Chakrabarti et al., https://doi.org/10.1083/jcb.201709111

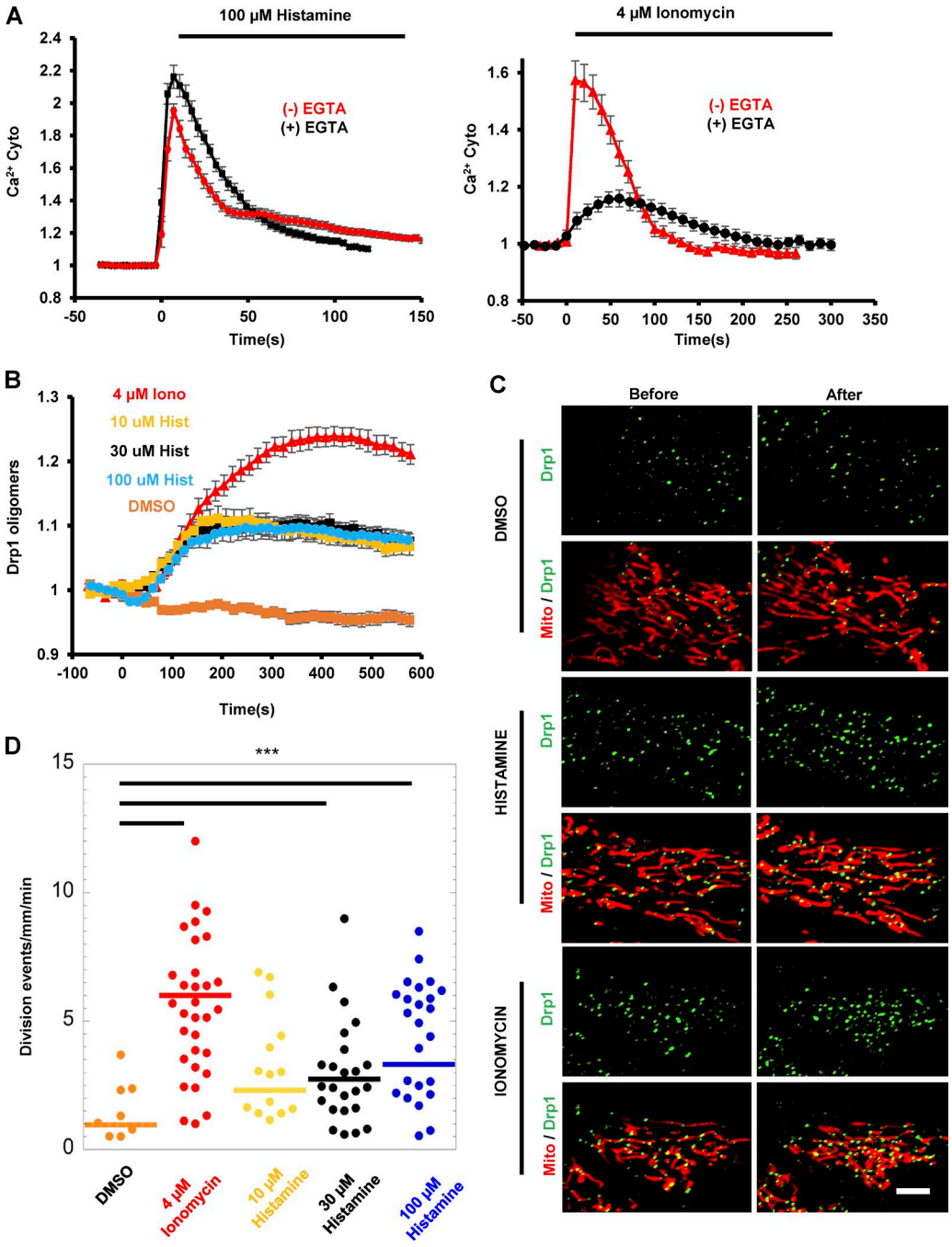


Figure S1. **Comparing responses to histamine and ionomycin for cytoplasmic calcium, Drp1 oligomerization, and mitochondrial division.** All experiments conducted in continuous presence of 10% calf serum. (A) Cytoplasmic calcium. U2OS cells (transfected with Cyto-R-Geco) were stimulated with 100 µM histamine (left) or 4 µM ionomycin (right) in the absence (red) or presence (black) of 5 mM EGTA; *n* = 12 cells (his), 15 cells (hist + EGTA), 14 cells (Iono), or 18 cells (Iono + EGTA). Error bars represent SEM. (B) Drp1 oligomerization. Quantification of total Drp1 oligomers (whole cell) in response to 4 µM ionomycin or 10 µM (yellow), 30 µM (black), or 100 µM (blue) histamine in U2OS cells (GFP-Drp1 knock-in cells transfected with mito-BFP); *n* = 10–15 cells. Error bars represent SEM. (C) Micrographs of ROIs from GFP-Drp1 knock-in U2OS cells transfected with mito-BFP (red) and treated with DMSO, 100 µM histamine, or 4 µM ionomycin as in B. Left, prestimulation; right, 300 s stimulation. Bar, 5 µm. (D) Mitochondrial division rate upon treatment with 4 µM ionomycin or varying concentrations of histamine (10, 30, or 100 µM) in U2OS cells. DMSO, 13 ROIs; ionomycin or histamine treated, at least 20 ROIs. Rates (division events per millimeter per minute): DMSO, 0.9 ± 1.1; 4 µM ionomycin, 5.9 ± 3.7; 10 µM histamine, 2.3 ± 2.2; 30 µM histamine, 2.7 ± 2.0; 100 µM histamine, 3.3 ± 2.7 (SD given). P-values are from an unpaired Student's *t* test.

