Burman et al., https://doi.org/10.1083/jcb.201612106

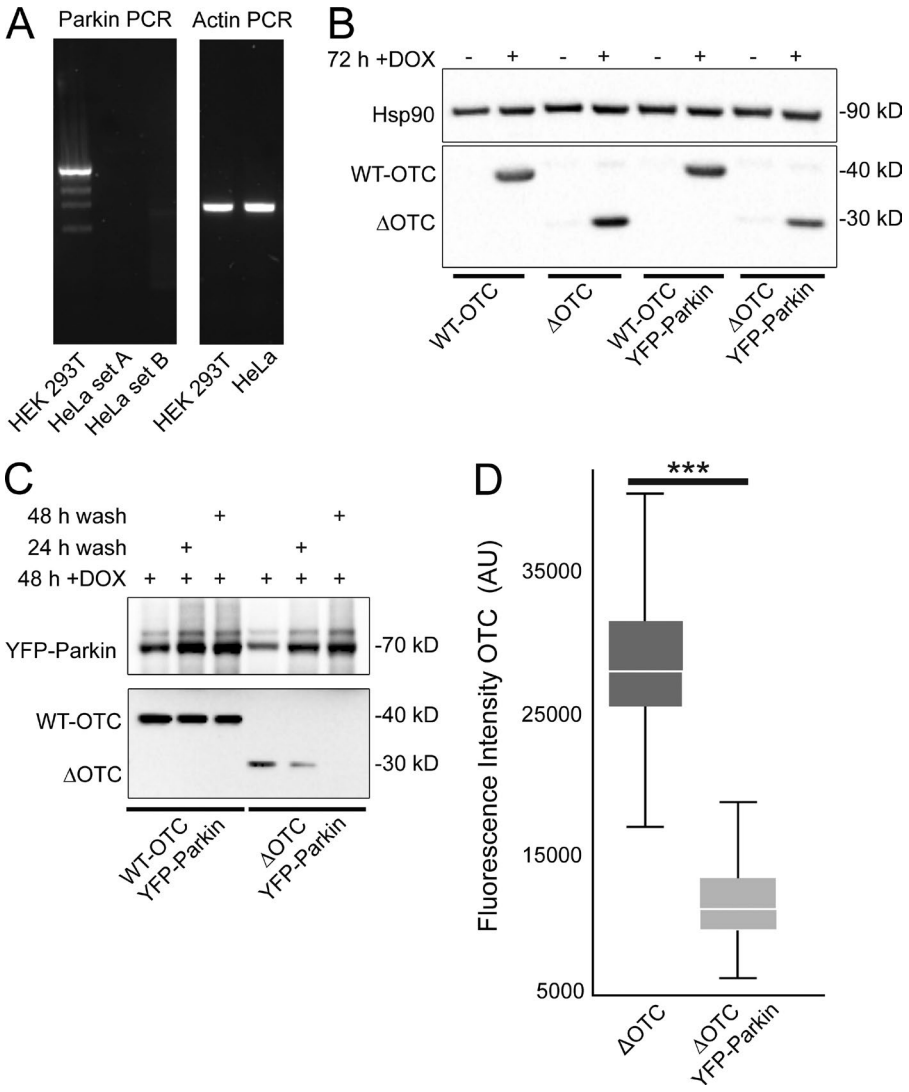


Figure S1. **Validation of selective clearance of ΔOTC from cells.** (A) 5′ ends of the *Parkin* gene cDNA in HEK293 or HeLa cells were amplified and run on agarose gels. At left, *Parkin* primers successfully amplified a PCR product of the expected size from HEK293 cells but were unable to detect *Parkin* mRNA in HeLa cells. At right, control primers for *Actin* successfully detected *Actin* mRNA in both HEK293 and HeLa cells. (B) Western blot of Tet ON: ΔOTC-expressing HeLa cells expressing YFP-Parkin after treatment with vehicle (H_20) or DOX for 72 h. (C) Western blot of Tet ON: WT or ΔOTC-expressing HeLa cells stably expressing YFP-Parkin treated with DOX for 48 h or 48 h with a 24- or 48-h washout of DOX. (D) Box plot of ΔOTC fluorescence intensity in AU from immunostaining of Tet-ON: ΔOTC cells treated with DOX for 48 h with a 24-h washout of DOX with or without YFP-Parkin expression. n = 1,409. ***, $P < 0.001$.

