Ji et al., https://doi.org/10.1083/jcb.201610057

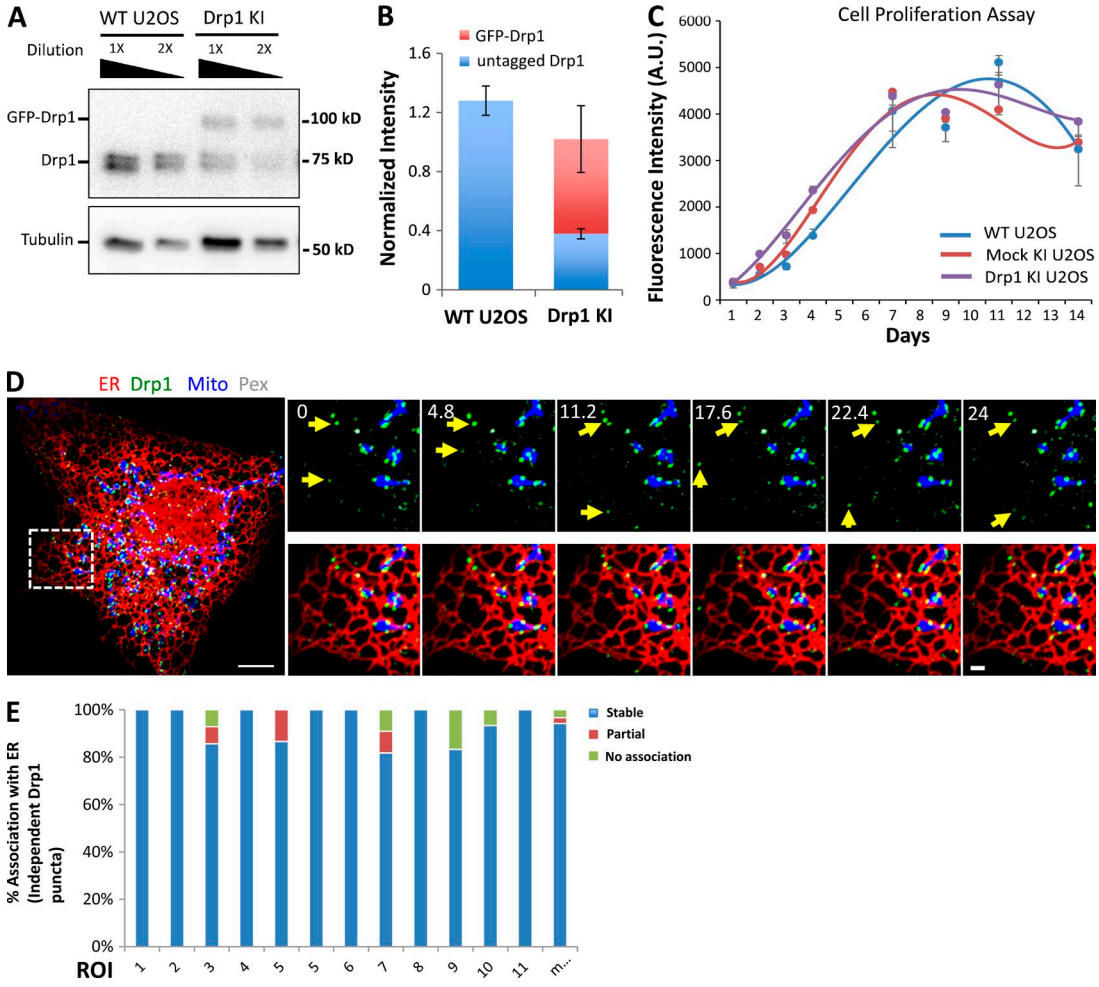


Figure S1. **Characterization of Drp1 KI U2OS cells and Drp1-independent punctae in Cos7 cells. (A)** Western blot of U2OS cells and Drp1 KI U2OS cells showing expression level of GFP-Drp1 and untagged Drp1 with two dilutions of extract loaded (1× and 2× dilution). **(B)** Quantification of untagged Drp1 and GFP-Drp1 in WT U2OS and Drp1 KI cells from Western blots (normalized to tubulin level). Error bars, SD. **(C)** Cell proliferation assay (Alamar blue). Three replicates taken for each time point (median shown, with error bars representing minimum and maximum). Starting density: 5,000 cells/24-well plate. Representative result from two independent experiments. Error bars represent SD. **(D)** Drp1-independent punctae in Cos7 cells. Left, merged image of a live COS7 cell transiently expressing mito-BFP (mitochondria, blue), eBFP2-PMP20 (peroxisome, gray), GFP-Drp1(green), and ER-TagRFP (ER, red). Right: Insets from boxed region. Yellow arrows denote independent Drp1 punctae associating with ER. **(E)** Graph depicting the degree of association between independent Drp1 punctae and ER in Cos7 cells, during 3-min movies imaged every 1.5 s. 175 independent Drp1 puncta were analyzed from 12 ROIs from 12 cells as shown in A. Stable association, 94.2 ± 7.6%; partial association, 2.5 ± 4.7%; no association, 3.3 ± 5.4%. Bars: (whole-cell image) 10 µm; (inset) 2 µm. Time in seconds.

