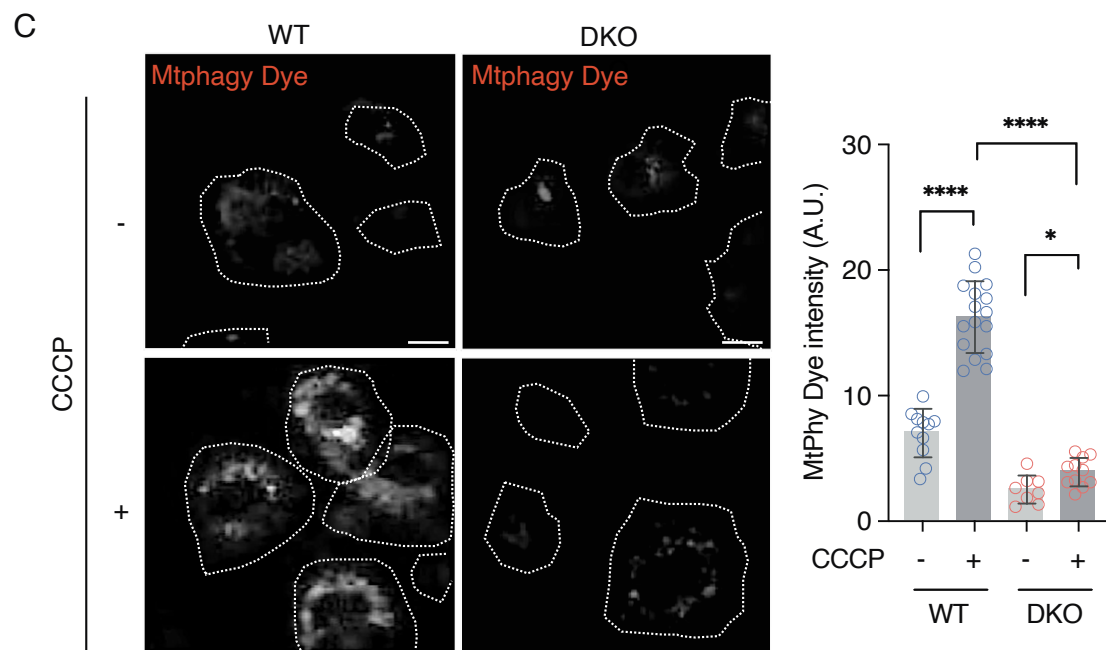
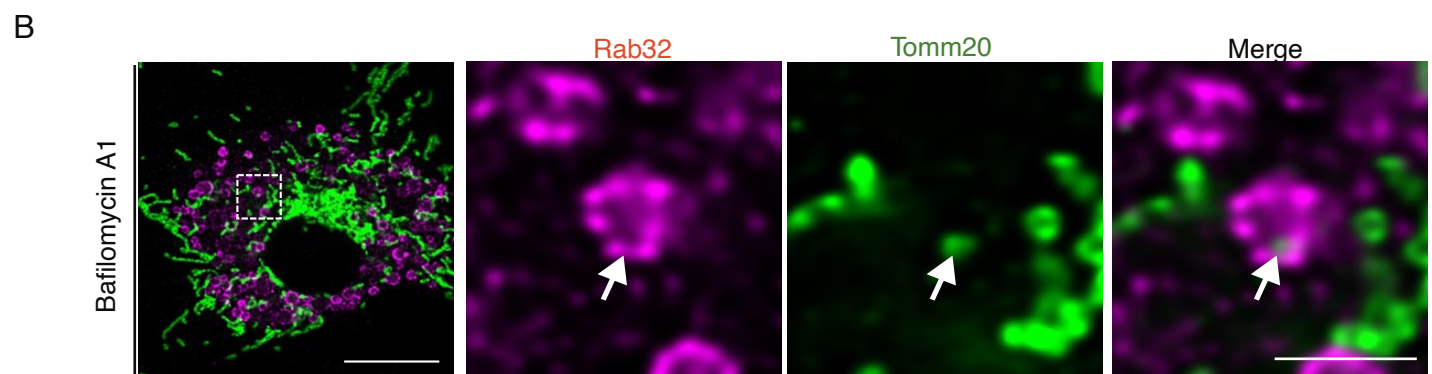
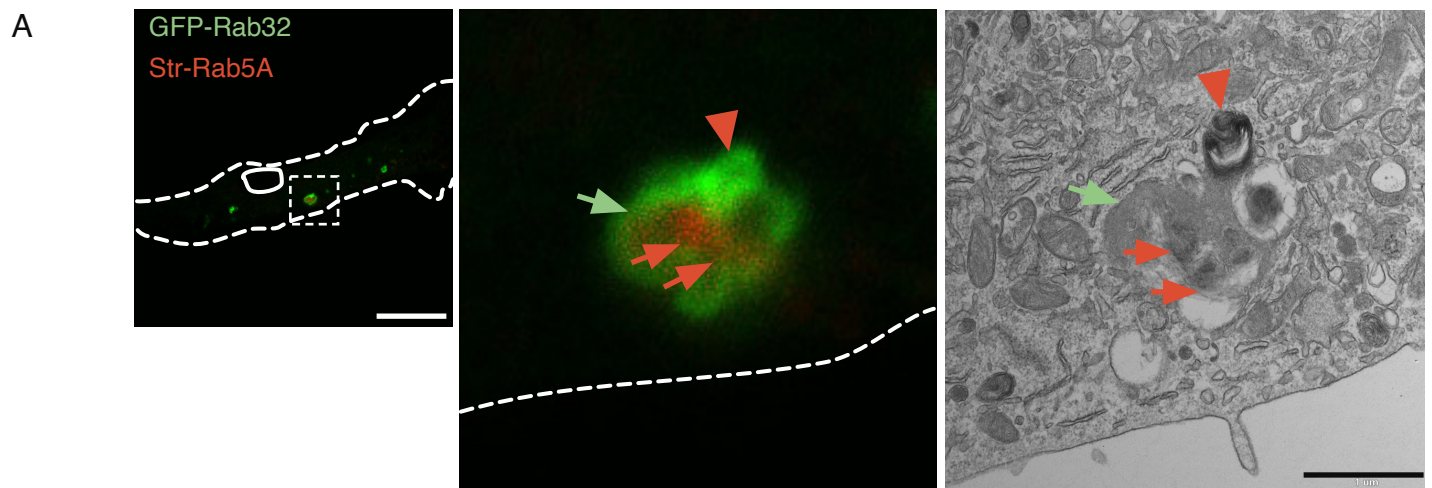
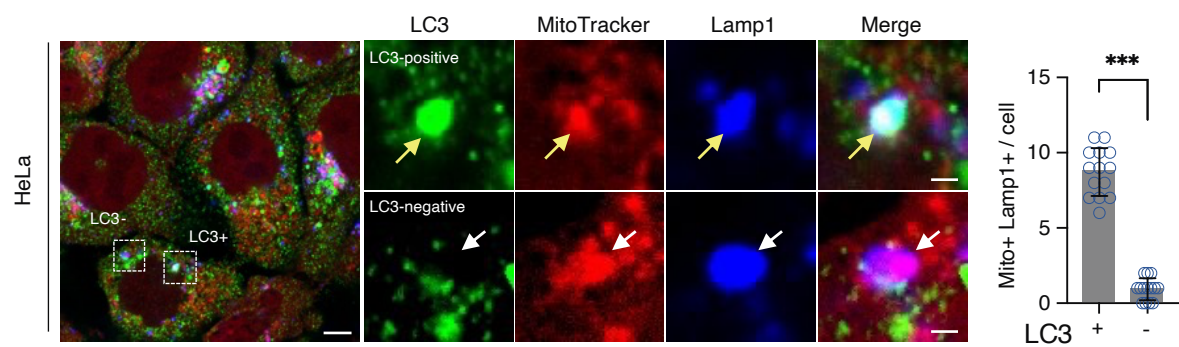