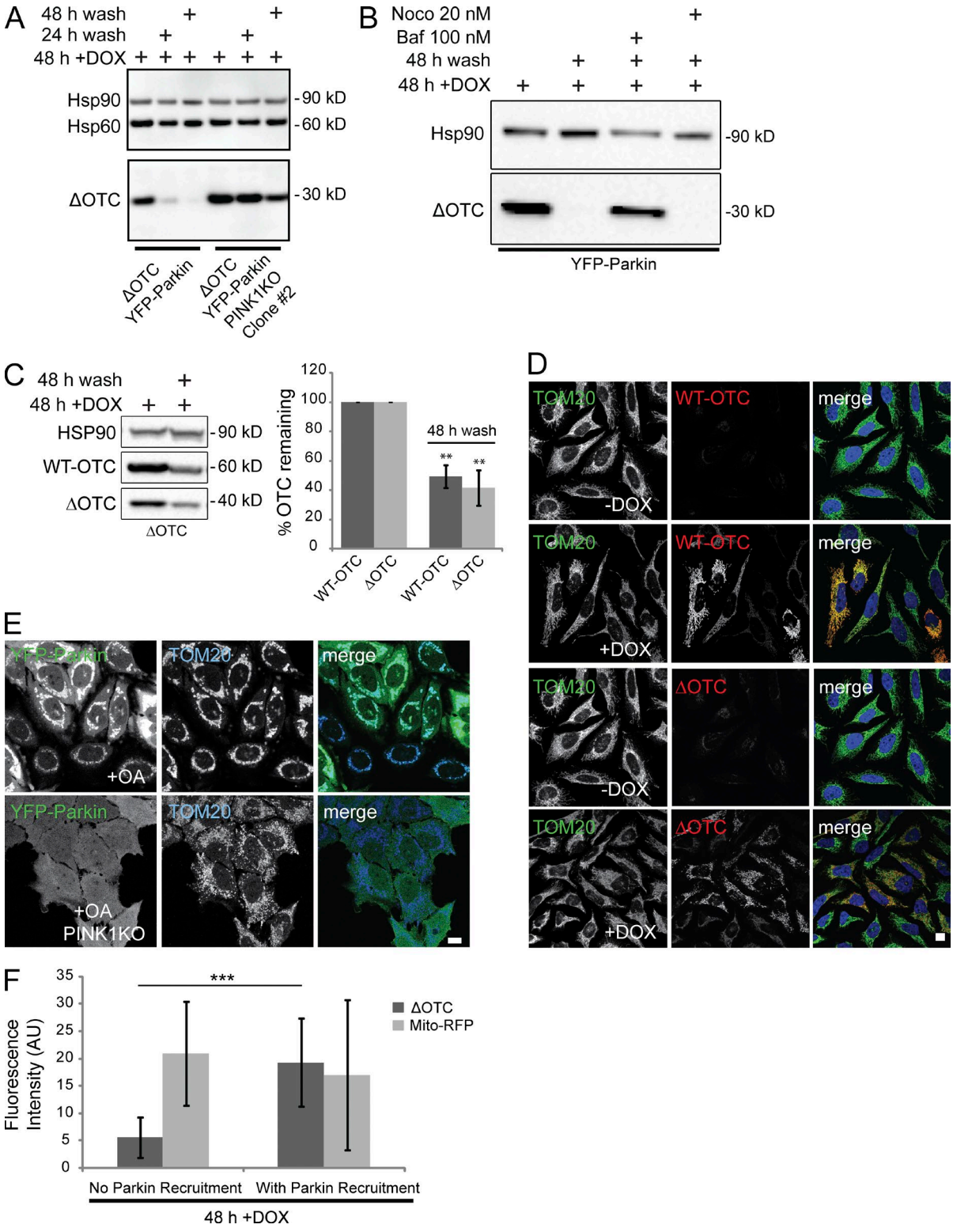


Figure S2. **Validation of the determinants for PINK1-mediated ΔOTC clearance.** (A) Western blot of Tet ON: ΔOTC-expressing HeLa cells expressing YFP-Parkin with or without a PINK1 KO background after 48 h DOX treatment and 48 h DOX treatment with a 24- or 48-h washout of DOX. (B) Western blot of Tet ON: ΔOTC-expressing HeLa cells stably expressing YFP-Parkin, treated with DOX for 48 h, or 48 h with a 48-h washout of DOX. After washout, cells were treated with 100 nM bafilomycin and 20 µM QVD or 20 nM nocodazole (Noco). (C) Western blot of Tet ON: WT and ΔOTC-expressing HeLa cells lacking Parkin expression treated with DOX for 48 h or 48 h with a 48-h washout of DOX. Western blots are quantified at right. *n* = 3. (D) Tet ON: WT or ΔOTC-expressing HeLa cells were treated with vehicle (H_20) or DOX for 48 h and processed for indirect immunofluorescence microscopy with antibodies against OTC (red) and TOM20 (green). (E) Tet ON: ΔOTC-expressing HeLa cells expressing YFP-Parkin (green) with or without a PINK1 KO background were treated with vehicle (DMSO) or OA for 3 h, fixed, and processed for indirect immunofluorescence microscopy with an antibody against TOM20 (blue). Bars, 10 µm. (F) Quantification of confocal images from Tet ON: ΔOTC-expressing HeLa cells expressing YFP-Parkin and mito-RFP treated with DOX for 48 h. *n* = 3. **, P < 0.01; ***, P < 0.001. Error bars indicate SD.

