MYO6 is targeted by *Salmonella* virulence effectors to trigger PI3-kinase signaling and pathogen invasion into host cells

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To establish infections, *Salmonella* injects virulence effectors that hijack the host actin cytoskeleton and phosphoinositide signaling to drive pathogen invasion. How effectors reprogram the cytoskeleton network remains unclear. By reconstituting the activities of the *Salmonella* effector SopE, we recapitulated Rho GTPase-driven actin polymerization at model phospholipid membrane bilayers in cell-free extracts and identified the network of Rho-recruited cytoskeleton proteins. Knockdown of network components revealed a key role for myosin VI (MYO6) in *Salmonella* invasion. SopE triggered MYO6 localization to invasion foci, and SopE-mediated activation of PAK recruited MYO6 to actin-rich membranes. We show that the virulence effector SopB requires MYO6 to regulate the localization of PIP3 and PI(3)P phosphoinositides and Akt activation. SopE and SopB target MYO6 to coordinate phosphoinositide production at invasion foci, facilitating the recruitment of cytoskeleton adaptor proteins to mediate pathogen uptake.

motor protein | type 3 secretion system | rho GTPase | macropinocytosis | infection

Mammalian cells use Rho GTPases Rac1, Cdc42, and RhoA as master regulators of the actin cytoskeleton to coordinate the formation of actin-rich structures such as lamellipodia and filopodia at the plasma membrane (1). Rho GTPases are anchored at the membrane by prenylation, where they are activated by guanine-nucleotide exchange factors (GEFs) that promote GTP binding to switch the GTPases from an inactive (GDP-bound) to an active (GTP-bound) conformation. GTP-bound Rho GTPases directly activate specific cellular effectors that coordinate the formation of distinct actin structures, e.g., Arp2/3 activators *N*-WASP and the WAVE complex (1). Rho effectors also include p21-activated kinase (PAK) that controls actin filament turnover by regulating the activity of actin-binding proteins (2). PAK also has been shown to phosphorylate MYO6, a unique member of the myosin superfamily that moves toward the minus end of actin filaments (3, 4). MYO6 has important roles in the endocytic pathway and in the regulation of plasma membrane dynamics and membrane ruffle formation (5, 6).

To establish infections, the intestinal pathogen *Salmonella enterica* Typhimurium subverts the actin cytoskeleton by injecting a mixture of virulence effector proteins into host epithelial cells to facilitate uptake by macropinocytosis (7–11). Of particular importance is the virulence effector SopE, a Rac1 and Cdc42 GEF that is required for generating membrane ruffles and bacterial macropinocytosis (7, 12). Indeed, deletion of SopE reduces *Salmonella* invasion by 60%, and in the absence of SopE and the known *Salmonella* Cdc42 activators SopE2 and SopB, the pathogen cannot activate Rho GTPases or invade host cells (8, 9). *Salmonella* likely highjacks a number of Rho GTPase effectors, but their identity, how they interact with each other, and how they contribute to *Salmonella* invasion remain unresolved. Because SopE activates both Rac1 and Cdc42, we set out to reconstitute SopE signaling at model membranes and identify the components of the Rho cytoskeleton network hijacked by *Salmonella*.

Results

Identification of the Cytoskeleton Protein Network Recruited via SopE. To identify the membrane-associated cytoskeleton network of proteins targeted by SopE, we first reconstituted SopE-mediated actin filament polymerization at immobilized phospholipid membrane bilayers in optimized cell-free brain extract, as previously described (10). Silica microspheres were coated with a phospholipid bilayer composed of equal concentrations of phosphatidylcholine and phosphatidylinositol (PC:PI), which have been used previously to reconstitute Rho-driven actin assembly (13, 14). When the microspheres were incubated in extract, the control PC:PI beads were unable to polymerize actin (Fig. 1*A*). In contrast, when the extract was supplemented with purified recombinant SopE, actin filaments were immediately assembled on the membrane surface and propelled the beads through the extract via actin comet tail formation (Fig. 1*A*). Inhibiting actin polymerization (cytochalasin D) or the Rho GTPases Rac1 and Cdc42 (the PBD domain of PAK or GDI, which binds and sequesters these Rho GTPases) (14) abrogated actin assembly (Fig. 1*C*). This effect confirmed that SopE-activation of Rho GTPases is necessary for the formation of actin comet tails.

