1   *Shigella flexneri* disruption of host cell-cell tension promotes intercellular spread

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18   secretion system, plasma membrane

19 **Summary (150 words)**

20 During infection, a subset of bacterial pathogens invades into the eukaryotic cytosol and

21 spreads between cells of an epithelial layer. This intercellular spread is essential for

22 disease and requires actin-based motility leading to the formation of plasma membrane

23 protrusions. Protrusions are engulfed by the adjacent cell in an active process requiring

24 both bacterial and eukaryotic proteins. Here, we demonstrate that the *Shigella* spp. type

25 3 secretion system protein IpaC promotes bacterial spread by reducing intercellular

26 tension. *S. flexneri* producing a point mutant of IpaC that cannot interact with the cell-

27 cell adhesion protein β-catenin were unable to reduce intercellular tension, form

28 protrusions, or spread, demonstrating that interaction of IpaC with β-catenin is required

29 for these processes. Spread was restored by chemical reduction of intercellular tension

30 or genetic depletion of β-catenin. This work defines a molecular mechanism by which

31 *Shigella* overcomes host cell-cell tension to mediate spread.

32   **Introduction**

33   Many cytosol-dwelling bacterial pathogens have evolved unique mechanisms of

34   spreading directly from the cytosol of an infected (donor) cell into an uninfected,

35   adjacent (recipient) cell. Spreading enables the pathogens to avoid immune clearance,

36   access new nutrients, and maintain an intracellular lifecycle (Sansonetti et al., 1991;

37   Weddle and Agaisse, 2018a). *Shigella flexneri*, a model cytosol-dwelling Gram-negative

38   bacterial pathogen, infects the colonic epithelium, replicating within the cytosol of

39   colonocytes and subsequently spreading through the epithelium (Labrec et al., 1964;

40   Sansonetti et al., 1986). Intercellular spread is required for *S. flexneri* to cause disease

41   and efficiently colonize the colon (Sansonetti et al., 1991; Yum et al., 2019). *S. flexneri*

42   polymerizes actin and moves in a directed manner to the cell periphery (Bernardini et

43   al., 1989; Egile et al., 1999; Goldberg and Theriot, 1995), where it remodels the plasma

44   membrane into pathogen-containing protrusions (Kadurugamuwa et al., 1991; Robbins

45   et al., 1999). These bacteria-containing protrusions are engulfed into a vacuole by

46   recipient cells in a clathrin-dependent process (Fukumatsu et al., 2012). Bacteria

47   escape this vacuole into the cytosol of the donor cell (Allaoui et al., 1992; Campbell-

48   Valois et al., 2015; Uchiya et al., 1995; Weddle and Agaisse, 2018b), enabling repeated

49   cycles of intercellular spread through the epithelium. Whereas the forces derived from

50   actin-based motility are necessary for protrusion formation (Monack and Theriot, 2001),

51   other pathogen and host factors, including the type 3 secretion translocon pore proteins

52   IpaB and IpaC and the type 3 effectors OspE1/2, IcsB, and VirA, are required for

53   efficient intercellular spread (Allaoui et al., 1995; Campbell-Valois et al., 2015;

54   Campbell-Valois et al., 2014; Heindl et al., 2010; Kuehl et al., 2014; Ogawa et al., 2003;

55   Page et al., 1999; Schuch et al., 1999; Yi et al., 2014; Yoshida et al., 2006).

56   Understanding the molecular mechanisms by which these proteins contribute to spread

57   will define the parameters necessary for bacterial spread.

58   Here, we show that the intercellular spread of *S. flexneri* is dependent upon IpaC. We

59   show that IpaC binds to the cell-cell adhesion protein β-catenin and that the IpaC-β-

60   catenin interaction enables intercellular spread by overcoming cell-cell tension;

61   overcoming cell-cell tension is required for the bacteria to generate protrusions of the

62   plasma membrane. Moreover, substitution of an arginine residue in the C-terminal tail of

63   IpaC abrogates the interaction of IpaC with β-catenin, consequently diminishing

64   protrusion formation and spread. Our results define a mechanism by which *S. flexneri*

65   subverts cell processes at the plasma membrane, enabling its spread between cells.

66 **Results**

67 **The IpaC C-terminal Tail is Required for Efficient Intercellular Spread of *S. flexneri***

68 During invasion by *S. flexneri*, the type 3 secreted protein IpaC interacts with

69 intermediate filaments; this interaction is necessary for efficient bacterial docking onto

70 host cells and for efficient translocation of effectors into the host cell cytosol, where the

71 effectors mediate bacterial invasion (Russo et al., 2016). In addition to being required

72 for invasion, type 3 secretion is required for *S. flexneri* intercellular spread (Campbell-

73 Valois et al., 2014; Page et al., 1999), and we hypothesized that the interaction between

74 IpaC and intermediate filaments is required at this step. We tested the efficiency of

75 spread for *S. flexneri ΔipaC* producing WT IpaC or an IpaC derivative that contains a

76 point mutation near the C-terminus (IpaC R362W) and is unable to interact with

77 intermediate filaments (Harrington et al., 2006; Russo et al., 2016; Terry et al., 2008).

78 IpaC R362W is efficiently secreted and forms normal-sized pores in the plasma

79 membrane during invasion (Russo et al., 2019; Russo et al., 2016). Bacterial plaques

80 formed in monolayers of mouse embryonic fibroblasts (MEFs) were significantly smaller

81 for *S. flexneri ΔipaC* producing IpaC R362W than for *S. flexneri ΔipaC* producing WT

82 IpaC (Figure 1A-B). In contrast, the presence of intermediate filaments did not affect

83 plaque size (Figure 1A-B). Similarly, plaques formed by wild-type *S. flexneri* in

84 monolayers of MEFs lacking vimentin (Vim$^{-/-}$) were similar to those formed by MEFs

85 producing vimentin (Vim$^{+/+}$) (Figure 1C); vimentin is the only intermediate filament in

86 these cells (Colucci-Guyon et al., 1994; Holwell et al., 1997), indicating that other

87 intermediate filaments are not compensating for the loss of vimentin. Together these

88 data show that the C-terminal region of IpaC at Arginine 362 is required for spread

89 independent of the intermediate filament vimentin.