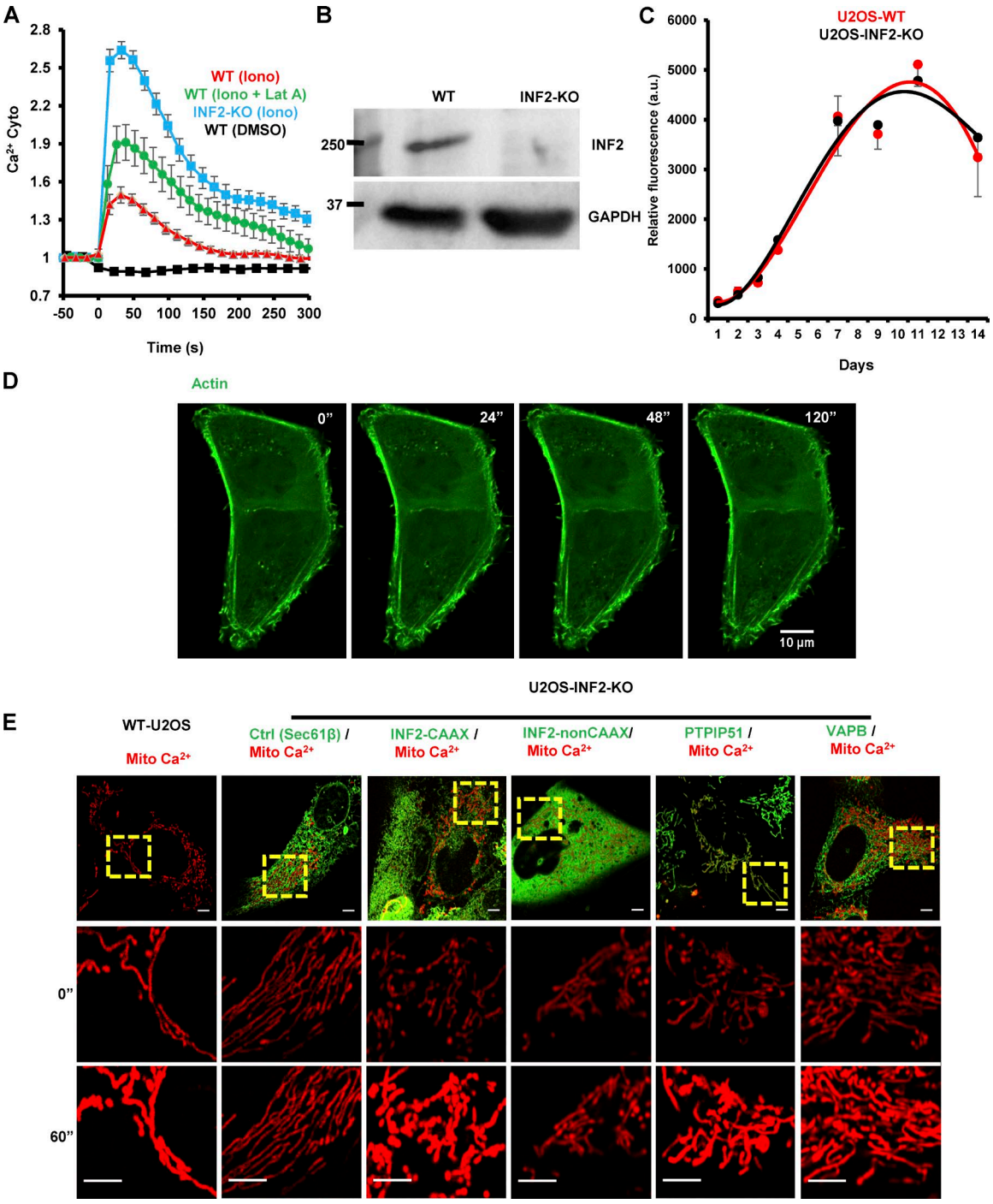


Figure S2. **Effects of LatA on cytosolic calcium, and INF2-KO U2OS cell characterization.** (A) Quantification of cytoplasmic calcium spike after 4 µM ionomycin treatment in U2OS-WT or INF2-KO cells, either with or without simultaneous 2 µM LatA treatment. Ionomycin treatment at time 0. *n* = 10–15 cells. Error bars represent SEM. (B) Western blot analysis of U2OS-WT and U2OS-INF2-KO cell lines probed with anti-INF2 (upper) and anti-GAPDH (lower). Mass in kilodaltons. (C) Cell proliferation assay (Alamar blue) of U2OS-WT or U2OS-INF2-KO cells. Three replicates taken for each time point (median shown, with error bars representing minimum and maximum). Starting density: 5,000 cells/well (24-well plate). Representative results from two independent experiments are shown. Error bars represent SD. (D) Representative confocal image montage of U2OS-INF2-KO cells transfected with GFP-Ftractin (filamentous actin, green) and stimulated with 4 µM ionomycin (in the presence of 10% serum) at time 0. Bar, 10 µm. Time in seconds. (E) Rescue of mitochondrial calcium response in INF2-KO cells by overexpression of ER–mitochondrial tethers. INF2-KO U2OS cells were transfected with a mitochondrial matrix calcium probe (Mito-R-GECO) along with either CFP-VAPB or GFP-PTPIP51 and then stimulated with 4 µM ionomycin. The effects of either GFP-INF2-CAAX or GFP-INF2-nonCAAX reexpression are shown for comparison. Insets show 0 and 60 s after ionomycin stimulation. Bars: (main) 5 µm; (insets) 2 µm.