Significance

***Salmonella* causes many different diseases including gastroenteritis and typhoid fever. For infection to take place, *Salmonella* must enter the epithelium in the gut by injecting a number of effector proteins that trigger dramatic actin rearrangements and membrane ruffles to engulf the pathogen. In this study we identified a myosin motor protein that translocates along actin filaments as one of the crucial host proteins that are targeted by two *Salmonella* effector proteins, SopE and SopB, at the onset of infection. SopE and SopB exploit MYO6 to facilitate membrane ruffle formation and phospholipid production at the invasion site to mediate pathogen uptake. Myosin motors are highly druggable targets, and therefore myosin inhibitors are attractive new tools to fight bacterial infections.**

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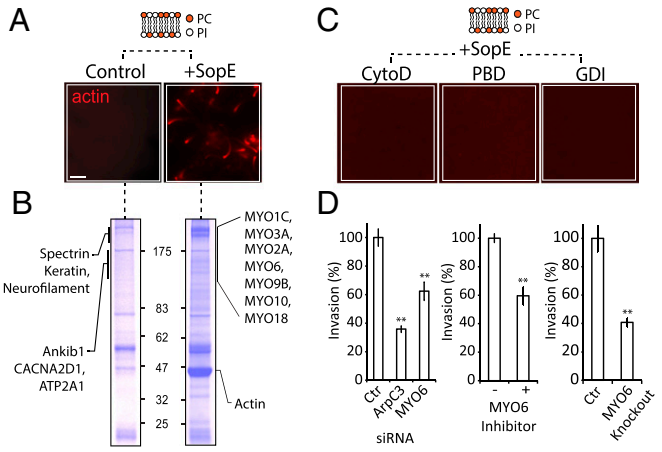

Fig. 1. Cytoskeleton proteins manipulated by *Salmonella* SopE. (*A*) Beads coated with 50% PC and 50% PI (depicted in cartoon) were incubated in cell-free extract containing rhodamine-labeled actin in the presence (+SopE) or absence (Control) of 1 μM SopE. (Scale bar: 5 μm.) (*B*) SDS/PAGE analysis and Coomassie blue staining of proteins recruited by the beads shown in *A*. SopE-recruited myosins (*Right*) are annotated with proteins of equivalent molecular mass recruited by the control (*Left*). Molecular mass markers are shown in kDa. (*C*) Experiment performed as *A* in extract containing inhibitors of actin polymerization (cytochalasin D, 5 μM), or Rho GTPase signaling (PBD or GDI, 1 μM). (Scale bars: 5 μm.) (*D*) *Salmonella* invasion into HeLa cells in which MYO6 was depleted by siRNA, inhibited by TIP, or knocked out by CRISPR/CAS9-based engineering. Error bars represent ± SEM; $^{**}P < 0.01$.

To identify the putative SopE-signaling network, the actin-based motility assays from Fig. 1*A* were scaled up. Proteins recruited from the cytosol to PC:PI were separated by SDS/PAGE (Fig. 1*B*) and identified by parallel mass spectrometry. More than 200 proteins were specifically recruited to PC:PI-coated beads directly or indirectly via SopE signaling through Rho GTPases (Table S1), including cellular effectors of Rac1 and Cdc42 that orchestrate actin filament polymerization (1), such as *N*-WASP and the WAVE complex (comprising Cyfip, Nap1, Abi, WAVE1, and homologs); formins (FMNL1, -2, and DIAPH2); PAK1 and -3; the BAR protein FNBP1; and CEP4/BORG4, as well as its cognate septin-binding partners (summarized in Table S2). Consistent with the presence of Rho GTPase effectors, the Arp2/3 complex and a number of actin-binding proteins that regulate filament dynamics and architecture were also recruited by SopE. In addition seven myosin motor proteins that were absent from control PC:PI beads were recruited in a SopE-dependent manner (Fig. 1*B* and Tables S1 and S2).

Myosin motors are actin-activated ATPases that translocate along actin filaments and are involved in many different cellular processes by anchoring intracellular cargoes and organelles, by mediating their short-range transport, and also by regulating the architecture of the actin cytoskeleton and plasma membrane dynamics (15). Given the importance of the myosin superfamily in these diverse cellular functions, we sought to address the role of myosin motors in *Salmonella* macropinocytosis.

MYO6 Facilitates *Salmonella* Invasion. To investigate the importance of these myosin motors in SopE-induced actin filament remodeling, we transfected siRNAs targeting the seven myosins listed in Fig. 1*B* and an additional six members of the myosin family expressed in HeLa cells (namely myosins 1B, 1E, 1G, 2B, 2C, 5A, and 5C) (16) before assessing *Salmonella* invasion (Fig. S1 and Table S3). Although we cannot completely exclude a possible role for the other myosins tested, our screen revealed that MYO1C, -5A, -5C, and -6 siRNA transfections significantly impaired invasion relative to cells transfected with a scrambled siRNA. However, only MYO1C and MYO6 were recruited to the PC:PI beads by SopE (Fig. 1*B*). Because MYO1C was recently reported to be required for *Salmonella* invasion (17), we further analyzed the requirement for MYO6 in pathogen uptake. Our results clearly demonstrate a key role for MYO6 in *Salmonella* invasion in cells treated with a small molecule inhibitor of MYO6 (18), in CRISPR-Cas9 MYO6-knockout cells, and in siRNA-transfected MYO6-depleted cells (Fig. 1*D*). Successful knockout and siRNA-mediated depletion of MYO6 were confirmed by immunoblotting (Fig. S1 *B* and *C*).

