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The host scaffolding protein Filamin A and the exocyst complex control exocytosis during InlB-mediated entry of *Listeria monocytogenes*

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Running title:

Control of host exocytosis during InlB-mediated entry

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18   **SUMMARY**

19   *Listeria monocytogenes* is a food-borne bacterium that causes gastroenteritis,

20   meningitis, or abortion. *Listeria* induces its internalization (entry) into some human

21   cells through interaction of the bacterial surface protein InlB with its host receptor,

22   the Met tyrosine kinase. InlB and Met promote entry, in part, through stimulation of

23   localized exocytosis. How exocytosis is upregulated during entry is not understood.

24   Here we show that the human signaling proteins mTOR, Protein Kinase C (PKC)-

25   α, and RalA promote exocytosis during entry by controlling the scaffolding protein

26   Filamin A (FlnA). InlB-mediated uptake was accompanied by PKC-α –dependent

27   phosphorylation of serine 2152 in FlnA. Depletion of FlnA by RNA interference

28   (RNAi) or expression of a mutated FlnA protein defective in phosphorylation

29   impaired InlB-dependent internalization. These findings indicate that phosphorylation

30   of FlnA by PKC-α contributes to entry. mTOR and RalA were found to mediate the

31   recruitment of FlnA to sites of InlB-mediated entry. Depletion of PKC-α, mTOR, or

32   FlnA each reduced exocytosis during InlB-mediated uptake. Because the exocyst

33   complex is known to mediate polarized exocytosis, we examined if PKC-α, mTOR,

34   RalA, or FlnA affect this complex. Depletion of PKC-α, mTOR, RalA, or FlnA

35   impaired recruitment of the exocyst component Exo70 to sites of InlB-mediated entry.

36   Experiments involving knockdown of Exo70 or other exocyst proteins demonstrated

37   an important role for the exocyst complex in uptake of *Listeria*. Collectively, our

38   results indicate that PKC-α, mTOR, RalA, and FlnA comprise a signaling pathway

39   that mobilizes the exocyst complex to promote infection by *Listeria*.

40

INTRODUCTION

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42   *Listeria monocytogenes* is a food-borne bacterium that causes gastroenteritis,

43   meningitis or abortion (1). Critical for disease is the ability of *Listeria* to induce its

44   internalization (entry) into nonphagocytic cells in the intestine, liver, or placenta (2).

45   A major pathway of *Listeria* entry is mediated by binding of the bacterial surface

46   protein InlB to its host receptor, the Met tyrosine kinase (3). Binding of InlB activates

47   Met, resulting in the stimulation of two host processes that promote bacterial uptake:

48   actin polymerization and exocytosis (4-6).

49   Actin polymerization is thought to contribute to entry of *Listeria* by providing

50   a protrusive force that drives the host plasma membrane around adherent bacteria (4,

51   7). Exocytosis is the fusion of intracellular vesicles with the plasma membrane (8).

52   How membrane flow through exocytosis controls InlB-dependent uptake is not fully

53   understood. One potential mechanism involves the delivery of the GTPase Dynamin 2

54   to the host plasma membrane (6). During InlB-mediated entry, Dynamin 2

55   translocates from an internal membrane compartment termed the recycling endosome

56   (RE) to sites in the plasmalemma near adherent bacteria. Dynamin 2 is known to

57   remodel membranes through a GTP-dependent scission activity and also through

58   interaction with membrane sculpting proteins containing BAR domains (9). These

59   membrane remodeling activities of Dynamin 2 are likely responsible for its role in

60   InlB-mediated entry (6, 10).

61   How are actin polymerization and exocytosis stimulated during InlB-

62   dependent uptake of *Listeria*? Substantial progress has been made on the mechanism

63   of actin polymerization, revealing that this process is mediated by the host Arp2/3

64   complex and the nucleation promoting factors N-WASP and WAVE (5, 11, 12). By

65   contrast, little is known about how exocytosis is induced during InlB-mediated

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66   internalization, except that induction requires the kinase activity of Met and the host

67   GTPase RalA (6).

68   Previous results demonstrated that the human serine/threonine kinases mTOR

69   and Protein Kinase C-α (PKC-α) are activated downstream of Met and play important

70   roles in InlB-mediated entry of *Listeria* (13). mTOR and PKC-α comprise a signaling

71   pathway involved in several biological events, including cell migration and survival

72   (14, 15). mTOR forms part of a multi-component complex called mTORC2 that

73   phosphorylates serine 657 in a hydrophobic motif in PKC-α, thereby upregulating

74   PKC kinase activity (16). Importantly, InlB-mediated entry of *Listeria* is

75   accompanied by mTOR-dependent phosphorylation of serine 657 in PKC-α,

76   indicating that mTOR functions upstream of PKC-α during entry (13). How mTOR

77   and PKC-α control internalization of *Listeria* is not well understood. Although PKC-

78   α has a minor role in actin polymerization during entry, mTOR has no detectable

79   effect on this process (13). It therefore seems likely that mTOR and PKC-α affect

80   uptake of *Listeria* by regulating host processes apart from the assembly of actin

81   filaments. One such process could be exocytosis.

82   In this work, we show that mTOR and PKC-α promote exocytosis during

83   InlB-mediated entry of *Listeria* by controlling the human scaffolding protein Filamin

84   A (FlnA). InlB-dependent internalization was accompanied by an increase in

85   phosphorylation of serine 2152 in FlnA, an event mediated by PKC-α. mTOR

86   promoted recruitment of FlnA to sites of InlB-mediated entry. The GTPase RalA was

87   also needed for FlnA recruitment. Depletion of FlnA by RNA interference (RNAi) or

88   expression of a mutated form of FlnA that is not phosphorylated on serine 2152

89   inhibited entry, indicating an important role for FlnA in infection by *Listeria*. Further

90   experiments revealed that mTOR, PKC-α, and RalA each control exocytosis during

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91   entry by recruiting the exocyst, a multicomponent complex known to promote

92   exocytosis by tethering vesicles to sites in the plasma membrane (17). RNAi

93   experiments demonstrated an important role for the exocyst complex in InlB-

94   mediated entry. Collectively, these findings identify a host signaling pathway that

95   controls the exocyst complex to promote infection of human cells by *Listeria*.

96

97

98   **RESULTS**

99   **The host scaffolding protein FlnA promotes InlB-mediated entry of *Listeria***

100 Previous results demonstrated that the host serine/threonine kinases mTOR and PKC-

101 α act together to control InlB-mediated entry of *Listeria* (13). In order to better

102 understand how these two kinases regulate InlB-dependent internalization, we

103 focused our attention on FlnA, a protein that bundles actin filaments and assembles

104 multi-molecular signaling complexes (18). This protein is known to interact with

105 PKC-α or the mTORC2 component Rictor, and is directly phosphorylated by PKC-α

106 (19-22).

107   We used RNAi to examine the role of FlnA in InlB-mediated invasion of

108 *Listeria* into the human epithelial cell line HeLa. In order to control for potential off-

109 target effects (23), three different siRNAs targeting distinct sequences in FlnA mRNA

110 were used. Control conditions included mock transfection in the absence of siRNA or

111 transfection with a control "non-targeting" siRNA that lacks complementarity to any

112 known mRNA. Importantly, each of the three siRNAs against FlnA reduced

113 expression of the target protein and inhibited invasion of *Listeria* into HeLa cells

114 compared to the control conditions (Fig. 1A,B).