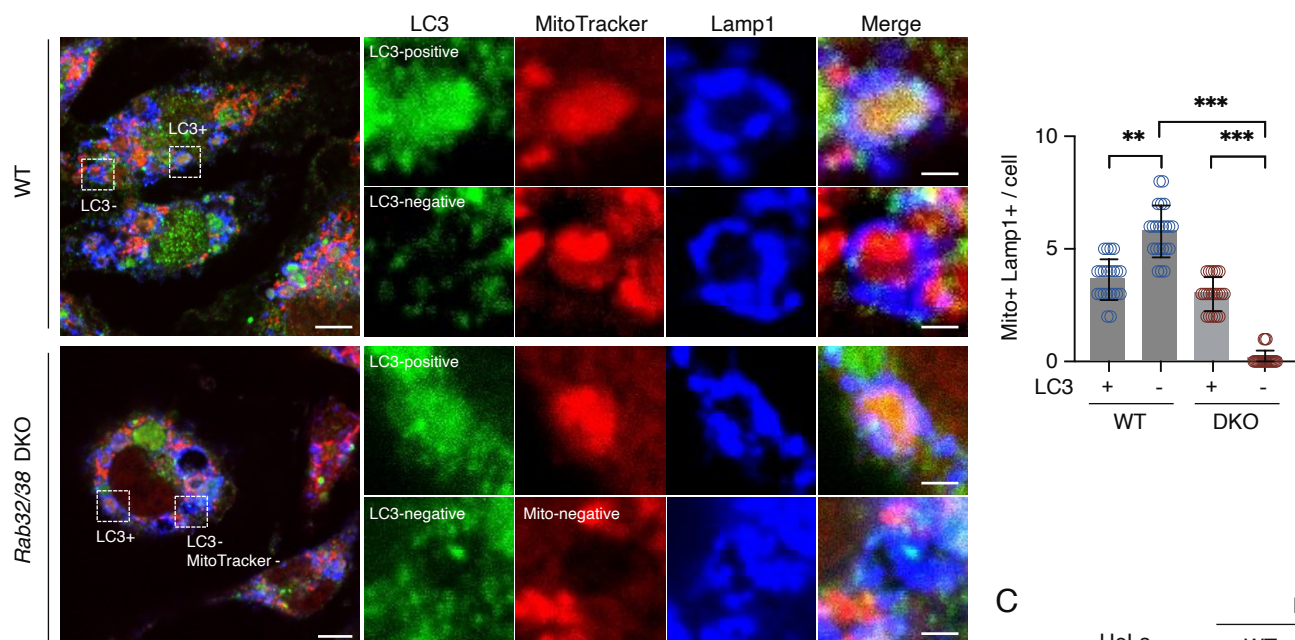
Supplementary figure 1 Incorporation of endosome and mitochondria into LRO

A. BMDMs expressing GFP-Rab32 and Str-Rab5A were fixed and organelles exhibiting fluorescence were analyzed using confocal fluorescence microscopy. The targeted regions were further examined by CLEM. Green arrows indicate Rab32-positive organelles, red arrows indicate Str-Rab5A-positive internal structures, and the red arrowhead marks late endosomes. The experiment was repeated twice. Scale bar: 10 μm (IF), 1 μm (EM). **B.** BMDMs were treated with bafilomycin A1 (50 nM) for 24 h and stained with anti-Rab32 and anti-Tomm20 antibodies. Cell samples were observed using confocal microscopy. White arrows indicate mitochondria inside Rab32-LRO. Scale bars: 10 μm (left), 2 μm (inset). The experiment was repeated twice. **C.** BMDMs isolated from WT and *Rab32/Rab38* DKO mice were treated with Mtpagy Dye and CCCP for 3 h. Cells were observed by confocal microscopy. Scale bar: 4 μm . The fluorescence signal intensities of Mtpagy Dye in WT and DKO BMDMs were quantified using ImageJ. The experiment was performed twice, and a representative result is shown. Each dot represents a measurement from an individual cell (N=11, 16, 8, 11 cells from left to right). Data are presented as mean values $\pm$ SD. A.U. denotes arbitrary units. Statistical significance was analyzed using an unpaired two tailed *t*-test (* $p < 0.05$; **** $p < 0.0001$).

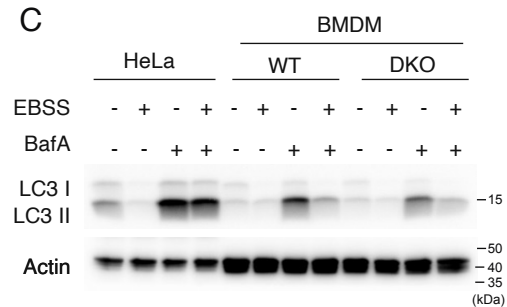
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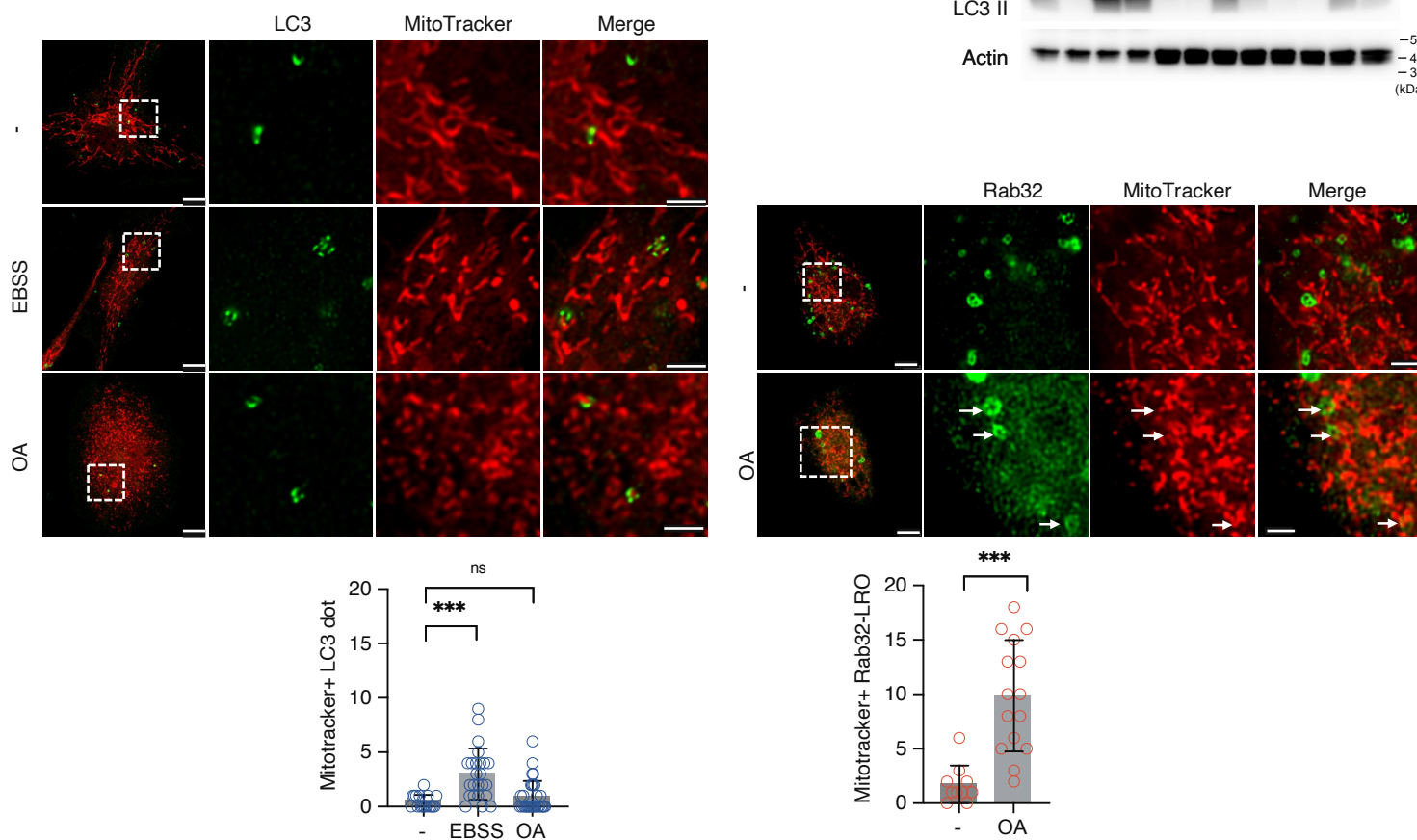
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C