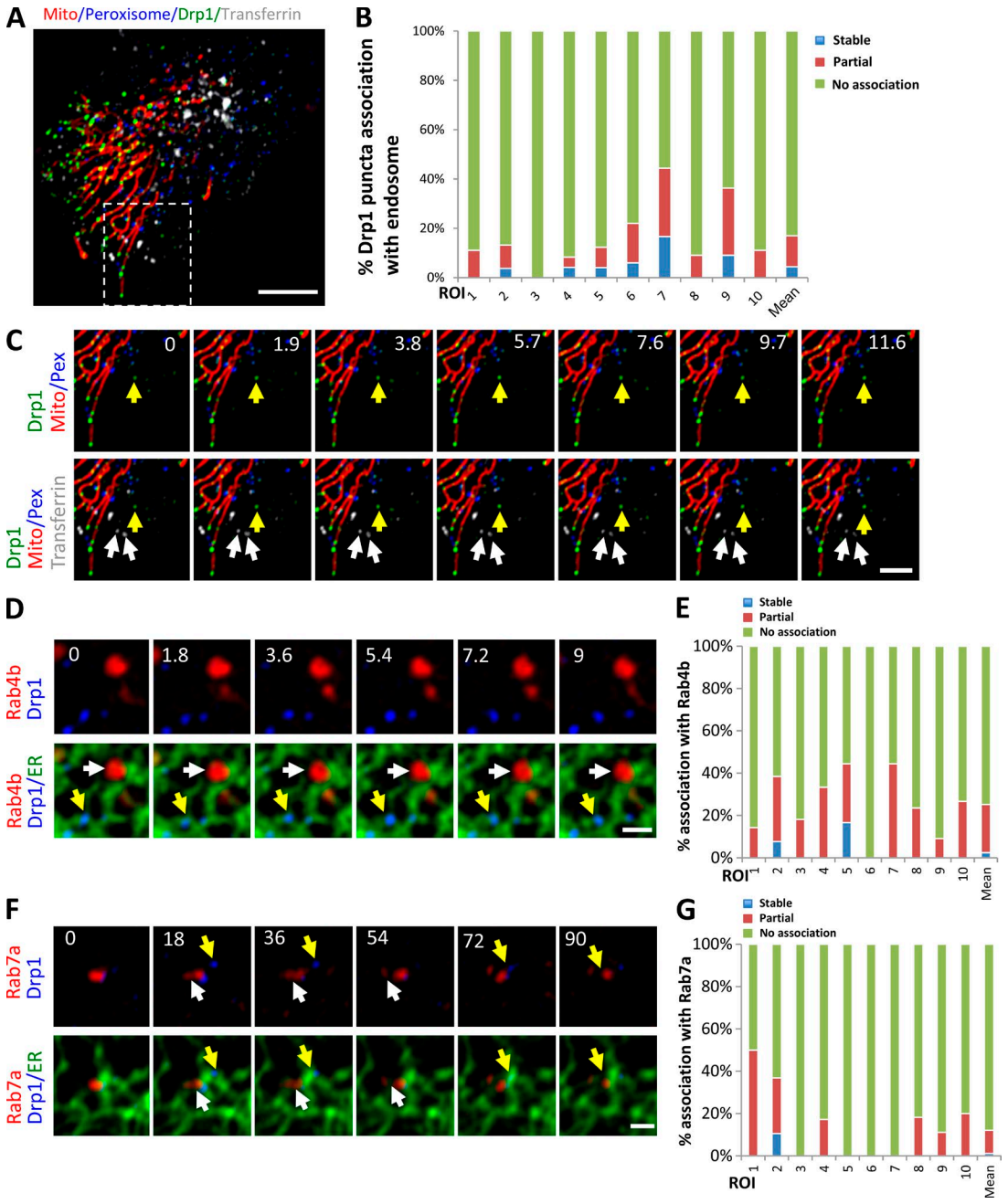


Figure S2. **Independent Drp1 punctae are not stably associated with endosomal membranes. (A)** Merged image of GFP-Drp1-KI cell expressing mCherry-mito3 (red) and eBFP2-peroxisome (blue) and treated with transferrin-Alex647 (endosomes, gray). GFP-Drp1 in green. **(B)** Graph depicting degree of association between independent Drp1 punctae and transferrin-labeled membranes during 3-min videos imaged every 1.7 s. 10 ROIs from 10 cells, 342 Drp1 punctae. **(C)** Time-lapse of inset from A showing independent Drp1 punctae distinct from transferrin-labeled endosomes. White arrows, endosomes; yellow arrow, independent Drp1 puncta. **(D)** Time-lapse ROI of a GFP-Drp1-KI cell expressing mStrawberry-Rab4b (red) and ER-eBFP2 (ER, green). Drp1 in blue. Arrows defined as in C. **(E)** Graph depicting the degree of association between independent Drp1 punctae and Rab4b-labeled membranes during 3-min videos imaged every 1.8 s. 10 ROIs from 10 cells, 152 Drp1 punctae. **(F)** Time-lapse of a Drp1 KI cell expressing mStrawberry-Rab7a (red) and ER-eBFP2 (ER, green). GFP-Drp1 is blue. Arrows defined as in C. **(G)** Graph depicting the degree of association between independent Drp1 punctae and Rab7a-labeled membranes during 3-min videos imaged every 1.5 s. 10 ROIs from 10 cells, 177 Drp1 punctae. Bars: (A) 10 µm; (C) 5 µm; (D and F) 2 µm. Time in seconds.