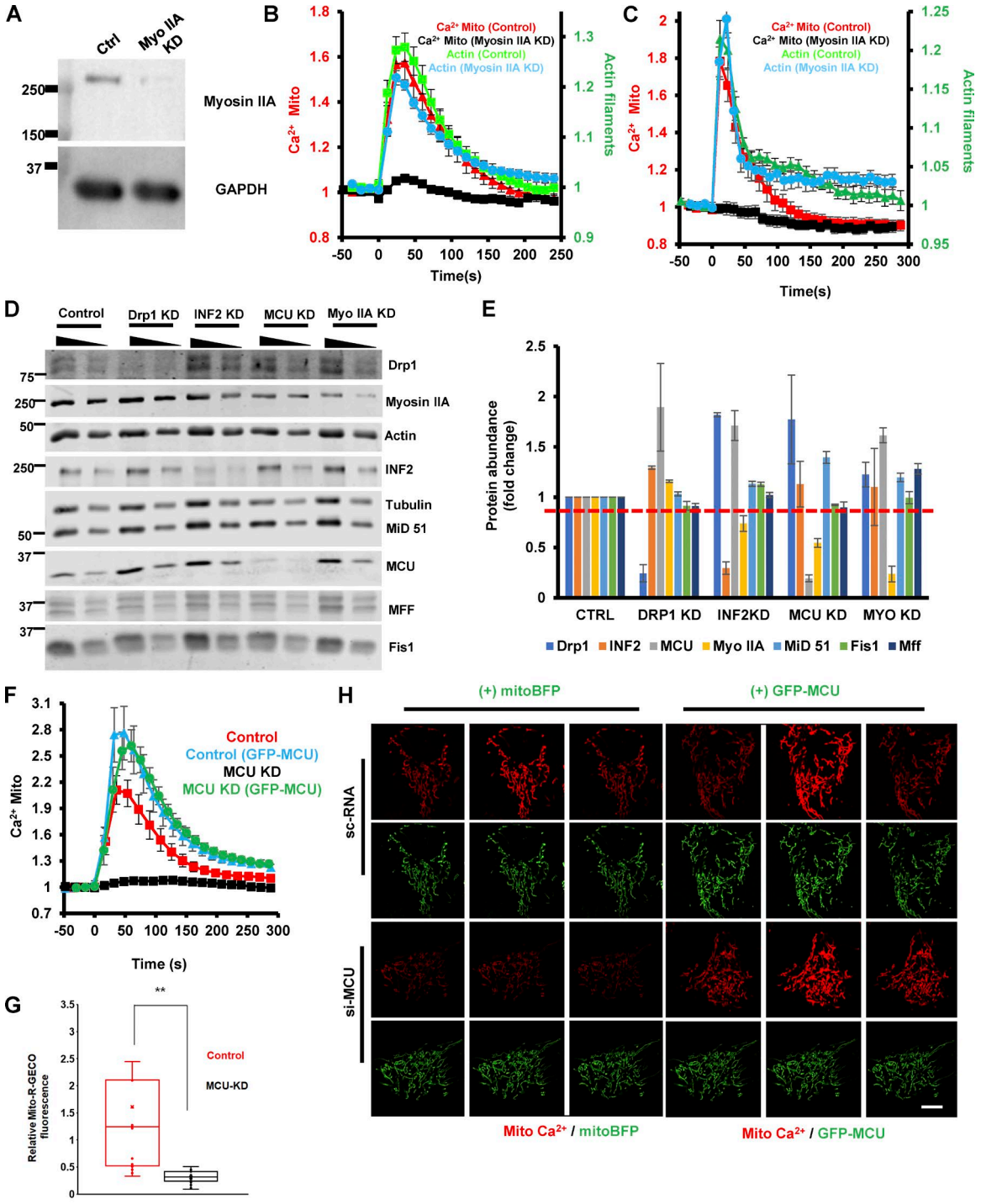


Figure S3. **Effects of myosin IIA, Drp1, MCU, and INF2 siRNA.** (A–C) Effect of Myosin IIA suppression on stimulus-induced mitochondrial calcium spike. U2OS cells were transfected with siRNA against myosin IIA for 72 h, then transfected with mito-R-GECO and GFP-Ftractin. (A) Western blot analysis of U2OS-WT and U2OS-Myosin IIA KD cells probed with anti-Myosin IIA (top) and anti-GAPDH (lower). Mass in kilodaltons. (B) Actin polymerization burst and mitochondrial calcium spike after 4 µM ionomycin treatment. Error bars represent SEM. (C) Actin polymerization burst and mitochondrial calcium spike after 100 µM histamine treatment. Error bars represent SEM. (D) Western blot analysis of protein levels for U2OS cells transfected with siRNAs against MCU, Drp1, INF2, and myosin IIA. Proteins analyzed also included the mitochondrial division factors Mff, MiD51, and Fis1. Wedges refer to the two volumes of extract loaded on the gel (100% and 50%). Molecular mass in kilodaltons. (E) Quantification of protein abundance from Western blot analysis. Data from two independent experiments consisting of two volumes of loaded cell extract analyzed. Abundance in each KD was normalized to the control cell value. Error bars represent SD. (F) Rescue of ionomycin-induced mitochondrial calcium spike by expression of GFP-MCU. U2OS cells were transfected with either scrambled siRNA (control) or MCU siRNA (MCU KD) for 72 h, and then transfected with mito-R-GECO with either mito-BFP or GFP-MCU. After 24 h, cells were stimulated with 4 µM ionomycin, and the mitochondrial calcium spike was measured. Error bars represent SEM. (G) Quantification of basal mito-R-GECO signal for un-stimulated control or MCU KD cells. The mito-R-GECO signal was normalized to signal for cotransfected mito-BFP. $n = 16$ cells (control) or 17 cells (MCU KD). Each point represents one cell. P-values are from an unpaired Student's t test. **, $P < 0.005$. Error bars represent SD. (H) Time-lapse montages of experiment quantified in B. Green represents mito-BFP (left) or GFP-MCU (right). Red represents mito-R-GECO. Bar, 10 µm.

