Supporting Information

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SI Materials and Methods

Immunofluorescence. For immunofluorescence, cells were fixed for 10 min in 4% (wt/vol) paraformaldehyde (Electron Microscopy Sciences) in PBS, washed in PBS, permeabilized for 5 min in 0.1% Triton X-100 in PBS, and blocked for at least 30 min in 1% BSA and 10% (vol/vol) goat serum. Primary antibodies were incubated for 60 min in blocking buffer, and unbound antibodies were washed off extensively before a 30-min incubation with DAPI, Phalloidin-488 where applicable, and Alexa-labeled secondary antibodies (Invitrogen) in blocking buffer. Eventually, the coverslips were washed extensively in PBS and mounted in Vectashield (Vector Laboratories). The following primary antibodies were used at the dilutions suggested by the manufacturers: rabbit anti-CoxIV (3E11; Cell Signaling), mouse anti-Drp1 (CT, i.e., against C terminus, 611112; BD Biosciences), rabbit anti-Drp1 (NT, i.e., against N terminus, NB110-55237; Novus Biologicals), mouse anti-Tom20 (clone 29; BD Biosciences), mouse anti-cytochrome *c* (clone 6H2.B4; BD Biosciences), rabbit anti-Mff (HPA010968; Sigma), rabbit anti-Fis1 (ALX210-907-R100; Alexis), and mouse anti HA 6E2 (2367; Cell Signaling). The following antibodies were used in Western blots only (in addition to the antibodies also used for immunofluorescence): mouse anti-actin (A5441; Sigma), mouse anti-tubulin (clone B5.1.2; Sigma), mouse anti-GAPDH (clone 6c5; Abcam), and mouse anti-Opa1 (clone 18; BD Biosciences).

Quantification of fragmented mitochondria in Drp1 knockout cells and upon actin depolymerization was performed as follows: random fields of view were chosen by using the DAPI channel. Cells were counted, and mitochondrial morphology was classified for each cell as elongated or fragmented. Intermediate cases were not considered. At least 150 cells were counted per experiment and ≥3 independent experiments were performed.

Live Cell Imaging. Imaging of live cells was performed with a Zeiss AxioObserver.Z1 inverted fluorescence microscope equipped with an Evolve EM-CCD camera (Photometrics) and a Yokogawa CSU-X1 spinning disk confocal system. Images were acquired at 37 °C with a 100× N.A. 1.4 oil objective by using MetaMorph. Cells were imaged in a single focal plane every 500 ms for up to 10 min for ER-mitochondrial fragmentation experiments and every 2 s for Mff and Drp1 decay experiments. All images were analyzed in Fiji (National Institutes of Health), including adjustment of brightness and contrast, generation of merged images, and addition of scale bars. To examine the decay in fluorescence of Mff-GFP or mCherry-Drp1, the mean fluorescence intensity in a region of interest (ROI) was measured over time by using the ImageJ/Fiji ROI Manager. Live cell imaging of Drp1-GFP was performed on a PerkinElmer spinning disk microscope driven by the Volocity software, fixed samples were analyzed on a Leica SP5 confocal microscope, and images were taken with a 63× oil objective.

For the quantification of fragmentations marked by ER-mitochondria contact sites, analysis was restricted to regions of cells where the ER and mitochondria were well resolved, typically the cell periphery. Mitochondrial fragmentations were analyzed in ImageJ/Fiji. A mitochondrial fragmentation was defined as a region of mitochondria that split into two separate fragments and were maintained as two separate fragments in subsequent images. An ER-mitochondrial contact site was defined as a region where the ER crossed over mitochondria (as opposed to merely running parallel to mitochondria). Cells expressing K38A Drp1 or Drp1 siRNA were identified by hyperfused mitochondria. The number of fragmentations that were quantified was as follows: WT: $n = 69$ from four independent experiments; K38A: $n = 47$ from three independent experiments; siRNApool: $n = 34$ from two independent experiments.

Statistical Analysis. Results are expressed as means of at least three independent experiments. The error bars represent the SEM. Analysis was performed with two-tailed Student t test unless otherwise specified.