SopE-Dependent Recruitment of MYO6 to *Salmonella*-Induced Ruffles. We next examined the recruitment of MYO6 by SopE in more detail. Immunoblotting of PC:PI beads isolated from actin-based motility assays confirmed that SopE (+) triggered the recruitment of MYO6 as well as Cdc42, Rac1, and actin (Fig. 2*A*), as was consistent with Rho-mediated actin comet tail formation (Fig. 1*C*). To investigate whether SopE regulated MYO6 localization to actin filaments at the pathogen entry site during invasion, HeLa

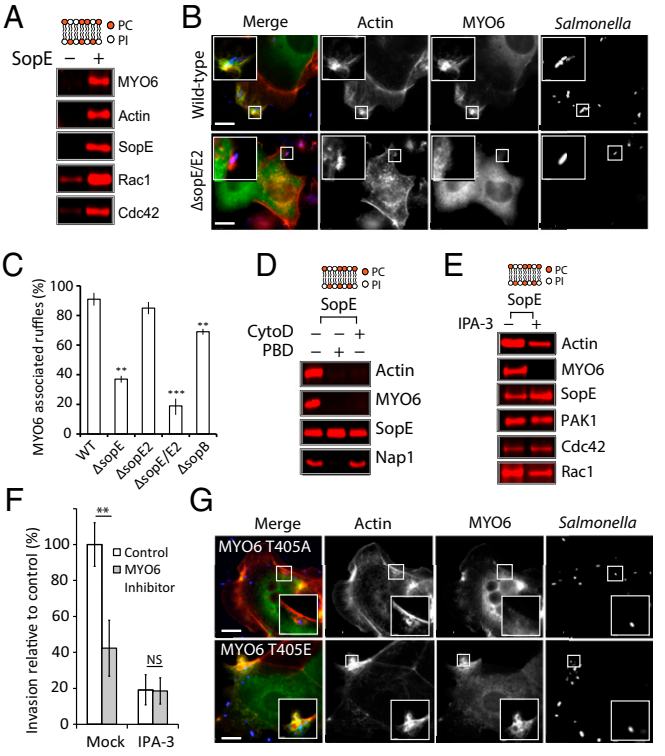

Fig. 2. SopE manipulation of MYO6 and PAK. (*A*) PC:PI beads incubated in cell extract in the absence (–) or presence (+) of SopE were analyzed by immunoblotting with the indicated antibodies. (*B*) Localization of GFP-MYO6 (green) in HeLa cells infected with wild-type or Δ*sopE Salmonella*. Bacteria were visualized using *Salmonella* antibodies (red) and actin filaments with Alexa-Fluor 350-conjugated phalloidin (blue). (Scale bars: 5 μm.) *Insets* 3× magnify *Salmonella*-induced ruffles. (*C*) Quantification of MYO6 localization to membrane ruffles. The experiment was performed as in *B* using wild-type, Δ*sopE*, Δ*sopE2*, Δ*sopE*/E2, and Δ*sopB Salmonella*. Error bars represent ± SEM; $^{**}P < 0.01$, $^{***}P < 0.001$. (*D*) SopE recruitment of MYO6 in the presence (+) or absence (–) of inhibitors of actin polymerization (CytoD) and Rho GTPase signaling (PBD). The experiment was performed as in *A*. (*E*) SopE recruitment of MYO6 in the presence (+) or absence (–) of the PAK inhibitor IPA-3. The experiment was performed as in *A*. (*F*) *Salmonella* invasion into HeLa cells in the absence (control) or presence of the MYO6 inhibitor TIP, with or without (Mock) the PAK inhibitor IPA-3 in combination. Error bars represent ± SEM; $^{**}P < 0.01$. NS, nonsignificant. (*G*) Localization of GFP-MYO6 (T405A) or phosphomimic (T405E) to *Salmonella*-induced ruffles. The experiment was performed as in *B*. *Insets* 3× magnify *Salmonella*-induced ruffles. (Scale bars: 5 μm.)

