

Atypical mitochondrial fission upon bacterial infection

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We recently showed that infection by *Listeria monocytogenes* causes mitochondrial network fragmentation through the secreted pore-forming toxin listeriolysin O (LLO). Here, we examine factors involved in canonical fusion and fission. Strikingly, LLO-induced mitochondrial fragmentation does not require the traditional fission machinery, as Drp1 oligomers are absent from fragmented mitochondria following *Listeria* infection or LLO treatment, as the dynamin-like protein 1 (Drp1) receptor Mff is rapidly degraded, and as fragmentation proceeds efficiently in cells with impaired Drp1 function. LLO does not cause processing of the fusion protein optic atrophy protein 1 (Opa1), despite inducing a decrease in the mitochondrial membrane potential, suggesting a unique Drp1- and Opa1-independent fission mechanism distinct from that triggered by uncouplers or the apoptosis inducer staurosporine. We show that the ER marks LLO-induced mitochondrial fragmentation sites even in the absence of functional Drp1, demonstrating that the ER activity in regulating mitochondrial fission can be induced by exogenous agents and that the ER appears to regulate fission by a mechanism independent of the canonical mitochondrial fission machinery.

mitochondrial dynamics | live cell imaging | actin

Mitochondria are essential organelles that perform a multitude of functions, ranging from the production of biosynthetic intermediates and energy to innate immune signaling and cellular calcium buffering or the storage of proapoptotic components (1). To perform these diverse functions, mitochondria respond to cellular cues and display a highly variable and dynamic morphology, constantly undergoing fusion and fission. It is becoming increasingly clear that mitochondrial dynamics and function are deeply interconnected, and mitochondrial dysfunction is associated with a range of diseases.

Wild-type mitochondrial morphology and function are maintained by a balance between mitochondrial fusion and fission. Fusion allows exchange of genetic material between single mitochondria and is mediated by two large guanosine triphosphate phosphohydrolases (GTPases) embedded in the outer membrane (mitofusin 1 and 2) and an inner membrane GTPase, Opa1 (2). Deletion mutants affecting these three proteins accumulate dysfunctional mitochondria, leading to neurodegenerative phenotypes and different forms of myopathy (1, 3).

Mitochondrial fusion is balanced by fission, which is essential to ensure proper distribution of mitochondria and energy supply to daughter cells in mitosis or within a single cell. This necessity is particularly evident in neurons, where fission defects prevent efficient mitochondrial transport to synapses, the crucial sites of energy consumption (4, 5). The physiological importance of mitochondrial fission is further highlighted by its essential role in embryonic development in mice and nematodes (6–8).

Mitochondrial fission is thought to be accomplished by the dynamin-like protein Drp1, a mainly cytosolic protein that is recruited to future fission sites, where it oligomerizes to form spirals that constrict mitochondria. Mitochondrial fission is regulated at several levels: by initial ER- and actin-mediated mitochondrial constriction (9, 10), leading to the accumulation of the membrane-bound Drp1 receptor Mff and by several posttranslational modifications of Drp1, which modulate its activity (11).

Listeria monocytogenes is a foodborne pathogen capable of invading nonphagocytic cells, where it can replicate and spread. The pathogenic potential of *L. monocytogenes* correlates with the expression of several virulence genes (12). One of the most important virulence factors is listeriolysin O (LLO), a highly regulated secreted pore-forming toxin (reviewed in ref. 13). LLO belongs to the family of cholesterol-dependent cytolysins (CDCs), most of which are produced by extracellular bacteria such as *Streptococci* or *Clostridia*. CDCs oligomerize on cholesterol-containing membranes to form nonselective ion-permeable pores of variable sizes (14) that act in concert with bacterial phospholipases to allow bacterial escape from the phagosome. More recently, LLO has been found to have several intracellular and extracellular roles that extend beyond phagosomal escape. For example, we have shown that infection with *L. monocytogenes* causes fragmentation of the host mitochondrial network by action of its pore-forming toxin LLO before bacterial entry (15).

In this study, we demonstrate that LLO-induced mitochondrial fragmentation does not follow canonical pathways, because it is independent of key fusion and fission components, such as Opa1 and Drp1. We demonstrate that the ER marks mitochondrial fragmentation sites even in the absence of functional Drp1, and that the actin cytoskeleton also facilitates fragmentation. LLO-induced fragmentation is distinct from that observed upon treatment with uncouplers [such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)] and apoptosis inducers (such as staurosporine), revealing a unique pathway for mitochondrial fragmentation that can be induced by an exogenous agent.

