti-cleaved PARP, pRip1, Rip1 and GAPDH antibodies (C). D. NRCMs were pretreated with Bapta-AM (5 and 10 μM) for 1 hour and then treated with Tat-Beclin 1 (1, 5 and 10 μM) for 3 hours. Relative cell death was quantified by CellTiter-Blue assays (mean values ± S.E., n=4, values were measured from more than 16 wells in each experiment; **p < 0.01 vs Scrambled, not significant (n.s.)) (2-way ANOVA) .

Supplementary Figure 6



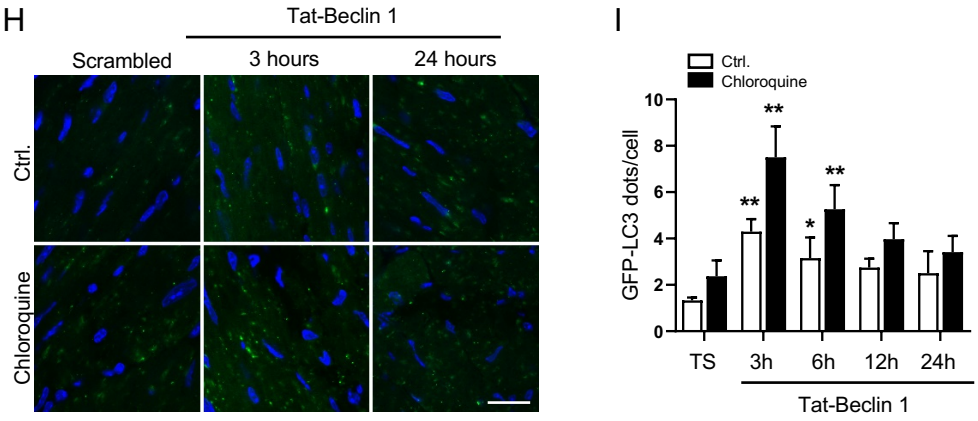
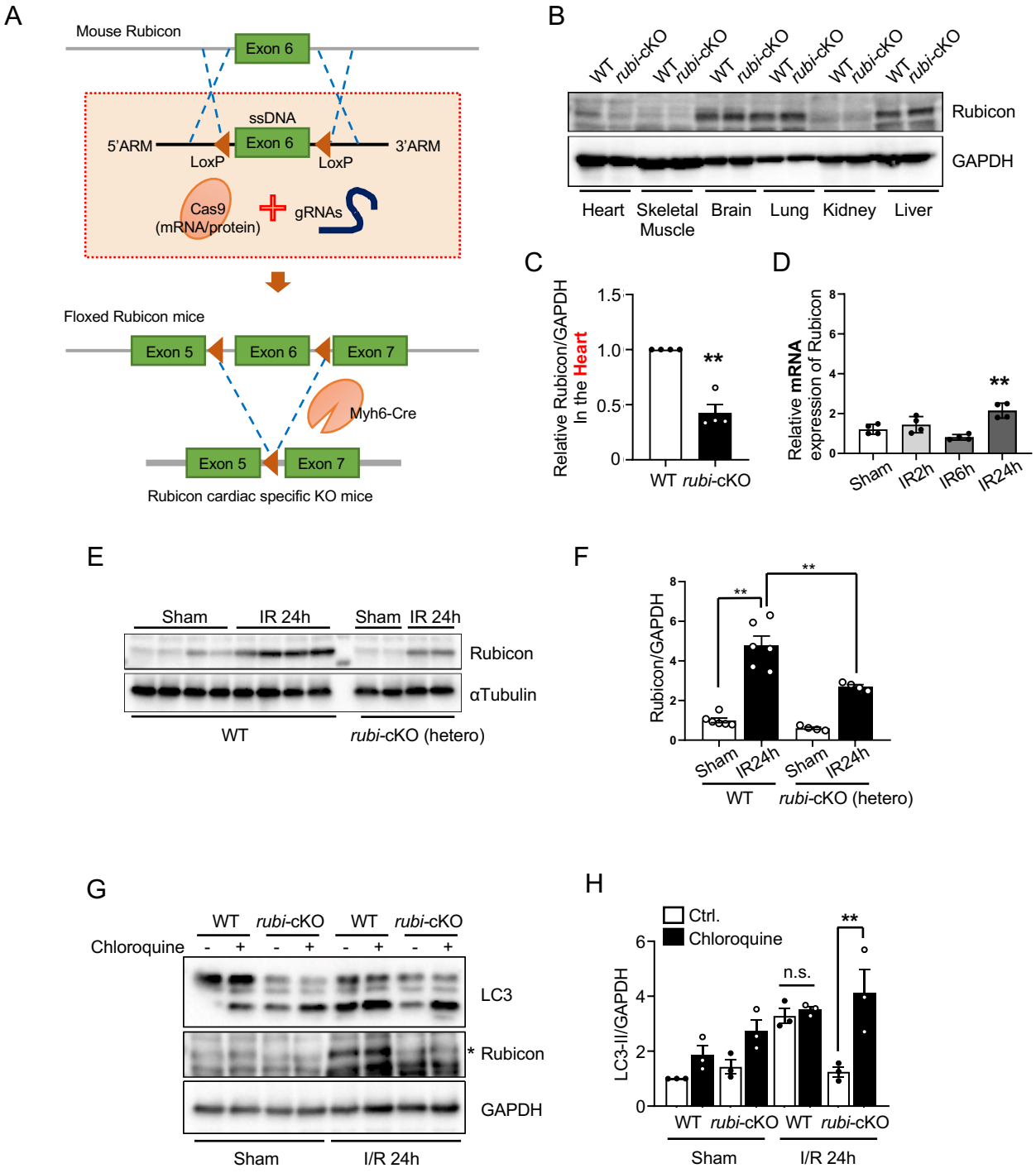