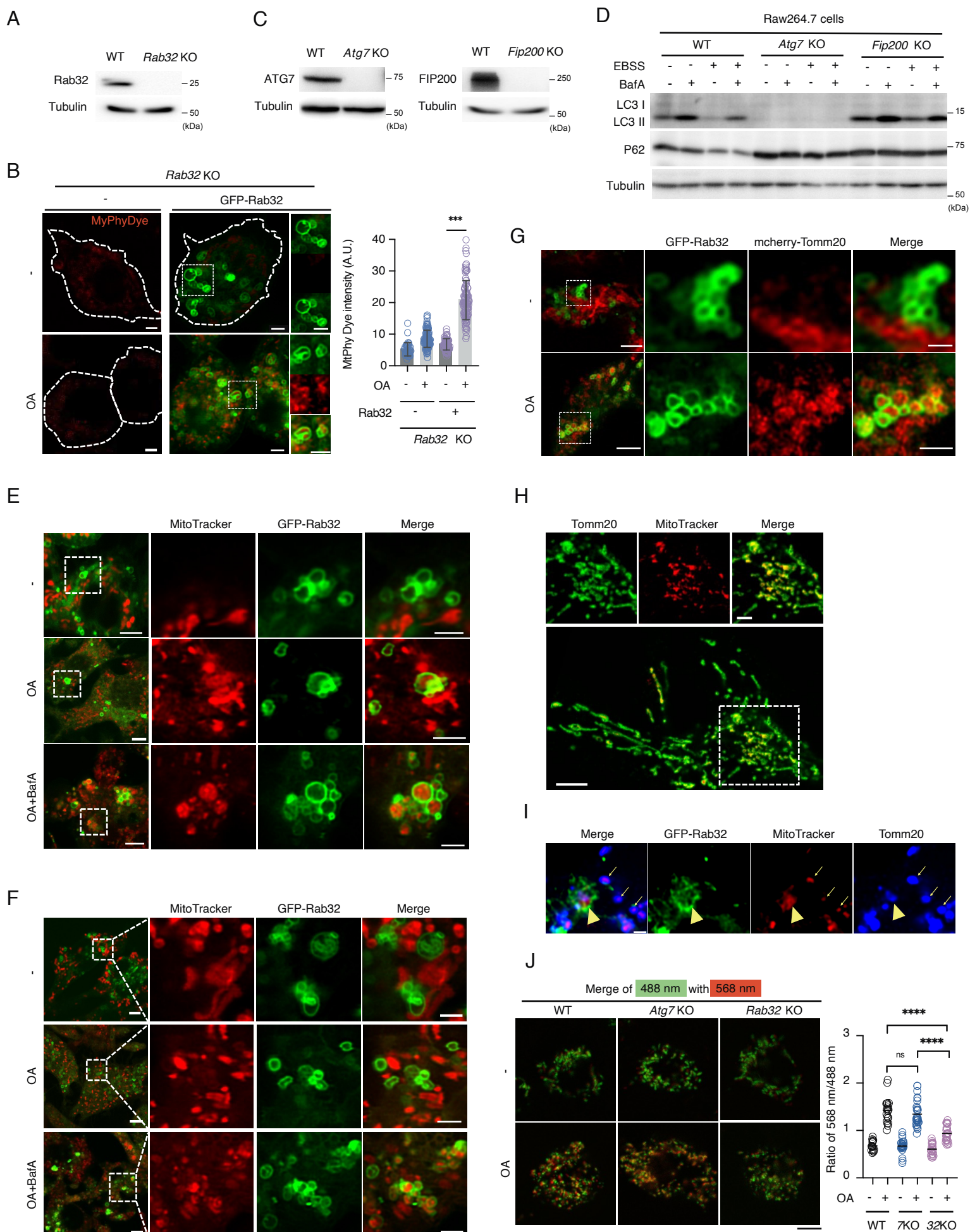


D



Supplementary figure 2 Macroautophagy-independent mitophagy

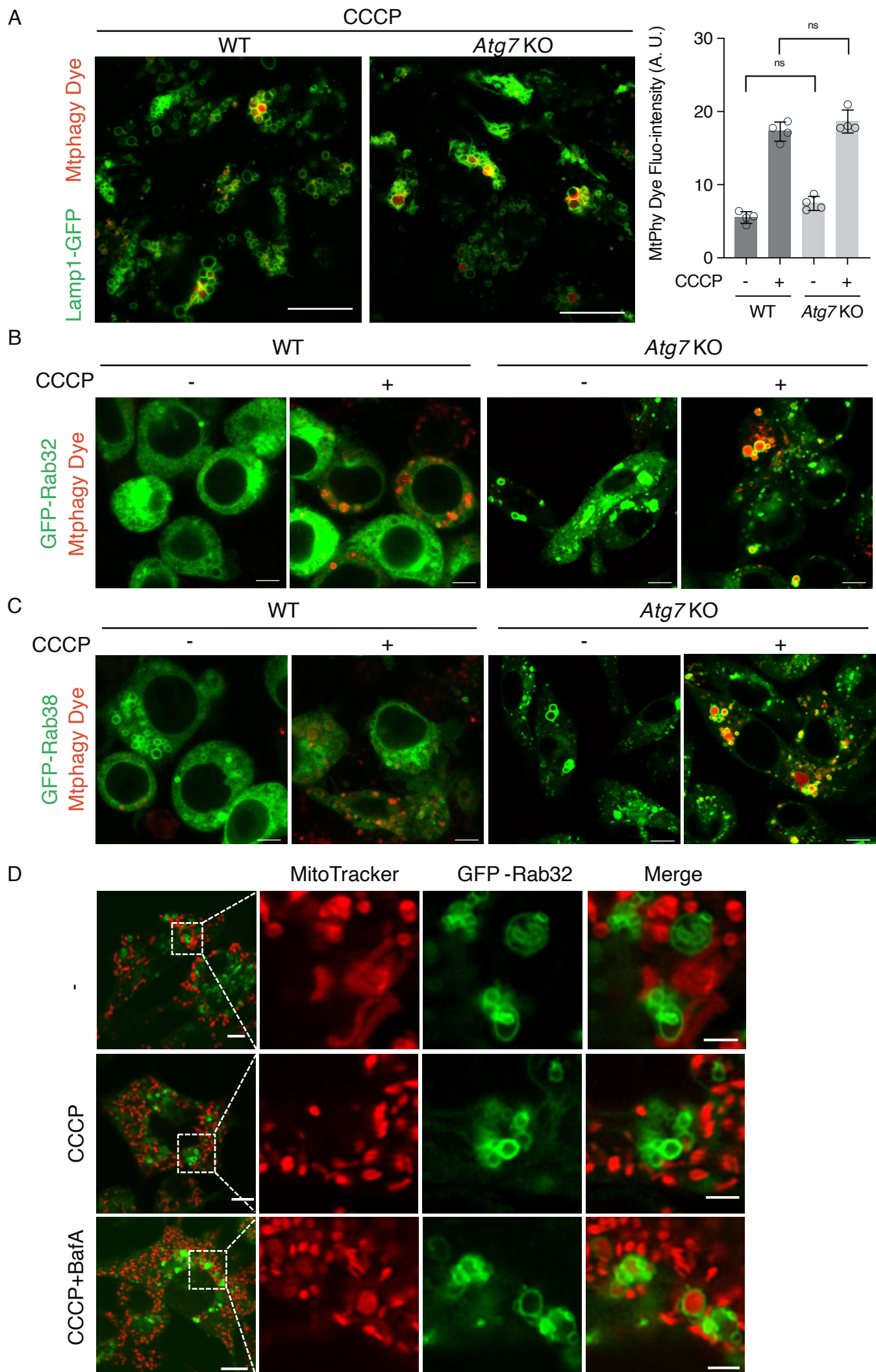
A. HeLa cells expressing Parkin were treated with MitoTracker and subsequently incubated with CCCP for 3 h. Following treatment, cells were fixed and stained with anti-LC3 and anti-Lamp1 antibodies. Samples were analyzed using confocal microscopy (scale bar: 2 μ m). The number of Lamp1-positive puncta with an overlapping region greater than half of the area with MitoTracker signals per cell was quantified in two groups: LC3-positive (any overlapping, yellow arrows) and LC3-negative (white arrows). N=14 cells for each group. Each condition was analyzed in a blinded manner, with quantification performed using the ImageJ Cell Counter tool. Data are presented as mean values $\pm$ SD. **B.** BMDMs isolated from WT or DKO mice were treated with MitoTracker and subsequently incubated with CCCP for 3 h. Following treatment, cells were fixed and stained with anti-LC3 and anti-Lamp1 antibodies, and then analyzed by confocal microscopy. Scale bars: 1 μ m. The number of Lamp1-positive puncta overlapping greater than half area with MitoTracker signals, as well as Lamp1-positive vacuoles containing any MitoTracker signals per cell, was quantified in two groups: LC3-positive (any overlapping) and LC3-negative. N=22 cells for each group. Each condition was analyzed in a blinded manner, with quantification performed using the ImageJ Cell Counter tool. Data are presented as mean values $\pm$ SD. **C.** Hela cells and WT or DKO BMDM cells were treated with EBSS with or without bafilomycin A1 (200 nM) for 3 h. Cells were collected for Western blot analysis of LC3 levels (#S9661, CST). All three cells were loaded with the sample protein levels (30 μ g/well) for SDS-PAGE separation. The experiments were repeated twice. **D.** WT BMDM cells were stained with MitoTracker and treated with EBSS or OA for 3 h. After fixation, cells were stained with anti-LC3 or anti-Rab32 antibodies and observed by confocal microscopy. The graph shows the number of mitochondria particles that co-localized within LC3 or Rab32-LRO in each cell counted in a blinded manner, with quantification performed using the ImageJ Cell Counter tool. N=17, 24, 31 cells for left and N=10, 15 cells for right. The experiments were performed twice, and representative results are presented. Data are presented as mean values $\pm$ SD. Statistical significance was analyzed using an unpaired two tailed *t*-test (***p* < 0.01; ****p* < 0.001).