Significance

Mitochondria are dynamic organelles that constantly fuse and fragment while acting as central hubs of energy production, apoptosis regulation, and Ca⁺⁺ signaling, therefore emerging as potential targets of pathogens. We previously showed that the foodborne bacterial pathogen *Listeria monocytogenes* interferes with the dynamics and function of the host cell mitochondrial network via the bacterial toxin listeriolysin O (LLO). In this study, we analyze the effects of LLO on key players known to be involved in mitochondrial dynamics and show that the fission protein dynamin-like protein 1 (Drp1) is not essential for LLO-induced fragmentation of the mitochondrial network, whereas the endoplasmic reticulum (ER) plays an important role, suggesting a unique Drp1-independent and ER-dependent mechanism that is different from the canonical fission machinery.

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The authors declare no conflict of interest.

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Results and Discussion

Opa1 Is Not Processed upon LLO-Induced Mitochondrial Fragmentation.

Mitochondrial fragmentation can result either from an increase in fission activity, an inhibition of fusion, or both. One possible pathway for mitochondrial fragmentation is rapid proteolytic processing of the inner membrane GTPase Opa1, as observed upon uncoupler [CCCP or trifluorocarbonyl cyanidephenylhydrazine (FCCP)]-induced mitochondrial fragmentation, which results in a fusion block and an ensuing mitochondrial fragmentation due to unopposed fission events (16–18). In contrast to CCCP, Opa1 is not processed upon LLO treatment (Fig. 1A), indicating that LLO-induced mitochondrial fragmentation is not caused by a block in inner membrane fusion. This finding is consistent with the rapid fission kinetics that we have described, which suggests an active fission mechanism (15). We therefore addressed whether LLO induces fragmentation by recruiting the key fission protein Drp1.

Mitochondrial Drp1 Oligomers Dissociate upon Infection or LLO Treatment.

Fission-inducing agents such as uncouplers or the apoptosis inducer staurosporine appear to act by recruiting Drp1 to fragmenting mitochondria (Fig. 1C; refs. 19–21). In contrast, LLO treatment and *L. monocytogenes* infection were characterized by a decrease in Drp1 puncta associated with mitochondria (Fig. 1C and D and Fig. S1A). This phenomenon was unique to LLO and *L. monocytogenes* infection because a number of other fission-inducing agents (the protonophore FCCP, the potassium ionophore valinomycin, and the detergent digitonin) did not show a decrease in mitochondria-associated Drp1 (Fig. S1B). Interestingly, potassium accumulation into mitochondria was invoked by Dimmer et al. to explain Drp1-independent fragmentation upon silencing of the inner membrane protein LETM1, because the potassium ionophore nigericin could restore tubular morphology of mitochondria in these cells (22). LLO may cause an intramitochondrial potassium imbalance that could impinge on mitochondrial morphology, although it does not explain the dissociation of Drp1 from mitochondria. Western blot analysis of cell extracts showed no decrease in total Drp1 levels in LLO-treated (Fig. 1B and Fig. S1C) cells. Use of two different antibodies against endogenous Drp1 on fixed samples (Fig. S1A) and live cell imaging of overexpressed Drp1-GFP (23) (Fig. S1D) confirmed a strong decrease in mitochondria-associated Drp1 staining upon LLO treatment.

LLO Effect on the Drp1 Receptors Fis1 and Mff. Drp1 requires several membrane-anchored receptor proteins to bind to the mitochondrial surface. We thus investigated whether the LLO-induced decrease in mitochondria-associated Drp1 staining was due to modulation of its receptors Fis1 (24–26) and Mff (27) at the mitochondrial outer membrane.

Upon LLO treatment or infection with *L. monocytogenes*, there was no detectable decrease in the association of Fis1 with mitochondria by immunofluorescence or total Fis1 protein levels by Western blotting (Fig. S2A and B), suggesting that loss of mitochondrial Drp1 does not depend on a decrease in Fis1. In contrast, LLO treatment and *L. monocytogenes* infection induced a decrease in Mff protein as observed in total cell extracts by Western blotting and at the single-cell level by immunofluorescence and live-cell imaging (Fig. 2). The level of Mff mRNA was unaltered, suggesting posttranscriptional modulation of Mff (Fig. S2C). Mff did not decrease upon uncoupling (Fig. 2A and B) nor when LLO treatment was performed in the absence of extracellular calcium (Fig. S2D). However, ionophore-induced calcium influx was not sufficient to induce Mff degradation (Fig. S2D), suggesting that calcium influx alone is not sufficient to cause Mff degradation upon LLO treatment. LLO-induced degradation of Mff appeared proteasome independent, because it was not prevented by treatment with MG132 and lactacystin (Fig. S2E),