115   We also determined the effect of siRNA-mediated depletion of FlnA on entry

116 of inert particles coated with InlB. Latex beads (3 µm in diameter) have been

117 extensively used as a model for InlB-dependent entry, since these particles lack other

118 bacterial factors and are efficiently internalized into mammalian cells in a manner that

119 depends on the Met receptor and other host proteins involved in *Listeria* uptake (6,

120 11-13, 24-28). As previously reported (6, 13, 26, 29), beads coupled to InlB were

121 efficiently internalized into HeLa cells, whereas control beads coupled to glutathione

122 S-transferase (GST), were not internalized (Fig. 1Ci). Next, the siRNA against FlnA

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123   that caused the largest inhibition in invasion of *Listeria* was used to deplete FlnA in

124   HeLa cells and the effect on uptake of InlB-coated beads was assessed. Internalization

125   of beads was inhibited by about 40% (Fig. 1Cii). Taken together, the results in Figure

126   1 indicate an important role for host FlnA in InlB-dependent entry.

127

128   **InlB induces PKC-α -dependent phosphorylation of FlnA**

129   FlnA is comprised of an amino-terminal actin binding domain and 24

130   immunoglobulin (Ig)-like domains (18). One of the phosphorylation sites for FlnA is

131   serine 2152, located in Ig domain 20. Phosphorylation of this residue controls several

132   biological events, including focal adhesion formation, lamellipodia formation, protein

133   trafficking, and cell migration (20, 22, 30-33).

134   We investigated whether the InlB-mediated pathway of entry involves

135   phosphorylation of serine 2152 in FlnA. The effects of InlB on FlnA phosphorylation

136   were assessed in two different conditions: treatment of mammalian cells with soluble

137   InlB protein and entry of InlB-coated beads. When used at low nanomolar

138   concentrations, soluble InlB is a potent agonist of the Met receptor and its associated

139   downstream signaling pathways (3, 13, 34, 35). Importantly, treatment of HeLa cells

140   with 4.5 nM of soluble InlB for 10 min caused an increase in reactivity of cell lysates

141   with antibodies generated against phosphorylated serine 2152 in FlnA (Fig. 2Ai).

142   Results from two types of control experiments indicated that these anti-phospho-FlnA

143   antibodies recognize predominantly phosphorylated serine 2152 in FlnA in HeLa

144   cells. First, transfection of HeLa cells with an siRNA targeting FlnA reduced

145   reactivity with the anti-phospho-FlnA antibodies (Fig. 2Ai). Secondly, substitution of

146   serine 2152 with an alanine residue (S2152A) decreased reactivity with these

7

147   antibodies (Fig. 2Aii). Collectively, the results in Figure 2A indicate that soluble InlB

148   stimulates phosphorylation of serine 2152 in FlnA.

149   Several serine/threonine kinases including PAK1, p70S6K, and PKC-α are

150   known to phosphorylate S2152 in FlnA (19 20, 30, 36). Since PKC-α plays an

151   important role in InlB-mediated entry of *Listeria* (13), we examined if this kinase

152   mediates phosphorylation of FlnA induced by InlB. Importantly, siRNA-mediated

153   depletion of PKC-α reduced phosphorylation of FlnA on serine 2152 in HeLa cells

154   treated with soluble InlB protein (Fig. 2B). The Met receptor is required for InlB-

155   induced activation (phosphorylation) of PKC-α (13). As expected, siRNA-mediated

156   knockdown of Met inhibited FlnA phosphorylation of serine 2152 (Fig. 2C). These

157   results demonstrate that Met and PKC-α are needed for efficient phosphorylation of

158   FlnA in response to InlB.

159   Experiments involving InlB-coated beads demonstrated that incubation with

160   these particles stimulated phosphorylation of FlnA on serine 2152 (Figure 2D). These

161   findings demonstrate that phosphorylation of FlnA increases during InlB-mediated

162   entry.

163

164   **Phosphorylation of FlnA contributes to InlB-mediated entry**

165   In order to determine if phosphorylation of FlnA on serine 2152 affects InlB-

166   dependent internalization, we used a FlnA mutant protein containing a serine-to-

167   alanine substitution at amino acid 2152. This S2152A mutation in FlnA causes defects

168   in membrane ruffling (30), cell migration (32), or trafficking of the lipid raft

169   component caveolin-1 (20), indicating that it perturbs FlnA function. We compared

170   the efficiency of InlB-mediated entry into HeLa cells transiently expressing myc-

171   tagged wild-type FlnA or FlnA.S2152A. The myc-FlnA wild-type and myc-

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172   FlnA.S2152A proteins were expressed at similar levels, as assessed by Western

173   blotting (Figure 3A) or confocal microscopy analysis (Fig. 3Bii). Importantly, entry

174   of InlB-coated beads was about 35% lower into cells expressing myc-FlnA.S2152A

175   compared to cells expressing the myc-FlnA wild-type protein (Fig. 3Bi). These results

176   indicate that phosphorylation of FlnA on serine 2152 contributes to InlB-dependent

177   uptake.

178

179   **mTOR and RalA mediate recruitment of FlnA during InlB-dependent entry**

180   Incubation of HeLa cells with InlB-coated beads resulted in accumulation of FlnA in

181   cup-like structures around particles (Fig. 4A). By contrast, incubation with control

182   GST-coated beads failed to induce FlnA accumulation. Accumulation of FlnA was

183   quantified by measuring fold enrichment (FE) values, essentially as described (6, 13,

184   28, 29). FE is defined as the mean fluorescence intensity of a host protein of interest

185   in a cup-like structure around beads normalized to the mean fluorescence intensity of

186   the protein throughout the entire human cell. An FE value greater than 1.0 indicates

187   enrichment of the host protein around particles. The mean FE value for FlnA in

188   control conditions involving HeLa cells that were mock transfected or transfected

189   with control siRNA was about 1.35, indicating enrichment (Figure 4B). By

190   comparison, the mean FE value for cells incubated with GST-coated beads was less

191   than 1.0. These results demonstrate that InlB induces a redistribution of FlnA, causing

192   this host protein to accumulate at sites of particle internalization.

193   We next examined the roles of mTOR, PKC-α, and Met in recruitment of

194   FlnA during entry. We previously reported that siRNAs targeting mTOR, PKC-α, or

195   Met inhibit entry of InlB-coated beads into HeLa cells (6, 13). Using these same

9

196   siRNAs, we found that RNAi against mTOR or Met, but not PKC-α, impaired

197   accumulation of FlnA around InlB-coated beads (Figure 4).

198   FlnA is known to interact with the activated form of the GTPase RalA, and

199   this interaction recruits FlnA to filopodia (37). We previously reported that RalA is

200   needed for efficient entry of *Listeria* and InlB-coated beads into HeLa cells (6). Here

201   we found that siRNA-mediated depletion of RalA prevented accumulation of FlnA

202   around InlB-coated beads (Fig. 4). These results suggest that RalA may control InlB-

203   dependent internalization, in part, through recruitment of FlnA. Taken together, the

204   results in Figures 4 indicate that mTOR and RalA act upstream of FlnA to localize

205   this protein to sites of InlB-mediated entry.

206

207   **mTOR, PKC-α, and FlnA control exocytosis during InlB-mediated entry**

208   RNAi-based studies indicate that localized exocytosis during InlB-dependent entry

209   requires the Met receptor and RalA (6). Since RalA controls recruitment of FlnA

210   (Fig. 4), we tested the possibility that FlnA and its regulators mTOR and PKC-α

211   might promote exocytosis during InlB-mediated entry. Exocytosis was detected using

212   a probe consisting of the v-SNARE protein VAMP3 fused to GFP (6, 38). Prior to

213   exocytosis, VAMP3-GFP resides in intracellular vesicles. When vesicles fuse with the

214   plasma membrane during exocytosis, the GFP moiety becomes extracellular

215   (exofacial) and can be labeled with antibodies without cell permeabilization.