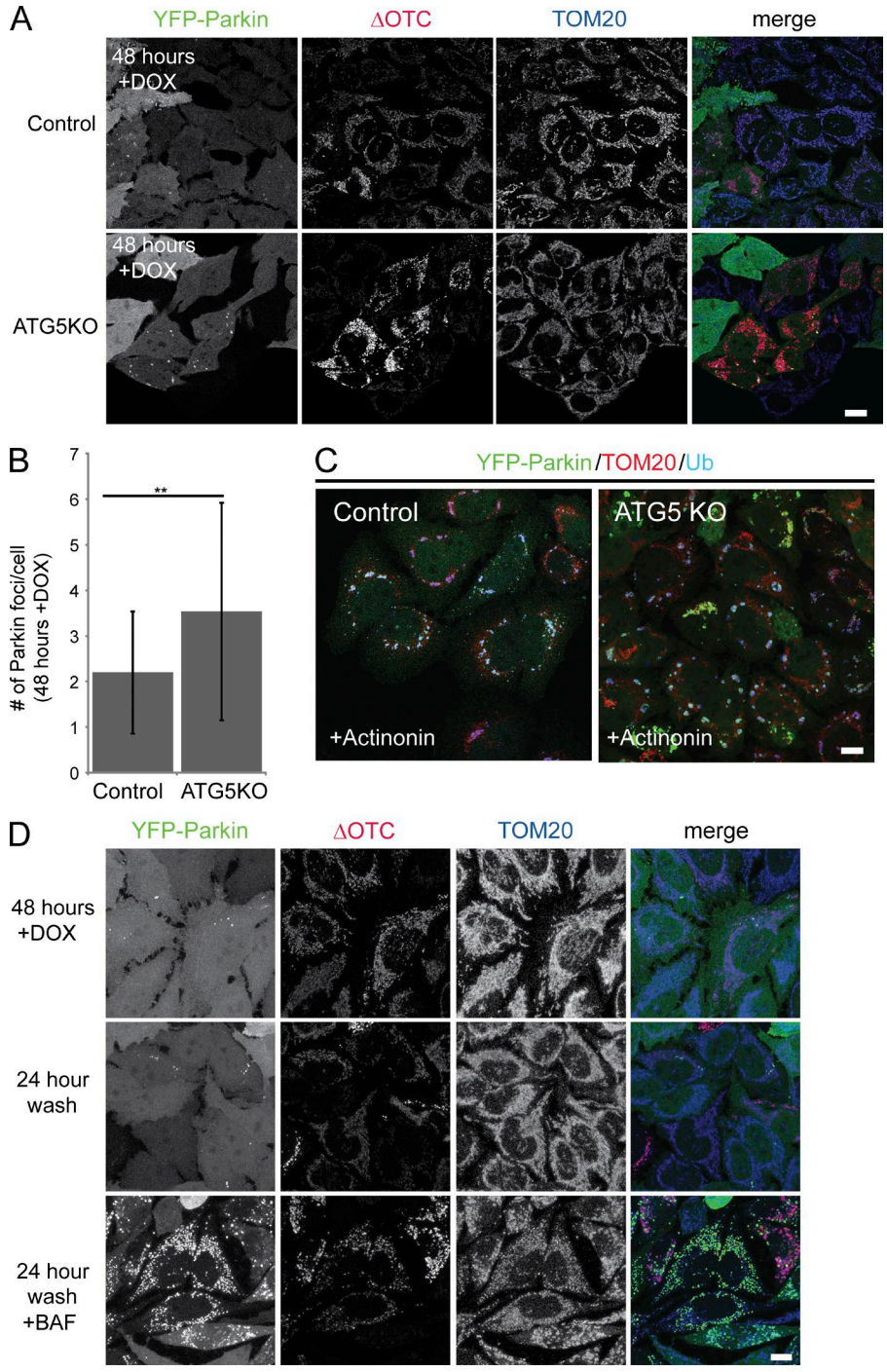


Figure S3. **ATG5 KO and bafilomycin treatment modify PINK1-dependent Parkin foci formation and subsequent mitophagy.** (A) Tet ON: ΔOTC-expressing HeLa cells expressing YFP-Parkin (green) with or without an ATG5 KO background were treated with DOX for 48 h and processed for immunostaining with antibodies against OTC (red) and TOM20 (blue). (B) Quantification of the number of Parkin foci/cell in Tet-ON: ΔOTC-expressing cells with or without an ATG5 KO background after treatment with DOX for 48 h. $n = 3$; $n \geq 30$. **, $P < 0.01$. Error bars indicate SD. (C) HeLa cells expressing YFP-Parkin (green) with or without an ATG5 KO background were treated with actinonin for 16 h and processed for immunostaining with antibodies against ubiquitin (blue) and TOM20 (red). (D) Tet ON: ΔOTC-expressing HeLa cells expressing YFP-Parkin (green) were treated with DOX for 48 h followed by a 24-h washout of DOX in the presence or absence of 200 nM bafilomycin and processed for immunostaining with antibodies against OTC (red) and TOM20 (blue). Bars, 10 µm.