90 *S. flexneri* infects epithelial cells of the intestine, in which the predominant intermediate

91 filaments are keratins rather than vimentin. Therefore, the ability of *S. flexneri* to spread

92 was tested in Caco-2 cells, which express keratin intermediate filaments. Similar to the

93 results with MEFs, plaques formed in monolayers of Caco-2 cells were significantly

94 smaller for *S. flexneri ΔipaC* producing IpaC R362W than for *S. flexneri ΔipaC*

95 producing WT IpaC (Figure 1D-E). The defect was again independent of intermediate

96 filaments, as wild type *S. flexneri* formed plaques of similar size in Caco-2 cells whether

97 or not the intermediate filaments keratin 8 or keratin 18 were present (Figure S1).

98 Altogether, these data show the spread of *S. flexneri* is independent of intermediate

99 filaments and dependent upon the presence of the IpaC C-terminal residue R362.

100 To distinguish whether IpaC aids in intercellular spread by contributing to a bacterial

101 process in the donor cell or the recipient cell, we directly assessed the efficiency of *S.*

102 *flexneri* spread from the initial donor cell to adjacent recipient cells. To do so, a

103 population of cells was sparsely seeded and infected. Uninfected cells were overlaid

104 onto the infected cells, and the ability of *S. flexneri* to spread into the second population

105 of cells was assessed at 4 hours of infection. Compared to bacteria producing WT IpaC,

106 bacteria producing IpaC R362W were impaired in movement out of the donor cells

107 (Figure 1F-G), demonstrating that IpaC, and specifically residue R362 adjacent to the

108 C-terminus, is required during exit from donor cells.

109 **IpaC is Necessary for Efficient Formation of Plasma Membrane Protrusions**

110   To ascertain the step of cellular exit at which IpaC R362W is defective, we assessed the

111   efficiency of *S. flexneri* Δ*ipaC* producing IpaC variants to form plasma membrane

112   protrusions. In HeLa cells, the percentage of intracellular bacteria located in membrane

113   protrusions was markedly decreased for bacteria producing IpaC R362W compared to

114   bacteria producing wild-type IpaC (Figure 2A–B), which demonstrates that IpaC is

115   required for the formation and/or maintenance of bacterial protrusions of the membrane

116   and that IpaC R362W is defective in this process.

117   Given that forces derived from actin-based motility are required for and under

118   heterologous experimental conditions can be sufficient for the formation of membrane

119   protrusions (Makino et al., 1986; Monack and Theriot, 2001), we hypothesized that

120   bacteria producing IpaC R362W might be defective in actin-based motility. To evaluate

121   the efficiency of actin polymerization, we measured the speed of intracellular bacteria

122   with actin tails using live microscopy in HeLa cells stably producing GFP-tagged actin

123   (LifeAct). The speeds were similar for bacteria producing IpaC R362W and bacteria

124   producing wild-type IpaC (Figure 2C–D), which indicates that IpaC plays a role in

125   protrusion formation in a manner independent of actin-based motility.

126   In support of the defect of IpaC R362W-producing bacteria occurring at the stage of

127   protrusion formation *per se*, we found no defect in other post-entry processes (Figure

128   S2). Bacteria producing IpaC R362W efficiently escaped from the vacuole into the

129   cytosol (Figure S2B). They regulated effector secretion through the T3SS similar to WT

130   IpaC both in the timing of effector secretion (Figure S2C-D) and in the magnitude of

131   secretion activation (Figure S2C and S2E). Unlike an *icsB* mutant, they avoided

132   recruitment of autophagy components similar to bacteria producing WT IpaC (Figure

133   S2F). Moreover, the percentage of bacteria that assembled an actin tail was similar to

134   WT IpaC (Figure S2G-H), indicating that the reduction in protrusion formation (Figure

135   2A-B and Figure S2I) was not due to a defect in initiation of actin tail formation by IpaC

136   R362W-producing bacteria. Altogether these data indicate that IpaC is required for

137   formation of plasma membrane protrusions *per se* and that *S. flexneri ΔipaC* producing

138   IpaC R362W is impaired in spread due to a defect in its ability to form protrusions.

139   Cell-cell Tension is Reduced by *S. flexneri* in an IpaC-Dependent Manner

140   *Listeria monocytogenes* and *Rickettsia parkeri* reduce cell-cell tension during spread.

141   The secreted proteins Internalin C (*L. monocytogene*) and Sca4 (*R. parkeri*) (Lamason

142   et al., 2016; Rajabian et al., 2009) decrease cell-cell tension, enabling the formation or

143   resolution of plasma membrane protrusions, respectively. We investigated whether IpaC

144   may similarly decrease intercellular tension. The impact of *S. flexneri* infection on the

145   junctional linearity of the plasma membrane was measured (Otani et al., 2006).

146   Whereas cells with normal membrane tension have nearly straight membranes between

147   adjacent cell-cell vertices, cells that have decreased cell-cell membrane tension contain

148   membranes that are more curved and thus have longer lengths of membrane between

149   the vertices. Caco-2 cells infected with *S. flexneri ΔipaC* producing wild-type IpaC

150   displayed membranes that curved more than membranes of either cells infected with *S.*

151   *flexneri ΔipaC* producing IpaC R362W or uninfected cells (Figure 3A–B), indicating that,

152   during infection, *S. flexneri* decreases cell-cell tension in an IpaC-dependent fashion.

153   Since the cortical actomyosin network maintains cell-cell tension at the adherens

154   junctions, we tested whether IpaC reduces actomyosin-mediated tension in a manner

155 that promotes protrusion formation. We assessed protrusion formation in the presence

156 of blebbistatin, a chemical inhibitor of myosin II; inhibition of myosin II relieves cell-cell

157 tension (Lamason et al., 2016; Rajabian et al., 2009). Blebbistatin treatment fully

158 rescued protrusion formation of *S. flexneri* that produce IpaC R362W (Figure 3C–D),

159 whereas it had no effect on the efficiency of protrusion formation by bacteria that

160 produce wild-type IpaC, demonstrating that efficient formation of protrusions requires a

161 reduction in actomyosin-mediated membrane tension.