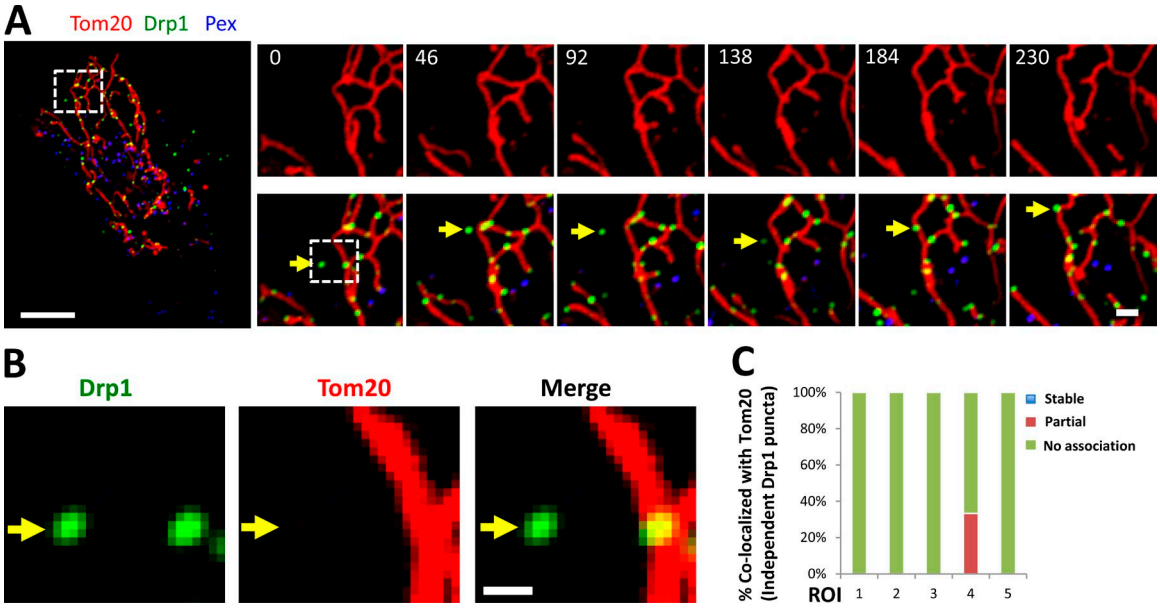


Figure S3. **Independent Drp1 punctae do not associate with Tom20. (A)** GFP-Drp1-KI cells transiently transfected with Tom20-mCherry (red) and eBFP2-PMP20 (peroxisomes, blue). Drp1 in green. Whole-cell overlay on left and time course of the indicated ROI on right (top, Tom20 alone; bottom, merged image). **(B)** Zoom of indicated region of 0-s time point in A, showing Drp1 and Tom20. No peroxisomes detected in this region. Yellow arrow, independent Drp1 puncta with no associated Tom20 signal. **(C)** Graph of percentage of independent Drp1 puncta overlaying with Tom20 (16 independent puncta from five ROIs analyzed). One instance of overlap observed in ROI 4. Bars: (A, whole-cell) 10 µm; (A, inset) 2 µm; (B) 1 µm. Time in seconds.

