The Gram-positive bacterium *Listeria monocytogenes* is a facultative intracellular pathogen whose virulence depends on its ability to spread from cell to cell within an infected host. Although the actin-related protein 2/3 (Arp2/3) complex is necessary and sufficient for *Listeria* actin tail assembly, previous studies suggest that other actin polymerization factors, such as formins, may participate in protrusion formation. Here, we show that Arp2/3 localized to only a minor portion of the protrusion. Moreover, treatment of *L. monocytogenes*–infected HeLa cells with a formin FH2-domain inhibitor significantly reduced protrusion length. In addition, the Diaphanous-related formins 1–3 (mDia1–3) localized to protrusions, and knockdown of mDia1, mDia2, and mDia3 substantially decreased cell-to-cell spread of *L. monocytogenes*. Rho GTPases are known to be involved in formin activation. Our studies also show that knockdown of several Rho family members significantly influenced bacterial cell-to-cell spread. Collectively, these findings identify a Rho GTPase–formin network that is critically involved in the cell-to-cell spread of *L. monocytogenes*.

**Keywords.** *Listeria monocytogenes*; diaphanous formins; mDia1, mDia2, mDia3; protrusion; *Listeria* cell-to-cell spread; Arp2/3; HeLa cells.
factors able to directly catalyze the formation of actin filaments are members of the formin family, which possess conserved formin-homology domains 1 (FH1) and FH2 [12]. In contrast to the actin filaments assembled by the Arp2/3 complex, formins drive polymerization of unbranched actin filaments [12]. Formins are ubiquitously expressed in mammalian cells and are known to play essential roles in various fundamental cell processes, including the formation of filopodia, lamellipodia, and cellular protrusions [12–15]. Moreover, efficient protrusion formation and intercellular spread of Shigella flexneri and Rickettsia parkeri, bacteria with life cycles similar to that of L. monocytogenes, were shown to depend on actin polymerization by formins or formin-like proteins [16, 17]. Collectively, these findings support the notion that formins may play an important role in L. monocytogenes–induced protrusion formation and cell-to-cell spread. However, which ones are involved and what factors may regulate them are not known.

Here, we investigated the involvement of formins in cell-to-cell spread by L. monocytogenes. Our findings demonstrate that the Diaphanous-related formins are involved in L. monocytogenes–induced protrusion formation and, in conjunction with members of the Rho GTPase family, are important facilitators of dissemination of these bacteria.

METHODS

Cell Culture and Bacterial Strains
HeLa epithelial cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Wisent) without antibiotics at 37°C and 5% CO2. Cells were seeded in 24-well tissue culture plates on glass coverslips at 2.5 × 104 cells/well and 5.0 × 104 cells/well for small interfering RNA (siRNA) and plasmid transfection experiments, respectively, involving direct visual analysis. Wild-type L. monocytogenes 10403S [18] and 10403S ΔactA [19] were used for infection studies as indicated.

Antibodies, Reagents, and Constructs
Details about these materials are available in the Supplementary Materials.

siRNA and Endoribonuclease-Prepared siRNA (esiRNA) Treatment
A complete listing of the siRNA and esiRNA oligos used in this study are specified in Supplementary Table 2, and details about these analyses are available in the Supplementary Materials. The efficiency of knockdown was confirmed for select factors (Supplementary Figure 1).

Bacterial Infection
L. monocytogenes were grown for approximately 16 hours in brain-heart infusion (BHI) broth at 30°C without shaking, subcultured 1:10 in BHI without antibiotics, and grown at 37°C for 2 hours with shaking (to an OD600 of approximately 0.3, which is equivalent to approximately 3 × 108 colony-forming units/mL). Bacterial inocula were prepared by pelleting at 10 000 × g for 1–2 minutes, washing twice, and resuspending in phosphate-buffered saline (PBS). Bacterial inocula were then diluted in DMEM without FBS. Cells were washed twice in PBS and infected at a multiplicity of infection of 100. Bacteria were centrifuged onto cells at 225g for 3 minutes at room temperature, and infected cells were incubated at 37°C with 5% CO2. Sixty minutes after infection, extracellular bacteria were removed by extensive washing with PBS. Gentamicin was added to the medium to achieve a final concentration of 10 µg/mL, which was maintained throughout the duration of the experiment.

Immunofluorescence
Details about this analysis are available in the Supplementary Materials.

Microscopy and Image Preparation
Details about these techniques are available in the Supplementary Materials.