216   HeLa cells were subjected to control conditions or transfected with siRNAs

217   against mTOR, PKC-α, FlnA, or Met. As a negative control for a condition

218   previously found to not affect exocytosis, HeLa cells were transfected with an siRNA

219   targeting the Arp3 component of the Arp2/3 complex (6, 29). After siRNA

220   transfection, cells were transfected with a plasmid expressing VAMP3-GFP,

10

221 incubated with beads coated with InlB or GST, fixed, and labeled for exofacial

222 VAMP3-GFP as described (6, 38). Images were acquired by confocal microscopy,

223 and exocytosis was quantified as FE values for exofacial VAMP3-GFP, as described

224 (6). The results indicate that siRNAs against mTOR, PKC-α, FlnA, or Met each

225 reduced exocytosis around InlB-coated particles (Fig. 5). By contrast, the siRNA

226 targeting Arp3 did not affect exocytosis around beads, consistent with previous

227 findings (6). Importantly, experiments in this study or in previously published work

228 indicate that each of the siRNAs used against mTOR, PKC-α, FlnA, Met, or Arp3

229 inhibit target protein expression and internalization of InlB-coated beads (Fig. 1A,C)

230 (6, 13, 26, 29). Taken together, the findings in Figure 5 demonstrate important

231 functions for mTOR, PKC-α, and FlnA in exocytosis during InlB-dependent

232 internalization.

233   Given the role of FlnA in exocytosis during entry, we next determined if

234 phosphorylation of S2152 affects this host process. HeLa cells were co-transfected

235 with plasmids expressing the exocytic probe VAMP3-GFP and myc-tagged wild-type

236 FlnA or FlnA.S2152A. After acquisition of images using confocal microscopy, the

237 degree of exocytosis in cells expressing either myc-tagged FlnA protein was

238 quantified as FE values for exofacial VAMP3-GFP. The results, presented in Figure

239 6, show that exocytosis was ~ 40% lower in cells expressing myc-FlnA.S2152A

240 compared to in cells expressing wild-type FlnA. These findings indicate that

241 phosphorylation of serine 2152 contributes to exocytosis during InlB-mediated entry.

242

243 **mTOR, PKC-α, RalA, and FlnA control exocytosis by recruiting the exocyst**

244 **complex**

11

245   RalA is known to stimulate exocytosis through the exocyst complex (17, 39). This

246   complex is comprised of eight proteins (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15,

247   Exo70, and Exo84) and tethers vesicles to the plasma membrane in a step preceding

248   fusion of these vesicles with the plasma membrane. RNAi studies indicated roles for

249   the exocyst components Sec3, Sec5, Sec8, and Exo70 in invasion of *Listeria* or entry

250   of InlB-coated beads into HeLa cells (Figures 7 and S1).

251   Experiments with constructs comprised of Exo70, Sec5, Sec8, or Sec15 fused to

252   GFP demonstrated that these host proteins accumulate around InlB-coated beads

253   during particle entry (Fig. S2A). Immunolabeling of endogenous Exo70, Sec5, or

254   Sec8 also indicated recruitment (Fig. S2B). Of all the endogenous or GFP-tagged

255   proteins examined, GFP-Exo70 displayed the most pronounced accumulation around

256   InlB-coated beads. We therefore assessed the roles of mTOR, PKC-α, RalA, and

257   FlnA in GFP-Exo70 recruitment. Importantly, treatment of HeLa cells with siRNAs

258   against mTOR, PKC-α, or FlnA each reduced accumulation of GFP-Exo70 around

259   InlB-coated beads (Fig. 8). An siRNA targeting Arp3 did not affect accumulation of

260   GFP-Exo70 around InlB-coated beads, consistent with the lack of effect of Arp3

261   RNAi on exocytosis (Fig. 8) (6). Collectively, these results demonstrate that mTOR,

262   PKC-α, RalA, and FlnA mobilize Exo70 to plasma membrane sites during particle

263   internalization.

264   Further experiments demonstrated that the exocyst complex mediates exocytosis

265   during InlB-dependent entry. RNAi-mediated depletion of Sec3, Sec5, Sec8, or

266   Exo70 each reduced the accumulation of exofacial VAMP3-GFP that normally occurs

267   around InlB-coated particles (Fig. 9). Taken together, the results in Figures 8 and 9

268   demonstrate that mTOR, PKC-α, RalA, and FlnA control recruitment of the exocyst

269   complex to promote exocytosis during InlB-dependent uptake.

270 **DISCUSSION**

271 In this study, we demonstrated that host mTOR, PKC-α, and FlnA each promote

272 exocytosis during InlB-mediated entry of *Listeria*. Our previous results indicate that

273 the host GTPase RalA also contributes to exocytosis during entry (6). The findings in

274 this work, combined with the previous results with RalA, suggest that mTOR, PKC-α,

275 RalA, and FlnA form a signaling pathway that controls exocytosis through

276 recruitment of Exo70, a component of the exocyst complex. Evidence for such a

277 pathway is that PKC-α is needed for efficient phosphorylation of FlnA on serine

278 2152, and that both mTOR and RalA mediate the recruitment of FlnA to sites of InlB-

279 mediated uptake. Collectively, these results suggest that FlnA acts downstream of

280 PKC-α, mTOR, and RalA during entry.

281 How do RalA and mTOR recruit FlnA the plasma membranes during InlB-

282 dependent internalization? FlnA has 24 immunoglobulin (Ig)-like repeats that interact

283 with at least 90 different binding partners, including various receptors, cytoskeletal

284 proteins, transcription factors, and cytoplasmic signaling proteins (18). Importantly,

285 activated RalA binds to Ig repeat 24 in FlnA, and this interaction recruits FlnA to

286 filopodia (37). It is plausible that the same interaction is responsible for the ability of

287 RalA to recruit FlnA during InlB-mediated uptake. In regard to mTOR, Ig repeat 21

288 in FlnA is known to associate with Rictor (21, 22), an essential component of the

289 mTOR-containing complex mTORC2 (16). mTORC2 has an important role in InlB-

290 dependent internalization of *Listeria* (13). Future work should reveal whether

291 mTORC2 contributes to recruitment of FlnA during InlB-mediated entry.

292 Our data indicate that phosphorylation of S2152 in FlnA participates in InlB-

293 mediated uptake and exocytosis. Although phosphorylation of this site controls

294 several biological processes including protein trafficking, cell adhesion, and cell

13

295   migration, how phosphorylation affects FlnA activity is not well understood (20, 22,

296   30-33). There is some evidence to suggest that phosphorylation of serine 2152

297   augments binding of ligands to Ig repeat 21 in FlnA (40). Serine 2152 is located in Ig

298   repeat 20 of FlnA (18). Structural and computer modeling studies provide evidence

299   that Ig repeat 20 controls the force-dependent interaction of ligands with Ig repeat 21

300   in FlnA (18, 41). FlnA is an actin filament bundling protein that is subjected to

301   mechanical forces exerted by actomyosin-mediated contractility (42). These forces

302   lead to the displacement of a beta strand from Ig repeat 20 that would otherwise

303   inhibit binding of ligands to Ig repeat 21 (42, 43). Interestingly, computer simulations

304   predict that phosphorylation of serine 2152 lowers the force needed to relieve

305   autoinhibition of binding to repeat 21 (40). Collectively, these structural, cell

306   biological, and modeling studies suggest that phosphorylation of serine 2152A might

307   enhance binding of ligands to Ig repeat 21 (40-43). This idea predicts that one or more

308   ligands of Ig repeat 21 might participate in exocytosis during InlB-mediated entry.