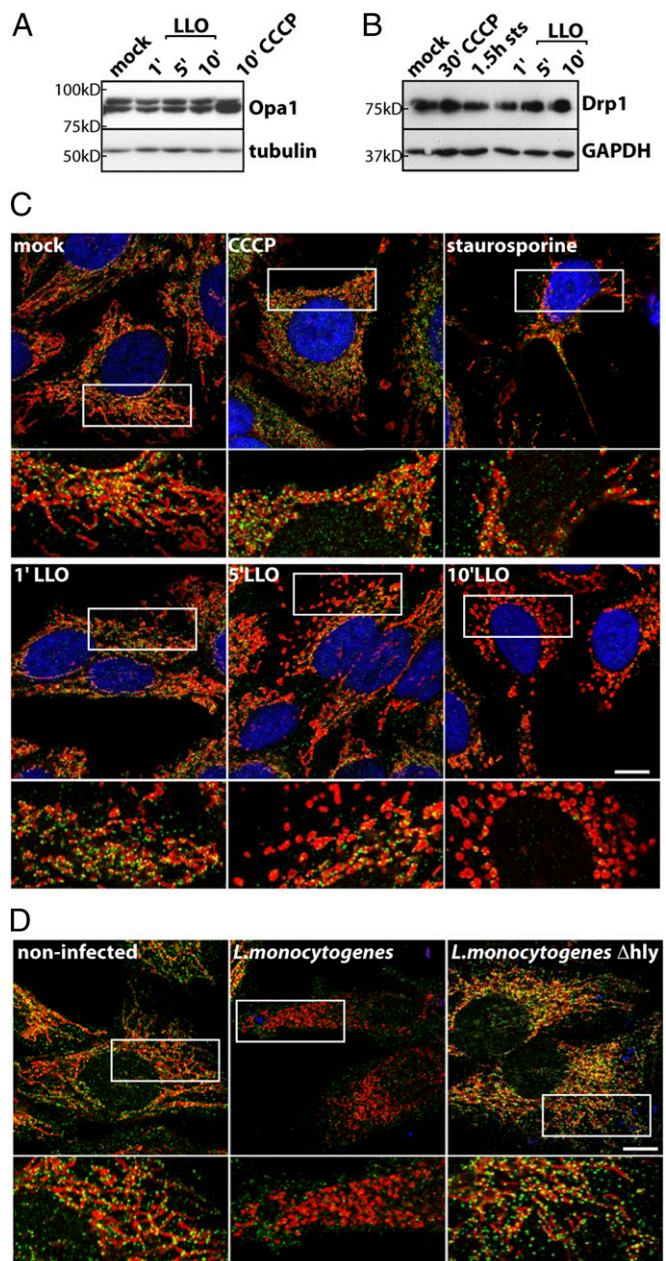


Fig. 1. Analysis of Opa1 and Drp1 upon LLO-induced mitochondrial fragmentation. (A) Western blot of HeLa cells treated for 1, 5, or 10 min with 6 nM LLO or for 10 min with 10 μ M CCCP and probed for Opa1, which is readily processed upon CCCP, but not LLO treatment. Tubulin was used as a loading control. (B) HeLa cells were treated for 30 min with 10 μ M CCCP, for 1.5 h with 1.2 μ M staurosporine (sts), or with 6 nM LLO for the indicated timepoints, and total cell lysates are analyzed by Western blotting, showing no apparent decrease in total Drp1 levels upon LLO or drug treatment. GAPDH was used as a loading control. (C) Immunofluorescence labeling of the same cells analyzed in B. Drp1 is shown in green (Drp1NT), mitochondria in red (Tom20), and nuclei in blue (DAPI). Staining reveals that mitochondrial Drp1 oligomers are lost upon LLO treatment (decrease in green puncta) but are retained upon CCCP or staurosporine treatment. (D) HeLa cells were infected for 1 h with *L. monocytogenes* or *L. monocytogenes* Δ hly (LLO⁻) at an MOI of 50 and stained for Drp1 (green), mitochondria (CoxIV; red), and nuclei/bacteria with DAPI (blue). Wild-type *L. monocytogenes* induces mitochondrial fragmentation and loss of mitochondria-associated Drp1, whereas infection with the Δ hly mutant does not overtly affect Drp1. For C and D, the white box indicates a 2 \times enlarged region, which is shown below. (Scale bars: 10 μ m.)