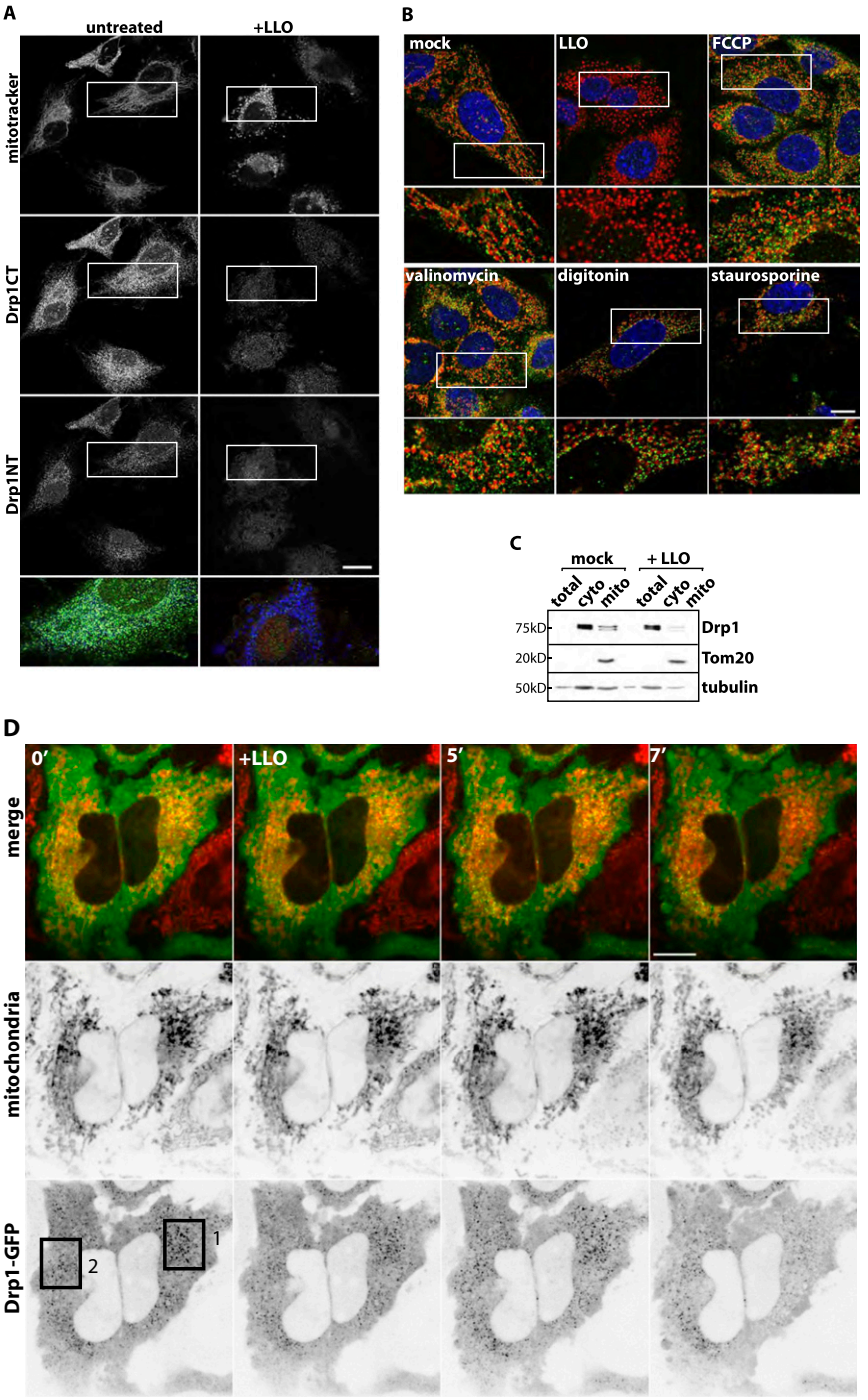

Fig. S1. Listeriolysin O (LLO) treatment causes a decrease in mitochondria-associated Drp1. (*A*) To rule out epitope masking as the origin of the observed decrease in the mitochondria-associated Drp1 signal, we assessed Drp1 localization upon LLO treatment using two different antibodies. Antibodies recognizing either the N or the C terminus detect loss of Drp1 staining from mitochondria. White boxes below indicate 2× enlarged regions and show an overlay of Drp1 labeled with two different antibodies (Drp1NT, green; DrpCT, red) with mitochondria (blue; Mitotracker Deep Red). (*B*) HeLa cells were treated with LLO or the indicated drugs as described in *Materials and Methods*, fixed, and stained for Drp1 (green) and mitochondria (CoxIV; red). All conditions (including digitonin, used as a control for plasma membrane permeabilization) induce mitochondrial fragmentation. However, loss of Drp1 staining at mitochondria is only observed upon LLO treatment. White box indicates region enlarged 2× and shown below. (*C*) As shown in Fig. 1*A*, there is a decrease in Drp1 puncta associated with mitochondria by immunofluorescence but no change in total Drp1 protein level by Western blotting. In an attempt to see whether there was degradation of a mitochondrial pool of Drp1 that would be masked in whole cell lysates, cell extracts were fractionated and the Drp1 was blotted in the cytosolic and mitochondrial fraction. There was not a striking difference in the Drp1 associated with cyto versus mito fraction. Tubulin was used as a loading control and Tom20 was used to verify the mitochondrial fraction. (*D*) HeLa cells were transfected with Drp1-GFP (green), stained with mitotracker-633 (red), and imaged at the indicated timepoints by timelapse spinning disk confocal imaging upon addition of LLO. Black and white images correspond to the single channels of either mitochondria or Drp1-GFP shown in the merge of the top tiles. Box 1 (t0) marks a region of the cell where Drp1 puncta are still present at time $t = 7$ min, whereas box 2 (t0) marks a region of the cell where Drp1 puncta have mostly disappeared by 7 min. (Scale bars: *A* and *B*, 10 μm; *D*, 23 μm.)