309   Finally, our results indicate an important role for the host exocyst complex in

310   entry of InlB-coated beads and in invasion of the wild-type *Listeria* strain EGD.

311   Interestingly, strain EGD expresses higher levels of InlB than some other commonly

312   studied strains of *Listeria* due the presence of an activated form of the transcription

313   factor PrfA (44). In future work, it will be of interest to examine if FlnA and the

314   exocyst complex play important roles in entry of *Listeria* strains apart from EGD.

315   Like the *Listeria* strain EGD, the bacteria *Salmonella enterica* serovar typhimurium

316   and *Staphylococcus aureus* exploit the exocyst complex in order to gain entry into

317   human cells (45-47). An interesting question for future research is whether subversion

318   of exocytosis through the exocyst is a general strategy used for internalization by

319   bacterial pathogens.

14

320 **MATERIALS AND METHODS**

321 *Bacterial strains, mammalian cell lines and media*

322 The *Listeria monocytogenes* strain BUG 947 was grown in brain heart infusion (BHI;

323 Difco) broth and prepared for infection as described (34). This strain is derived from

324 the wild-type strain EGD and contains an in-frame deletion in the *inlA* gene and is

325 internalized into mammalian cells in a manner dependent on the *Listeria* protein InlB

326 and its host receptor Met (3, 26, 48).

327 The human epithelial cell line HeLa (ATTC CCL-2) was grown in Dulbecco's

328 modified Eagle's medium (DMEM) with 4.5 g of glucose per liter and 2 mM

329 glutamine (catalog no. 11995-065; Life Technologies), supplemented with 5 or 10%

330 fetal bovine serum (FBS). Cell growth, bacterial infections, incubations with latex

331 beads, and stimulation with InlB protein were performed at 37°C under 5% CO2.

332

333 *Antibodies, inhibitors, and purified proteins*

334 Rabbit antibodies used were anti-InlB(3), anti-Met (4560; Cell Signaling

335 Technology), anti-myc (PRB-150P; Covance), and anti-phospho-Filamin A (Serine

336 2152) (Cell Signaling Technology; 4761). Mouse monoclonal antibodies used were,

337 anti-Exo70 (ED2001; Kerafast), anti-Filamin A (Millipore; CBL228), anti-

338 glutathione-S-transferase (GST) (G1160; Sigma-Aldrich), anti-GFP (11814460001;

339 Sigma-Aldrich), anti-myc (9E10) (626802; Biolegend), normal mouse IgG (sc-2025;

340 Santa Cruz Biotechnology), anti-Sec3 (HPA037706; Sigma-Aldrich); anti-Sec5

341 (ED2002; Kerafast), anti-Sec8 (610658; Becton Dickenson) and anti-tubulin (T5168;

342 Sigma-Aldrich). Horseradish peroxidase (HRPO)-conjugated secondary antibodies

343 were purchased from Jackson Immunolabs. Secondary antibodies or phalloidin

344 coupled to Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 647 were obtained from

15

345 Life Technologies. 6XHis-tagged InlB or glutathione S-transferase proteins were

346 expressed in *E. coli* and purified as previously described (34, 49). Okadaic acid and

347 sodium orthovanadate were purchased from Sigma-Aldrich.

348

349 *siRNAs*

350 The sequences of short interfering RNAs (siRNAs) used were 5'-

351 GGAAUUGAGUGGUGGUAGAtt-3' (Arp3), 5'-

352 GGUUAAAGGUGACUGAUUAuu-3' (Exo70-1), 5'-

353 CAGACAACAUCAAGAAUGAtt-3' (Exo70-2), 5'-

354 GACUGGCGUGUCAUUGGACAGAUAAtt-3' (Exo70-3), 5'-

355 CAGUCAAGUUCAACGAGGAtt-3' (FlnA #1), (2)

356 GUGACCGCCAAUAACGACAuu (FlnA #2), 5'-CGAAGAAAGCCCGUGCCUAtt-

357 3' (FlnA #3), 5'-CCAGAGACAUGUAUGAUAAuu-3' (Met), 5'-

358 GGAAAUGGGUUGAUGAACUtt-3' (mTOR), 5'-

359 GCUCCACACUAAAUCCGCAtt-3' (PKC-α), 5'-

360 CUGCAAUUAGAGACAACUAtt-3' (RalA), 5'-GAUUCAGUGAUUUGCGAGAtt-

361 3' (Sec3-1), 5'-CACUAAACCUUGUGAAAGAtt-3' (Sec3-2), 5'-

362 GAUUGCAUGGGCCCUUCGAtt-3' (Sec3-3), 5'-

363 CUCAAUGUGCUUCAGCGAUtt-3' (Sec5-1), 5'-

364 GUUAGCAUGGCCUCAUUGAtt-3' (Sec5-2), 5'-

365 GUAAUUGCUGCUAUCUAGAtt-3' (Sec5-3), 5'-

366 AGAACCUGCUUUCAUGCAAuu-3' (Sec8-1), 5-

367 CUUGAUACCUCUCACUAUUtt-3' (Sec8-2), and 5'-

368 CCAGAAACAGUUAAGGCAAtt-3' (Sec8-3). These siRNAs were obtained from

369 Sigma-Aldrich. The negative, non-targeting control siRNA molecule #1 (catalog no.

16

370   D-001210-01) was purchased from Dharmacon. This siRNA has two or more

371   mismatches with all sequences in the human genome, indicating that it should not

372   target host mRNAs.

373

374   *Mammalian expression plasmids*

375   Mammalian expression vectors used were EGFPC1 (Clontech), pcDNA-myc-FlnA.wt

376   (Addgene # 8982; gift of John Blenis), pcDNA-myc-FlnA.S2152A (Addgene # 8983;

377   gift of John Blenis), pEGFP-C3-Exo70 (Addgene #53761; gift of Channing Der), and

378   VAMP3-GFP (38).

379

380   *Transfection*

381   HeLa cells grown in 24 well plates or on 22- by 22-mm coverslips were transfected

382   with siRNAs or plasmid DNA using lipofectamine 2000 (Life Technologies) as

383   previously described (26, 50).

384

385   *Coupling of proteins to latex beads*

386   InlB or GST proteins were coupled to carboxylate-modified latex beads 3 μm in

387   diameter (Polysciences; catalog no. 09850) using either passive binding or covalent

388   linkage as described (6, 26).