162 ***S. flexneri* spread requires an interaction of IpaC with β-catenin**

163 Catenin-cadherin networks maintain cell-cell tension and are integral to protecting

164 against membrane stress (Ray et al., 2013). Since IpaC interacts with the cell-cell

165 adhesion protein β-catenin (Shaikh et al., 2003), a component of these catenin-cadherin

166 networks, we hypothesized that the role of IpaC in disruption of cell-cell tension may

167 depend on its interaction with β-catenin and that IpaC R362 may be required for this

168 interaction. In an orthogonal yeast-based protein-protein interaction assay that has been

169 successfully used in the study of host-pathogen protein interactions (de Groot et al.,

170 2011; Russo et al., 2016; Schmitz et al., 2009; Yi et al., 2014), we observed that IpaC

171 R362W is defective for interaction with β-catenin. β-catenin (bait) was fused to the

172 reovirus scaffold protein μNS, which forms inclusions bodies, and IpaC derivatives

173 (prey) were tagged with mCherry. Protein-protein interaction results in the formation of

174 puncta of fluorescent signal, whereas lack of interaction between bait and prey proteins

175 results in diffuse mCherry signal throughout the cell (Figure 4A). Co-expression of IpaC-

176 mCherry and β-catenin fused to μNS resulted in fluorescent foci in most yeast, whereas

177 co-expression of IpaC R362W-mCherry and β-catenin fused to μNS resulted diffuse

178   mCherry signal with few yeast displaying foci (Figure 4B–C). These data confirm that

179   IpaC interacts with β-catenin and demonstrate that the IpaC R362W point mutation

180   disrupts it.

181   We tested the impact of β-catenin depletion on the efficiency of *S. flexneri* spread.

182   Stable knock down of β-catenin in HeLa cells and Caco-2 cells (Figure S3A–D) rescued

183   the spread of *S. flexneri ΔipaC* producing IpaC R362W (Figures 4D-E and S3E). As

184   expected, in cells expressing a scrambled shRNA, *S. flexneri ΔipaC* producing IpaC

185   R362W spread less efficiently than bacteria producing wild-type IpaC (Figure 4D–E).

186   Knockdown of β-catenin on its own was insufficient to alter membrane linearity (Figure

187   S3F), indicating that additional stresses associated with bacterial infection or other

188   bacterial proteins are likely required to reduce membrane tension. These data

189   demonstrate that β-catenin is a negative regulator of *S. flexneri* intercellular spread and

190   that the interaction of IpaC with β-catenin disrupts β-catenin function in membrane

191   tension.

Discussion

192

193 For cytosol-dwelling bacterial pathogens, direct spread from an infected cell into

194 adjacent uninfected cells is essential for dissemination during infection and disease

195 pathogenesis. To traverse the plasma membranes, bacteria remodel the cellular cortical

196 cytoskeleton. Here, we show that the *S. flexneri* protein IpaC decreases cell-cell tension

197 and promotes the formation of plasma membrane protrusions, enabling intercellular

198 spread, and that cell-cell tension was alleviated by the binding of IpaC to the cell-cell

199 adhesion protein β-catenin.

200 β-catenin and cadherins are components of adherens junctions, which function to

201 anchor one cell to another. β-catenin contains a stretch of 12 armadillo repeats. IpaC

202 binds β-catenin within this armadillo repeat region, and its binding requires the ninth

203 armadillo repeat (Figure S4) (Shaikh et al., 2003). E-cadherin binds via its cytoplasmic

204 domain to β-catenin; in a co-crystal structure (PDB:1I7X), the interaction of E-cadherin

205 with β-catenin extends across all 12 armadillo repeats (Figure S4) (Huber and Weis,

206 2001). Thus, the site of IpaC binding to β-catenin overlaps with the region of β-catenin

207 to which E-cadherin binds (Figure S4), suggesting that IpaC may alter the interaction of

208 E-cadherin and β-catenin. We speculate that IpaC disruption of cell-cell tension is

209 mediated at least in part by modulation of the E-cadherin interaction with β-catenin.

210 Our data highlight that for efficient spread, *S. flexneri* must simultaneously maintain cell-

211 cell contact but release membrane tension. Previous work shows cell-cell contact is

212 necessary for intercellular spread; cell-cell adhesion proteins, including cadherin,

213 tricellulin and occludin were required for efficient intercellular spread by *S. flexneri*

214   (Fukumatsu et al., 2012; Sansonetti et al., 1994). In contrast, our findings show that the

215   cell junction protein β-catenin restricts intercellular spread. Our findings that β-catenin

216   restricts bacterial spread appear to oppose the role of E-cadherin in promoting spread.

217   A model to explain these observations is that the interaction of IpaC with β-catenin may

218   generate slack in cell-cell junctions while maintaining sufficient homotypic interactions of

219   E-cadherin molecules to maintain cell-cell contact. Thus, by targeting catenin-cadherin

220   interactions via β-catenin, IpaC may selectively alter membrane tension while

221   maintaining cell-cell contact.

222   Our findings demonstrate that at different stages of *S. flexneri* infection, the arginine

223   immediately adjacent to the C-terminus of IpaC (R362) is required for interactions with

224   two distinct cellular proteins. In addition to being required for the formation of

225   protrusions during intercellular spread, IpaC R362 is required for interactions with

226   intermediate filaments to support docking during bacterial entry (Russo et al., 2016).

227   Whether the binding preference of IpaC is determined by the distinct sub-cellular niche

228   of the bacterium at these two stages of infection or by other factors is at present

229   unclear. Of note, other *S. flexneri* type 3 secreted proteins contain sequences that bind

230   more than one host protein, for example, during entry, vinculin binding site 3 of IpaA

231   separately binds talin and vinculin (Valencia-Gallardo et al., 2019).

232   Like *S. flexneri*, *R. parkeri* and *L. monocytogenes* diminish cell-cell tension but do so by

233   targeting different host proteins. *R. parkeri* prevents vinculin-mediated cell tension via

234   the type 4 secretion system effector protein Sca4, whereas the secreted *L.*

235   *monocytogenes* protein Internalin C decreases cell-cell tension by binding the focal

236   adhesion protein Tuba (Lamason et al., 2016; Rajabian et al., 2009). These findings

237   provide evidence of convergent evolution by a cadre of pathogens upon mechanisms to

238   decrease cell-cell tension and underscore that cell-cell tension is a critical barrier that

239   must be subverted for successful bacterial infection.

240   **Acknowledgements**

250   **Author Contributions**

251   This project was conceived of and experiments were designed by J.K.D., M.B.G., and

252   B.C.R. All experiments were performed by J.K.D., A.L.W., and B.C.R. The manuscript

253   was written by J.K.D., A.L.W., M.B.G., and B.C.R.