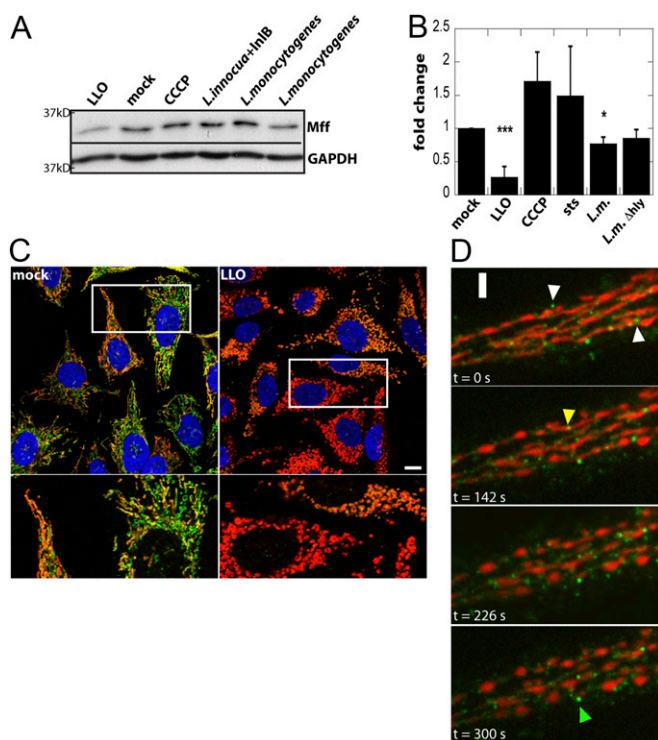


Fig. 2. LLO treatment and *L. monocytogenes* infection cause a decrease in the Drp1 receptor Mff. (A) HeLa cells were treated for 10 min with 6 nM LLO, 30 min with 10 μ M CCCP, or infected at an MOI of 50 with the indicated strains for 1 h. Representative Western blot of Mff and GAPDH (loading control) on total cell lysates shows a decrease in Mff levels upon LLO treatment or infection with wild-type *L. monocytogenes*. (B) Quantification of Western blots from >3 independent experiments (** $P < 0.001$; * $P < 0.05$). (C) Immunofluorescence of HeLa cells treated for 10 min with LLO and stained for Mff (green), mitochondria (Tom20; red), and DNA (DAPI; blue). Although there is heterogeneity in staining of endogenous Mff, there is a significant decrease in Mff staining upon LLO treatment. White box indicates a 4 \times enlarged region that is shown below. (D) Heterogeneous behavior of mitochondria-associated Mff puncta observed by live cell imaging. Timelapse spinning disk confocal images of HeLa cells expressing Mff-GFP (green) and TagBFP-mito (red). Data were collected every 2 s 2 nM LLO was added at time $t = 50$ s. White arrowheads mark Mff-GFP puncta that are rapidly lost from mitochondria. Yellow arrowhead marks an Mff dot that disappears before fragmentation and green arrowhead marks one that remains associated with mitochondria over the time course. (Scale bars: C, 10 μ m; D, 2.6 μ m.)

consistent with previous reports suggesting that LLO induces protein degradation through an as-yet-unknown pathway (28, 29). Timelapse studies of Mff-GFP in HeLa cells treated with LLO indicate that Mff degradation begins almost immediately after LLO addition (Fig. 2D and Fig. S2F) and before mitochondrial fragmentation. Indeed, LLO induced mitochondrial fragmentation in Mff^{-/-} HCT116 cells (Fig. S2G). Given that Mff serves as one of the primary receptors for anchoring Drp1 at mitochondria, this result indicates Drp1 may be lost from mitochondria before fragmentation, further suggesting that Drp1 is not required at fragmentation sites.