389

390   *Stimulation of mammalian cells with soluble InlB or beads coated with InlB*

391   HeLa cells were starved by incubation in DMEM without FBS for 9-10 h followed by

392   addition of 300 ng/ml (4.5 nM) soluble InlB for 10 min at 37°C in 5%. In the case of

393   experiments with beads, particles coated with InlB or GST were added at a ratio of

394   approximately 5 beads per HeLa cell. Cells were centrifuged at 1000 rpm for 2 min to

395   enhance contact between beads and cells, and then incubated for 10 min at 37˚C in

396   5% CO_2. After incubation with soluble InlB or beads, cells were then washed in cold

397   PBS, and lysates were prepared for Western blotting or immunoprecipitation.

398

399   *Western blotting and immunoprecipitation*

400   For experiments involving Western blotting of total cell lysates, HeLa cells were

401   solubilized in radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100,

402   0.25% sodium deoxycholate, 0.05% SDS, 50 mM Tris-HCl [pH 7.5], 2 mM EDTA,

403   150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 10 mg/liter each of

404   aprotinin and leupeptin. For experiments assessing phosphorylation of FlnA, cells

405   were solubilized in RIPA buffer containing 3 mM sodium orthovanadate and 1 μM

406   okadaic acid. Protein concentrations of lysates were determined using a bicinchoninic

407   acid (BCA) assay kit (Pierce), and equal protein amounts of each sample were

408   migrated on 7.5% SDS/polyacrylamide gels. For analysis of phosphorylation of myc-

409   tagged FlnA proteins, cells were solubilized in lysis buffer containing 50 mM Tris-

410   HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 1 μM

411   okadaic acid, 1 mM vanadate, and 10 μg per ml each of aprotinin and leupeptin.

412   Lysates were used to prepare immunoprecipitates with anti-FlnA antibody or normal

413   mouse IgG as a control. Immunoprecipitations were performed using protein A/G

414   agarose beads (Santa Cruz Biotechnology) as described (34). Immunoprecipitates

415   were migrated on 7.5% SDS/polyacrylamide gels and Western blotted with anti-

416   phospho-Filamin A (Serine 2152) antibodies. All Western blotting experiments

417   involved transfer of samples to PVDF membranes, incubation with primary antibodies

418   or secondary antibodies coupled to horse radish peroxidase, and detection using

419   enhanced chemiluminescence (ECL) or ECL Plus reagents (GE Healthcare), as

18

420   described previously (3). Chemiluminescence was detected using an Odyssey imaging

421   system (Li-Cor Biosciences). Bands in Western blot images were quantified using

422   ImageJ software as described (51).

423

424   *Bacterial invasion assays*

425   Invasion of *Listeria* was measured using gentamicin protection assays, as previously

426   described (3, 29). HeLa cells were infected with *Listeria* approximately 48 h after

427   transfection with siRNAs. Cells were infected for 1 h in the absence of gentamicin

428   using a multiplicity of infection of 30:1, and then incubated in DMEM with 20 μg/ml

429   gentamicin for an additional 2 h. Bacterial invasion efficiencies were first expressed

430   as the percentage of the inoculum that survived gentamicin treatment. To obtain

431   relative invasion values, absolute percent entry values in a given experiment were

432   normalized to the value in cells subjected to mock transfection in the absence of

433   siRNA.

434

435   *Quantification of internalization of beads*

436   Beads coated with InlB or GST were added to HeLa cells growing on 22- by 22-mm

437   coverslips. A ratio of approximately 5 particles to human cells was used. Cells were

438   centrifuged at 1000 rpm for 2 min at room temperature and then incubated for 30 min

439   at 37˚C in 5% CO_2 to allow internalization of beads. Cells were then washed in PBS

440   and fixed in PBS containing 3% paraformaldehyde (PFA). Samples were labeled with

441   anti-InlB or anti-GST antibodies, using a previously described approach that

442   distinguishes extracellular or intracellular particles (26). In the case of experiments

443   involving myc-tagged FlnA proteins (Fig. 3A,B,C), samples were also labeled with

444   mouse anti-myc antibodies to allow identification of transfected cells. Secondary

19

445   antibodies used for labeling were coupled to Alexa Fluor 488, Alexa Fluor 555, and

446   Alexa Fluor 647. Labeled samples were mounted in Molwiol with 1,4-

447   diazabicyclo[2.2.2]octane (DABCO) as an anti-fade agent. Samples were analyzed for

448   intracellular and extracellular beads using an Olympus BX51 epifluorescence

449   microscope equipped with a 20x 0.75 NA dry objective lens and an Olympus DP80

450   CCD camera, using Olympus cellSens software (version 1.13). The data shown in

451   Figures 1C, 3B, and S1 are from three experiments. In each experiment, at least 100

452   intracellular beads were scored for the control conditions involving mock transfection

453   in the absence of siRNA. A similar number of total (intracellular plus extracellular)

454   beads were analyzed for all other conditions. Data were initially expressed as the

455   percentage of total cell-associated beads that were internalized. These data were then

456   converted to relative internalization values by normalizing to percent internalization

457   data from controls lacking siRNA.

458

459   *Confocal microscopy analysis*

460   For studies involving exocytosis in Figures 5 and 9, HeLa cells grown on 22- x 22-

461   mm coverslips were transfected with siRNAs and then transfected again 24 h later

462   with a plasmid expressing VAMP3 fused to GFP (VAMP3-GFP). Approximately 24

463   h after addition of plasmid DNA, cells were washed, placed in serum-free DMEM,

464   and incubated for 5 min in serum-free DMEM with InlB- or GST-coated beads, as

465   described above. HeLa cells were washed in PBS and incubated with mouse anti-GFP

466   antibodies for 1 h at 4°C. Cells were then fixed in PBS with 3% PFA, and incubated

467   with anti-mouse antibodies coupled to Alexa Fluor 647 for 1 h. This method resulted

468   in labeling of exofacial VAMP3-GFP (6, 38). Extracellular beads were labeled by

469   incubation with anti-InlB or anti-GST antibodies, followed by secondary antibodies

20

470   conjugated to Alexa Fluor 555. Experiments to determine effect of myc-tagged FlnA

471   proteins on exocytosis (Fig. 6) were performed similarly to the exocytosis studies

472   described above, except that HeLa cells were co-transfected with plasmids expressing

473   VAMP3-GFP and either myc-FlnA.wt or myc-FlnA-S2152A. After exofacial labeling

474   of VAMP3-GFP with mouse anti-GFP antibodies, cells were permeabilized in PBS

475   containing 0.4% Triton X-100, and myc-tagged proteins were labeled with rabbit anti-

476   myc antibodies and anti-rabbit-Alexa Fluor 555. Experiments assessing recruitment of

477   GFP-Exo70 (Fig. 8) were performed similarly to the exocytosis experiments, except

478   that the exofacial labelling step with anti-GFP antibodies was omitted. For labeling of

479   endogenous Exo70, Sec5, or Sec8 (Fig. S2B), cells were fixed by incubation in

480   methanol for 5 min at -20˚C. Samples were then incubated overnight at 4˚C with

481   primary antibodies in PBS with 1.0% BSA and 0.1% Tween 20.

482   All samples analyzed by confocal microscopy were mounted in Molwiol

483   supplemented with DABCO. Imaging was performed with an inverted Olympus

484   FV1200 laser scanning confocal microscope, using a 60x 1.35 NA oil immersion

485   objective, laser lines of 488 nm, 543 nm, and 633 nm, and photomultiplier tubes for

486   detection. Images from serial sections spaced 1.0 μm apart were used to ensure that

487   all cell-associated beads were detected. Image J (version 1.51e) software was

488   employed to determine fold enrichment (FE) values for each cell-associated bead. FE

489   is defined as the mean pixel intensity in a ring-like structure around the bead,

490   normalized to the mean pixel intensity throughout the human cell (6, 13, 28, 29). The

491   thresholding function of Image J was used to measure mean pixel intensities in ring-

492   like structures of FlnA, exofacial VAMP3-GFP, or GFP-Exo70 around beads. This

493   function was also used to measure mean pixel intensity throughout the cell. In each

494   experiment, approximately 50-100 extracellular, cell-associated beads were analyzed

495   for each condition. The data shown in Figures 4B, 5B, 6B, 8B, and 9B are pooled FE

496   values from three or four independent experiments.