254   **Declaration of Interests**

255   The authors declare no competing interests.

256 **Figure titles and legends.**

257 **Figure 1. IpaC C-terminal tail is required for efficient intercellular spread of *S.***

258 ***flexneri***

259 (A-B) Plaques formed in cell monolayers by *S. flexneri* Δ*ipaC* producing wild-type IpaC

260 or IpaC R362W. Representative Images. (B) Plaque size from experiments represented

261 in A. (C) Plaques formed by wild-type *S. flexneri* in Vim$^{+/+}$ and Vim$^{-/-}$ MEFs.

262 Representative images. (D) Plaques formed by *S. flexneri* strains in Caco-2

263 monolayers. Representative images. (E) Plaque size from experiments represented in

264 panel D, (F) Infectious foci formed in HeLa cells by *S. flexneri* strains. Red, *S. flexneri*;

265 green, bacteria with active T3SS; blue, DAPI. Representative images. (G) Percentage

266 of bacteria that escape into adjacent cells from experiments represented in panel F. For

267 box plots, Dots represent independent experiments (B, C, and E); data are mean ±

268 SEM. Dots represent individual infectious foci of one experiment from two independent

269 experimental replicates (G); boxes outline the 25th and 75th percentiles, midlines

270 denote medians, and whiskers show minimum and maximum values. Scale bars, 500

271 μm (A, D) or 10 μm (F). N.S., not significant; *, $p<0.05$; **, $p<0.01$.

272 **Figure 2. IpaC is required for protrusion formation by *S. flexneri***

273 Infection of HeLa cells with *S. flexneri* Δ*ipaC* producing wild-type IpaC or IpaC R362W.

274 (A) Formation of plasma membrane protrusions. Green, *S. flexneri*; red, t-farnesyl-red

275 fluorescent protein (RFP), which labels plasma membranes; blue, DAPI. Arrowheads,

276 bacteria in protrusions. Representative images. (B) Percentage of intracellular bacteria

277 located within protrusions from experiments represented in panel A, mean ± SEM. (C)

278   Live cell imaging snapshots of actin-based motility of designated *S. flexneri* strains.

279   Yellow, *S. flexneri*; purple, actin. Arrowheads, motile bacteria with unipolar polymerized

280   actin. Representative images. (D) Speeds of bacteria with polymerized actin at one

281   pole. Data is from two (C and D) or three (A and B) independent experiments. Dots

282   represent independent experiments (B) or individual bacteria (D). Scale bars, 10 µM.

283   N.S., not significant; **, $p<0.01$.

284   **Figure 3. IpaC C-terminal tail arginine is required for *S. flexneri*-mediated**

285   **reduction of host membrane tension**

286   Infection of polarized Caco-2 cells with *S. flexneri* Δ*ipaC* producing wild-type IpaC or

287   IpaC R362W. (A) Cell-cell junctions of Caco-2 cells delineated by ZO-1 staining. White,

288   ZO-1; green, *S. flexneri*; orange dots, membrane junctions; orange dotted lines, linear

289   distances between junctions. Representative images. (B) Membrane length from

290   experiments represented in panel A, mean ± SEM. (C) Formation of plasma membrane

291   protrusions after treatment with blebbistatin. Arrowheads, bacteria in protrusions. Red, t-

292   farnesyl-RFP; green, *S. flexneri*; blue, DAPI. Representative Images. (D) Percentage of

293   bacteria in protrusions from experiments represented in panel C, mean ± SEM. Dots

294   represent independent experiments (B and D). Scale bars,10 µM. *, $p<0.05$; **, $p<0.01$.

295   **Figure 4. IpaC interactions with β-catenin are associated with *S. flexneri* cell to**

296   **cell spread**

297   (A) Schematic of yeast protein-protein interaction platform. Interaction of the mCherry-

298   tagged prey protein (red) with the bait protein fused to the inclusion-body forming

299   protein µNS (blue) results in puncta of red fluorescence. In contrast, lack of interaction

300   between prey and bait proteins results in diffuse mCherry fluorescence throughout the

301   cytosol of the yeast cells. (B) Protein interaction assay. Arrowheads, fluorescent puncta.

302   Representative images. (C) Percentage of yeast displaying puncta indicating an

303   interaction, mean ± SEM. (D) Bacterial plaques formed in monolayers stably expressing

304   β-catenin-targeting (#1, #2) or control shRNA. Infection with *S. flexneri* Δ*ipaC* producing

305   wild-type IpaC or IpaC R362W. Green, *S. flexneri*; blue, DAPI. Scale bar, 50 mm.

306   Representative images. (E) Quantification of plaque size (area of spread) from

307   experiments represented in panel D. Dots represent data from three or more

308   independent experiments (C and E). *, p<0.05; **, p<0.01; *** p<0.001.

Supplemental Figure Legends

Figure S1. Intermediate filaments keratin 8 and 18 are dispensable for intercellular spread of *S. flexneri*. (Related to Figure 1).

Plaque formation by wild-type *S. flexneri* in monolayers of Caco-2 cells stably expressing keratin 8 shRNA, keratin 18 shRNA, or non-targeting shRNA. (A) Representative images of plaques. (B) Plaque size (area of spread) from experiments represented in panel A, mean ± SEM. a.u., arbitrary units. Dots represent independent experiments.

Figure S2. Vacuolar escape, regulation of the T3SS, autophagic escape, and actin tail formation of *S. flexneri* producing IpaC R362W are similar to that of *S. flexneri* producing wild-type IpaC. (Related to Figure 2).

Infections of cells with *S. flexneri* Δ*ipaC* producing wild-type IpaC or IpaC R362W. (A) Schematic of *S. flexneri* intracellular lifestyle. (I) After invasion, the *S. flexneri* T3SS ruptures the uptake vacuole, releasing bacteria into the cytosol. (II) In the cytosol, *S. flexneri* utilizes host actin machinery to propel itself to the periphery. (III) At the periphery, motile bacteria form plasma membrane protrusions. (IV) The membranous protrusion is engulfed into a double membrane vacuole in an adjacent cell. (V) The bacteria then rupture the secondary vacuole, thereby escaping into the cytosol, wherein the process is repeated. Type 3 secretion is active during invasion and during spread (stages I, III, IV). Green bacteria, active type 3 secretion. (B) Percent of bacteria that escape the vacuole of Vim$^{+/+}$ MEFs, determined by chloroquine resistance. Intravacuolar bacteria are killed by the high vacuolar concentration of chloroquine,

331 whereas bacteria that escape the vacuole into the cytosol survive. Mean ± SEM. (C)

332 Vim$^{+/+}$ MEFs infected for 60 or 150 minutes with bacteria containing TSAR, a

333 fluorescent reporter of T3SS activity. Red, *S. flexneri*; green, *S. flexneri* with active