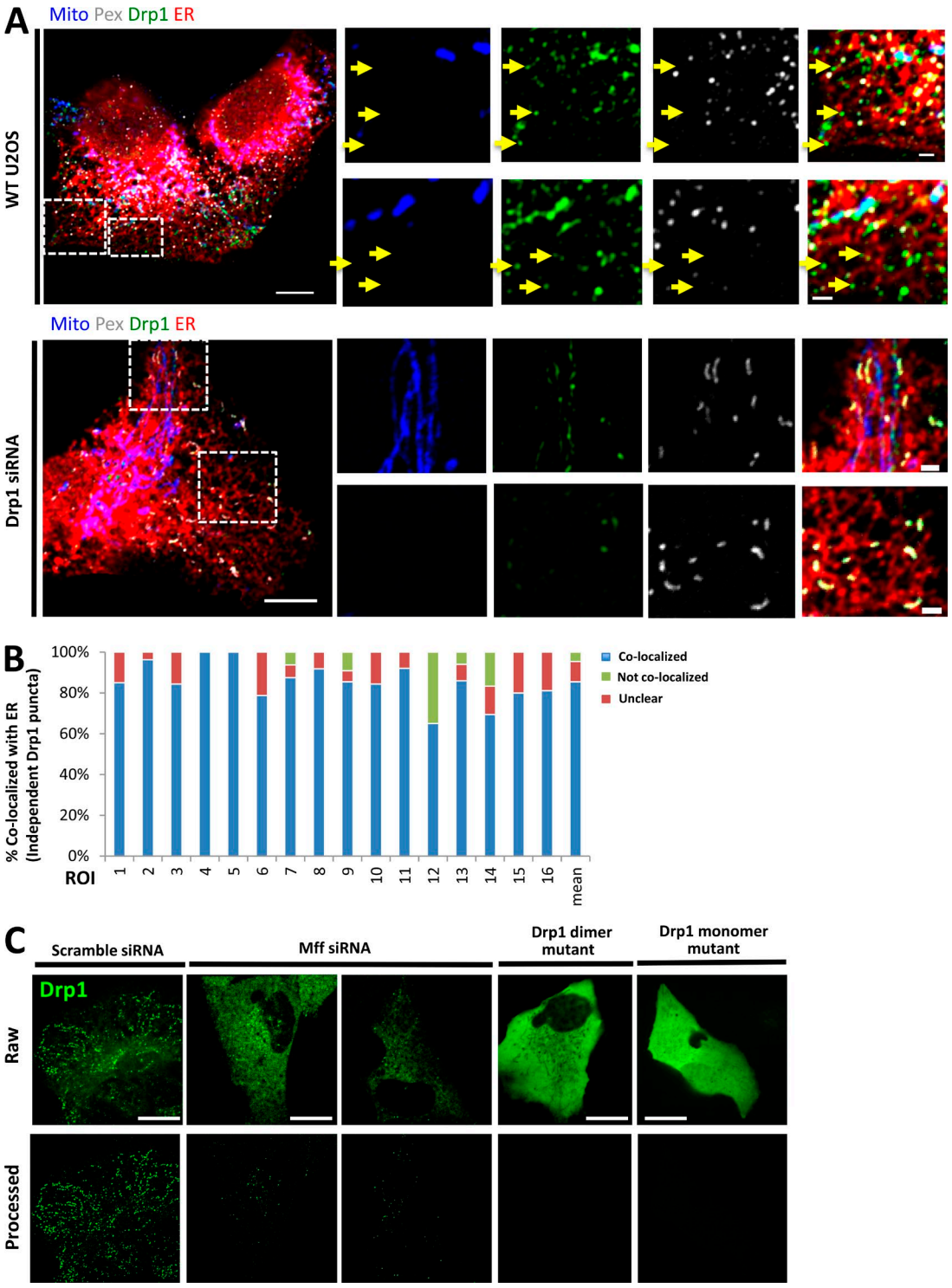


Figure S4. **Experiments to test Drp1 aggregation. (A)** Endogenous Drp1 staining by immunofluorescence in WT (top) and Drp1 KD U2OS cells (bottom). Also stained are mitochondria (blue), peroxisomes (gray), and ER (red). Yellow arrows, independent Drp1 puncta. WT and KD images acquired and processed identically. **(B)** Quantification of colocalization between endogenous Drp1 punctae and ER in WT U2OS cells. 562 independent puncta counted from 16 cells. Colocalized, 85.5 ± 9.7%; not colocalized, 10.0 ± 7.3%; unclear, 4.6 ± 9.4%. **(C)** Comparison of GFP-Drp1 distribution in U2OS cells under three conditions: GFP-Drp1-KI cells transfected with a scrambled siRNA (left), siRNA for Mff (center), and U2OS cells overexpressing GFP-Drp1 dimer or monomer mutant (right). Top row represents raw images, and bottom row shows processed images to reveal Drp1 punctae, as described in Materials and Methods (background subtracted and smoothed using ImageJ). Bars: (A and C, whole-cell images) 10 µm; (A, zoomed images) 2 µm.