497

498   *Statistical analysis*

499   Statistical analysis was performed using Prism (version 6.0c; GraphPad Software). In

500   comparisons of data from three or more conditions, analysis of variance (ANOVA)

501   was used. The Tukey-Kramer test was used as a posttest. For comparisons of two data

502   sets, Student's t-test was used. A P-value of 0.05 or lower was considered significant.

503

511
512
513

514 **FIGURE LEGENDS**

515 **Figure 1.** *Host FlnA promotes InlB-mediated entry*. HeLa cells were either mock

516 transfected in the absence of siRNA, transfected with a control non-targeting siRNA,

517 or transfected with three different siRNAs against FlnA. About 48 h after transfection,

518 cell lysates were prepared for analysis of target gene expression by Western blotting,

519 or cells were incubated with *Listeria* or InlB-coated beads for assessment of invasion

520 or entry, respectively. A. Effect of siRNAs against FlnA on target protein expression.

521 A representative blot showing depletion of FlnA is displayed. After reaction with anti-

522 FlnA antibodies, the membrane was stripped and probed with anti-tubulin antibodies

523 to confirm equivalent loading. The adjacent bar graph displays mean +/- SEM values

524 of quantified Western blotting data from three independent experiments. B. Effect of

525 siRNAs targeting FlnA on invasion of *Listeria* expressing InlB. Results are mean +/-

526 SEM relative entry values from three to six independent gentamicin protection

527 experiments, depending on the condition. C. Effect of an siRNA targeting FlnA on

528 entry of InlB-coated beads. (i). Internalization of beads coated with InlB or GST into

529 HeLa cells. Data are the mean percentage of total cell-associated beads internalized

530 +/- SEM from three independent experiments. 'ND' indicates that no internalized

531 beads were detected. (ii). Decreased entry of InlB-coated beads into HeLa cells

532 transfected with FlnA siRNA. The siRNA against FlnA used was #2. Results are

533 mean relative entry values +/- SEM from three independent experiments. *, P < 0.05

534 compared to the control siRNA condition, as determined by ANOVA and the Tukey-

535 Kramer posttest.

536

537 **Figure 2.** *InlB stimulates PKC-α -dependent phosphorylation of FlnA*. A.

538 Specificity of anti-phospho-FlnA antibodies. (i). Effect of FlnA RNAi on reactivity

24

539   with antibodies. HeLa cells were either transfected with a control siRNA or with an

540   siRNA targeting FlnA. Cell lysates were prepared and used for Western blotting with

541   antibodies that recognize phosphorylated serine 2152 in FlnA. The left panel shows a

542   representative Western blot and the right panel displays quantified Western blotting

543   data as mean +/- SEM values from three independent experiments. (ii). Effect of

544   mutation of serine 2152 on antibody reactivity. HeLa cells were transfected with

545   plasmids expressing myc-tagged wild-type (wt) FlnA or FlnA containing a serine-to-

546   alanine substitution in residue 2152 (S2152A). After transfection, lysates were

547   prepared and used for immunoprecipitation with anti-myc antibodies or mock

548   precipitation with control IgG. Precipitates were Western blotted using anti-phospho-

549   FlnA (serine 2152) antibodies. Shown is a representative Western blot from one of

550   two experiments performed. (B). Effect of depletion of PKC-α on phosphorylation of

551   FlnA. After transfection of HeLa cells with control siRNA or an siRNA against PKC-

552   α, lysates were prepared and used for Western blotting with antibodies against

553   phosphorylated serine 2152 in FlnA, total FlnA, PKC-α, or tubulin. (i).

554   Representative Western blots are shown. (ii). Quantified Western blotting data

555   expressed as mean +/- SEM values from six independent experiments are presented.

556   (C). Effect of depletion of Met on phosphorylation of FlnA. (i). Representative

557   Western blotting results are shown. (ii). Quantified Western blotting data expressed as

558   mean +/- SEM values from seven independent experiments are displayed. (D).

559   Phosphorylation of FlnA during InlB-mediated entry. HeLa cells were incubated with

560   latex beads coupled to InlB or GST for 10 min, followed by solubilization in lysis

561   buffer. Lysates were Western blotted with anti-phospho-FlnA (serine 2152)

562   antibodies. (i). A representative Western blot is shown. (ii). Quantified Western

25

563   blotting data expressed as mean +/- SEM values from three independent experiments

564   are presented. *, P < 0.05, as determined by ANOVA and the Tukey-Kramer posttest.

565

566   **Figure 3.** *Phosphorylation of FlnA on serine 2152 contributes to InlB-mediated*

567   *entry*. HeLa cells were transfected with plasmids expressing myc-tagged wild-type

568   (WT) FlnA or FlnA containing a serine-to-alanine substitution in residue 2152

569   (S2152A). About 24 h after transfection, lysates were prepared for evaluation of

570   tagged FlnA protein expression by Western blotting or fixed samples were made for

571   fluorescence microscopy analysis of entry of InlB-coated beads. A. Expression of

572   myc-tagged FlnA proteins assessed by Western blotting. The panel on the left shows a

573   representative Western blot, whereas the graph on the right displays quantified

574   Western blotting data as mean +/SEM values from three independent experiments. B.

575   Entry of InlB-coated beads. (i). The percentage of cell-associated beads that were

576   internalized into HeLa cells expressing myc-tagged FlnA.WT or FlnA.S2152A

577   proteins is shown. (ii). Expression of myc-tagged FlnA proteins in the same samples

578   used for analysis of internalization of InlB-coated beads. Pixel intensities in HeLa

579   cells associated with InlB-coated beads were quantified using Image J software. The

580   data in (i) and (ii) are mean +/- SEM values from four independent experiments. *, P,

581   < 0.05, as determined by Student's t-test.

582

583   **Figure 4**. *FlnA is recruited during entry in a manner that depends on mTOR, RalA,*

584   *and Met*. HeLa cells were mock transfected in the absence of siRNA, transfected with

585   a control siRNA, or transfected with siRNAs targeting mTOR, PKC-α, RalA, or Met.

586   Cells were then incubated for 10 min with beads coupled to InlB or to GST, followed

587   by fixation and labeling for confocal microscopy. A. Confocal microscopy images of

26

588   localization of endogenous FlnA. Panels on the left show FlnA localization in HeLa

589   cells, with locations of beads indicated with arrows. Regions near beads are expanded

590   in the middle and right panels. Middle panels show FlnA labeling, whereas right

591   panels are differential interference contrast (DIC) images displaying beads. Scale bars

592   indicate 5 micrometers. B. Quantification of recruitment of FlnA. Data are pooled

593   fold enrichment (FE) values from four independent experiments. Each dot represents

594   a single FE value. Horizontal bars are means and error bars are SD. *, P, < 0.05

595   compared to the no siRNA and control siRNA conditions.

596

597   **Figure 5.** *mTOR, PKC-α, and FlnA control exocytosis during InlB-mediated entry.*

598   HeLa cells were mock transfected in the absence of siRNA, transfected with a control

599   siRNA, or transfected with siRNAs targeting mTOR, PKC-α, FlnA, Met, or Arp3.

600   Cells were then transfected with a plasmid expressing the exocytic probe VAMP3-

601   GFP and incubated for 5 min with beads coupled to InlB or to GST. Samples were

602   fixed and labeled for confocal microscopy. A. Representative confocal microscopy

603   images. Total VAMP3-GFP is green, exofacial VAMP3-GFP is red, and beads are

604   blue. Panels on the left are merged images of single HeLa cells, with locations of

605   beads indicated with arrows. Regions near beads are expanded in panels to the right.