Protrusion and Comet Tail Analysis
Images of so-called primary infected cells (ie, host cells containing >50 intracellular bacteria) were acquired, and the following parameters were analyzed using Volocity: (1) number of host cells, (2) total number of bacteria, (3) number of protrusions and actin (ie, comet) tails, and (4) lengths of protrusions and actin tails. A protrusion was defined as a bacteria-associated extension of the plasma membrane that stained positive for ezrin and actin. A comet tail was defined as a bacteria-associated actin tail that stained negative for ezrin. Only protrusions and comet tails that were >1 µm long were included in the analysis.

Plaque Assay
A total of 2.0 × 105 HeLa cells were seeded per well in 6-well tissue culture plates. Cells were transfected with siRNA 24 hours later. Cells were infected with 4.0 × 104 bacteria 48 hours after transfection. After 1 hour of infection, cells were washed 3 times with PBS and were overlaid with a 0.7% agarose–DMEM mixture containing 50 µg/mL gentamicin. At 96 hours after infection, a second agarose-medium overlay containing 6% neutral red (Sigma; N2889) and 50 µg/mL gentamicin was added. After 8 hours, plaques were imaged using a Fluorchem E scanner (Proteinsimple). Plaque diameter was measured using Adobe Photoshop, and the area was calculated as 3.14159 × [diameter/2]². Plaque area was normalized to control siRNA/esiRNA-treated cells infected with wild-type bacteria for each experiment.

Western Blotting and Real-Time Reverse-Transcription Polymerase Chain Reaction Analysis
Details about these analyses are available in the Supplementary Materials.
Data were analyzed using GraphPad Prism v5.0 for Mac OS X (GraphPad Software). Data are expressed as mean ± standard error of the mean. Results were analyzed using analysis of variance with a post hoc test (described in detail in the figure legends) or an unpaired t test. Differences were considered statistically significant at P values of <.05.

RESULTS

The Arp2/3 Complex Differentially Localizes Along L. monocytogenes Actin Comet Tails and Protrusions

The available evidence in the literature implicates the involvement of actin polymerization factors with the capacity to generate linear filaments in the formation of L. monocytogenes protrusions [10]. To investigate this possibility, we examined localization of Arp p34, a subunit of the Arp2/3 complex [20], to protrusions and comet tails. To this end, HeLa cells were transfected with LifeAct-RFP (to visualize F-actin) and infected for 8 hours, and then the presence of the Arp2/3 complex on protrusions and comet tails was examined by immunofluorescence analysis. The membrane-cytoskeleton linker ezrin has been shown to localize to L. monocytogenes–induced protrusions but not to comet tails [10, 21]. Thus, for the purpose of analysis, protrusions were identified as bacteria-associated actin tails enriched with ezrin (ie, actin+ ezrin+), while comet tails were identified as bacterial-associated actin tails that stained negative for ezrin (ie, actin− ezrin−; Figure 1A). Arp p34 localized, to some extent, to all protrusions and comet tails observed (Figure 1A).

In the case of protrusions, we noted that Arp p34 enrichment was largely limited to approximately one-third the length of the protrusion (Figure 1B), emanating from the bacterial-actin interface and proceeding down the tail. In contrast, Arp p34 staining was often found along the large majority, if not the entirety, of the comet tail. The reduction in Arp p34 staining among protrusions, compared with comet tails, was further confirmed on the basis of Arp p34 staining intensity measurements (data not shown).

Formins Are Required for Protrusion Formation

To ascertain whether formins may be involved in the formation of protrusions, we infected HeLa cells with L. monocytogenes for 8 hours and treated them with a formin FH2-domain inhibitor (SMIFH2) during the latter 4 hours of infection. Our intent was to begin treatment with SMIFH2 at a time when primary infection was fully established (ie, approximately 1 hour after infection), but protrusion formation was not overt (ie, approximately 6 hours after infection). This small-molecule inhibitor of formin FH2 domains has been shown to prevent formin-mediated actin nucleation and processive barbed-end elongation [22]. Importantly, these effects were specific to formins as this compound did not appear to interfere with Arp2/3 complex-driven processes at the dose used in our study [22].