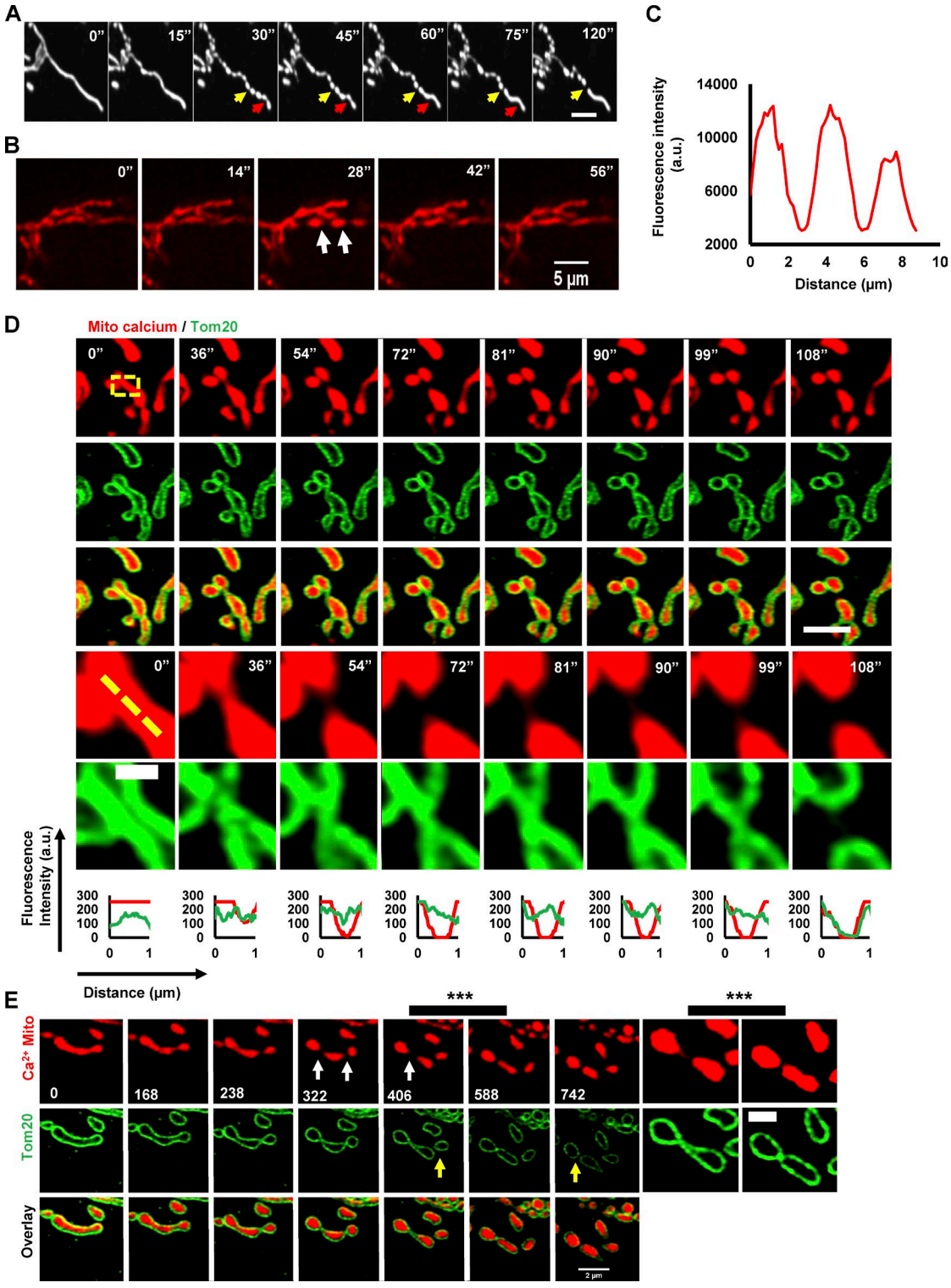


Figure S4. **Mitochondrial constrictions and IMM division.** (A–C) Spontaneous mitochondrial constrictions corresponding to increased mitochondrial calcium. (A) Confocal image montage of spontaneous mitochondrial constrictions in U2OS cell transfected with mitoDsRed construct. Red arrows point to constriction that relaxes back without dividing, and yellow arrows point to constriction resulting in division. Bar, 5 µm. Time in seconds. (B) Representative confocal image montage of spontaneous mitochondrial constrictions in U2OS cell transfected with mito-R-GECO (mitochondrial calcium) construct showing calcium spike associated with constriction (arrow). Bar, 5 µm. Time in seconds. (C) Line scan showing periodicity of spontaneous constrictions from U2OS cells transfected with mito-R-GECO construct. (D and E) Matrix division before OMM division in U2OS