LLO-Induced Mitochondrial Fragmentation Is Drp1 Independent. Drp1 is described as the key and essential factor mediating mitochondrial fission. Given that we observed a decrease rather than an augmentation of the Drp1 signal at mitochondria upon infection or LLO treatment, we tested whether Drp1 is essential for LLO-induced mitochondrial fragmentation by using dominant-negative K38A Drp1 in HeLa cells, Drp1^{-/-} mouse embryonic fibroblasts (MEFs) (6), and HCT116 Drp1^{-/-} cells. Strikingly, there was no difference in mitochondrial fragmentation compared with

controls in cells transfected with K38A Drp1, as well as both Drp1 knockout cell lines (Fig. 3), indicating that LLO- and *L. monocytogenes*-induced mitochondrial fragmentation is truly Drp1 independent. In contrast, CCCP-induced mitochondrial fragmentation was substantially reduced in the Drp1 knockout cell lines. Staining of fixed cells, and live cell imaging with fluorescent protein-tagged markers, with both matrix and outer-membrane markers confirmed complete abscission of mitochondria upon LLO treatment (Fig. 3C and Fig. S3A and B). Furthermore, upon incubation of wild-type and knockout cells with LLO for different amounts of time, similar percentages of cells with fragmented mitochondria were scored, indicating that the loss of Drp1 did not delay the kinetics of LLO-induced mitochondrial fragmentation (Fig. S3C).

The Drp1 dependence of mitochondrial fission induced by uncouplers or activators of apoptosis is a matter of debate. For example, it has been demonstrated that transfection of K38A Drp1 does not block uncoupling-induced formation of disk-shaped mitochondria (30), and deletion of Drp1 does not affect the number of cells with fragmented mitochondria upon induction of apoptosis (8). However, several other groups found that overexpression of K38A Drp1 or Drp1 depletion prevented or diminished apoptosis- or CCCP-induced mitochondrial fragmentation (6, 31–33). It has been suggested that this discrepancy may arise because fragmentation depends both on the cell type and the physiological cue used to induce apoptosis (6). In contrast, we see no measureable perturbation of fragmentation upon knock out of Drp1 or expression of dominant-negative Drp1, indicating that fragmentation induced by LLO is truly independent of functional Drp1. Moreover, LLO-induced fragmentation is not associated with cytochrome *c* release and activation of canonical apoptosis (15), suggesting that this mechanism of fragmentation is distinct from the one that activates uncouplers or apoptosis inducers. Opening of the mitochondrial transition pore (MTP) may be invoked to explain LLO-induced mitochondrial fragmentation. We do not favor this hypothesis because of previous experiments confirming that the mitochondrial inner membrane remains intact upon LLO treatment, as respiration can resume (15). Furthermore, we could not inhibit the fragmentation phenotype with the MTP inhibitor cyclosporine A (15).

The ER Marks Sites of Mitochondrial Fragmentation Even in the Absence of Functional Drp1. It has recently been shown that mitochondrial fission occurs at contact sites with the ER, and that mitochondria are constricted at such contact sites (9). Although the mechanism of how the ER regulates fission was not identified, it was suggested that ER proteins may participate in fission and/or that mitochondrial constriction by the ER facilitates recruitment of the mitochondrial fission machinery (i.e., Drp1). We thus explored whether LLO-induced and Drp1-independent mitochondrial fragmentation was marked by ER-mitochondrial contact sites. Timelapse studies of U2OS epithelial cells expressing the ER marker Sec61 β -GFP (34) upon treatment with LLO revealed that the ER marked sites of mitochondrial fragmentation in 87 \pm 8% of events (Fig. 4). Transfection of U2OS cells with K38A Drp1 or siRNA to knockdown Drp1 did not prevent LLO-induced mitochondrial fragmentation. Intriguingly, there was no change in the percent of fragmentations marked by ER-mitochondria contact sites (96 \pm 6% for K38A; 82 \pm 6% for siRNA), demonstrating that the ER plays an active role in regulating mitochondria fission even in the absence of Drp1.

Actin Is Involved in LLO-Induced Mitochondrial Fragmentation. Actin has been shown to play a role in drug-induced mitochondrial fission in mammalian cells, although the effect appears to be stimulus dependent because oligomycin and cyclosporine A, but not CCCP, depended on F-actin for efficient mitochondrial network fragmentation (30). We tested whether the action of