Treatment of L. monocytogenes–infected HeLa cells with SMIFH2 resulted in a significant decrease in average protrusion length (Figure 2A and 2G). Although the effect was modest, treatment with the formin FH2-domain inhibitor clearly influenced the size distribution of protrusions, as nearly 70% of protrusions were <4 μm in length in the SMIFH2-treated group, compared with approximately 45% of protrusions in vehicle-control-treated infected cells (Figure 2B). No differences were observed in terms of protrusion frequency (ie, number of protrusions per infected host cell) between L. monocytogenes–infected cells treated with SMIFH2 and those treated with vehicle control (Figure 2C). Treatment of infected cells with SMIFH2 did not influence the average length, size distribution, or frequency of comet tails (Figure 2D–F).

mDia1, mDia2, and mDia3 Selectively Localize to Protrusions and Are Required for Efficient Cell-to-Cell Spread of L. monocytogenes

Given that protrusion formation is critical to L. monocytogenes cell-to-cell spread and that treatment with a general formin inhibitor influenced protrusion length, we sought to specifically identify the formins that may be involved in cell-to-cell spread of L. monocytogenes. To this end, we screened the approximately 15 mammalian formins for localization to protrusions. We transfected HeLa cells with epitope-tagged constructs of formin proteins and examined their association with protrusions in HeLa cells 8 hours after infection. Of the 15 formins we examined, only the mDia proteins were enriched along protrusions (>95% of protrusions), while none of the other formins were present above background levels on these structures (<5% of protrusions; Figure 3A and data not shown). In addition, mDia1-3 were rarely, if ever, observed to associate with comet tails (<5% of comets; Figure 3B and data not shown).

Next, we investigated whether the mDia formins were required for L. monocytogenes cell-to-cell spread in a plaque assay, using esiRNA. esiRNAs are a pool of siRNA-like oligonucleotides that cover a larger region of the messenger RNA being targeted for depletion. The different siRNAs that constitute this pool are present in comparable quantities and share the same on-target gene. As a result, the silencing capacity is augmented, and off-target effects are reduced [23]. Knockdown of mDia1, mDia2, and mDia3 led to a substantial reduction in plaque area, indicative of diminished cell-to-cell spread (Figure 4A and 4B).

We also examined the influence of mDia1, mDia2, and mDia3 knockdown on protrusion formation. Depletion of mDia1 and mDia3 significantly reduced the average protrusion length, while knockdown of mDia2 had only a minor, statistically insignificant effect, compared with control esiRNA–treated cells (Figure 4C). No differences were observed with respect to protrusion frequency, comet tail length, or comet tail frequency between any of the mDia formin-depleted groups and the control group (Figure 4C and data not shown).
Figure 1. The actin-related protein 2/3 (Arp2/3) complex is primarily associated with comet tails. A, HeLa cells were transfected with LifeAct-RFP (F-actin probe), infected with *Listeria monocytogenes* for 8 hours, and immunostained with DAPI (to visualize bacteria) and antibodies to Arp p34 and ezrin. Representative confocal images are shown of a protrusion (inset 1: actin+ ezrin+) and comet tail (inset 2: actin+ ezrin−). Arrowheads denote the bacterium associated with the protrusion/comet tail identified by the dashed line. Scale bar, 10 µm for low magnification and 3 µm for I1 and I2. B, The length along the protrusion/comet tail demonstrating Arp p34 enrichment (based on signal thresholding) was quantified and expressed as a percentage of the total protrusion/comet tail length. Arp p34 staining was measured on at least 50 protrusions and comet tails. Data show individual measurements along with average values (± standard error of the mean). Statistical analysis was performed using an unpaired t test. ***P<.001.
Rho GTPases Participate in *L. monocytogenes* Cell-to-Cell Spread

Several reports have demonstrated that the mDia subfamily of formins function, at least in part, as Rho GTPase effectors. Thus, in an effort to identify the involvement of Rho GTPases in *L. monocytogenes* cell-to-cell spread, we screened a limited group of Rho GTPases that have been previously implicated in mDia activation. Using siRNA, we investigated the influence of individually depleting select GTPases on *L. monocytogenes* cell-to-cell spread by plaque assay. Of the GTPases examined, knockdown of Rac1, Cdc42, RhoA, RhoC, and RhoD led to considerable decreases in plaque size (Figure 5). Treatment with siRNA against RhoB, RhoG, and Rac2 had no effect on plaque size.

**DISCUSSION**

The mechanisms used by *L. monocytogenes* to drive intracellular motility within an infected host cell are well understood. Comparatively less is known regarding the processes and factors involved in the formation of protrusions and cell-to-cell spread. Given that the host Arp2/3 complex is important for...
L. monocytogenes comet tail formation and intracellular motility, we examined the localization of this complex to protrusions. In agreement with a recent report, we noted that in contrast to comet tails, where Arp2/3 is localized along the majority of the tail, this complex is enriched along a small portion of the protrusion [24], indicating to us that other host actin nucleating factors may contribute to protrusion formation.