cells. (D) Second example of a phenomenon shown in Fig. 8 (A and B) shows matrix division before OMM division after ionomycin stimulation of U2OS cells transfected with mito-R-GECO (mitochondrial matrix calcium, red) and GFP-Tom20 (OMM, green). Bars: (main) 2 µm; (insets) 0.5 µm. (E) Example of matrix division before OMM division after CGP37157 treatment of U2OS cells transfected with mito-R-GECO (mitochondrial matrix calcium, red) and GFP-Tom20 (OMM, green). White arrows show matrix division events, and yellow arrows show the subsequent OMM division event. Images at the right show two of these time points (***) as zoomed and enhanced images for mito-R-GECO, in an attempt to detect a lingering matrix tether. Bars: (main) 2 µm; (zoom) 1 µm. Corresponding to Video 10.

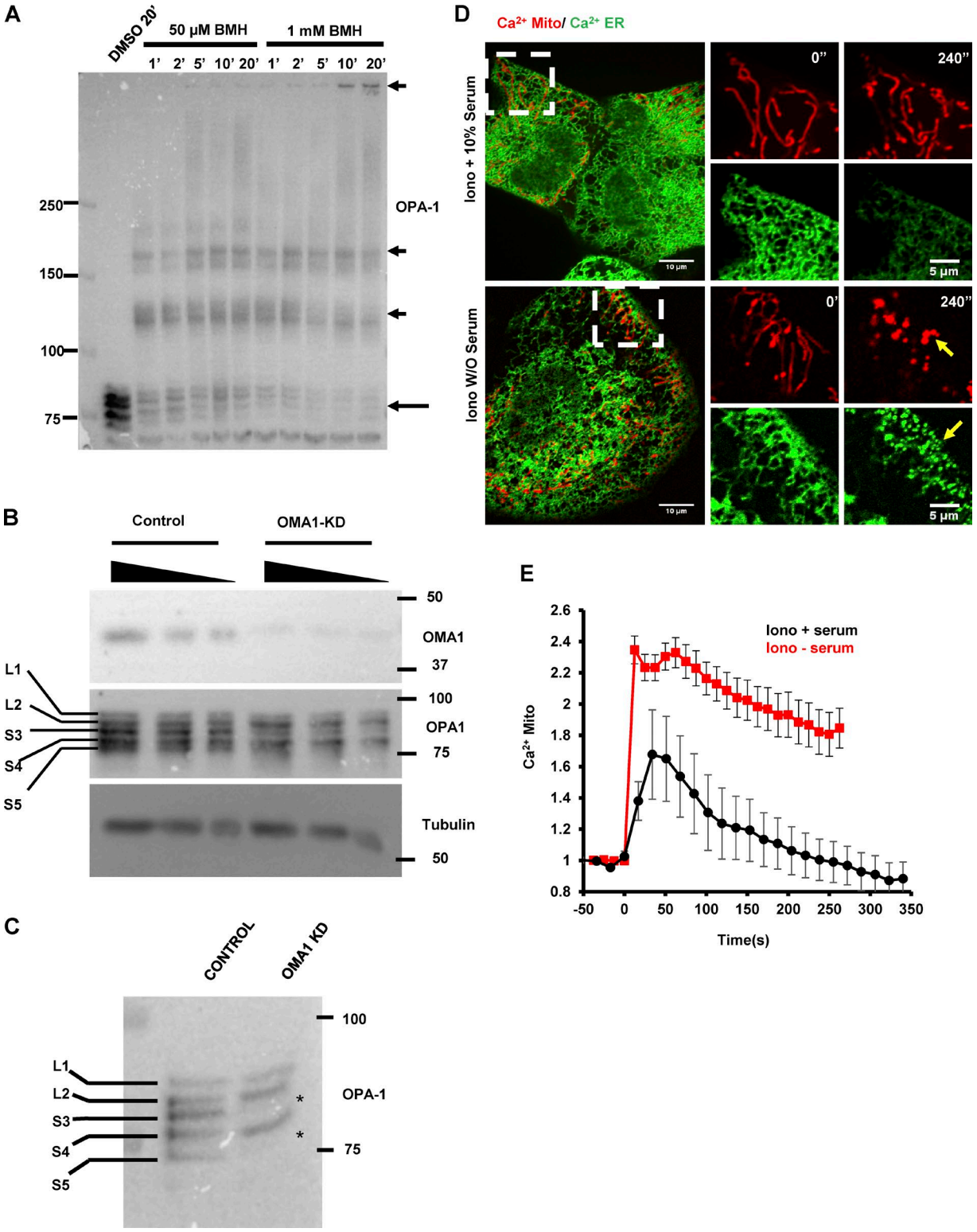
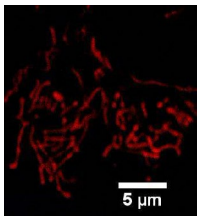
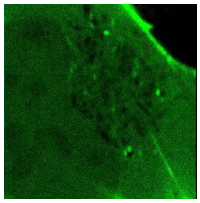