Supplementary figure 3

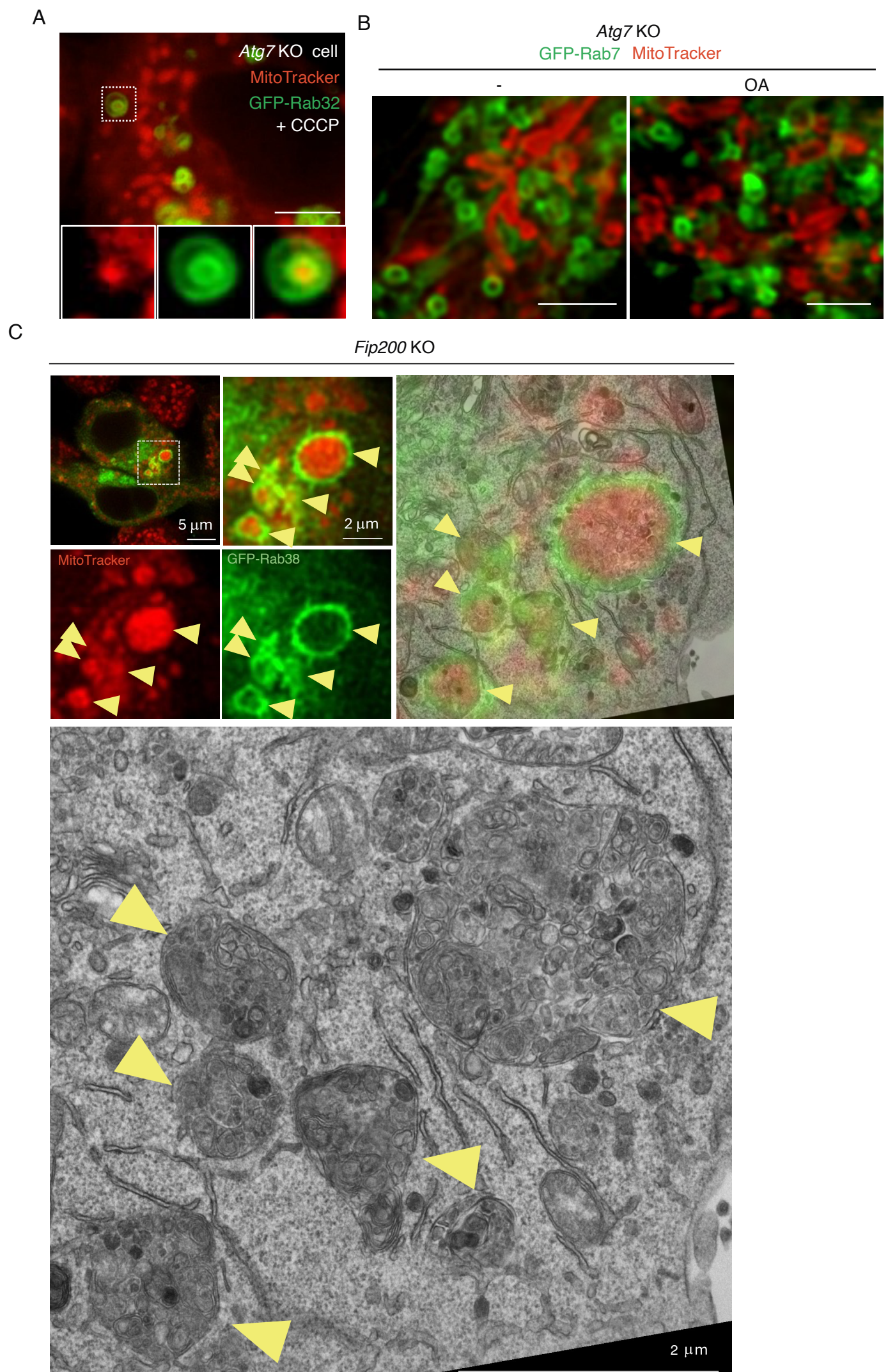
Supplementary figure 3 Macroautophagy-independent mitophagy