The notion that formins are involved in the dissemination of L. monocytogenes is in line with previous studies that have demonstrated the importance of formins or formin-like factors...
in the spread of various other human pathogens. Indeed, FHOD1 has recently been shown to facilitate robust actin-based motility of host-cell extracellular-associated vaccinia viral particles, leading to protrusion formation and dissemination to neighboring cells in vitro [25]. Notably, recruitment of FHOD1 and its involvement in actin tail formation was shown to be dependent on Rac1 activity [25]. In addition, the surface cell antigen 2 (Sca2) of Rickettsia is critically required for actin-tail formation and virulence [26]. Of significance, Sca2 functions, similarly to eukaryotic formins, as a profilin-dependent barbed-end elongation factor that directly mediates polymerization of host actin into unbranched filaments [17] to propel bacteria throughout the host cell. In the case of S. flexneri, studies performed by Heindl et al showed that, upon depletion of mDia1 and mDia2, the frequency of protrusions was decreased relative to controls and that this effect was associated with impaired spread [16]. Codepletion of mDia1 and mDia2 did not further influence protrusion formation. In contrast, we did not observe a difference in the number of protrusions formed following treatment of L. monocytogenes–infected HeLa cells with either an FH2-inhibitor or an esiRNA against mDia1–3. These findings suggest that, in the context of L. monocytogenes, other factors in addition to formins may participate in protrusion elongation and that the initiation of these structures may not be impaired by formin inhibition. As neither SMIFH2 treatment nor mDia1–3 esiRNA influenced comet tail length or number, perhaps it is not unexpected that L. monocytogenes was able to contact the plasma membrane and initiate protrusion assembly to the same extent as in control-treated cells. Interestingly, although it did not reach statistical significance,
inhibition of mDia1 or mDia2 showed a trend toward decreased Shigella actin tail length within the host cell body (ie, comets), indicating that, in addition to their involvement in protrusions, these formins may also participate in the assembly of Shigella comet tails [16]. Therefore, mDia proteins may influence Shigella cell-to-cell spread by regulating protrusion frequency and, perhaps, comet tail length, while in the case of L. monocytogenes, mDia proteins may facilitate cell-to-cell spread by mediating protrusion elongation.

At present, the possibility that mDia inhibition indirectly influenced L. monocytogenes cell spread in some fashion, such as by affecting cell-cell interactions, cannot be excluded. However, the reduction in protrusion length following targeted depletion of mDia1, mDia3, and, to a small extent, mDia2, coupled with the observation that mDia formins localize to protrusions, argues against the contribution of indirect effects. Moreover, decreased protrusion length has been previously shown to correlate with reduced cell-to-cell spread [27].

Yeast 2-hybrid-based analyses conducted over a decade ago identified mDia family members as downstream effectors of the Rho family of small GTPases [28, 29]. Binding of activated Rho proteins to the Rho GTPase–binding domain of mDia formins induces these formins to nucleate and polymerize actin [12]. Since these discoveries, a network of interactions between these 2 groups of proteins has been described, and various processes were shown to involve cooperation between Rho GTPases and mDia proteins [29–34] including, stress fiber formation, vesicle movement, and filopodia and lamellipodia protrusion [13, 22, 30, 34–38]. To begin to discover the potential network of Rho GTPases that may be involved in L. monocytogenes cell-to-cell spread, we screened a subset of the Rho GTPase family by using a conventional plaque assay. Several of the GTPases screened proved to be required for efficient cell-to-cell spread. Our observation that Cdc42 knockdown led to diminished L. monocytogenes spread is, seemingly, in disagreement with a previous report [39]. Rigano et al noted that efficient protrusion formation and spread of Listeria required the effector protein internalin C (InlC), which mediated the downregulation of activated Cdc42. Of significance, there were several well-recognized differences among the methods used that could account for these discordant results. Overexpression of a dominant-negative construct was used by Rigano et al to interfere with Cdc42, and their studies were conducted in the context of a polarized cell-line (Caco-2 BBE1 cells), whereas we used an siRNA-based approach and a nonpolarized cell line (HeLa cells). Alternatively, it is conceivable that Listeria drives local inhibition of Cdc42 at sites where InlC or other virulence factors are localized. But there may be a general requirement for Cdc42 (eg, for trafficking processes) at other cellular sites that are required for productive spread. On the basis of the experimental approach used here, we cannot rule out the possibility that some of the GTPases we examined may affect cell-to-cell spread through involvement in 1 or more events upstream and/or downstream of protrusion formation (eg, invasion, comet tail formation, and uptake of L. monocytogenes by neighboring uninfected host cells). It is worth noting that the findings of some studies, but not all, suggest that select Rho GTPases may play a role in promoting L. monocytogenes invasion, although perhaps not intracellular motility [40–45]. Noteworthy, the evidence suggests that involvement of Rho GTPases in these processes may depend, in part, on the cell type examined and the mechanism of invasion used by L. monocytogenes.