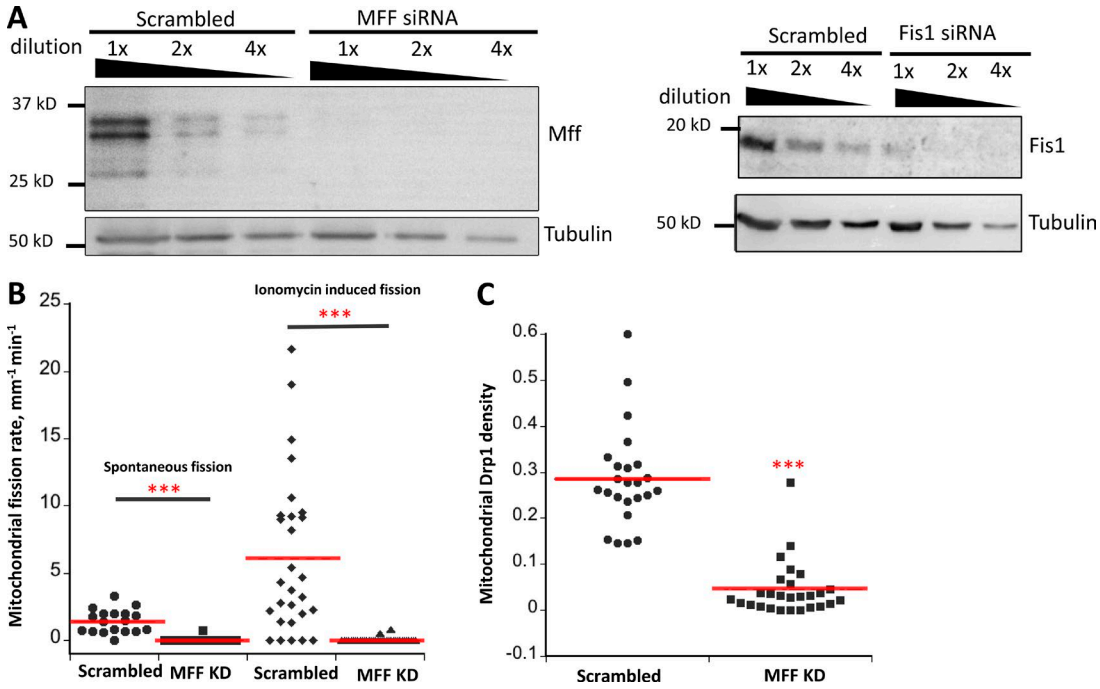


Figure S5. **siRNA treatments for Mff and Fis1 in U2OS cells. (A)** Western blots showing effectiveness of siRNA against Mff and Fis1. **(B)** Division rate quantification for scrambled siRNA and Mff siRNA in GFP-Drp1-KI U2OS cells, in both the unstimulated and ionomycin-stimulated states. In quantification of spontaneous division, 18 scrambled siRNA cells and 17 Mff siRNA cells were analyzed. In quantification of ionomycin induced division, 30 scrambled