A and C. WT, *Rab32* KO, *Atg7* KO, or *Fip200* KO RAW264.7 cells were subjected to Western blot analysis to confirm successful knockout. **B.** *Rab32* KO RAW264.7 cells, either stably expressing GFP-Rab32 or not, were pre-stained with MtpHagy Dye and treated with or without OA for 3 h. Live cells were observed using confocal microscopy. Scale bar: 5 μ m (left) and 2 μ m (inset). The fluorescence signal intensities of MtpHagy Dye were quantified using ImageJ. Each dot represents the fluorescence signal intensity of an individual cell. N=40, 105, 58, 99 cells from left to right. The experiment was performed twice, and a representative result is shown. Data are presented as mean values $\pm$ SD. **D.** WT, *Atg7* KO, or *Fip200* KO RAW264.7 cells were treated with EBSS with or without bafilomycin A1 (200 nM) for 3 h. Cells were collected for Western blot analysis of LC3 II (PM036, MBL) and p62 levels. **E and F.** GFP-Rab32-expressing *Rab32* KO (E) or *Fip200* KO (F) RAW264.7 cells were pre-stained with MitoTracker and treated with or without OA and bafilomycin A1 for 3 hours. Live cells were observed using confocal microscopy. Scale bar: 5 μ m (left) and 2 μ m (inset). **G.** GFP-Rab32 expressing *Atg7* KO RAW264.7 cells were transfected with plasmids for the transient expression of mCherry-Tomm20. Forty-eight hours post-transfection, cells were treated with OA for 3 h and observed using a confocal microscope. Scale bars: 5 μ m (left), 2 μ m (inset). **H.** BMDM cells were stained with MitoTracker for 30 minutes, fixed, and immuno-stained for Tomm20 (11802-1-AP, Proteintech). Images were acquired using a confocal microscope. Scale bars: 5 μ m (bottom) and 2 μ m (inset). **I.** *Atg7* KO RAW264.7 cells expressing GFP-Rab32 were pre-stained with MitoTracker and treated with OA for 3 hours. Cells were then fixed and stained for Tomm20 (11802-1-AP, Proteintech), followed by a secondary antibody conjugated to Alexa-647. Images were acquired using a confocal microscope. Scale bars: 2 μ m. **J.** WT, *Atg7* KO, or *Rab32* KO RAW264.7 cells stably expressing mito-mKeima were treated with OA for 6 h and observed using confocal microscopy. The same cell images were captured under laser 488 nm or laser 568 nm excitation with emission detected in the 560–650 nm range. Each dot represents the ratio of two images from a single cell. N=16, 21, 25, 29, 24, 26 cells from left to right. The experiment was performed twice, and a representative result is shown. Data are presented as mean values $\pm$ SD. Statistical significance was analyzed using an unpaired two tailed *t*-test ($***p < 0.001$).



Supplementary figure 4 Macroautophagy-independent mitophagy

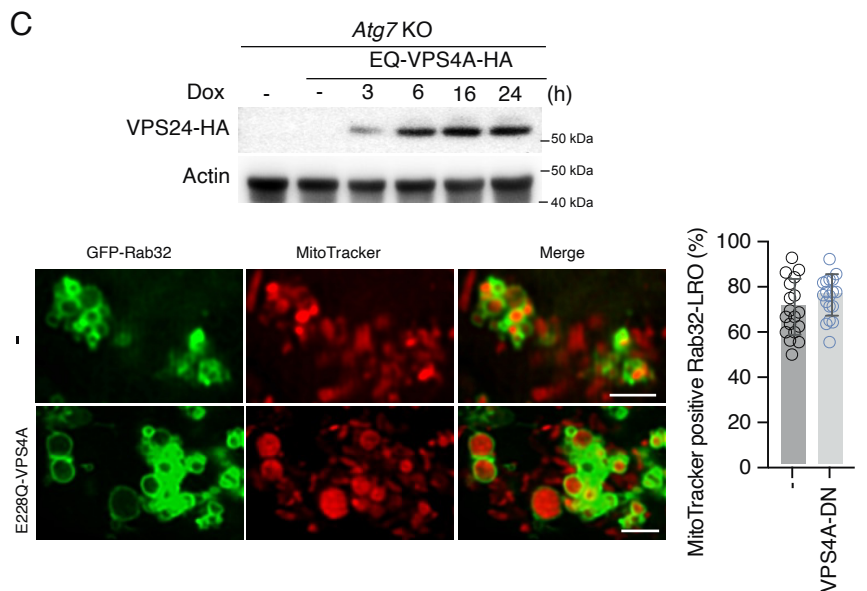
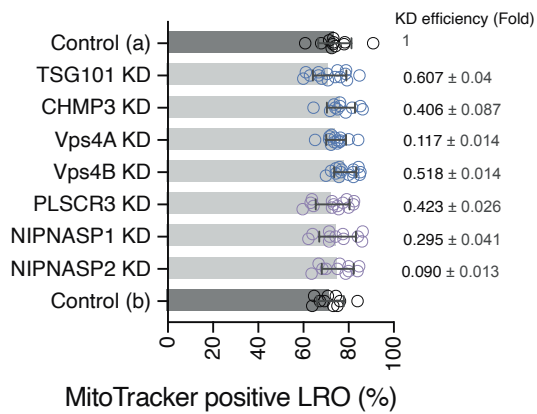
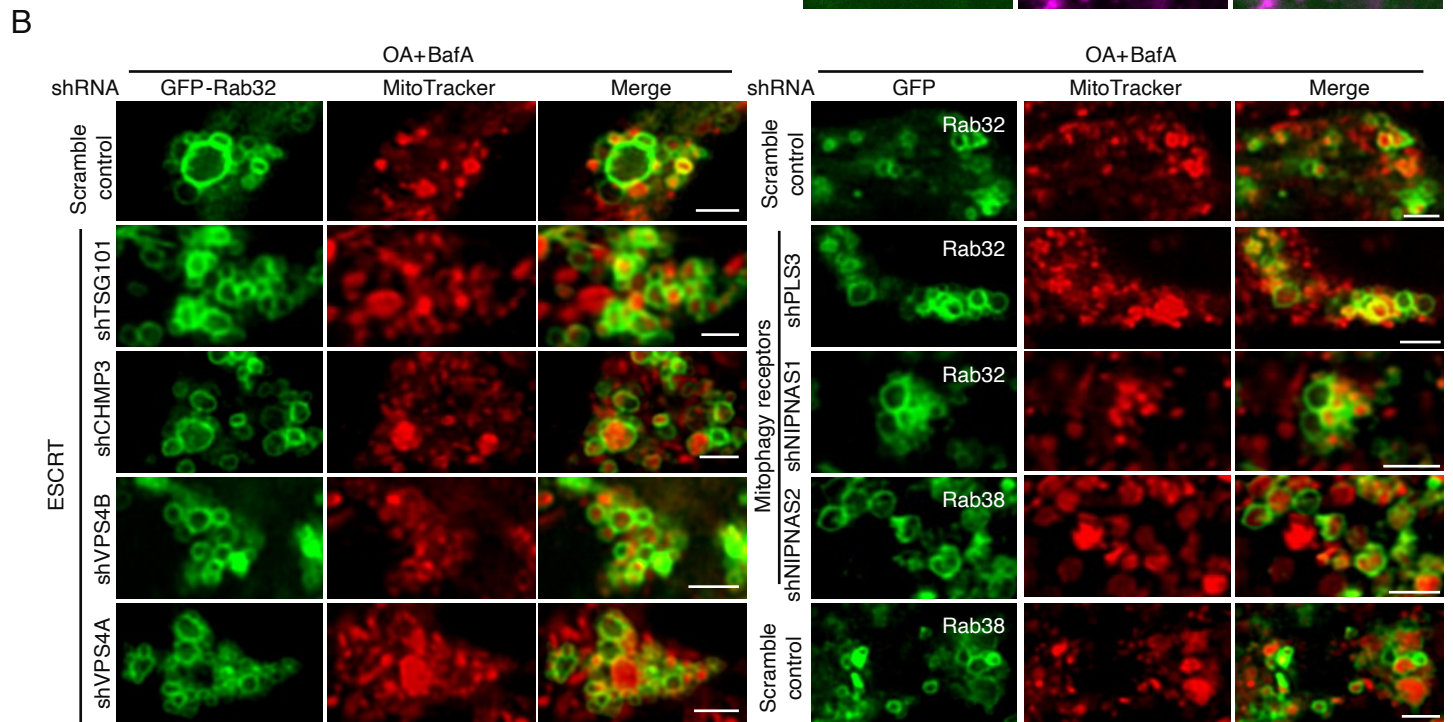
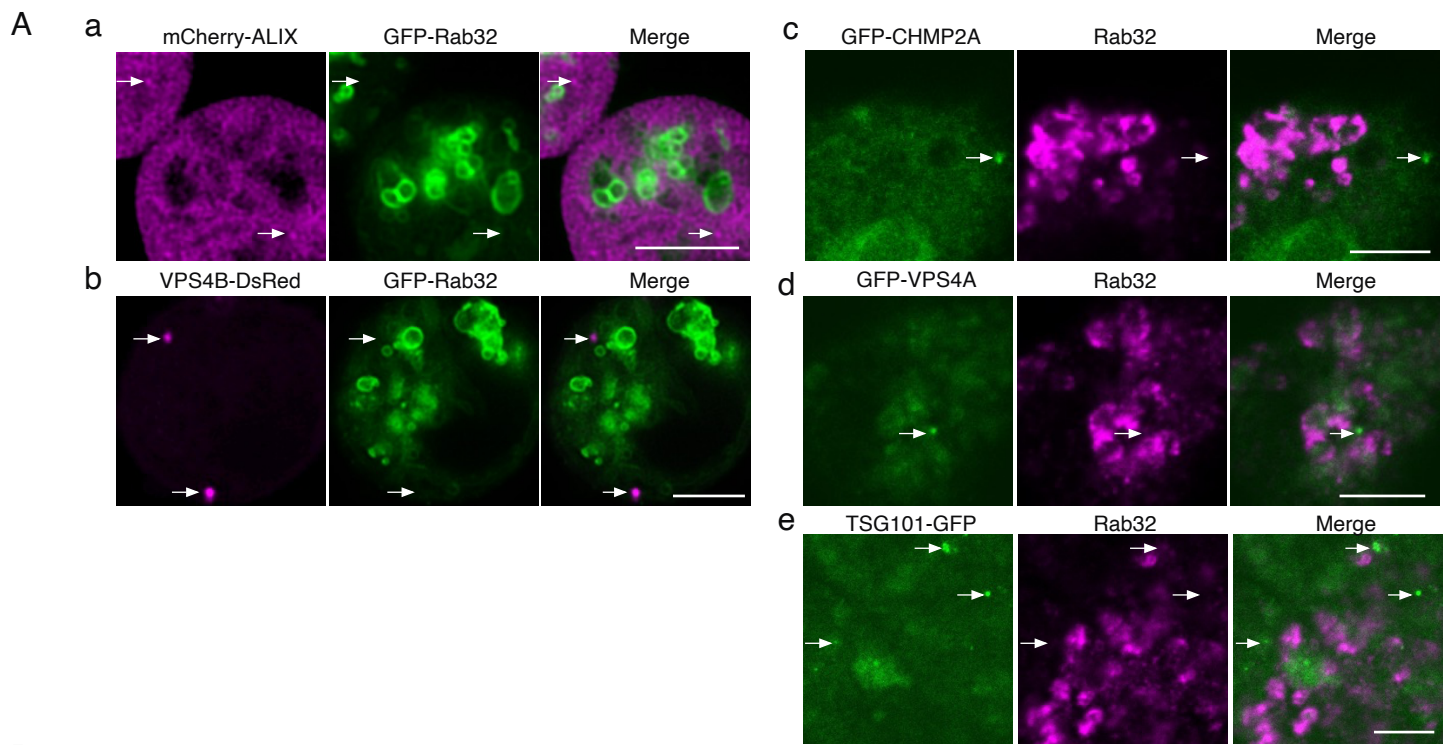
A. WT and *Atg7* KO RAW264.7 cells expressing Lamp1-GFP were stained with Mtpagy Dye and treated with CCCP for 3 h. Scale bar: 10 μ m. The fluorescence intensity of Mtpagy Dye in each cell was measured using ImageJ. Each point represents the fluorescence intensity of Mtpagy Dye per image, normalized to the number of cells. N=4 images containing over 20 cells per condition. The experiment was repeated twice, and a representative result is shown. Data are presented as mean values $\pm$ SD. **B and C.** WT or *Atg7* KO RAW264.7 cells stably expressing GFP-Rab32 or GFP-Rab38 were stained with Mtpagy Dye and treated with CCCP for 3 h. Scale bar: 4 μ m. **D.** *Fip200* KO RAW264.7 cells stably expressing GFP-Rab32 were stained with MitoTracker and treated with CCCP with or without bafilomycin A1 for 3 h. The inset image was captured as a live-cell image immediately following acquisition of the corresponding wide-field image. The untreated set of images is the same as in Supplementary figure 3F. Scale bar: 5 μ m (left), 2 μ m (inset). All the experiments were repeated twice, and a representative result is shown.