Figure S6. Marked accumulation of autophagosomes during the late phase of ischemia/reperfusion is accompanied by upregulation of Rubicon. A and B. NRCMs were treated with Ad-tf-LC3 for 48 hours and then treated with 5 μM Tat-Beclin 1 time-dependently. Cells were fixed with 4% paraformaldehyde and then observed under a confocal microscope (A). Scale bar: 50 μm. The quantification of autophagosomes (yellow puncta) and autolysosomes (red puncta) is shown (mean values ±S.D., n=5 different areas; **p < 0.01) (2-way ANOVA) (B). C and D. NRCMs were transduced with Ad-Rubicon for 24 hours, treated with 2.5 or 5 μM Tat-Beclin 1 for 3 hours and treated with 20 nM Bafilomycin A_1 2 hours before collecting cells as indicated. Lysates were analyzed by Western blotting using anti-LC3, anti-Rubicon, and anti-GAPDH antibodies (C). The relative ratio of LC3II to GAPDH (mean values ±S.D., n=5; ***p* < 0.01, non-significant (n.s.)) (2-way ANOVA) (D). E and F. NRCMs were treated with Ad-tf-LC3 for 24 hours and then treated with 5 μM Tat-Beclin 1 for 3 hours. Cells were fixed with 4% paraformaldehyde and then observed under a confocal microscope (E). The quantification of autophagosomes (yellow puncta) and autolysosomes (red puncta) is shown (mean values ±S.D., n=5; ***p* < 0.01) (2-way ANOVA) (F). G. NRCMs were treated with Rubicon virus for 48 hours. After then cells were applied 3 hours hypoxia and 3 hours reoxygenation. Cell death was quantified by CellTiter-Blue assays (mean values ±S.D., n=4 values were measured from more than 16 wells per each experiment; **p < 0.01 vs lacZ) (2-way ANOVA). H and I. Tg-GFP-LC3 mice were injected with 5 mg/kg of Tat-Beclin 1 through the jugular vein. Heart sections were prepared at various time points after injection. In some animals, chloroquine was administered (10 mg/kg i.p.) 3 hours before sacrifice. Representative images of GFP-LC3 dots (H) and the quantification of the number of dots/field (I) are shown. n=5 per group. (2-way ANOVA)Scale bar = 20 μm.

Supplementary Figure 7



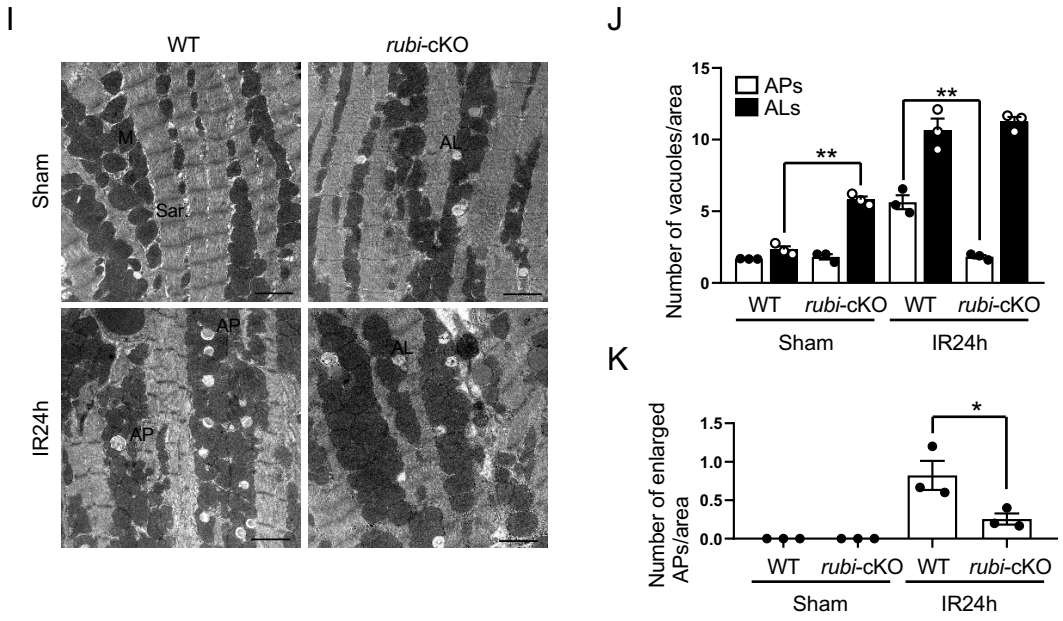
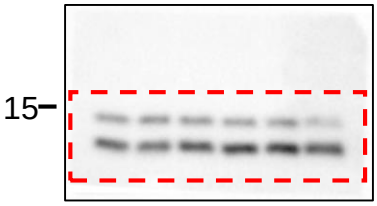
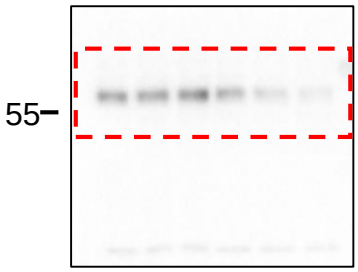