siRNA cells and 32 Mff siRNA cells were analyzed. ***, $P < 0.005$, unpaired Student's *t* test. **(C)** Mitochondrial Drp1 punctae density quantification (units, Drp1 puncta per micrometer) for scrambled siRNA and Mff siRNA in GFP-Drp1-KI U2OS cells. 24 ROIs from 20 control cells and 27 ROIs from 25 MFF KD cells are analyzed. ***, $P < 0.005$, unpaired Student's *t* test.

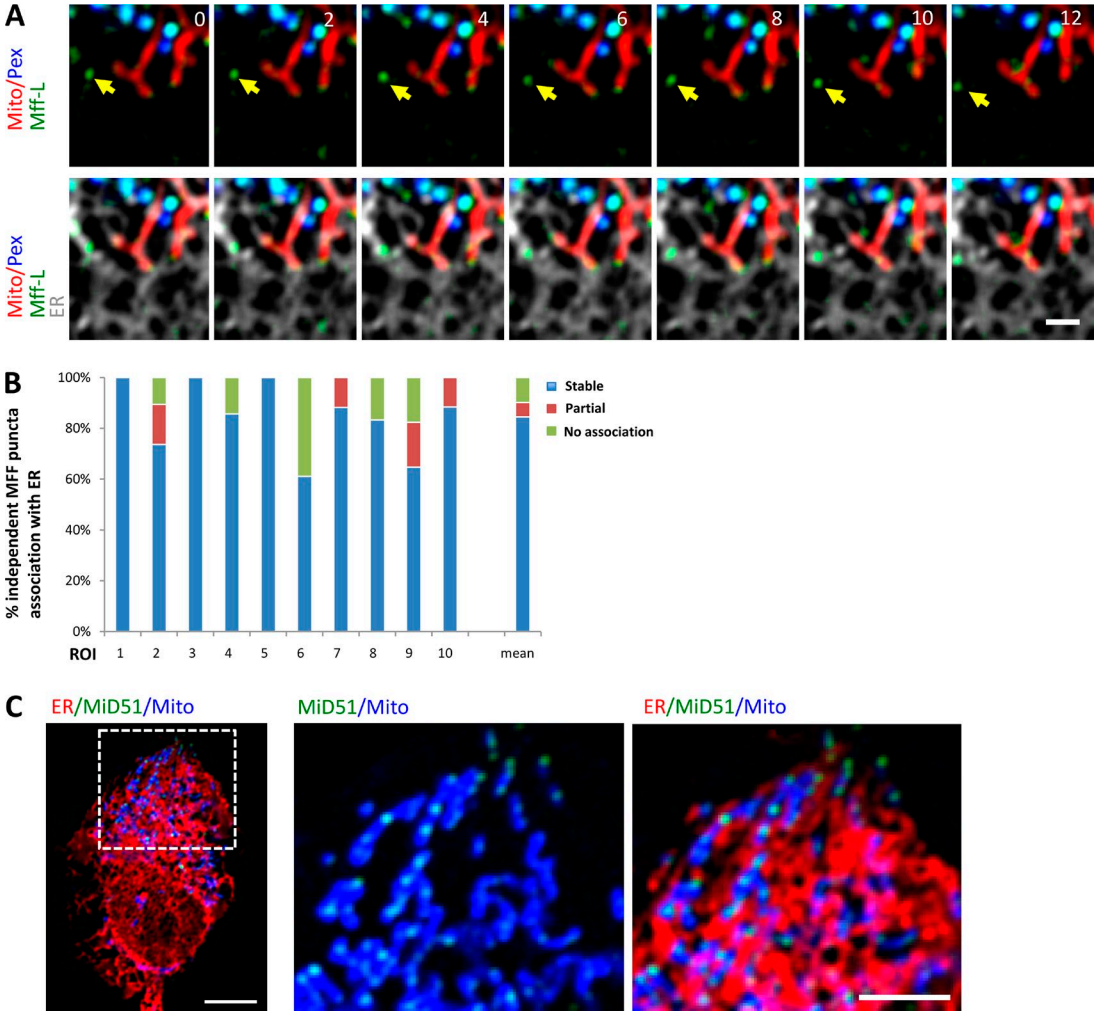
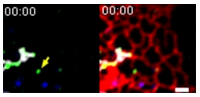
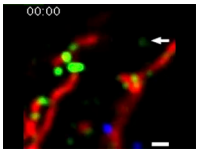