334 T3SS; blue, DNA. Representative images. (D) Percent of *S. flexneri* with active T3SS

335 from experiments represented in panel C and including additional time points at 90 and

336 120 minutes, mean ± SEM. (E) Intensity of the GFP signal from *S. flexneri* with active

337 T3SS from panel C, mean ± SEM. (F) Percent of designated *S. flexneri* strains

338 associated with GFP-LC3 during infection of GFP-LC3 HeLa cells. Positive control, *S.*

339 *flexneri* Δ*icsB*. Mean ± SEM. (G) Actin tail and protrusion formation in Vim$^{+/+}$ MEFs

340 infected with designated *S. flexneri* strains for 180 minutes. Red, phalloidin: green, *S.*

341 *flexneri*; blue, DAPI. Representative images. (H) Percentage of bacteria with unipolar

342 actin (tails) from experiments represented in panel G, mean ± SEM. (I) Percentage of

343 bacteria in protrusions from panel G, mean ± SEM. Dots represent independent

344 experiments. Scale bars, 10 μM (C), 5 μM (G). N.S., not-significant; *, p<0.05; **,

345 p<0.01.

346 **Figure S3. β-catenin in intercellular spread of *S. flexneri*. (Related to Figure 4).**

347 (A) β-catenin levels in cell lysates from HeLa cells stably transfected with control or β-

348 catenin targeting shRNAs. β-actin, loading control. Representative western blots. (B)

349 Densitometric analysis of β-catenin depletion in HeLa cells from experiments

350 represented in panel A. (C) β-catenin levels in cell lysates from Caco-2 cells stably

351 transfected with control or β-catenin targeting shRNAs. β-actin, loading control.

352 Representative western blots. (D) Densitometric analysis of β-catenin depletion in Caco-

353 2 cells from experiments represented in panel C. (E) Plaque size (area of spread) of

354 Caco-2 cells depleted of β-catenin and infected with *S. flexneri ΔipaC* producing wild-

355 type IpaC or IpaC R362W. (F) Quantification of membrane length for Caco-2 cells stably

356 transfected with control or β-catenin targeting shRNAs. Mean ± SEM. Dots represent

357 independent experiments. a.u., arbitrary units. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

358 **Figure S4. IpaC binding site on β-catenin overlaps with the E-cadherin binding**

359 **pocket. (Related to Figures 1-4).**

360 Region of β-catenin to which E-cadherin and IpaC bind. E-cadherin binds throughout

361 the armadillo repeats (Huber and Weis, 2001). IpaC interaction with β-catenin occurs

362 within the armadillo repeat region of β-catenin and requires the ninth armadillo repeat

363 (Shaikh et al., 2003).

364 **STAR methods**

365 Contact for Reagent and Resource Sharing

366 Further information and requests for reagents may be directed to Marcia Goldberg

367 marcia.goldberg@mgh.harvard.edu or Brian Russo brusso1@mgh.harvard.edu.

368 **Bacterial Culture**

369 The wild-type *S. flexneri* strain used in this study is *S. flexneri* 2457T (Labrec et al.,

370 1964), and all mutants were isogenic derivatives of it. *S. flexneri* strains were cultured in

371 trypticase soy broth at 37°C. *ipaC* derivatives were cloned into the plasmid pBAD33,

372 and their expression was driven from the pBAD promoter, induced with 1.2% arabinose.

373 **Cell Lines**

374 HeLa, Caco-2, and MEFs were maintained at 37°C in 5% CO_2. All cells were grown in

375 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine

376 serum (FBS). Caco-2 and HeLa cell lines stably expressing farnesylated TagRFP-T (gift

377 of R. Lamason; HeLa only), shRNAs targeting β-catenin [Broad Institute;

378 TRCN000314921 and TRCN000314991(Yang et al., 2011)], or a control (Addgene,

379 Cat# 10879; (Moffat and Sabatini, 2006) were generated using retroviral transduction

380 with selection with 10 μg/ml puromycin.

381 **Plaque Assays**

382 For confluent monolayers, $8x10^5$ (Caco-2) or $6x10^5$ (MEF) cells per well were seeded in

383 six-well plates. The next day, monolayers were infected with bacteria in mid-exponential

384 phase at a multiplicity of infection (MOI) of 0.002 for MEFs and a MOI of 0.02 for Caco-2

385 cells in DMEM. Multiplicity of infection (MOI) was adjusted to compensate for the known

386 decrease in efficiency of invasion by *S. flexneri* in the absence of interaction of IpaC

387 with intermediate filaments. Bacteria were centrifuged onto cells at 800 x*g* for 10

388 minutes and incubated at 37°C in 5% CO_2 for 50 minutes. Media was replaced with

389 0.5% agarose in DMEM containing 25 µg/mL gentamicin, 10% FBS, 1.2% arabinose

390 (for *ipaC* expression from the arabinose promoter), and 0.45% glucose and incubated

391 an additional two days. An additional overlay formulated as before but containing 0.7%

392 agarose and 0.1% neutral red was added. Following incubation for at least 4h, the

393 plates were imaged with an Epson Perfection 4990 photo scanner. ImageJ was used to

394 quantify plaque area.

Discrimination of primarily infected and secondarily infected cells

396 HeLa cells were seeded at $1x10^4$ cells per well on coverslips and, the next day, were

397 infected at a MOI of 200, as above, with strains constitutively expressing a RFP reporter

398 and a GFP whose expression is induced by T3SS secretion (pTSAR). At 30 min of

399 infection, media was replaced with DMEM supplemented with 0.45% glucose, 1.2%

400 arabinose, 25 µg/mL gentamicin, and 10% FBS. $6x10^4$ naïve HeLa cells were overlaid

401 and centrifuged on onto the infected cells at 100 x*g* for 5 minutes. After an additional 3

402 hours, cells were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS)

403 for 20 minutes and stained for 5 minutes with 1:10,000 Hoescht 33342 in PBS.

404 Coverslips were mounted with Prolong Diamond Antifade Mountant (Thermo Fisher)

405 and imaged the next day.