Figure S7. Cardiac-specific knockout of Rubicon rescued ischemia/reperfusion injury and reduced autosis in the mouse heart. A. A schematic representation of the cardiac specific *rubicon* KO (*rubi*-cKO) mice. B and C. Heart, skeletal muscle, brain, lung, kidney and liver samples were prepared from 3-month-old WT and *rubi*-cKO mice at baseline and analyzed by Western blotting using anti-Rubicon and anti-GAPDH antibodies (B). The relative level of Rubicon normalized by GAPDH in the heart is shown (C) (mean values ±S.E., n=4; **$p < 0.01$) (unpaired t test). D. The mouse heart samples were subjected to qPCR analyses using the Rubicon primer sets (mean values ±S.E., n=4/each group; **p<0.01) (one-way ANOVA with Dunnett's post hoc). E and F. Three-month-old WT and *rubi*-cKO mice were subjected to 30 minutes of ischemia and 24 hours of reperfusion and heart samples were analyzed by Western blotting using anti-Rubicon and anti-GAPDH antibodies (E). The relative ratio of Rubicon to GAPDH was quantified (mean values ±S.E., n=6 (WT group); n=4 (*rubi*-cKO group); *p<0.05, **p<0.01) (one-way ANOVA with Tukey's post hoc) (F). G and H. Three-month-old WT and *rubi*-cKO mice were subjected to 30 minutes ischemia and 24 hours reperfusion. Some mice were injected with 10 mg/kg chloroquine (i.p., 3 hours before sacrifice). Heart lysates were subjected to immunoblot analyses with anti-LC3, anti-Rubicon and anti-GAPDH antibodies (G). The level of LC3-II relative to GAPDH was quantified (mean values ± S.E., n=3; **p < 0.01) (2-way ANOVA) (H). I - K. Heart samples from mice subjected to either sham operation or ischemia/reperfusion were subjected to electron microscopic analyses. Autolysosomes (AL), autophagosomes (AP), mitochondria (M) and sarcomere (Sar) are indicated (I). The numbers of cytoplasmic ALs and APs (J), and enlarged APs with non-degraded materials (K) were counted (mean values ±S.E., n=3/each; *p<0.05, **p<0.01, values were measured from 20 different areas per mouse) (one-way ANOVA with Tukey's post hoc) .

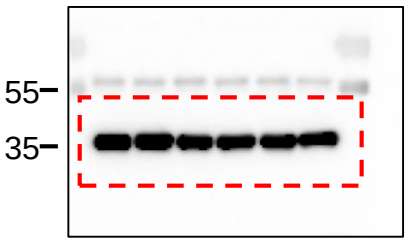
Full unedited gel for Figure 1C: LC3



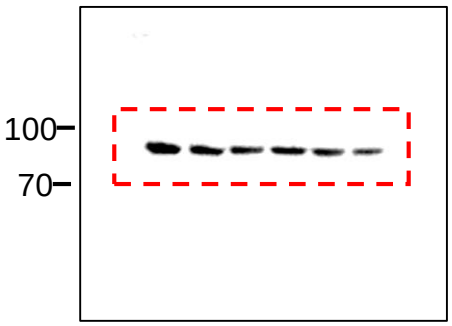
Full unedited gel for Figure 1C: p62



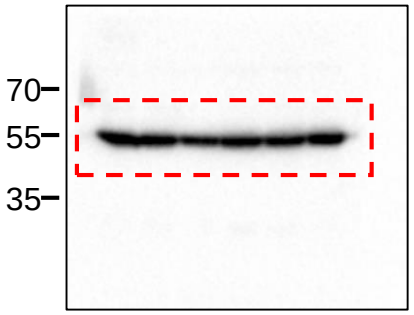
Full unedited gel for Figure 1C: GAPDH



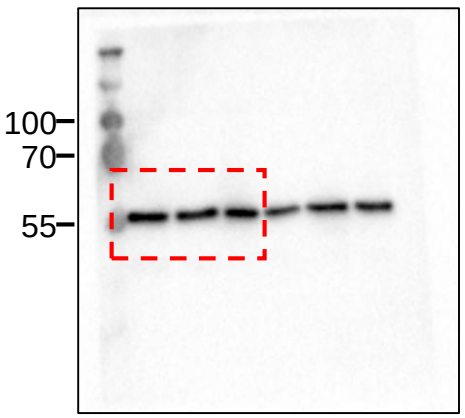
Full unedited gel for Figure 1J: Na^+,K^+-ATPase α1



Full unedited gel for Figure 1J: αTubulin



Full unedited gel for Figure 2A: αTubulin



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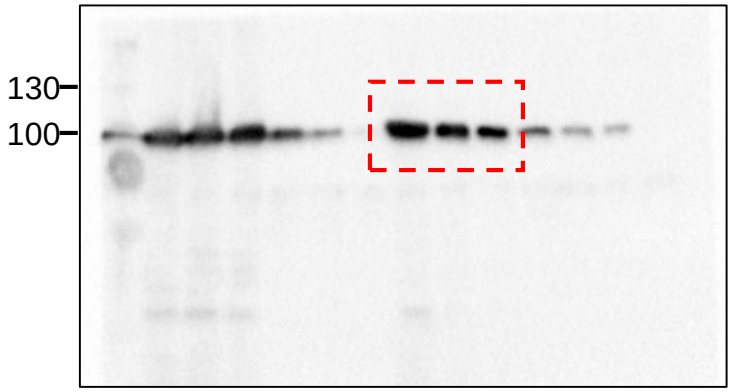