Supplementary figure 5

Supplementary figure 5 Macroautophagy-independent mitophagy

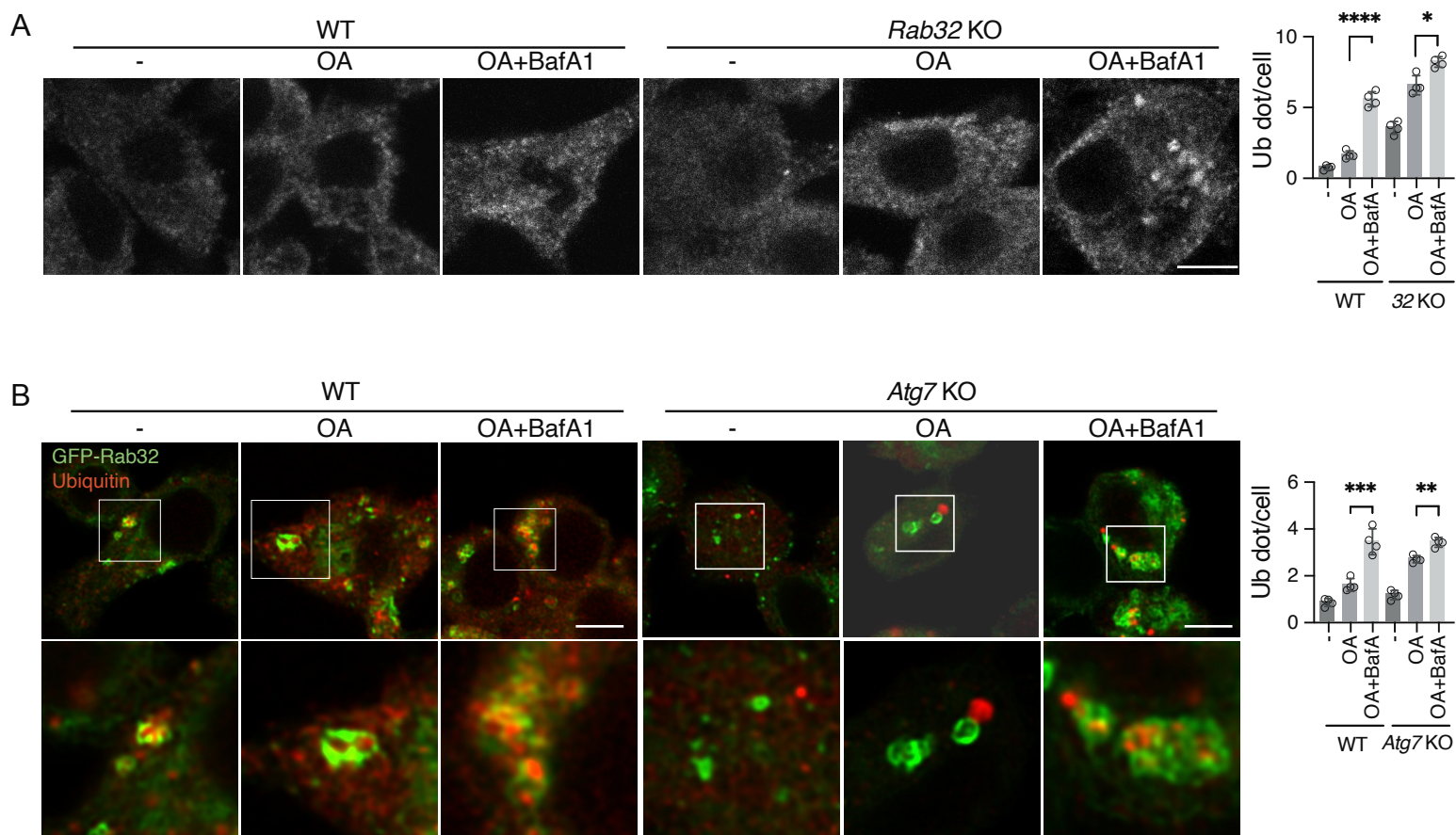
A. GFP-Rab32-expressing *Atg7* KO RAW264.7 cells were stained with MitoTracker and treated with CCCP for 1 h. Cells were observed by confocal super-resolution microscopy (SpinSR10). The experiment was repeated at least three times. Scale bar: 5 μ m. **B.** GFP-Rab7-expressing *Atg7* KO RAW264.7 cells were stained with MitoTracker and treated with OA for 3 h. Cells were observed using confocal microscopy. The experiment was repeated twice. Scale bar: 5 μ m. **C.** *Fip200* KO RAW264.7 cells expressing GFP-Rab38 were treated with CCCP for 1.5 h. After fixation, cells were observed by confocal microscopy, and then further analyzed by electron microscopy. Yellow arrowheads indicate the MitoTracker-positive bodies inside Rab38-LROs, which exhibited poorly defined cristae, suggesting potential damage induced by OA treatment as in Figure 3C. Total 3 cells were observed.