It is becoming increasingly clear that the formation of membrane protrusions by L. monocytogenes and related pathogens is an active process and not simply a byproduct of the forces generated by actin-based cytosolic propulsion. Indeed, Escherichia coli expressing IcsA, the molecule that is necessary and sufficient for Shigella actin-based motility, was adequate to promote protrusion formation in a manner that was largely related to the direction but not the speed of intracellular travel [46]. This finding is evidence that a primary objective of intracellular motility may be to contact the membrane and not necessarily to provide the forces required to project through it. This is not to say that the

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Knockdown of select Rho GTPases reduces cell-to-cell spread of Listeria monocytogenes. HeLa cells were transfected with control small interfering RNA (siRNA) or siRNA against the indicated Rho GTPases. Forty-eight hours after transfection, cells were infected with L. monocytogenes, and a plaque assay was performed as described in Materials and Methods. Data show the average plaque area relative to control siRNA-treated plaques (± standard error of the mean) for 3 independent experiments. The average plaque areas, by treatment group, were as follows: 5.93 mm² for control, 3.50 mm² for Rac1, 5.95 mm² for Rac2, 2.14 mm² for Cdc42, 3.50 mm² for RhoA, 5.77 mm² for RhoB, 2.72 mm² for RhoC, 2.41 mm² for RhoD, and 5.63 mm² for RhoG. Statistical analysis was performed using 1-way analysis of variance with the Dunnett multiple comparison post-hoc test. *P<.05, **P<.01, and ***P<.001, compared with the control siRNA–transfected group.
force of contact is not an important factor for protrusion formation, but rather that it is not likely to be the sole contributing factor determining whether a protrusion develops. In this regard, disruption of ezrin, a key membrane-cytoskeleton linker that accumulates at *L. monocytogenes*–induced protrusions, specifically reduces the number and morphology (shortened and collapsed) of protrusions, resulting in impaired cell-to-cell spread [21]. Myosin-X has also been shown to play a critical role in the development of protrusions [27]. As a consequence of Myo10 depletion, *Shigella* protrusions were stunted, and cell-to-cell spread was dramatically diminished. Myo10 was observed to concentrate at the base of *Shigella* and to cycle along its sides within a protrusion as the protrusion lengthened. Similarly, Myo10 accumulated in *L. monocytogenes*–induced protrusions, and knockdown of this protein also impaired bacterial cell-to-cell spread [27]. In addition, InlC of *L. monocytogenes* has been shown to facilitate protrusion formation by regulating cell membrane tension [47]. InlC was found to interfere with the interaction between the mammalian adaptor protein Tuba and the actin regulator N-WASP2, which together regulate cortical actin tension at apical junctions. Disruption of the Tuba-N-WASP interaction by binding of InlC to Tuba, via a C-terminal SH3 domain, resulted in a loss of membrane tension, creating slack that permits protrusion formation by *L. monocytogenes*. Disruption of components of the host cell actin network disassembly machinery has also been reported to impair *L. monocytogenes* spread. Specifically, codepletion of actin-interacting protein 1 and coflin 1 dramatically reduced the velocity of *Listeria* in protrusions, led to altered protrusion morphology, and decreased bacterial spread to adjacent uninfected host cells [24]. Collectively, these findings strongly suggest that bacterial effector proteins in conjunction with host cell factors actively drive the formation of bacterial-associated protrusions.

The observations presented here identify a potential network of formins and Rho GTPases that aid in active protrusion formation (Figure 6). *L. monocytogenes* forms ActA- and Arp2/3-dependent comet tails within the host cell cytoplasm [2, 5, 6]. Similar to the involvement of N-WASP and FHOD1-Rac1 pathways in the spread of vaccinia virus [25], processes related to actin-based motility and/or contact with the cell membrane may activate Rho GTPases, which, in turn, recruit and induce the activation of formins, such as mDia1–3. Subsequently, these mDia proteins contribute to the elongation of actin filaments in protrusions, independent of Arp2/3, thereby promoting *L. monocytogenes* cell-to-cell spread. Thus, our findings show that *L. monocytogenes* exploits the mDia subfamily of formins to promote effective cell-to-cell spread. Characterization of the precise array of host factors involved specifically in the protrusion phase may help to identify novel strategies through which the spread and resulting pathologies of intracellular pathogens such as *L. monocytogenes* can be abrogated.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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