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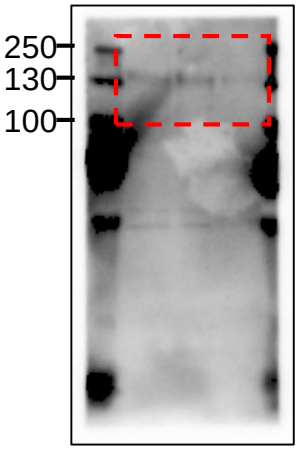

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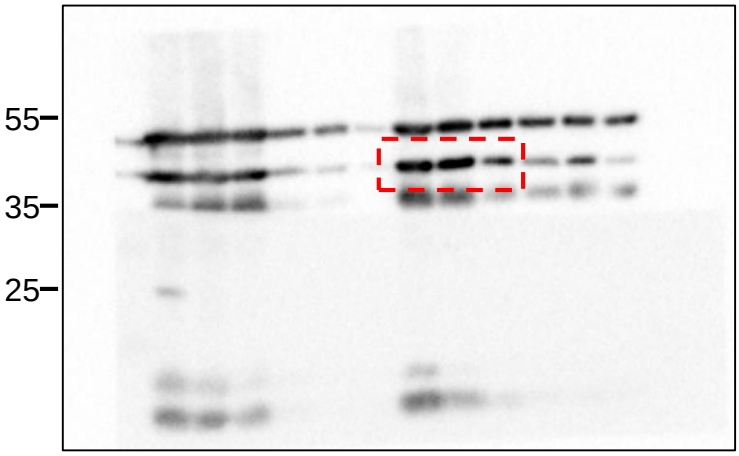

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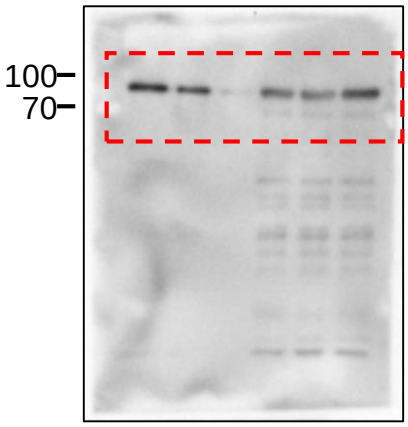

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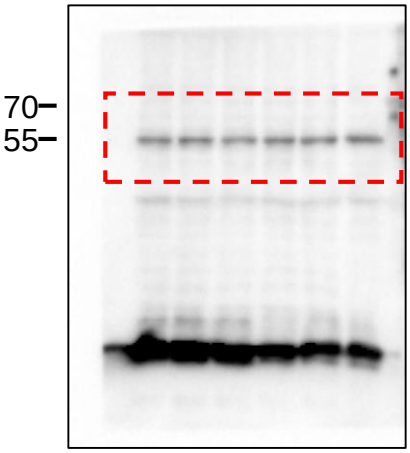

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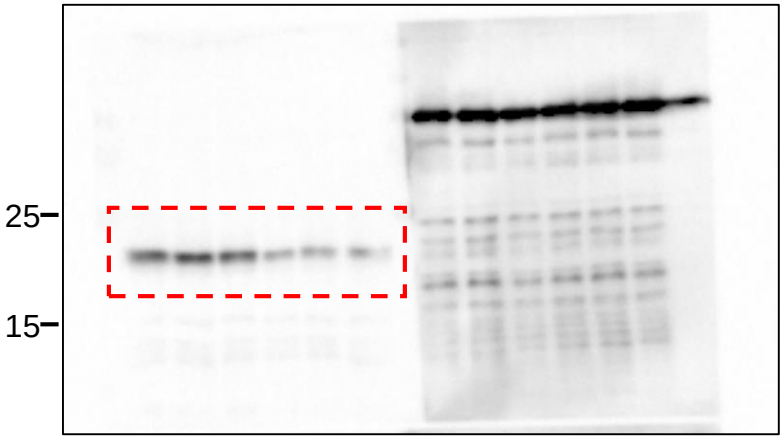
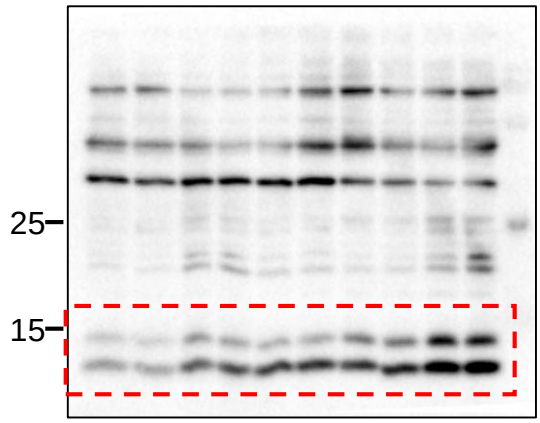
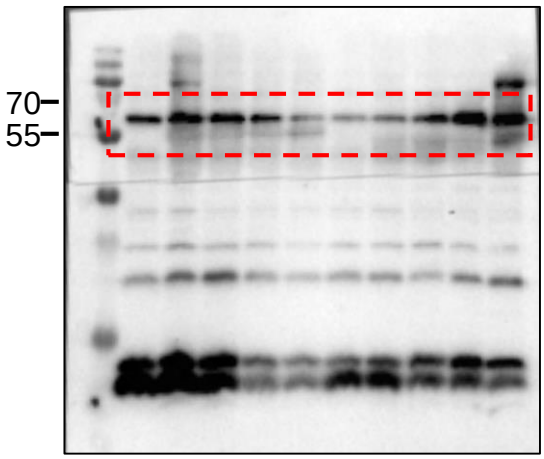