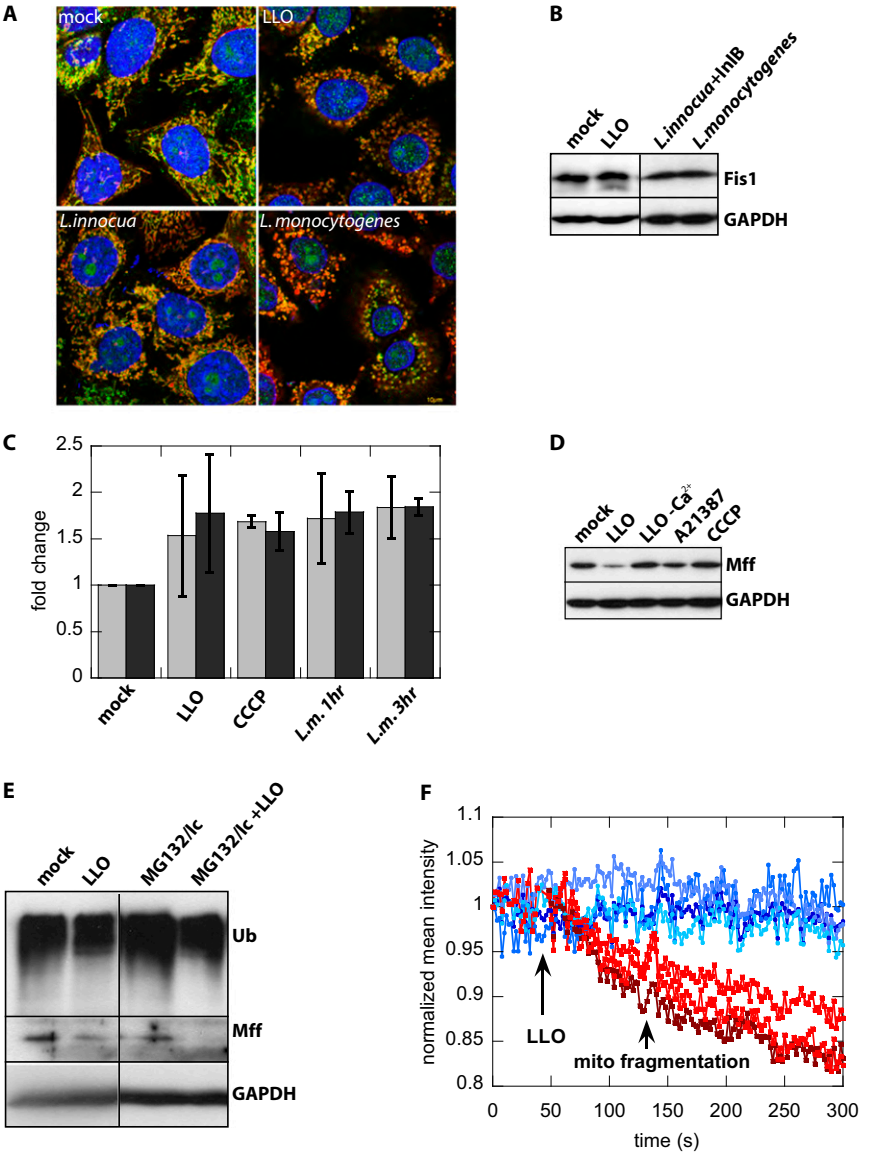

Fig. S2. (Continued)

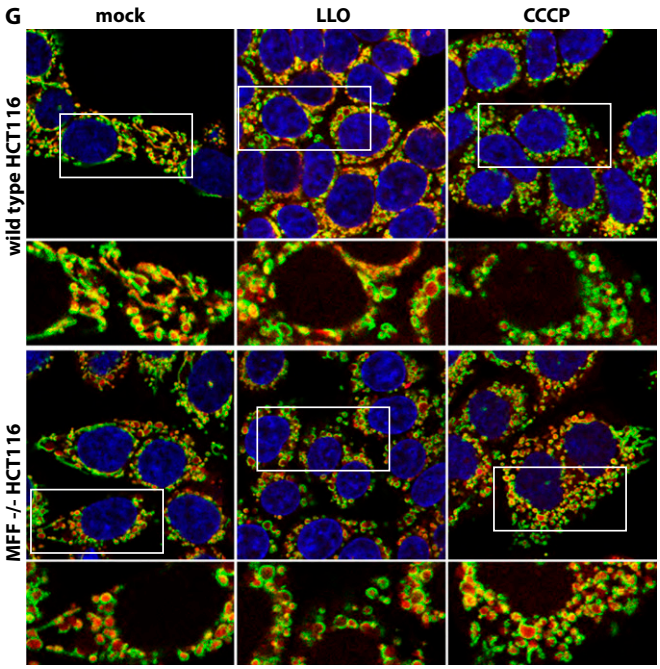

Fig. S2. The effect of LLO on Drp1 receptors Fis1 and Mff. (*A*) HeLa cells were treated for 10 min with 6 nM LLO and stained for Fis1 (green), mitochondria (Mitotracker Deep Red; red), and DAPI (blue). Immunofluorescence images show no detectable decrease in the association of Fis1 with mitochondria. (Scale bar: 10 μm.) (*B*) Total cell lysates were analyzed by Western blot for Fis1 and GAPDH (loading control), showing that total levels and size of Fis1 are not affected by LLO treatment or infection. (*C*) Quantitative RT-PCR of Mff mRNA levels using two different sets of primers (MFF 1 and MFF 2), for HeLa cells treated with LLO, carbonyl cyanide m-chlorophenylhydrazone (CCCP), or infected with *Listeria monocytogenes* for 1 h or 3 h. Values are reported relative to the mock-treated cells. (*D*) Total lysates of cells treated for 10 min with 6 nM LLO in the presence or absence of extracellular calcium or treated for 30 min with 10 μM CCCP or with 1 μM of the calcium ionophore A23187 were analyzed by Western blot for Mff and GAPDH (loading control). (*E*) Cells were left untreated or pretreated for 1.5 h with 20 μM MG132 and 20 μM lactacystin and then incubated for 10 min with LLO, loaded on a Western blot probed for ubiquitin, Mff, and GAPDH. Note that proteasome inhibition leads to an increase in the ubiquitin signal without preventing Mff degradation. (*F*) Live-cell analysis of Mff-GFP fluorescence intensity over time in HeLa cells treated with LLO (red traces) or untreated (blue traces), but exposed to the same image acquisition protocol. Each trace represents the mean intensity in a region of interest on an individual cell. Data are normalized to the starting intensity in a given cell. There is negligible photobleaching of GFP under the image acquisition conditions (blue traces). In the red traces, there is little fluorescence decay before addition of LLO, whereas substantial decay starts immediately after addition of LLO and before fragmentation of mitochondria. Data are representative of four independent experiments. (*G*) Wild-type or $Mff^{-/-}$ HCT116 cells were treated with LLO or CCCP for the indicated amount of time and stained with an outer mitochondrial membrane marker (Tom20) and an inner mitochondrial membrane marker (CoxIV). White box indicates 2× enlarged region shown below.