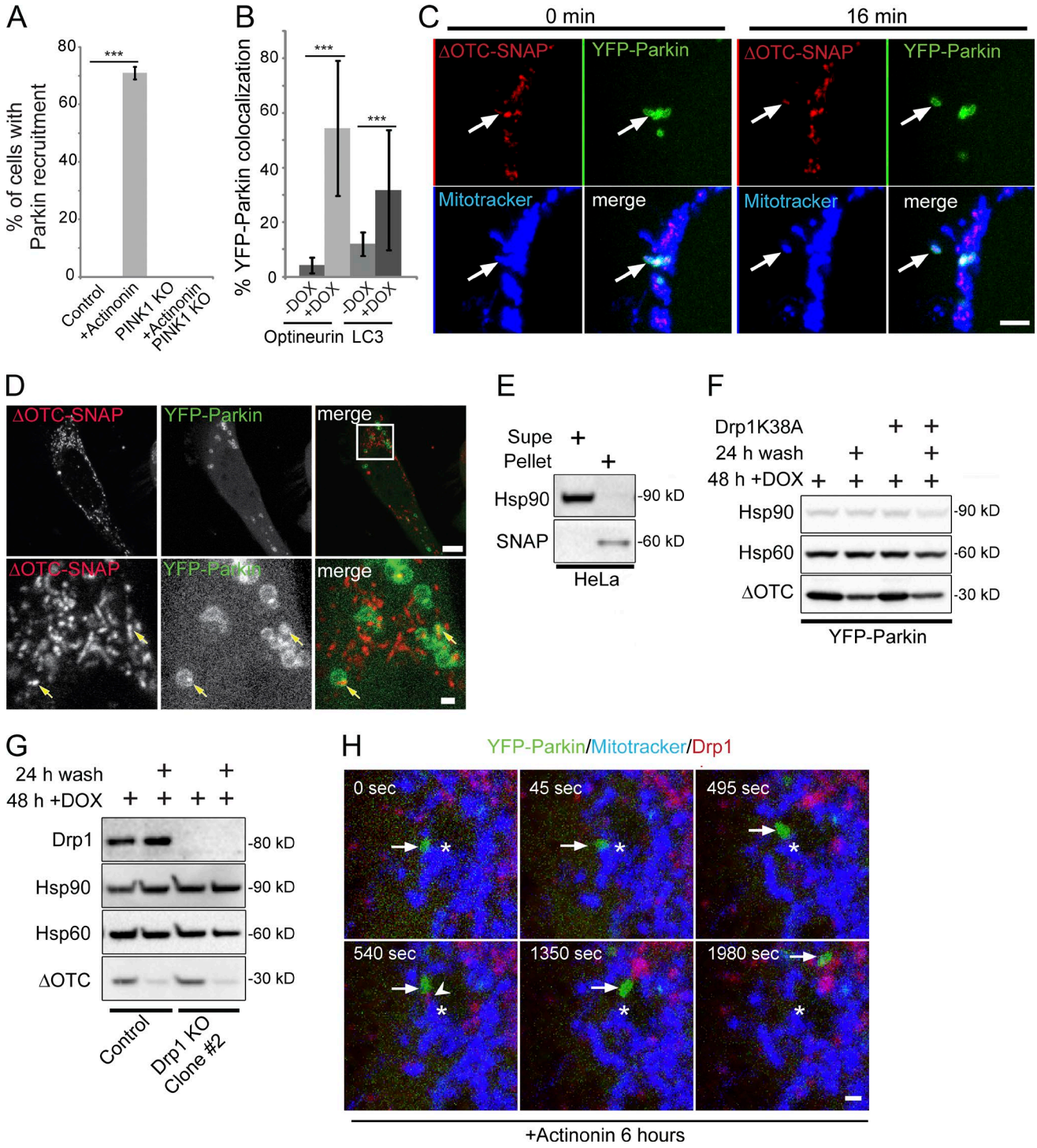


Figure S4. **Drp1 is not required for ΔOTC clearance but is recruited to Parkin foci before mitochondrial fission.** (A) Quantification of the percentage of cells with Parkin recruitment in HeLa cells with or without a PINK1 KO background after treatment with 150 µM actinonin for 6 h. $n = 3$; $n \geq 500$. (B) Quantification of the percentage of cells that displayed colocalization between Parkin and optineurin or LC3 with or without 48 h DOX treatment. $n = 3$; $n \geq 28$ for optineurin; $n = 3$; $n \geq 26$ for LC3. ***, $P < 0.001$. Error bars indicate SD. (C) Tet ON: ΔOTC-expressing HeLa cells expressing YFP-Parkin (green) and ΔOTC-SNAP (red) were treated with DOX for 48 h, labeled with SNAP-TMR and