Full unedited gel for Figure 3A: LC3



Full unedited gel for Figure 3A: p62



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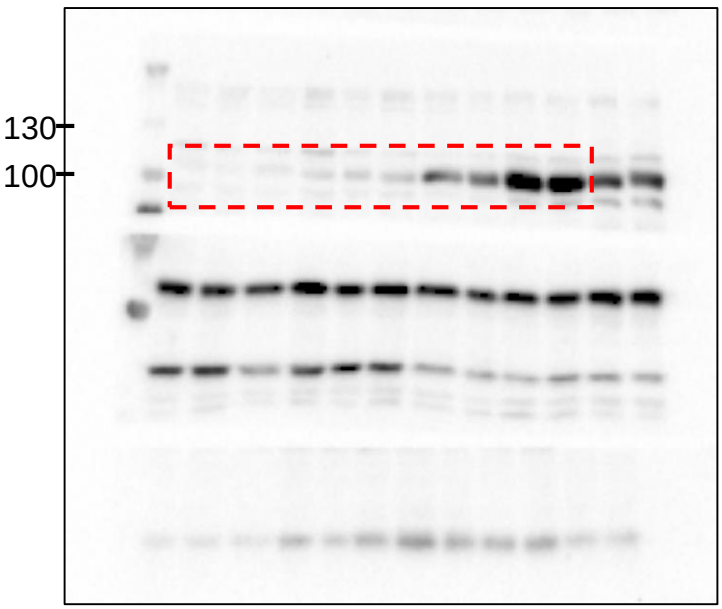
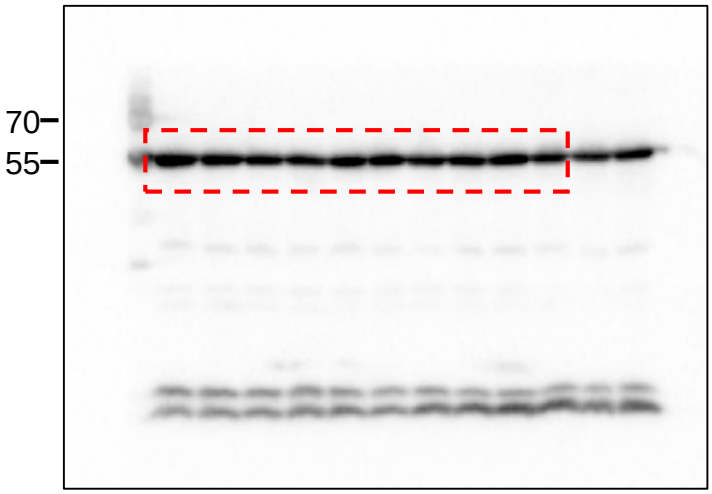


Full unedited gel for Figure 3A,Figure 6A: αTubulin



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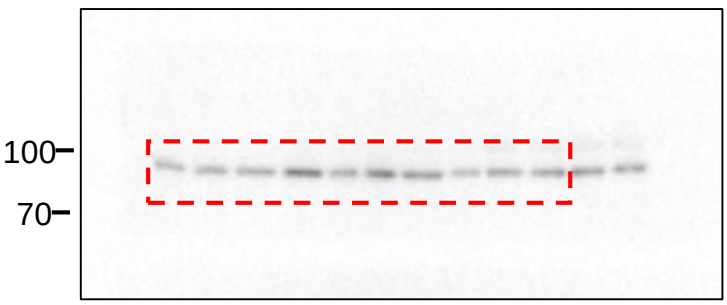

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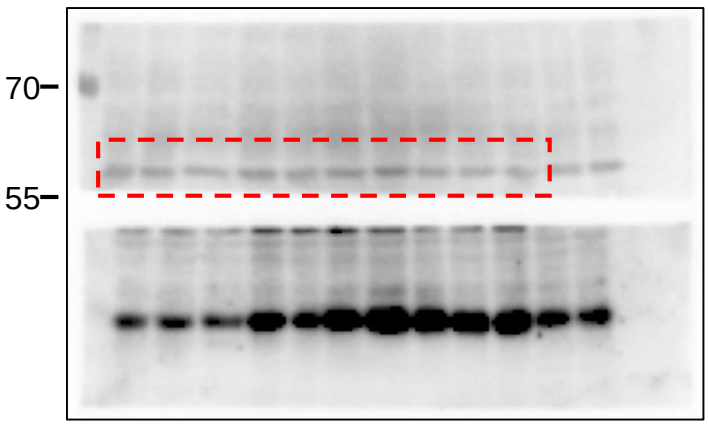

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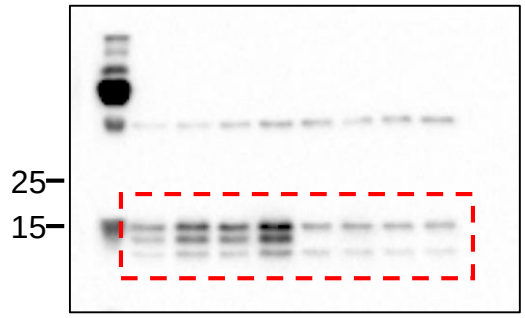