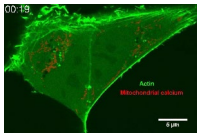
Figure S5. **Opa1 and Oma1 experiments.** (A) BMH cross-linking assay to examine Opa1 oligomers. Control experiment to test time and concentration of BMH. Cross-linker was added to live U2OS cells in medium for the indicated times, then cells were lysed by medium removal and addition of SDS-containing buffer. Samples were analyzed by anti-Opa1 Western blot. Mass is in kilodaltons. (B) Western blot of U2OS transfected with siRNA for Oma1. Top panel shows Oma1 depletion. Middle panel shows depletion of Opa1 S3 and S5 bands, and increase in L2 and S4 bands, upon Oma1 depletion, as shown previously (Anand et al., 2014). Mass in kilodaltons. (C) Western blot of Opa1 bands in control and Oma1 KD cells at high resolution, showing depletion of S3 and S5 bands and increases in L2 and S4 bands upon Oma1 depletion. Mass is in kilodaltons. (D) Comparison of ionomycin stimulation in the presence or absence of serum. Top: representative confocal images of U2OS cells transfected with mito-R-GECO (mitochondrial calcium, red) and ER-GCaMP6-150 (ER calcium, green), stimulated with 4 µM ionomycin in the absence or presence (10%) of serum at time 0. Bars: (main) 10 µm; (insets) 5 µm. Time in seconds. Yellow arrows indicate fragmented mitochondria and ER. (E) Quantification of mitochondrial calcium spike after 4 µM ionomycin treatment in the absence or presence of 10% serum in U2OS cells transfected with mito-R-GECO and treated at time 0; *n* = 10 cells. Error bars represent SEM.



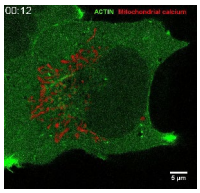
Video 1. **Ionomycin-stimulated increases in cytoplasmic calcium (Cyto-R-GECO), actin polymerization (GFP-Ftractin), and mitochondrial calcium (Mito-R-GECO) in U2OS cells (4 µM ionomycin).** Movie at 5 frames/s showing the first 25 s. Arrows indicate the incidence of each of the events. Time in seconds. Bar, 5 µm. Corresponds to graphs in Fig. 1 B.



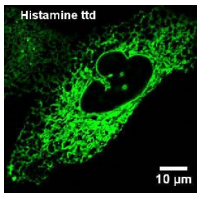
Video 2. **Histamine-stimulated increases in cytoplasmic calcium (Cyto-R-GECO), actin polymerization (GFP-Ftractin), and mitochondrial calcium (Mito-R-GECO) in U2OS cells (100 µM histamine).** Movie at 5 frames/s showing the first 15 s. Arrows indicate the incidence of each of the events. Time in seconds. Bar, 5 µm. Corresponds to graphs in Fig. 1 E.