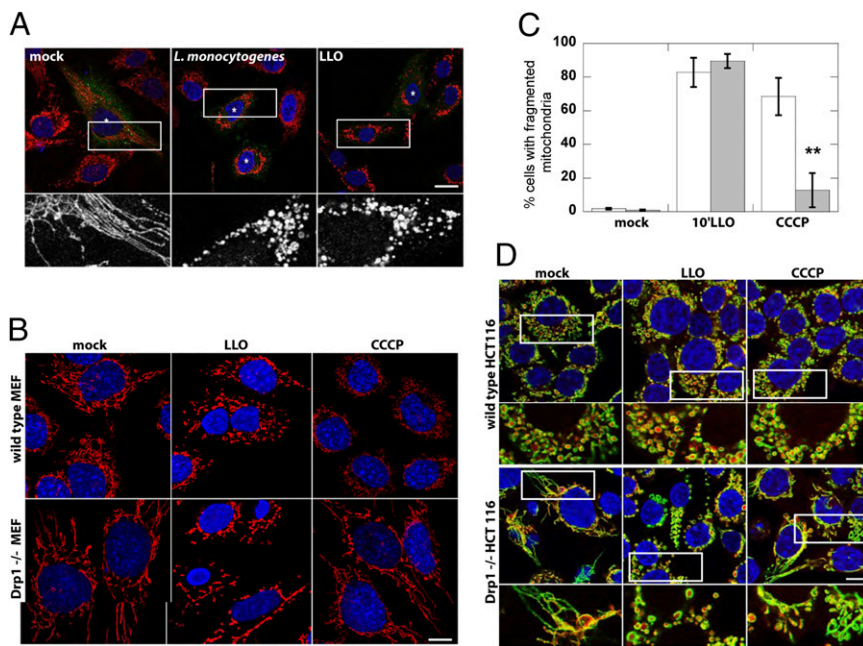


Fig. 3. LLO and *L. monocytogenes* induce mitochondrial-fragmentation in Drp1^{-/-} cells. (A) Infection of HeLa cells transfected with K38A-HA Drp1 with *L. monocytogenes* for 1 h or treatment with 6 nM LLO for 10 min induces mitochondrial fragmentation. Immunofluorescence images show mitochondria (CoxIV; red) and K38A-HA Drp1 (anti-HA; green). Asterisk marks transfected cells, as identified by immunofluorescence. The white box indicates the portion of the image enlarged 4× and shown below. (Scale bars: 20 μm.) (B) Immunofluorescence of mitochondria (CoxIV; red) and nuclei (DAPI; blue) upon treatment of WT or Drp1^{-/-} MEFs with either 6 nM LLO (10 min) or 10 μM CCCP (30 min). (C) The percentage of cells displaying a fragmented mitochondrial network upon CCCP or LLO treatment was quantified in five or six randomly chosen fields of view (*n* > 150 cells) per experiment. Pooled data from three independent experiments is shown (***P* < 0.003, one-tailed Student *t* test). (D) Immunofluorescence of mitochondrial matrix (cytochrome *c*; red), mitochondrial outer membrane (Tom20; green), and nuclei (DAPI; blue) upon treatment of WT or Drp1^{-/-} HCT116 cells with either 6 nM LLO (10 min) or 10 μM CCCP (30 min). White box indicates 2× enlarged region shown below. (Scale bars: 10 μm.)

LLO on mitochondria required the presence of F-actin and found that actin depolymerization by cytochalasin D reduces the number of cells that display a fragmented mitochondrial network upon LLO treatment (Fig. 5). Given that Drp1 is dispensable for LLO-induced mitochondrial fragmentation, we hypothesize that, in addition to contributing to Drp1 recruitment to mitochondria as observed by ref. 30, actin may also facilitate mitochondrial fragmentation by precontracting or physically stretching mitochondria independently of Drp1.

In summary, we have shown that in contrast to uncouplers and apoptosis-inducing agents, mitochondrial fragmentation caused by *Listeria* via its toxin LLO proceeds in a Drp1-independent manner.

LLO-induced mitochondrial fragmentation concurs with degradation of the Drp1-receptor Mff, causing a loss of Drp1 at mitochondria. Drp1 has been proposed to be a multifunctional protein that contributes to peroxisomal and possibly also to ER fission (35, 36), as well as possessing a putative GTPase-independent role in cell death (37). Its modulation by bacterial infection might therefore represent a means to act on several organelles and cellular functions in a coordinated fashion.

The data presented in this study suggests that actin and the ER play an active role in regulating LLO-induced mitochondrial fission. Recently, the ER-localized formin INF2 has been proposed as a link between the ER and actin at mitochondrial constriction sites (10), but it remains to be clarified whether this protein plays a role in LLO-induced mitochondrial fragmentation. Although LLO acts on mitochondrial dynamics before bacterial invasion and we could not detect overt LLO accumulation on intracellular structures by confocal imaging (15), we cannot exclude that a fraction of LLO inserts into mitochondria-associated membranes enriched in cholesterol (38), where it could modulate mitochondria-ER contact sites or their protein components, such as Mfn2 (39). Upon LLO treatment, ER-mediated mitochondrial fission occurs even in the absence of Drp1, indicating that either ER proteins may contribute to mitochondrial abscission or that the ER recruits another as-yet-unidentified mitochondrial fission machinery that exists in mammalian cells.