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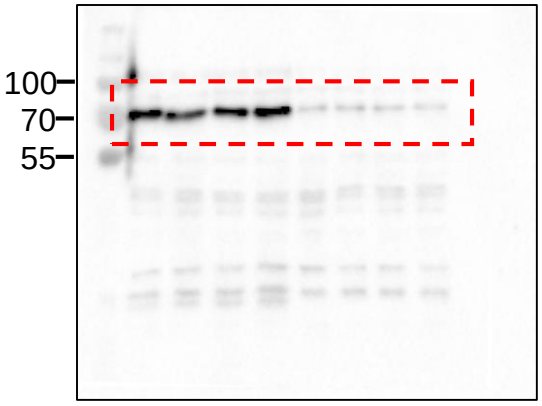

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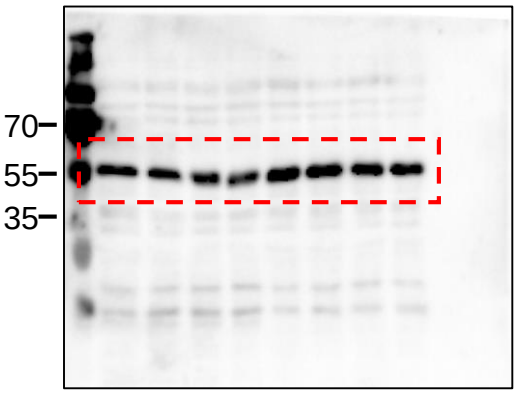

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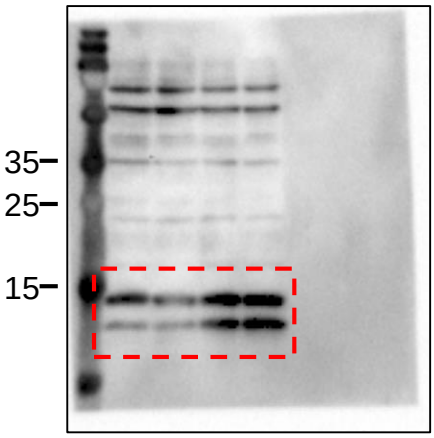

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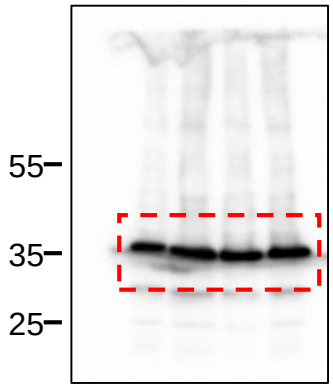

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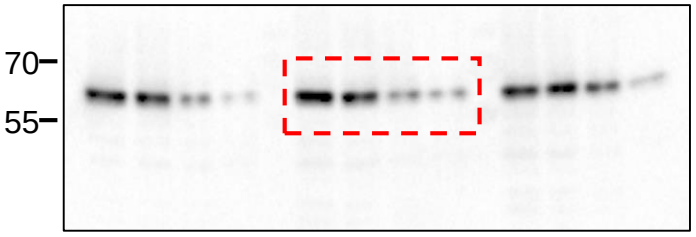

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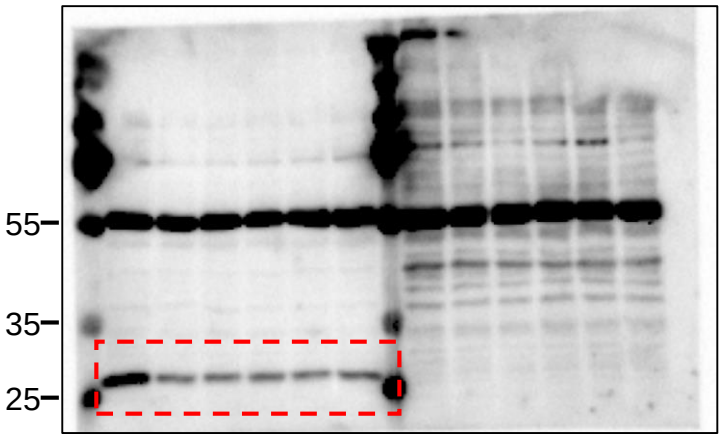
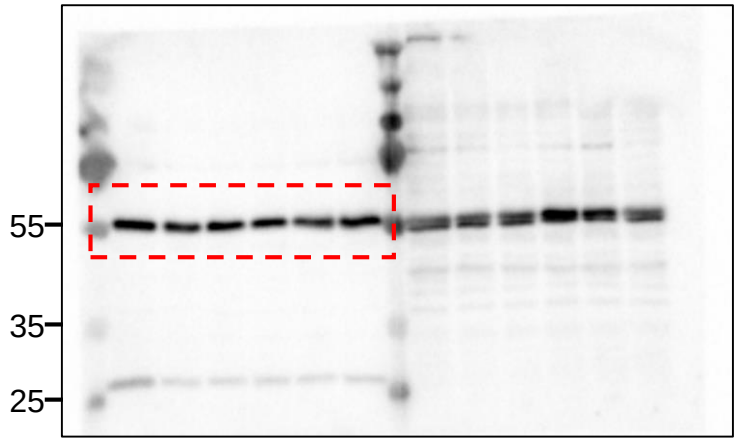
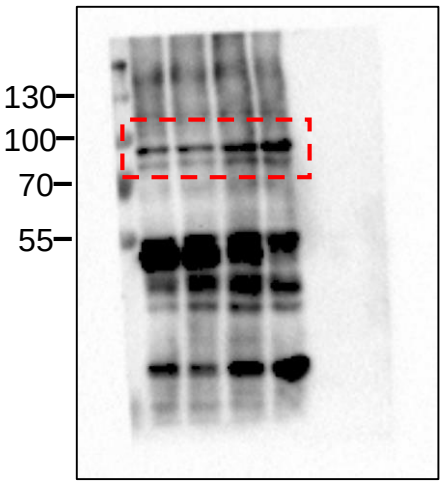