Protrusion Assays

406

407 HeLa cells expressing t-farnesyl-RFP were seeded at 6×10^5 cells per well on coverslips.

408 The next day, cells were infected at an MOI of 100, as above, with designated strains

409 expressing pROEX-Aqua [Addgene; plasmid #42889, (Erard et al., 2013)]. At 30

410 minutes of infection, media was replaced with DMEM supplemented with 0.45%

411 glucose, 1.2% arabinose, 25 µg/mL gentamicin, 1 mM IPTG, and 10% FBS. After an

412 additional 3 hours, cells were fixed with 3.7% paraformaldehyde in PBS and stained

413 with for 5 minutes with 1:10,000 Hoescht 33342 in PBS. Coverslips were mounted with

414 Prolong Diamond Antifade Mountant and imaged the next day.

Live-cell Imaging Analysis

415

416 HeLa cells expressing *LifeAct-GFP* were seeded at 2×10^5 cells per well on 20 mm

417 MatTek glass bottom dishes. The next day cells were infected at a MOI of 100, as

418 above, with *S. flexneri* expressing a constitutive RFP and a GFP reporter of T3SS

419 activity (pTSAR). At 30 minutes of infection, media was replaced with DMEM

420 supplemented with 0.45% glucose, 1.2% arabinose, 25 µg/mL gentamicin, 50 mM

421 HEPES, and 10% FBS. Samples were imaged with a Nikon Eclipse TE-300 at 37°C at

422 5-second intervals for 5 minutes. To calculate actin-tail mediated velocities, the ImageJ

423 plugin particle tracker was used to track in-frame bacteria over the course of the video.

424 Speed was determined for each bacterium by measuring the distance traveled over the

425 duration of time the bacteria was in focus.

Membrane Linearity Assay

426

427   Membrane linearity was performed as previously described (Lamason et al., 2016).

428   Caco-2 cells were seeded onto fibronectin treated coverslips at a density of 8×10^5 cells

429   per well and grown for 7 days to polarize the cells. After 4 days, the media was changed

430   daily. Cells were infected as above with *S. flexneri* strains expressing the uropathogenic

431   *E. coli* Afa-1 pilus (Labigne-Roussel et al., 1984), which binds to decay accelerating

432   factor (CD55) on the surface of human cells (Nowicki et al., 1993) and enhanced

433   infection at a MOI of 10. At 30 minutes of infection, media was replaced with DMEM,

434   supplemented with 0.45% glucose, 1.2% arabinose, 25 µg/mL gentamicin, and 10%

435   FBS. After an additional 3 hours, cells were fixed with 3.7% paraformaldehyde in PBS,

436   permeabilized with 0.5% Triton X-100 in PBS, and stained with mouse anti-ZO-1

437   antibody (Invitrogen, cat# 339100; 1:100 dilution) and Alexa Fluor 568 conjugated

438   secondary, with anti-*Shigella* antibody (ViroStat cat# 0903; 1:100 dilution), and with

439   Hoescht 33342. Coverslips were mounted with Prolong Diamond Antifade Mountant and

440   imaged the next day. The junction length was determined by measuring with ImageJ the

441   linear distance between cell vertices and the actual length of the plasma membrane

442   between the same vertices. Junction length was expressed as a ratio of the actual

443   length of the membrane divided by the linear inter-vertex distance.

444   **Yeast Protein-Protein Interaction Assay**

445   Yeast protein interaction assays were performed as previously described (Schmitz et

446   al., 2009). Yeast carrying plasmids encoding mCherry-tagged IpaC variants and µNS

447   alone (negative control) or µNS-tagged β-catenin were cultured overnight in complete

448   synthetic media lacking histidine and leucine supplemented with 2% raffinose. The next

449   morning, strains were back diluted to OD_{600} 0.5 and grown for 2h at 30°C with 2%

450 raffinose. Then, to induce protein synthesis, the media was changed to 2% galactose,

451 and growth was allowed to proceed for 4h at 30°C. Yeast were wet mounted and

452 imaged. To determine the percentage of yeast with puncta, the total number of yeast

453 cells was determined by brightfield and puncta of red fluorescent protein were counted.

454 **Infectious Foci Assay**

455 HeLa cells were seeded at a density of 6 x 10^5 cells per well on coverslips in 6 well

456 plates. The next day, cells were infected as above at a MOI of 0.05 with designated

457 strains. At 50 minutes of infection, media was replaced with DMEM supplemented with

458 0.45% glucose, 1.2% arabinose, 25 μg/mL gentamicin, and 10% FBS. Cells were

459 incubated at 37°C with 5% CO_2 for 12 hours at which point they were rinsed once and

460 fixed with 3.7% paraformaldehyde in PBS for 20 minutes. Cells were washed three

461 times with PBS, incubated with 1 M glycine in PBS for 15 minutes, washed three

462 additional times with PBS and then permeabilized with 0.5% Triton X-100 for 20

463 minutes. Cells were washed five times with PBS and incubated overnight at 4°C with

464 Alexa Fluor 488 conjugated anti-*Shigella* antibody (ViroStat, 1:1000 dilution). Cells were

465 washed three times with PBS, stained with Hoescht 33342, washed twice with PBS and

466 mounted with Prolong Diamond Antifade Mountant. Foci were randomly imaged across

467 the coverslip, and the number of infected cells within each focus was determined by

468 counting nuclei that co-localized with bacteria.

469 **Vacuolar Escape**

470 The resistance of intracellular bacteria to chloroquine, which accumulates within

471 vacuoles and kills intra-vacuolar bacteria but remains at sub-bactericidal concentrations

472 within the cytosol, was tested as previously described (Zychlinsky et al., 1994). Notably,

473 IpaC is an important factor in vacuolar escape ((Du et al., 2016), Briefly, 1×10^4 Vim$^{+/+}$

474 MEFs were seeded into wells of a 96-well plate. The next day, cells were infected as

475 above at a MOI of 100 with designated strains. At 50 minutes of infection, media was

476 replaced with DMEM supplemented with 0.45% glucose, 1.2% arabinose, 25 µg/mL

477 gentamicin, 10% FBS ± 200 µg/mL chloroquine. After 1 hour, cells were washed three

478 times and lysed with 1% Triton X-100 in PBS. Bacteria were serially diluted 1:5 and the

479 number of intracellular bacteria were enumerated by plating dilutions. Percent cytosolic

480 bacteria were the ratio of colony forming units (CFU) in the presence of gentamicin and

481 chloroquine to CFU in the presence of gentamicin without chloroquine multiplied by 100.