MitoTracker deep red (blue), and imaged live. The arrow tracks Parkin foci containing ΔOTC-SNAP after a fission event. Bars, 5 µm. (D) Tet ON: ΔOTC-expressing HeLa cells expressing YFP-Parkin (green) and ΔOTC-SNAP (red) were treated with DOX for 72 h, labeled with SNAP-TMR, and imaged live. Arrows denote SNAP-tagged ΔOTC within autophagosome-like Parkin-coated structures. Bars: (top) 5 µm; (bottom) 2 µm. (E) HeLa cells expressing ΔOTC-SNAP. After detergent extraction and centrifugation, the Triton X-100–soluble fraction (supe) and insoluble fraction (pellet) were analyzed by Western blot. (F) Tet ON: ΔOTC HeLa cells expressing Drp1 K38A and YFP-Parkin were treated with DOX for 48 h or for 48 h with a 24-h washout of DOX and analyzed by Western blot. (G) Western blot of Tet ON: ΔOTC-expressing HeLa cells with or without a Drp1 KO background treated with DOX for 48 h or for 48 h with a 24-h washout of DOX. (H) Tet ON: ΔOTC-expressing HeLa cells expressing YFP-Parkin (green) and mCherry-Drp1 (red) were treated with 150 µM actinonin for 6 h, labeled with MitoTracker deep red (blue), and imaged live. The arrow tracks a YFP-Parkin focus that colocalized adjacent to mCherry-Drp1 before a mitochondrial fission event. The arrowhead shows Drp1 accumulation proximal to YFP-Parkin. Stars represent a portion of the mitochondrial network that did not undergo a fission event. Bar, 2 µm.

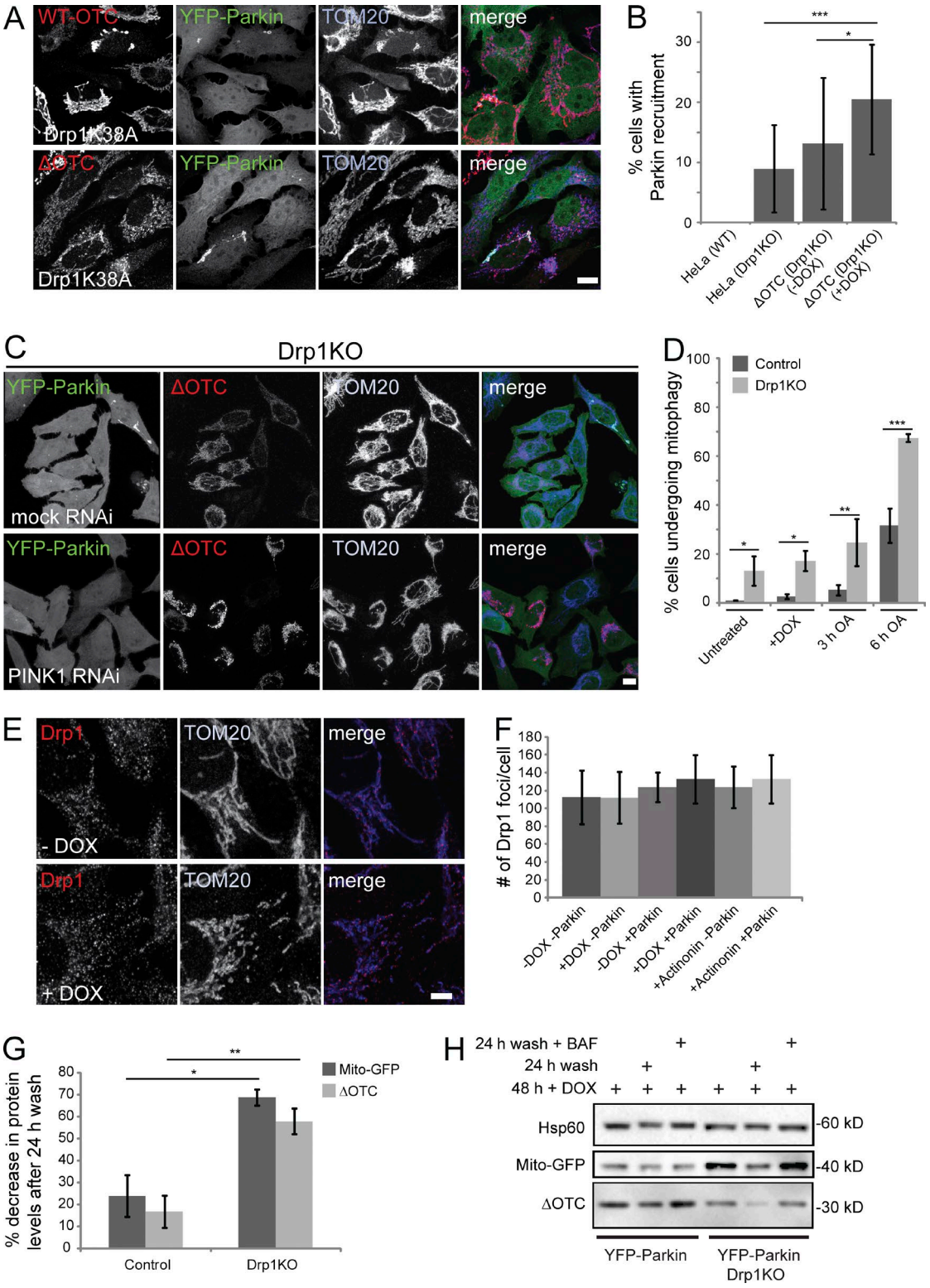
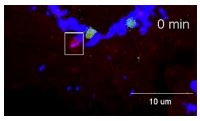
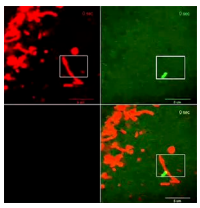