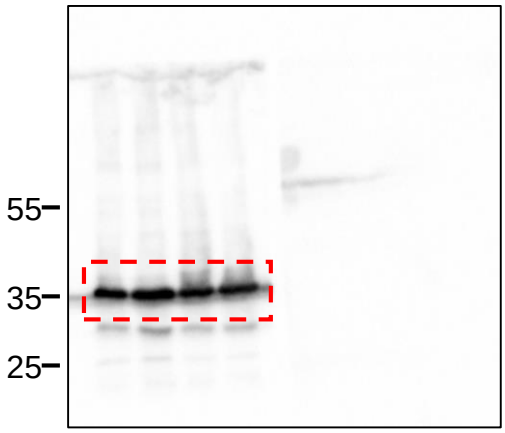
Full unedited gel for Figure S2D: αTubulin



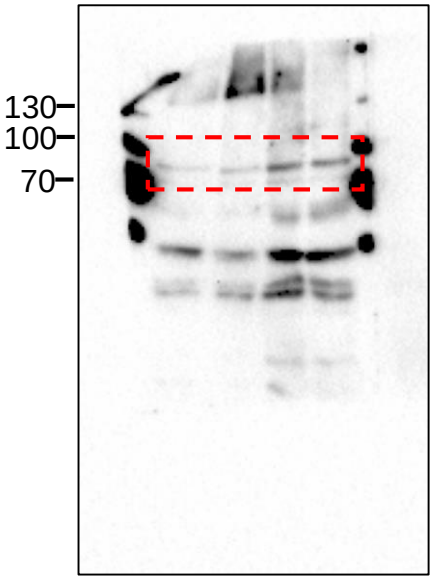
Full unedited gel for Figure S5C: Cleaved PARP



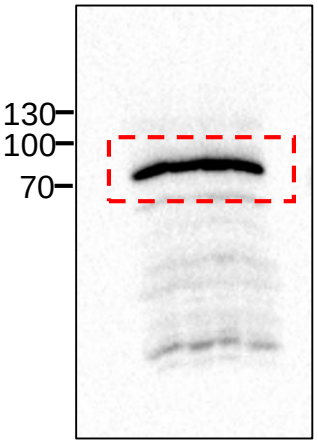
Full unedited gel for Figure S5C: GAPDH



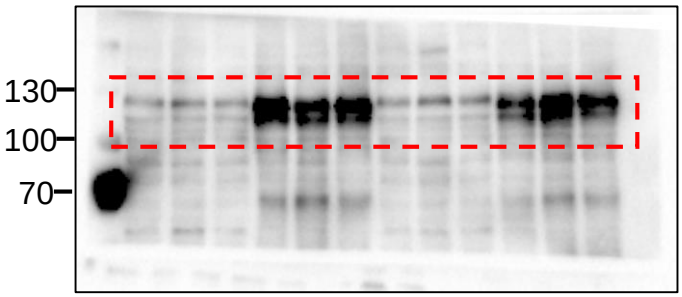
Full unedited gel for Figure S5C: phosphor-RIP1



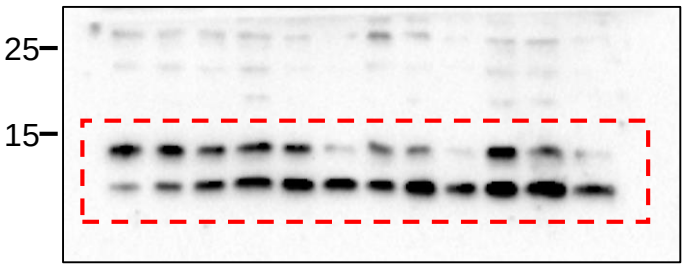
Full unedited gel for Figure S5C: RIP1



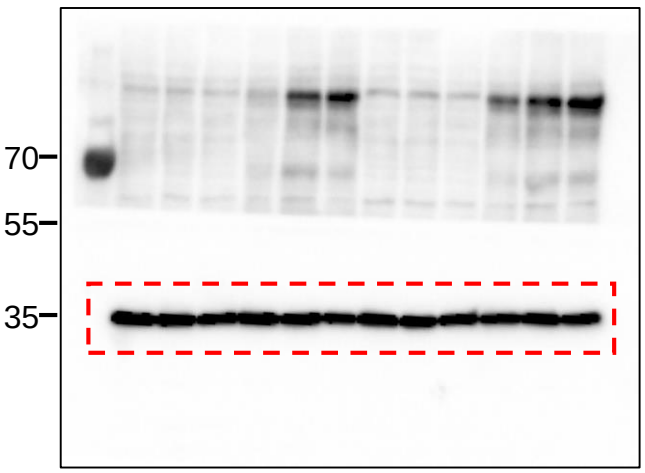
Full unedited gel for Figure S6A: Rubicon