482 **LC3 Co-localization**

483 To quantify intracellular bacteria associated with LC3, HeLa cells stably expressing a

484 GFP-LC3 construct were seeded at a density of 4×10^5 cells per well in a 6-well plate

485 on coverslips. The next day, cells were infected as above at a MOI of 100 with indicated

486 strains. At 50 minutes of infection, media was replaced with DMEM supplemented with

487 0.45% glucose, 1.2% arabinose, 25 µg/mL gentamicin, and 10% FBS. After an

488 additional 3 hours, cells were washed once with PBS, fixed with 3.7%

489 paraformaldehyde and stained with Hoechst 33342. Coverslips were mounted with

490 Prolong Diamond Antifade Mountant, and cells were imaged randomly across the

491 coverslip. LC3-positive bacteria were counted as those that co-localized with strong

492 GFP signal.

493 **Type 3 Secretion System Activity**

494   To measure type 3 secretion system activity, Vim$^{+/+}$ MEFs were seeded at a density of 4

495   x 10^5 cells per well in 6-well plates on coverslips. The next day, cells were infected, as

496   above, with indicated strains carrying the plasmid pTSAR [GFP expression is driven by

497   the mxiE box, which requires type 3-mediated secretion of the effector OspD1, and

498   mCherry is driven by the constitutive rpsM promoter (Campbell-Valois et al., 2014;

499   Parsot et al., 2005). At 20 minutes of infection, media was replaced with DMEM

500   supplemented with 0.45% glucose, 1.2% arabinose, 25 µg/mL gentamicin, and 10%

501   FBS. At indicated time points, cells were washed once with PBS, fixed with 3.7%

502   paraformaldehyde, and stained with Hoechst 33342. Cells were washed two additional

503   times and mounted with Prolong Diamond Antifade Mountant. Cells were randomly

504   imaged across the coverslip, and the percent of bacteria with active type 3 secretion

505   systems was determined by enumerating the ratio of GFP-positive bacteria (T3SS

506   active) to RFP-positive bacteria (total). To determine the intensity of GFP signal,

507   bacteria were identified by an RFP signal, and the intensity of GFP signal was

508   measured with imageJ (Schneider et al., 2012).

509   **Actin Tail Formation**

510   To quantify actin-tail formation, Vim$^{+/+}$ MEFs were seeded at a density of 4 x 10^5 cells

511   per well in 6-well plates on coverslips. The next day, cells were infected as above with

512   indicated strains. At 50 minutes of infection, media was replaced with DMEM

513   supplemented with 0.45% glucose, 1.2% arabinose, 25 µg/mL gentamicin, and 10%

514   FBS. After an additional 3 hours, cells were washed once with PBS, fixed with 3.7%

515   paraformaldehyde, and stained with anti-*Shigella* antibody conjugated to Alexa Fluor

516   488 (ViroStat, 1:1000 dilution). The next day, cells were washed three times with PBS

517   and stained with phalloidin conjugated to Alexa Fluor 568 (Invitrogen, cat# A12380).

518   Cells were washed three times with PBS and stained with Hoescht 33342. Cells were

519   washed twice more with PBS and mounted with Prolong Diamond Antifade Mountant.

520   The percentage of bacteria with actin-tails was determined by counting the number of

521   total bacteria (GFP-positive) to the number of bacteria with actin-tails.

522   **Quantification and Statistical Analysis**

523   Statistical differences between means was determined with GraphPad Prism. Statistical

524   differences between two means was tested by unpaired Student's t-test. Differences

525   between the means of three or more groups was tested by either two-way ANOVA with

526   Sidak *post hoc* test (Figures S2E-F) or one-way ANOVA with Tukey *post hoc* test.

527   Microscopic images were pseudocolored and assembled using Adobe Photoshop or

528   ImageJ.

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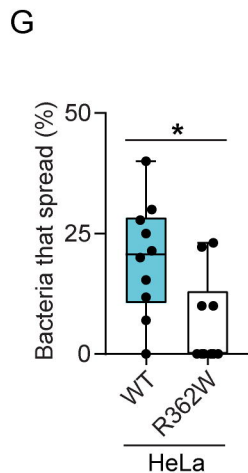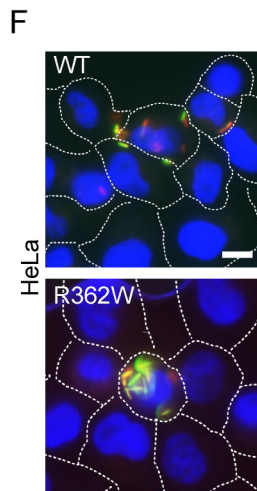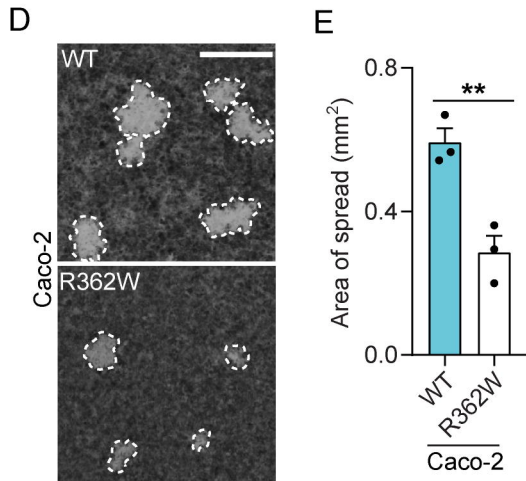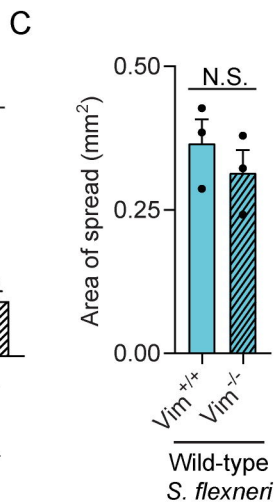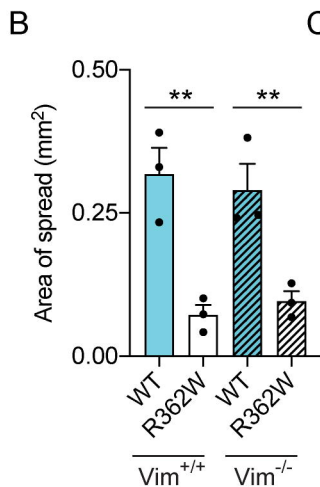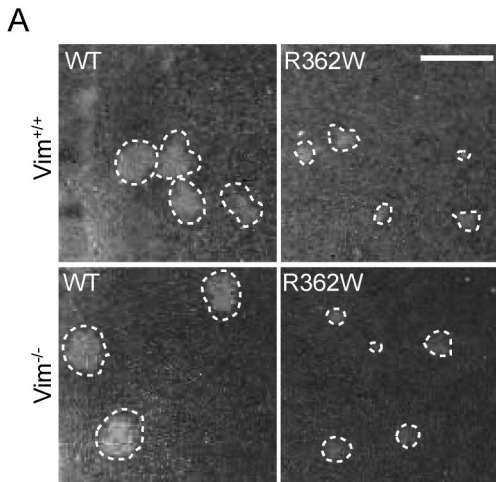671   LC3-Dependent Autophagy in Shigella flexneri Dissemination. Infect Immun *86*.