Given that among the exogenous agents that induce mitochondrial fragmentation LLO is unique in its ability to act rapidly, without activating canonical apoptosis, and in a Drp1-independent manner, we suggest it could serve as an unprecedented tool to

understand the regulation and function of mitochondrial dynamics proteins. Indeed, efforts in this field should aim toward the identification of the molecule(s) that facilitate mitochondrial fragmentation upon LLO treatment in the absence of Drp1 and the signaling cascade that activates such fragmentation.

Materials and Methods

Reagent references, detailed experimental protocols, image and statistical analysis, and nonstandard abbreviations used in this study are provided in *SI Materials and Methods*.

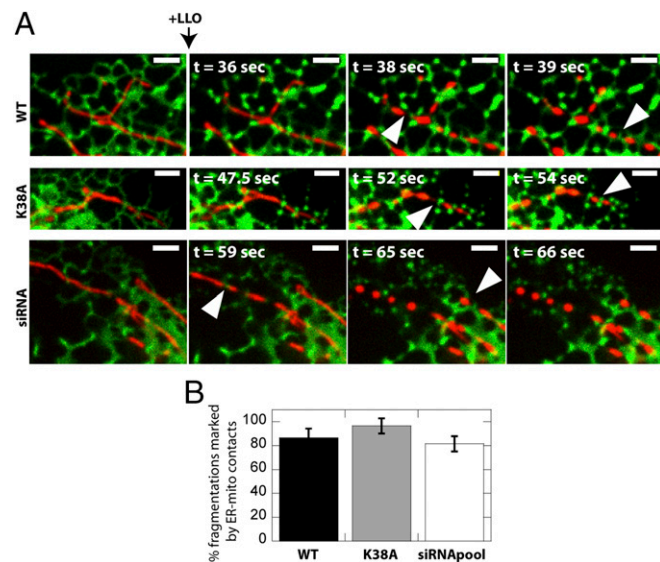


Fig. 4. The ER marks sites of mitochondrial fragmentation independent of Drp1. (A) U2OS Sec61β-GFP cells were treated with 2 nM LLO and examined live by confocal microscopy. Representative images of mitochondria (DsRed2-mito; red) and ER (Sec61β-GFP; green) are shown. Arrowheads indicate mitochondrial fragmentation sites marked by ER-mitochondria contact sites. Experiments were repeated a minimum of three times. (Scale bars: 2.6 μm.) (B) Quantification of mitochondrial fragmentations marked by ER-mitochondria contact sites. Analysis was carried out as described in *Materials and Methods*.

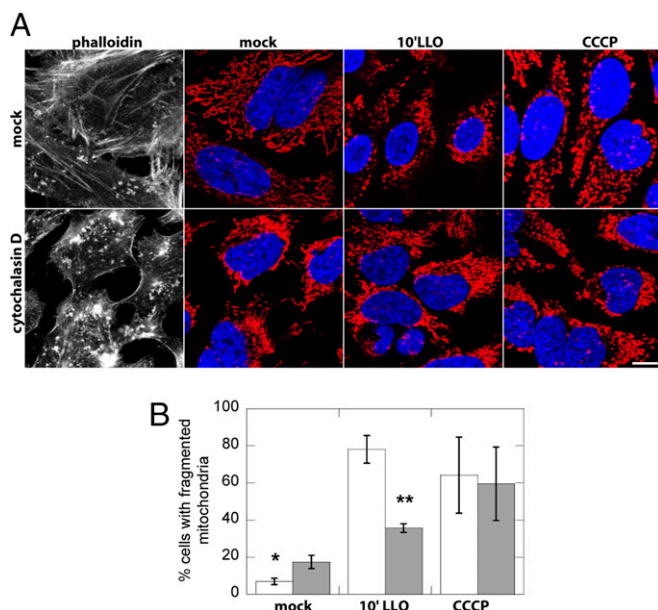


Fig. 5. Actin contribution to LLO-induced mitochondrial fragmentation. (A) Immunofluorescence images of HeLa cells mock-treated or preincubated with 2.5 μM cytochalasin D for 30 min to depolymerize actin and then treated for 10 min with 6 nM LLO or for 30 min with 10 μM CCCP, showing phalloidin staining of actin in the grayscale images, mitochondria (red; CoxIV), and nuclei (blue; DAPI). Actin depolymerization caused partial perinuclear aggregation of mitochondria, but mitochondria retained a tubular morphology. (B) Pooled data from three independent experiments ($n > 150$ cells per experiment, randomly chosen fields of view) showing that cytoD treatment decreases the number of cells with fragmented mitochondria upon LLO treatment. * $P < 0.01$, ** $P < 0.001$ (one-tailed Student t test).