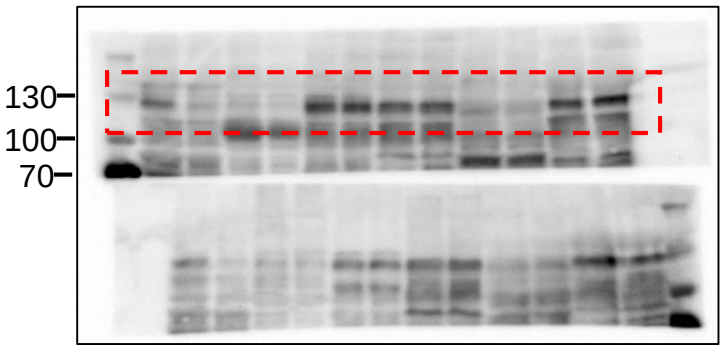
Full unedited gel for Figure S6A: LC3



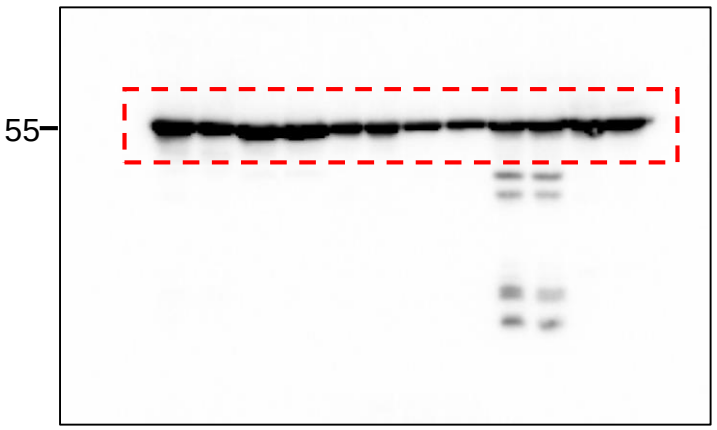
Full unedited gel for Figure S6A: GAPDH



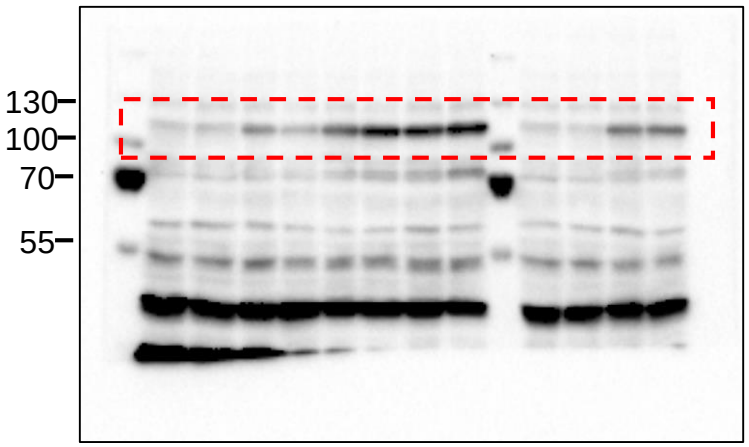
Full unedited gel for Figure S7B: Rubicon



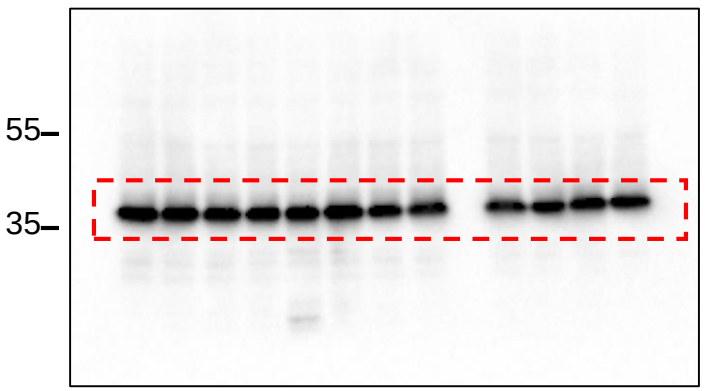
Full unedited gel for Figure S7B: αTubulin



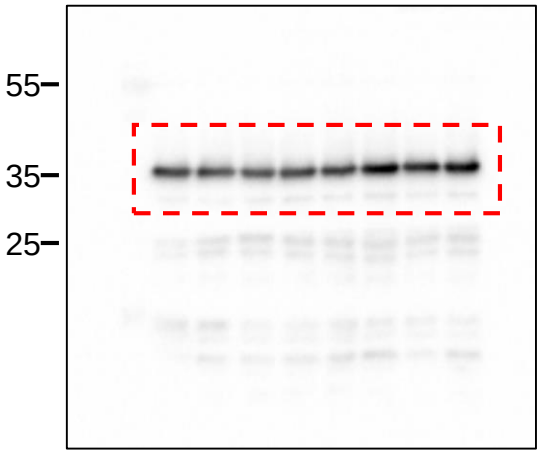
Full unedited gel for Figure S7D: Rubicon



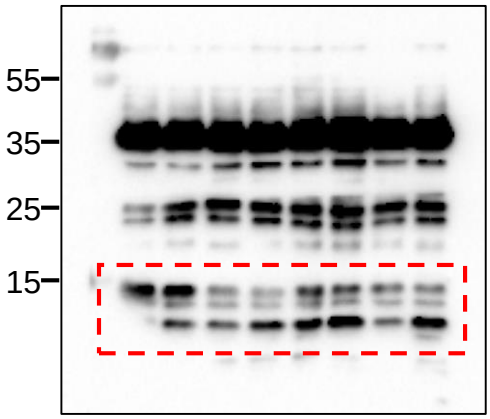
Full unedited gel for Figure S7D: GAPDH



Full unedited gel for Figure S7F: GAPDH



Full unedited gel for Figure S7F: LC3



Full unedited gel for Figure S7F: Rubicon