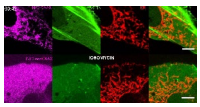
Video 3. **Ionomycin-stimulated actin burst (GFP-Ftractin) and mitochondrial calcium spike (Mito-R-GECO) in the same cell.** Movie at 1 frame/s. Time in minutes:seconds. Bar, 5 µm. Corresponds to Fig. 1 G.



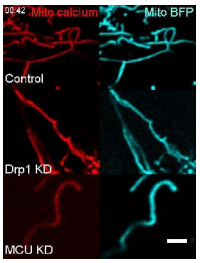
Video 4. **Histamine-stimulated actin burst (GFP-Ftractin) and mitochondrial calcium spike (Mito-R-GECO) in the same cell.** Movie at 2 frames/s. Time in minutes:seconds. Bar, 5 µm. Corresponds to Fig. 1 H.



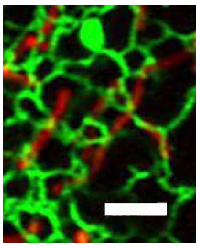
Video 5. **Effects of DMSO, ionomycin (4 µM), or histamine (100 µM) on ER calcium release.** U2OS cells transfected with (ER-GCaMP6-150) and treated with either of the drugs listed above as indicated. Time in minutes:seconds. Bar, 10 µm Corresponds to Fig. 2 A.



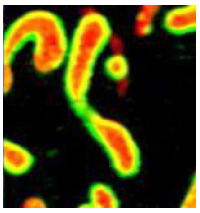
Video 6. **Ionomycin-induced actin morphology in INF2-KO cells reexpressing GFP-INF2-CAAX or GFP-INF2-nonCAAX.** Cells transfected with INF2 construct, actin marker (mApple-Ftractin) and ER marker (ER-BFP) and treated with 4 µM ionomycin. Arrows indicate actin filaments on ER (INF2-CAAX) and non-ER regions (INF2-nonCAAX). Blue arrowhead denotes actin filament formation on the nuclear envelope for INF2-CAAX. Time in minutes:seconds. Bar, 2 µm. Corresponds to Fig. 3 F.



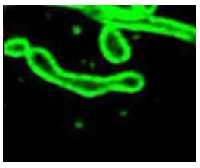
Video 7. **Comparison of ionomycin-induced mitochondrial matrix constrictions in WT, Drp1-KD, or MCU-KD U2OS cells using Mito-R-GECO (mitochondrial matrix calcium-sensor, red, left) and MitoBFP (matrix marker, blue, right).** Time in minutes:seconds. Bar, 5 µm. Corresponds to Fig. 7 B.



Video 8. **Mitochondrial constrictions at ER contact sites. U2OS cell transfected with GFP-Sec61β (green, ER marker) and mito-R-GECO (red, mitochondrial calcium) and imaged live during ionomycin stimulation (4 µM).** Time in minutes:seconds. Bar, 1 µm. Corresponds to Fig. 7 C.



Video 9. **Matrix division before OMM division during ionomycin-stimulated mitochondrial division.** U2OS cells transfected with mito-R-GECO (mitochondrial matrix calcium, red) and GFP-Tom20 (OMM, green). Cells imaged live after addition of 4 µM ionomycin. Arrow indicates apparent matrix separation, and arrowhead indicates OMM separation. Time in minutes:seconds. Bar, 2 µm. Corresponds to Fig. 8 A.



Video 10. **Matrix division before OMM division during CGP37157 (80 µM)-stimulated mitochondrial division. U2OS cells transfected with mito-R-GECO (mitochondrial matrix calcium, red) and GFP-Tom20 (OMM, green).** Arrows indicate apparent matrix separation, and arrowheads indicate OMM separation. Time in minutes:seconds. Bar, 2 µm. Corresponds to Fig. S4 E.

References

Anand, R., T. Wai, M.J. Baker, N. Kladt, A.C. Schauss, E. Rugarli, and T. Langer. 2014. The i-AAA protease YME1L and OMA1 cleave OPA1 to balance mitochondrial fusion and fission. *J. Cell Biol.* 204:919–929. https://doi.org/10.1083/jcb.201308006