Supplementary figure 6

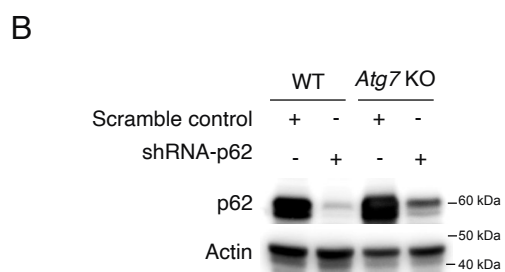
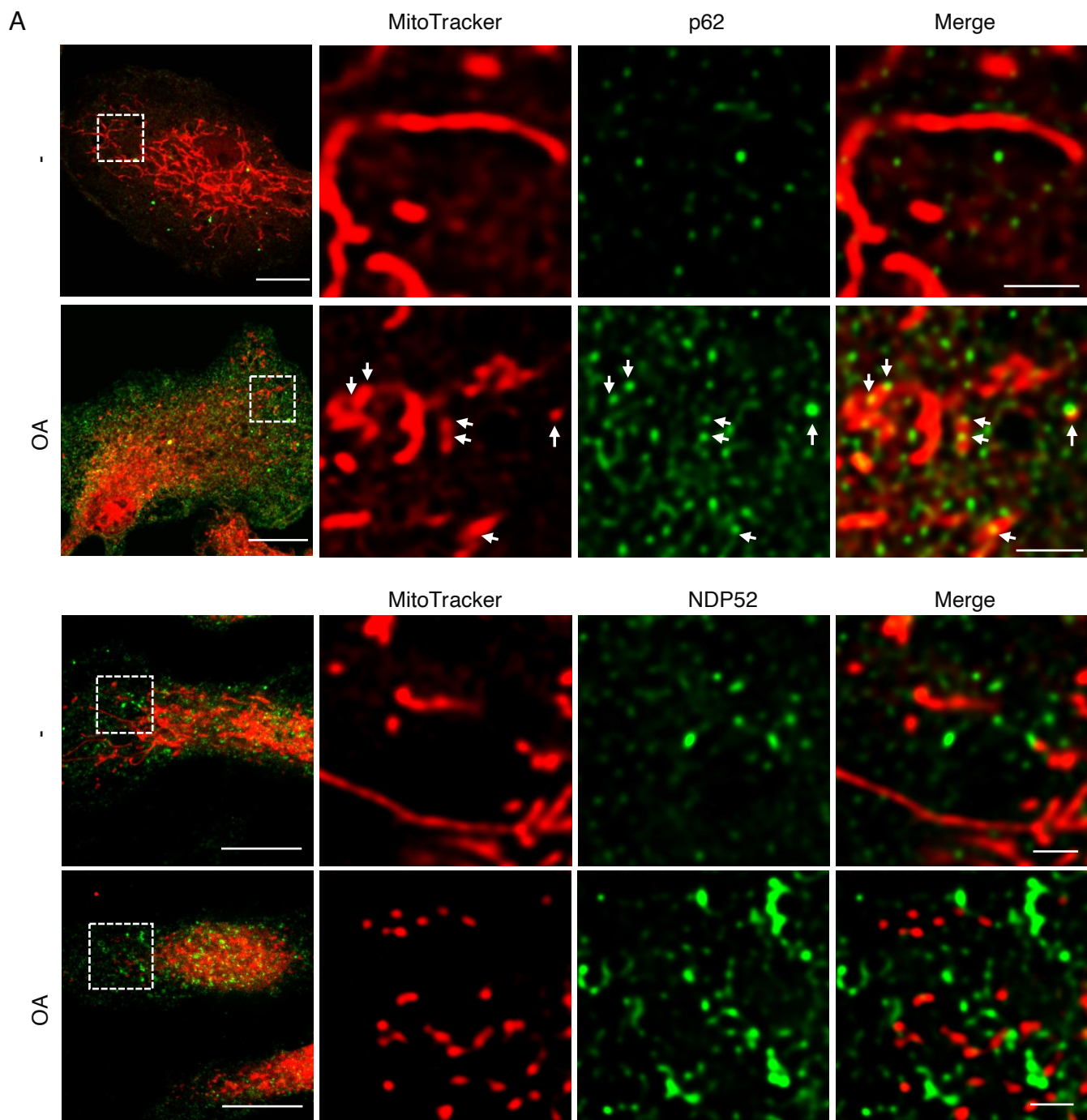
Supplementary figure 6 ESCRT is less dispensable for micromitophagy

A. *Atg7* KO RAW264.7 cells, either expressing GFP-Rab32 (a, b) or not (c, d, e), were transfected with mCherry-ALIX (a), pCAG-DsRed-VPS4B (b), pCAG-GFP-CHMP2A (c), pCAG-GFP-VPS4A (d), or pCAG-TSG101-GFP plasmids. Forty-eight hours post-transfection, cells were treated with OA and bafilomycin A1 for 2 h. GFP-Rab32-expressing cells were directly observed by confocal microscopy, while non-expressing cells were fixed, and stained with anti-Rab32 antibody, and observed by confocal microscopy. White arrows indicate ESCRT component-positive dots. The experiment was repeated twice. Scale bar: 5 μ m. **B.** RAW264.7 cells were transduced with a lentivirus encoding shRNA targeting specific genes. After puromycin selection for 5 days, knockdown efficiency was confirmed by qPCR. Cells were then transduced with GFP-Rab32 or -Rab38 using a retrovirus. After 24 h of transduction, cells were stained with MitoTracker and treated with OA and bafilomycin A1 for 2 h. Live cells were observed using confocal microscopy. The percentage of MitoTracker-positive Rab32-LRO per cell was quantified, with over 10 cells analyzed per condition. The fold change in mRNA levels for each target gene, compared to control cells, is shown with standard deviation, based on technical triplicates. The experiment was performed twice, and a representative result is shown. Data are presented as mean values $\pm$ SD. Scale bar: 2 μ m. **C.** *Atg7* KO RAW264.7 cells, with or without E228Q-VPS4A-HA virus transduction and G418 selection, were treated with doxycycline (1 μ g/ml) for the indicated time points. Cells were then subjected to Western blot analysis. A 16-hour treatment induced E228Q-VPS4A-HA to a sufficient level comparable to the 24-hour condition, after which cells were used for further experiments. Cells were then stained with MitoTracker and treated with OA and bafilomycin A1 for 2 h. Live cells were observed by confocal microscopy. The percentage of MitoTracker-positive Rab32-LRO per cell was quantified, with 18 cells analyzed per condition. The experiment was performed twice, and a representative result is shown. Data are presented as mean values $\pm$ SD. Scale bar: 2 μ m.