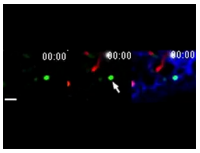
Figure S6. **The Mff-L isoform displays ER-associated punctae, whereas Mid51 does not localize to ER. (A)** Time-lapse from region of U2OS cell expressing mCherry-mito3 (red), eBFP2-peroxisome (blue), GFP-Mff-L (green), and E2-Crimson-ER (gray). Yellow arrow denotes independent Mff puncta associating with ER tubules. **(B)** Graph depicting the degree of association between independent Mff-L punctae and ER during 3-min videos imaged every 2 s. 10 ROIs from eight U2OS cells, 167 independent Mff punctae. **(C)** GFP-MiD51 does not display ER-associated punctae independent of mitochondria. Left, merged image of a live cell expressing MiD51-GFP (green), mitoBFP (blue), and ER-tagRFP (ER, red). Right, insets. Bars: (A) 2 µm; (C, whole cell) 10 µm; (C, inset) 5 µm. Time in seconds.



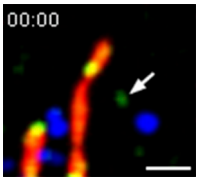
Video 1. **Confocal time-lapse of independent Drp1 puncta stably associating with ER tubules.** GFP-Drp1-KI cell transiently expressing mPlum-mito3 (gray), eBFP2-peroxisome (blue), and ER-tagRFP (red). Drp1 in green. Left, without ER; right, with ER. Yellow arrow, independent Drp1 puncta. Time-lapse taken in single *z*-plane every 1.77 s. Time min:s. Bar, 2 µm. See also Fig. 1 C.



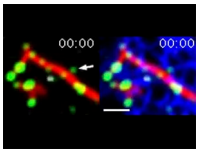
Video 2. **Confocal time-lapse of independent Drp1 puncta transferring to mitochondria.** GFP-Drp1-KI U2OS cell transiently expressing mCherry-mito-7 (mitochondria, red) and eBFP2-peroxisome (peroxisome, blue). Drp1 in green. White arrow, independent Drp1 puncta. Time-lapse taken in single *z*-plane in dorsal region of cell every 2.1 s. Time min:s. Bar, 2 µm. See also Fig. 2 A.



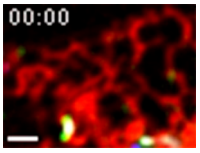
Video 3. **Confocal time-lapse of independent Drp1 puncta transferring from ER to mitochondria.** GFP-Drp1-KI U2OS cell transiently expressing mito-BFP (Mito in red), mPlum-peroxisome (Pex in gray), and ER-tagRFP (ER in blue). Drp1 in green. White arrow, independent Drp1 puncta. Time-lapse taken in single *z*-plane in dorsal region of cell every 3 s. Left, Drp1 only; middle, without ER; right, with ER. Time min:s. Bar, 2 µm. See also Fig. 2 B.