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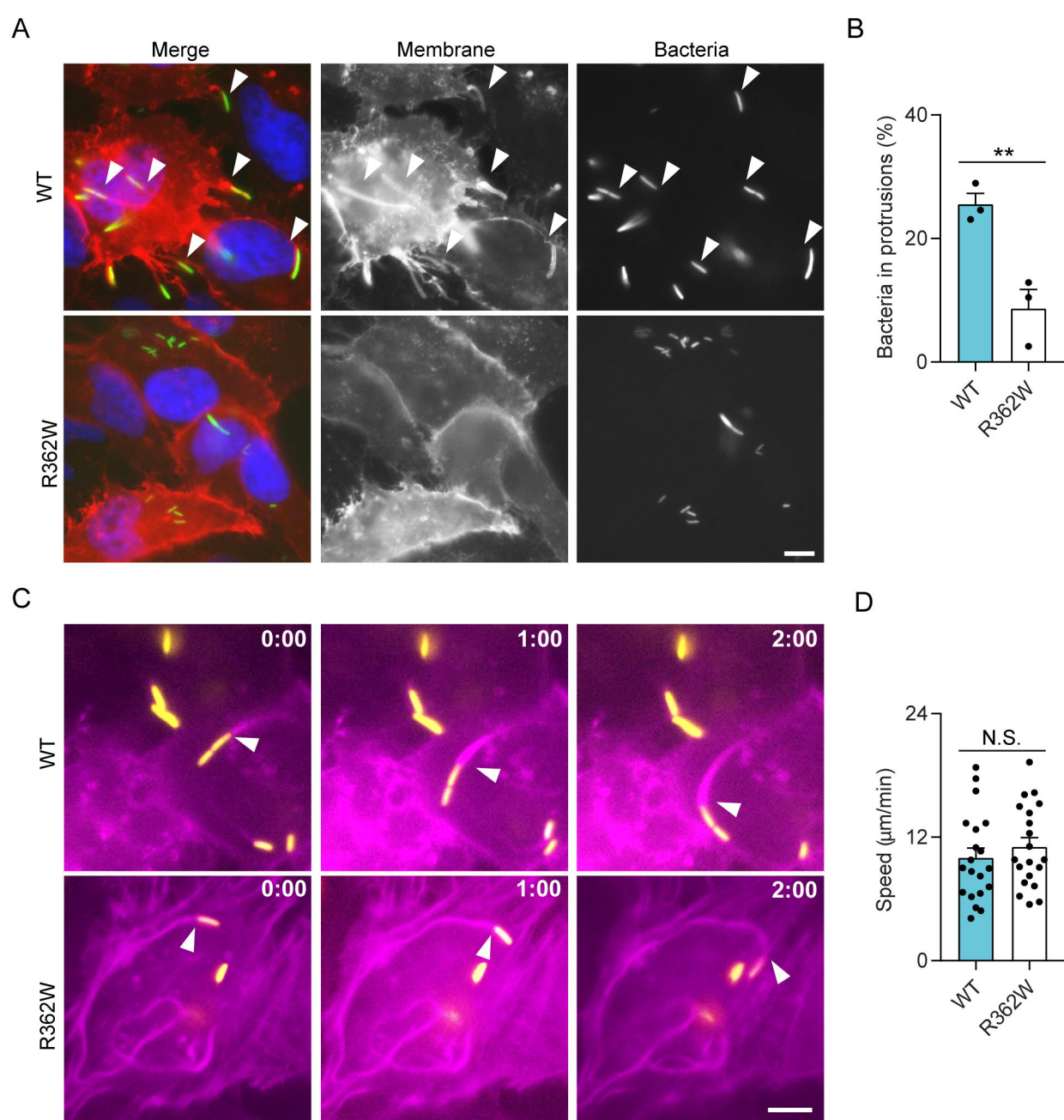675   Yi, C.R., Allen, J.E., Russo, B., Lee, S.Y., Heindl, J.E., Baxt, L.A., Herrera, B.B.,

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A

WT | R362W (Vim$^{+/+}$)
WT | R362W (Vim$^{-/-}$)

B

Area of spread (mm^2)

** | **

WT | R362W | WT | R362W
Vim$^{+/+}$ | Vim$^{-/-}$

C

Area of spread (mm^2)

N.S.

Vim$^{+/+}$ | Vim$^{-/-}$
Wild-type *S. flexneri*

D

Caco-2
WT
R362W

E

Area of spread (mm^2)

**

WT | R362W
Caco-2

F

HeLa
WT
R362W

G

Bacteria that spread (%)

*

WT | R362W
HeLa

A

	Merge	Membrane	Bacteria
WT			
R362W			

B

Bacteria in protrusions (%)

WT   R362W

**

C

	0:00	1:00	2:00
WT			
R362W			

D

Speed (µm/min)

WT   R362W

N.S.

A

Uninfected | WT | R362W

B

Junction Length (relative to vertex distance)

Uninfected | WT | R362W

** *

C

Merge | Membrane | Bacteria

No Blebbistatin

WT

R362W

Blebbistatin

WT

R362W

D

Bacteria in protrusions (%)

WT | R362W | WT | R362W

− | +

Blebbistatin

* * *

A

protein-protein interaction | no interaction

mCherry-tagged prey ● | bait ⬤

B

| Prey: | IpaC WT | IpaC WT | IpaC R362W |
| Bait: | Empty | β-catenin | β-catenin |

C

Yeast with interactions (%)

Prey	Bait
IpaC WT	Empty
IpaC WT	β-catenin
IpaC R362W	β-catenin

D

WT | R362W

Control

shRNA #1

β-catenin

#2

E

Area of spread (# cells)

shRNA: | WT | R362W | WT | R362W | WT | R362W
Control | #1 | #2
β-catenin