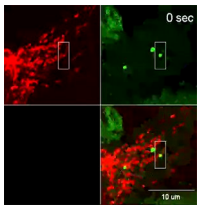
Figure S5. **Loss of Drp1 promotes mitophagy.** (A) Tet ON: WT OTC or ΔOTC-expressing HeLa cells expressing Drp1 K38A and YFP-Parkin (green) were fixed and stained with antibodies to OTC (red) and TOM20 (blue). (B) Quantification of the percentage of cells with Parkin recruitment in the cell lines and conditions indicated. $n = 3$; $n \geq 75$. (C) Tet ON: ΔOTC-expressing HeLa cells expressing YFP-Parkin (green) in a Drp1 KO background were treated with 20 nM mock RNAi or *pink1* RNAi for 1 d and then treated with DOX for 48 h and fixed and stained with antibodies to OTC (red) and TOM20 (blue). (D) Quantification of the FACS-based mito-Keima assays described in Fig. 9 A. $n = 3$. (E) Tet ON: ΔOTC-expressing HeLa cells lacking Parkin expression with and without DOX treatment for 48 h were fixed and immunostained with the indicated antibodies. Bars, 10 µm. (F) Quantification of the number of Drp1 foci on mitochondria in the indicated cell lines. $n = 3$. (G) Quantification of Western blots as described in Fig. 8 G. $n = 3$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars indicate SD. (H) Tet ON: ΔOTC-expressing HeLa cells expressing YFP-Parkin and transiently overexpressing mito-GFP with or without a Drp1 KO background were treated either with DOX for 48 h, with or without a 24-h DOX washout, or with or without 200 nM bafilomycin/20 µM QVD treatment and then analyzed by Western blot.



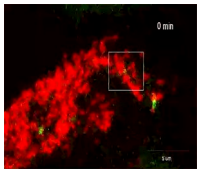
Video 1. **LC3 is recruited to mitochondrial subdomains coated in Parkin.** Live imaging of Tet-ON: ΔOTC-expressing HeLa cells expressing mCherry-Parkin (red) and GFP-LC3 (green) and labeled with MitoTracker deep red (blue) after 48 h of DOX treatment. The white box denotes a mitochondrial subdomain coated in mCherry-Parkin prior to LC3B-GFP recruitment and mitochondrial fission. Frame rate, two frames/s.