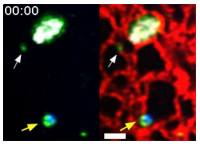
Video 4. **Confocal time-lapse of independent Drp1 puncta transferring to mitochondria, followed by mitochondrial division.** GFP-Drp1-KI U2OS cell transiently expressing mCherry-mito-7 (Mito in red) and eBFP2-peroxisome (Pex in blue). Drp1 in green. White arrow, independent Drp1 puncta. Time-lapse taken in single *z*-plane in dorsal region of cell every 1.5 s. Cells were treated with ionomycin (4 µM) to stimulate division at time 0. Time min:s. Bar, 2 µm. See also Fig. 2 C.



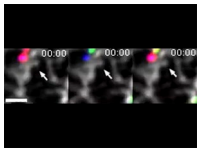
Video 5. **Confocal time-lapse of independent Drp1 puncta transferring from ER to mitochondria, followed by mitochondrial division.** GFP-Drp1-KI U2OS cell transiently expressing mito-BFP (Mito in red), mPlum-peroxisome (Pex in gray), and ER-tagRFP (ER in blue). Drp1 in green. White arrow, independent Drp1 puncta. Time-lapse taken in single *z*-plane in dorsal region of cell every 3 s. Left, without ER; right, with ER. Cells were treated with ionomycin (4 µM) to stimulate division at time 0. Time min:s. Bar, 2 µm. See also Fig. 2 D.



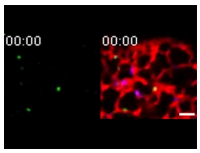
Video 6. **Confocal time-lapse of independent Mff punctae on ER.** U2OS cell transiently expressing mCherry-mito7 (gray), GFP-Mff-S (green), eBFP2-peroxisome (blue), and ER-E2-Crimson (Red). Time-lapse was taken in single *z*-plane every 1.8 s. Time min:s. Bar, 2 µm. See also Fig. 4 C.



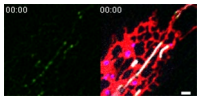
Video 7. **Airyscan time-lapse of independent Mff puncta transfer from ER to mitochondrion.** U2OS cell transiently expressing mPlum-mito-3 (gray), GFP-Mff-S (green), eBFP2-peroxisome (blue), and ER-tagRFP (red). Left, without ER; right, with ER. White arrow denotes independent Mff. Yellow arrow denotes peroxisome-associated Mff. Taken in single *z*-plane every 24 s. Time min:s. Bar, 2 µm. See also Fig. 6 A.



Video 8. **Confocal time-lapse of Drp1 appearance and maturation at Mff-enriched site on ER.** GFP-Drp1-KI cell transiently expressing mito-BFP (blue), eBFP2-peroxisome (blue), mStrawberry-Mff-S (red), and ER-E2-Crimson (white). Drp1 in green. Left, Mff only; middle, Drp1 only; right, both Mff and Drp1. White arrow, Mff/Drp1 puncta. Time-lapse was taken every 1.7 s. Time min:s. Bar, 2 µm. See also Fig. 7 A.



Video 9. **Confocal time-lapse of Drp1 oligomerization upon ionomycin treatment.** GFP-Drp1-KI U2OS cell transiently expressing mPlum-mito-3 (gray), eBFP2-peroxisome (blue), and ER-tagRFP (red). Drp1 in green. Left, Drp1 only; right, Drp1 Mito Pex with ER. Taken in single *z*-plane in every 23 s. Time min:s. Ionomycin treatment (4 µM) at 1:30. Bar, 2 µm. See also Fig. 9 A.



Video 10. **Confocal time-lapse of Drp1 oligomerization after LatA pretreatment followed by ionomycin treatment.** Drp1 KI U2OS cell transiently expressing mPlum-mito-3 (gray), eBFP2-peroxisome (blue), and ER-tagRFP (Red). Left, Drp1 only; right, Drp1 Mito Pex with ER. LatA, 10 min pretreatment at 1 µM; ionomycin, 4 µM. Time-lapse was taken in single *z*-plane in every 23.6 s. Time min:s. Ionomycin treatment at 1:34. Bar, 2 µm. See also Fig. 9 B.