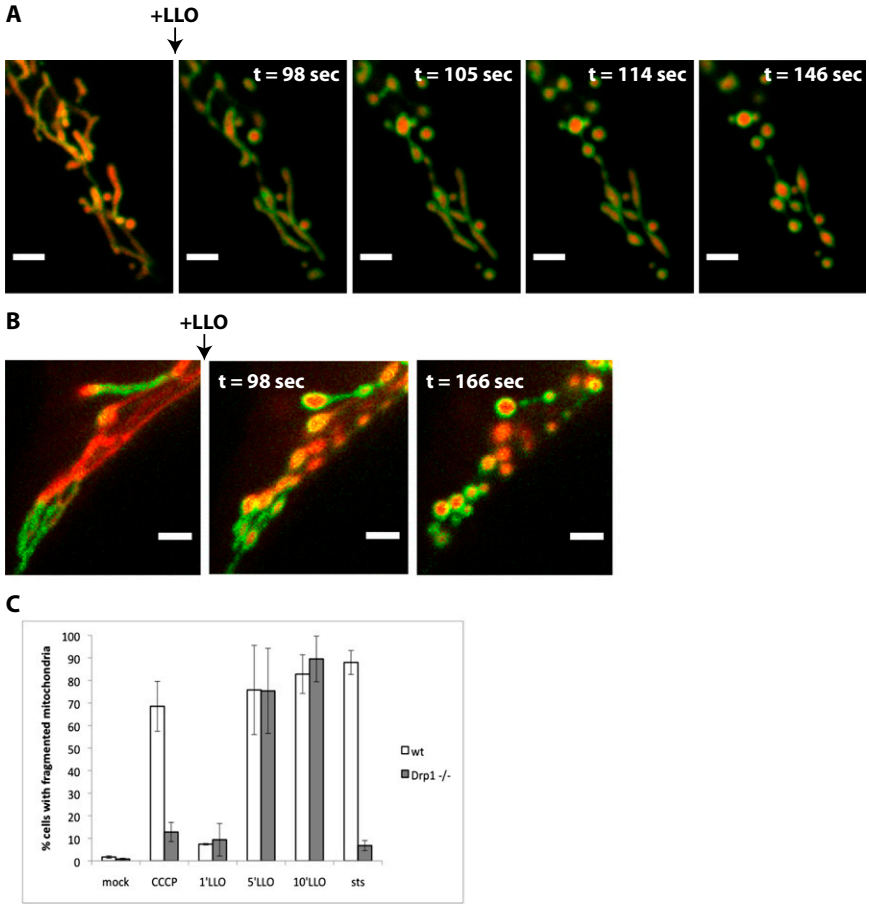

Fig. S3. Timelapse images of LLO-induced mitochondrial fragmentation demonstrate abscission of the outer mitochondrial membrane. (*A*) HeLa cells transfected with OM-GFP (green) and DsRed2-mito (red) and treated with 2 nM LLO. Confocal fluorescence images were collected every 2 s. (*B*) HCT116 Drp1$^{-/-}$ cells transfected with OM-GFP (green) and DsRed2-mito (red) and treated with 2 nM LLO. Confocal fluorescence images were collected every 2 s. (*C*) Wild-type or Drp1$^{-/-}$ MEFs were loaded with mitotracker 633 and treated for the indicated timepoints with 6 nM LLO for 30 min with 10 μM CCCP or for 1.5 h with 1.2 μM staurosporine. The percentage of cells with fragmented mitochondria was assessed as in Fig. 3*C*.