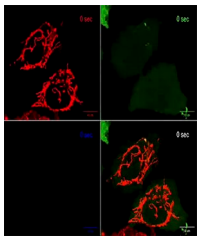
Video 2. **Parkin foci dynamically undergo asymmetric mitochondrial division.** Live imaging of Tet-ON: ΔOTC-expressing HeLa cells expressing YFP-Parkin (green) and mito-RFP (red) after 48 h of DOX treatment. The white box denotes a mitochondrial subdomain coated in YFP-Parkin. Frame rate, five frames/s.



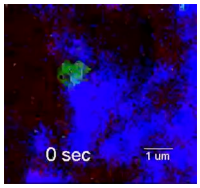
Video 3. **Parkin foci dynamically undergo asymmetric mitochondrial division.** Live imaging of Tet-ON: ΔOTC-expressing HeLa cells expressing YFP-Parkin (green) and mito-RFP (red) after 48 h of DOX treatment with a 24-h washout of DOX. The white box denotes a mitochondrial subdomain coated in YFP-Parkin. Frame rate, five frames/s.



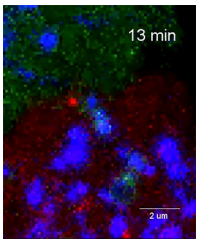
Video 4. **Parkin foci are associated with dynamic mitochondrial subdomains that undergo asymmetric division.** Live imaging of HeLa cells expressing YFP-Parkin (green) labeled with MitoTracker deep red (red) after 4 h treatment with 150 µM actinonin. The white box denotes a polarized mitochondrial subdomain coated in YFP-Parkin. Frame rate, two frames/s.



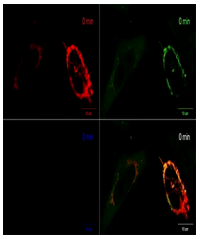
Video 5. **Loss of Drp1 function inhibits fission of Parkin foci and associated mitochondrial subdomains.** Live imaging of Tet-ON: ΔOTC-expressing HeLa cells expressing YFP-Parkin (green), Drp1K38A, and mito-RFP (red) after 48 h of DOX treatment. Frame rate, one frames/s.



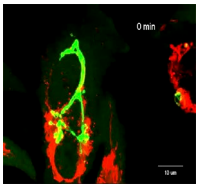
Video 6. **Drp1 can localize to Parkin foci before mitochondrial fission.** Live imaging of HeLa cells expressing YFP-Parkin (green) and mCherry-Drp1 (red) treated with actinonin for 4–6 h and labeled with MitoTracker deep red (blue). Frame rate, one frames/s.



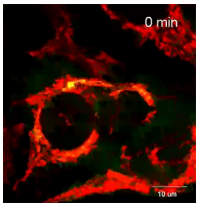
Video 7. **Drp1 can remain associated with Parkin foci after mitochondrial fission.** Live imaging of HeLa cells expressing YFP-Parkin (green) and mCherry-Drp1 (red) labeled with MitoTracker deep red (blue) and treated with actinonin for 4–6 h. Frame rate, two frames/s.



Video 8. **Identification of a Drp1-independent fission pathway after proteotoxic stress.** Live imaging of Drp1 KO HeLa cells expressing YFP-Parkin (green) and mito-RFP (red) treated with actinonin for 6 h. Frame rate, three frames/s.



Video 9. **Drp1-independent wholesale mitochondrial fission after Parkin recruitment to mitochondria.** Live imaging of Tet-ON: ΔOTC-expressing Drp1 KO HeLa cells expressing YFP-Parkin (green), labeled with TMRM (red), treated with DOX for 48 h, and labeled with TMRM (red). Time is indicated. Frame rate, four frames/s.



Video 10. **Mitochondrial membrane potential fluctuations are observed in Drp1 KO cells expressing YFP-Parkin followed by wholesale recruitment of Parkin to mitochondria.** Live imaging of Drp1 KO HeLa cells expressing YFP-Parkin (green), labeled with TMRM (red), and treated with DOX for 48 h. Frame rate, four frames/s.