606   Scale bars indicate 5 micrometers. B. Quantification of exocytosis. Data are pooled

607   FE values of exofacial VAMP3-GFP from three to four independent experiments,

608   depending on the condition. Dots represent individual FE values. Horizontal bars are

609   means and error bars are SD. *, P < 0.05 compared to the control siRNA condition, as

610   determined by ANOVA and the Tukey-Kramer posttest.

611

612   **Figure 6.** *Phosphorylation of serine 2152 in FlnA contribute to exocytosis.*

27

613   HeLa cells were co-transfected with plasmids expressing VAMP3-GFP and either

614   myc-tagged wild-type (wt) FlnA or FlnA.S2152A. Cells were then incubated with

615   InlB-coated beads for 5 min, followed by labeling for exofacial VAMP3-GFP and

616   myc-tagged proteins. Confocal microscopy was performed to acquire images for

617   quantification of exocytosis. A. Representative microscopy images. Myc-tagged FlnA

618   proteins are colored blue, VAMP3-GFP is green, exofacial VAMP3-GFP is red, and

619   beads are detected using differential interference contrast (DIC) microscopy. Panels

620   on the left are merged images of single HeLa cells, with locations of beads indicated

621   with arrows. Regions near beads are expanded in panels to the right. Scale bars

622   indicate 5 micrometers. B. (i). Quantification of exocytosis. Data are pooled FE

623   values of exofacial VAMP3-GFP from three independent experiments. Dots represent

624   individual FE values. Horizontal bars are means and error bars are SD. (ii).

625   Expression of myc-tagged FlnA proteins in the same samples used for analysis of

626   exocytosis. Pixel intensities in HeLa cells associated with InlB-coated beads were

627   quantified using Image J software. The data are mean pixel intensities +/- SEM from

628   three independent experiments. *, $P < 0.05$, as determined by Student's t-test.

629

630   **Figure 7.** *The exocyst complex promotes InlB-dependent invasion of Listeria.* HeLa

631   cells were mock transfected in the absence of siRNA, transfected with a control

632   siRNA, or transfected with siRNAs against the exocyst components Sec3, Sec5, Sec8,

633   or Exo70. About 48 h after transfection, cells were solubilized for assessment of

634   target protein expression or infected with wild-type *Listeria* for analysis of invasion.

635   A. Effect of siRNAs on Sec3 expression and invasion of *Listeria*. (i). Sec3 expression.

636   The left panel shows a representative Western blot indicating depletion of Sec3 by

637   siRNAs. The right panel is quantified Western blotting data showing mean relative

28

638   Sec3 expression +/- SEM from three independent experiments. (ii). Invasion of

639   *Listeria*. Data are mean relative entry values +/- SEM from three to eight independent

640   experiments, depending on the siRNA. B. siRNA-mediated inhibition in Sec5

641   expression and invasion of *Listeria*. (i). Sec5 expression. In the left panel, a

642   representative Western blot is shown. The right panel displays quantified Western

643   blotting data from three independent experiments. (ii). Invasion of *Listeria*. Data are

644   means +/- SEM from three to six independent experiments. C. Reduction in

645   expression of Sec8 and invasion of *Listeria* by siRNAs. (i). Sec8 expression. (i). In

646   the left panel, a representative Western blot is presented. The right panel shows

647   quantified Western blotting data from three independent experiments. (ii). Invasion of

648   *Listeria*. Data are means +/- SEM of three to six independent experiments. D. siRNA-

649   mediated inhibition in expression of Exo70 and invasion of *Listeria*. (i). Exo70

650   expression. The left panel shows a representative Western blot and the right panel

651   displays quantitative Western blotting data from three independent experiments. (ii).

652   Invasion of *Listeria*. Data are means +/- SEM from three independent experiments. *,

653   P < 0.05, as determined by ANOVA and the Tukey-Kramer posttest.

654

655   **Figure 8. *mTOR, PKC-α, RalA, and FlnA mediate recruitment of Exo70 during***

656   ***InlB-mediated entry***. HeLa cells were subjected to control conditions or transfected

657   with siRNAs against mTOR, PKC-α, RalA, FlnA, Met, or Arp3. Cells were then

658   transfected with a plasmid expressing Exo70 fused to GFP (GFP-Exo70). After

659   transfection, cells were incubated for 5 min with beads coupled to InlB or to GST,

660   followed by fixation and labeling for confocal microscopy. A. Representative

661   confocal microscopy images. GFP-Exo70 is green and beads are red. Panels on the

662   left are merged images of single HeLa cells. Arrows indicate beads. Regions near

29

663   beads are expanded in the panels to the right. Scale bars indicate 5 micrometers. B.

664   Quantification of recruitment of GFP-Exo70. Data are pooled FE values of GFP-

665   Exo70 from three independent experiments. Dots represent individual FE values.

666   Horizontal bars are means and error bars are SD. *, P < 0.05 compared to the control

667   siRNA condition, as assessed by ANOVA and the Tukey-Kramer posttest.

668

669   **Figure 9.** *The exocyst complex promotes exocytosis during InlB-mediated entry.*

670   HeLa cells were subjected to control conditions or transfected with siRNAs against

671   Sec3, Sec5, Sec8, or Exo70. Cells were then transfected with a plasmid expressing

672   VAMP3-GFP. After transfection, cells were incubated for 5 min with beads coupled

673   to InlB, followed by fixation and labeling for confocal microscopy. Scale bars

674   indicate 5 micrometers. A. Representative confocal microscopy images. Total

675   VAMP3-GFP is green, exofacial VAMP3-GFP is red, and beads are blue. Panels on

676   the left are merged images of single HeLa cells, with beads being indicated by arrows.

677   Regions near beads are expanded in panels to the right. B. Quantification of

678   exocytosis. Data are pooled FE values of exofacial VAMP3-GFP from three

679   independent experiments. Dots represent individual FE values. Horizontal bars are

680   means and error bars are SD. *, P, < 0.05 compared to the no siRNA or control

681   siRNA conditions, as determined by ANOVA and the Tukey-Kramer posttest.

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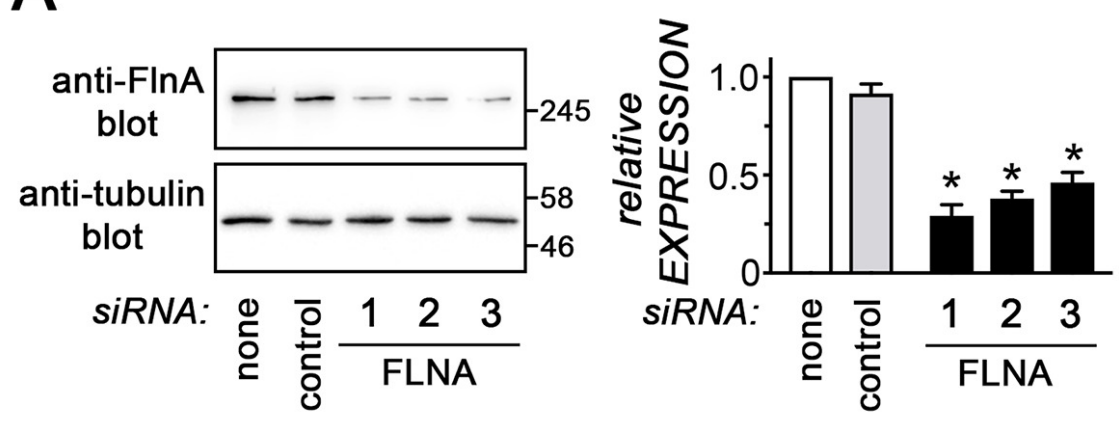810   https://doi.org/10.1128/MCB.24.7.3025-3035.2004.