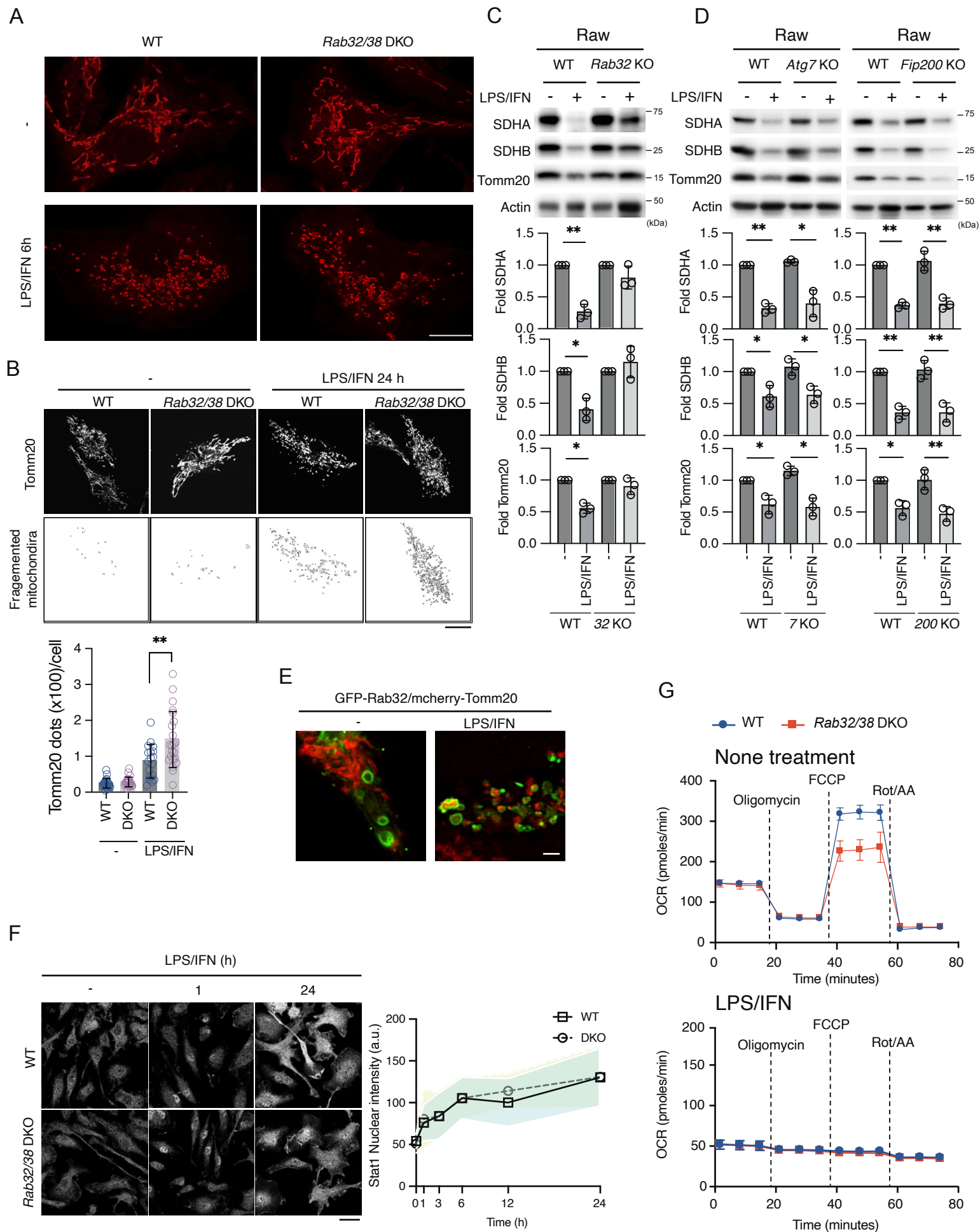
Supplementary figure 7 Ubiquitination is associated with micromitophagy

A. WT or *Rab32* KO RAW264.7 cells were treated with OA, with or without bafilomycin A1, for 6 h. After fixation, cells were stained with an anti-ubiquitin antibody and observed using confocal microscopy. Scale bar: 5 μ m. Each point represents the number of ubiquitin signals per image, normalized to the number of cells. N= 4 images for each condition. More than 100 cells were analyzed using the ImageJ 'Find Maxima' tool, applying a defined threshold in a blinded manner. The experiment was performed twice, and a representative result is shown. Data are presented as mean values $\pm$ SD. **B.** WT or *Atg7* KO RAW264.7 cells stably expressing GFP-Rab32 were treated under the same condition as in (A). Scale bar: 5 μ m. Each point represents the number of ubiquitin signals per image, normalized to the number of cells. N= 4 images for each condition. More than 300 cells were analyzed using the ImageJ 'Find Maxima' tool, applying a defined threshold in a blinded manner. The experiment was performed twice, and a representative result is shown. Data are presented as mean values $\pm$ SD. Statistical significance was analyzed using an unpaired two tailed *t*-test (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001).



Supplementary figure 8 p62 is associated with micromitophagy

A. BMDMs were stained with MitoTracker and treated with OA for 3 h. After fixation, cells were stained with anti-p62 or anti-NDP52 antibodies and observed using confocal microscopy. White arrows indicate p62-positive mitochondria. This experiment was repeated twice. Scale bar: 5 μ m (left), 1 μ m (inset). **B.** WT or *Atg7* KO RAW264.7 cells were transduced with a lentivirus encoding shRNA targeting the *p62* gene or a scramble control. After 5 days of puromycin selection, knockdown efficiency was confirmed by Western blot analysis. This experiment was done once.



Supplementary figure 9

Supplementary figure 9 Micromitophagy is associated with M1 polarization

A. WT and DKO BMDMs were stained with MitoTracker and treated with or without LPS/IFN- γ to induce M1 macrophage polarization. After 6 h of treatment, cells were observed using confocal microscopy. This experiment was repeated twice. Scale bar: 10 μ m. **B.** WT and DKO BMDMs were and treated with or without LPS/IFN- γ to induce macrophage M1 polarization. After 24 h of treatment, cells were fixed, stained with an anti-Tomm20 antibody, and analyzed using confocal microscopy (upper images). Scale bar: 2 μ m. Small and fragmented mitochondria were extracted using the 'Analyze Particles' tool of ImageJ with a defined threshold and circularity (bottom images). Each dot represents the number of fragmented mitochondria per cell, quantified using the 'Analyze particle' tool of ImageJ. N=20, 21, 18, 22 cells. The experiment was performed twice, and a representative result is shown. Data are presented as mean values $\pm$ SD. **C and D.** WT, *Rab32* KO, *Atg7* KO or Fip200 KO RAW264.7 cells were treated with LPS/IFN- γ for 24 h. Cells were then collected for Western blot analysis to detect SDHA, SDHB, and Tomm20 protein levels. Quantitative data were collected from three independent experiments. Data are presented as mean values $\pm$ SD. Statistical significance was analyzed using a paired two tailed *t*-test. **E.** GFP-Rab32-expressing *Atg7* KO RAW264.7 cells were transfected with a plasmid for transient expression of mCherry-Tomm20. Forty-eight hours post-transfection, cells were treated with LPS/IFN- γ for 3 h and observed using a confocal microscope. This experiment was repeated twice. Scale bar: 2 μ m. **F.** WT or DKO BMDMs were treated with LPS/IFN- γ at indicated time points and then fixed and stained with an anti-STAT1 antibody and DAPI (not shown). Cells were observed through confocal microscopy. Scale bar: 10 μ m. Quantitative data represent the mean signal intensity of STAT1 in the nucleus for each condition. More than 300 cells were measured in each cell line. The blue and yellow background areas indicate standard deviation (SD) ranges. The experiment was performed twice, and a representative result is shown. **G.** WT or DKO BMDMs were treated with or without LPS/IFN- γ for 24 h, and the oxygen consumption rate (OCR) was determined using the Seahorse XF Analyzer. The experiments were performed three times, and representative data are shown from a technical triplicate. Data are presented as mean values $\pm$ SD.