Bacterial Infections. For infection experiments, bacteria were grown overnight in brain-heart infusion medium (Difco; BD Biosciences) at 37 $^{\circ}\text{C}$, subcultured at a 1:10 dilution until an optical density of 0.8–1 was reached, washed three times in medium without serum, and added to cells for 1 h at a multiplicity of infection (MOI) of 50. The strains used were as follows: wild-type *L. monocytogenes* (EGD, BUG600), *L. monocytogenes* Δhly (EGD, BUG2132), and *L. innocua* overexpressing InIB (BUG1642).

Cell Culture, Drug Treatments, and Transfection. HeLa CCL2 cells were cultured according to American Type Culture Collection guidelines. MEF (kind gift from K. Mihara, Kyushu University, Japan) and U2OS Sec61 β -GFP cells (kind gift from Yoko Shibata and Tom Rapoport, Harvard Medical School, Boston; for the Sec61 β -GFP plasmid, see ref. 34) were maintained in DMEM supplemented with 10% (vol/vol) FCS. HCT116 cells were maintained in McCoy's 5A medium supplemented with 10% FCS, nonessential amino acids, and glutamine. All cells were maintained under 10% (vol/vol) CO_2 . Media/additives

were purchased from Invitrogen. Drugs were purchased from Sigma-Aldrich and incubated in full medium unless otherwise stated. Drugs were incubated at the following concentrations and times: 10 μM CCCP for 30 min, 0.1 μM valinomycin for 30 min, 1.2 μM staurosporine for 90 min, 0.05% (wt/vol) digitonin for 5 min, 20 μM MG132 and 20 μM lactacystine for 90 min, 2.5 μM cytochalasin D for 30 min (HeLa) and 1 μM for 20 min (MEF), and 2–6 nM LLO for 10 min (in absence of FCS). DNA transfection (3.5-cm wells) was performed with FuGENE HD (Roche) according to the manufacturer's instructions. The following plasmids were used for live-cell imaging: pDsRed2-mito (Clontech; 0.2 μg), Mff-GFP (ref. 9; 0.125 μg), mCherry-Drp1 (ref. 9; 0.1 μg), Drp1-GFP (ref. 23; 0.5 μg), BFP-mito (ref. 9; 0.5 μg), OM-GFP ("Mito-GFP", ref. 40; 1 μg), and K38A-HA Drp1 (ref. 23; 4 μg). siRNA transfection was performed with Oligofectamine (Invitrogen) according to the manufacturer's instructions, using Drp1-specific sequences described in ref. 15.

After treatment, cells were either analyzed with a spinning disk microscope for live-cell imaging or processed for immunofluorescence with the antibodies indicated in the respective figure legends (see *SI Materials and Methods* for antibody catalog numbers and for detailed live cell imaging immunofluorescence procedures).

Quantitative RT-PCR. Total RNA was extracted using the RNeasy kit (Qiagen) and treated with TurboDNase (Ambion). cDNA was synthesized from 500 ng of RNA with the RT² cDNA synthesis kit (Qiagen). Standard quantitative RT-PCR was performed with the EvaGreen kit (Bio-Rad) on a Bio-Rad CFX machine, and gene expression was calculated by the 2^{- $\Delta\Delta$ Ct} method. Two independent primer sets were used for MFF. Data represent the mean of two biological replicates and three technical replicates.

Western Blotting. Cells were lysed with 2 \times Laemmli loading buffer (124 mM Tris-HCl at pH 6.8, 4% (vol/vol) SDS, 20% (wt/vol) glycerol, 0.02% bromophenol blue, and 0.03% DTT), and genomic DNA was digested with Benzonase (Novagen). Proteins were separated by SDS/PAGE (Bio-Rad), transferred by wet transfer to a nitrocellulose membrane (GE Healthcare) that was blocked in 10% (wt/vol) milk; antibodies were incubated in milk at dilutions indicated by the manufacturers. Quantification of Western blots was accomplished by using the Gel Analyzer plugin in ImageJ. The Mff signal was normalized to GAPDH signal, and ≥ 3 independent experiments were analyzed.

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