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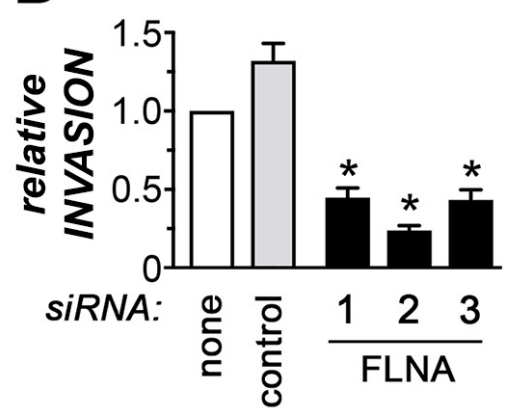860   Vanbennekom N, Ireton K. 2012. Identification of components of the type IA

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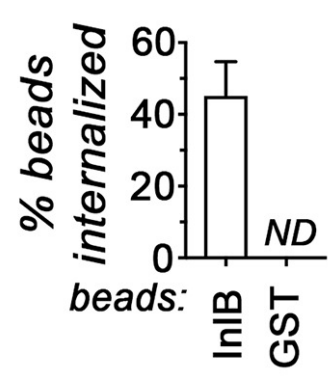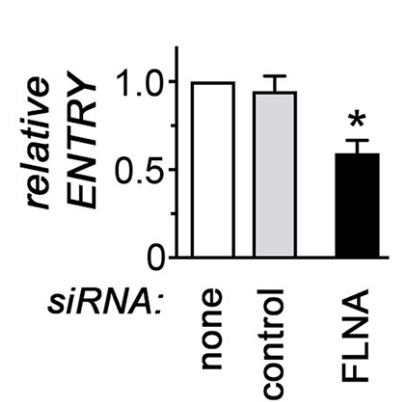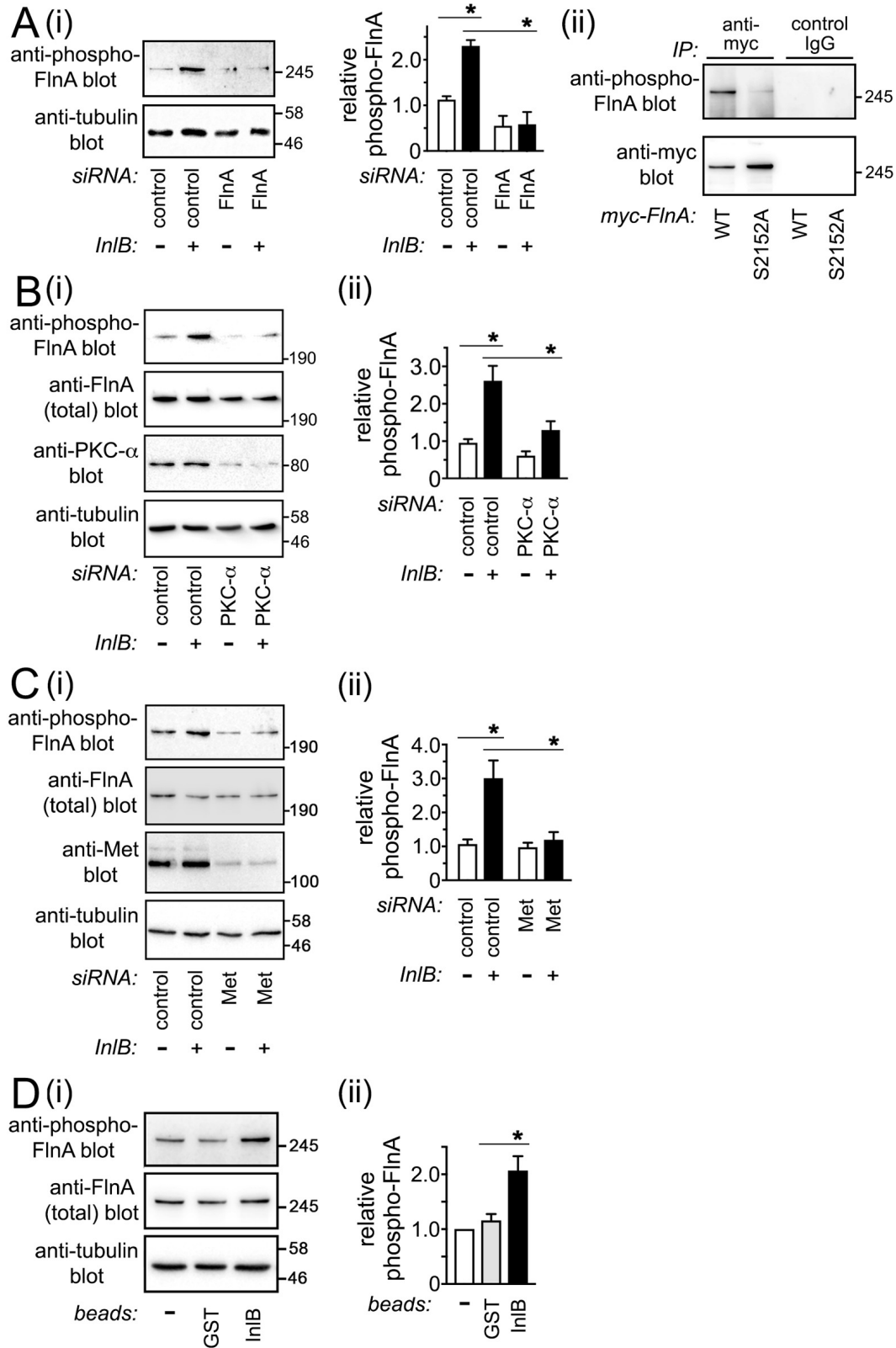867   Listeria monocytogenes. Cell Microbiol 17:876-892.

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Figure 1

A

anti-FlnA
blot

anti-tubulin
blot

— 245

— 58

— 46

siRNA: none control 1 2 3
FLNA

relative EXPRESSION

siRNA: none control 1 2 3
FLNA

B

relative INVASION

siRNA: none control 1 2 3
FLNA

C (i)

% beads internalized

beads: InlB GST

ND

(ii)

relative ENTRY

siRNA: none control FLNA

Figure 2

Figure 3

Figure 4

A

B

Figure 5

Figure 6

A

myc-FlnA:

	merge	*expanded*				
		merge	DIC (bead)	myc-FlnA protein	total VAMP3-GFP	exofacial VAMP3-GFP
WT						
S2152A						

B (i)

FE exofacial VAMP3-GFP

myc-FlnA: WT S2152A

(ii)

mean pixel intensity

myc-FlnA: WT S2152A

Figure 7

Figure 8

A

expanded

merge | GFP-Exo70; beads | beads | GFP-Exo70

no siRNA; InlB beads

control siRNA; InlB beads

mTOR siRNA; InlB beads

PKC-α siRNA; InlB beads

FlnA siRNA; InlB beads

RalA siRNA; InlB beads

Met siRNA; InlB beads

Arp3 siRNA; InlB beads

control siRNA; GST beads

B

beads: InlB | GST

FE EGFP-Exo70

siRNA: none control mTOR PKC-α FlnA RalA Met Arp3 control

Figure 9

A

B