cells expressing GFP-tagged MYO6 were infected with either wild-type *Salmonella* or the isogenic Δ*sopE* mutant (Fig. 2*B*). Both wild-type and Δ*sopE Salmonella* induced actin-rich membrane ruffles at pathogen foci (Fig. 2*B*, *Insets*), as expected given the role of multiple SPI-1 effectors in cytoskeleton remodeling (19). In cells with wild-type *Salmonella*, robust MYO6 localization was observed at ∼90% of pathogen-induced ruffles (Fig. 2*C*). However, when cells were infected with Δ*sopE*, MYO6 was absent from the majority of ruffles (Fig. 2*B*), with only ∼35% showing colocalization with MYO6 (Fig. 2*C*). We next tested whether, in addition to the Rac1/Cdc42 GEF SopE, other effectors such as SopE2 and SopB, which directly and indirectly activate Cdc42, also promote MYO6 localization to ruffles (8, 11). Cells infected with Δ*sopE2* showed no reduction in MYO6 recruitment, presumably because of the presence of SopE. However, MYO6 localization at pathogen-induced ruffles was reduced to ∼20% in cells infected with the double mutant Δ*sopE*/*E2* and to ∼70% in cells infected with Δ*sopB* (Fig. 2*C*). Consistent with their role in activating Rho GTPases, chemical inhibition of Rac1 and Cdc42 impeded MYO6 recruitment to ruffles induced by wild-type *Salmonella* (Fig. S1*D*).

Rho GTPase subversion is a central virulence strategy of many bacterial pathogens. For example, *Shigella flexneri* also invades host cells using Rho GTPases that trigger membrane ruffling (20), and, indeed, MYO6 also was present in *Shigella*-induced ruffles (Fig. S1*E*). Because MYO6 binds actin filaments, it remained possible that this motor is recruited to actin filaments assembled at the plasma membrane independently of Rho signaling, e.g., to actin pedestals generated beneath the extracellular pathogen enteropathogenic *Escherichia coli* (EPEC) (21). When we examined MYO6 localization in EPEC-infected host cells, MYO6 was absent from actin pedestals (Fig. S1*E*), as previously reported (22). Taken together, our data suggest that MYO6 localizes specifically to pathogen foci where Rho GTPases are activated to induce membrane ruffling.

PAK Recruits MYO6 to the Membrane. Given the importance of Rho GTPases in mediating MYO6 localization, we next addressed the mechanism by which MYO6 is recruited to actin-rich membrane ruffles. Inhibition of Rho GTPases (+PBD) or actin polymerization (+CytoD) in vitro blocked SopE-dependent recruitment of MYO6 to the membrane (Fig. 2*D*). We noticed that components of the WAVE regulatory complex (i.e., nap1), a Rac1 effector) were still recruited in the presence of cytochalasin D but not in the presence of PBD, indicating that MYO6 targeting is not solely regulated by Rho GTPases or membrane interactions but also requires Rho GTPase-induced actin filaments for recruitment.

Interestingly, our proteomics data also highlight the presence of the PAK family of Rac1 and Cdc42 effectors within the cytoskeleton network recruited by SopE (Tables S1 and S2). *Salmonella* is known to trigger activation of PAK (23), and PAK also has been reported to phosphorylate MYO6 in the motor domain (4). A potential phosphorylation site is the threonine in position 405 at a conserved site within the head domain of MYO6 (24). To determine whether PAK regulates MYO6 recruitment, actin filament polymerization was triggered at the membrane by SopE in extract supplemented with IPA-3 (Fig. 2*E*), an inhibitor of PAK1–3 activation. IPA-3 completely ablated SopE recruitment of MYO6 to membranes while still recruiting Rho GTPases and triggering actin polymerization, thereby distinguishing MYO6 recruitment from actin filament formation. Thus, MYO6 recruitment required the combination of actin filaments and Rho GTPase-activated PAK (Fig. 2 *D* and *E*). Furthermore, inhibiting PAK with IPA-3 in host cells reduced *Salmonella* invasion by ∼80% (Fig. 2*F*). Consistent with PAK regulation of MYO6, no further reduction in invasion was observed when inhibitors of PAK and MYO6 were used in combination (Fig. 2*F*), showing that PAK and MYO6 mediate pathogen uptake via the same pathway.

To verify the link between PAK and MYO6, we assessed the significance of the putative MYO6 phosphorylation site at threonine 405 for its localization to *Salmonella*-induced ruffles (Fig. 2*G*). GFP-tagged MYO6 mutants mimicking the dephosphorylated (T405A) or phosphorylated (T405E) form were expressed in HeLa cells before *Salmonella* infection. MYO6 (T405E) was strongly recruited to *Salmonella*-induced ruffles, but the T405A mutant was not (Fig. 2*G* and Fig. S2*A*), demonstrating that PAK-dependent phosphorylation in the motor domain may regulate MYO6 localization to foci of *Salmonella* invasion. Consistent with this suggestion, only the GFP-MYO6 head domain (residues 1–835), but not the tail domain alone, was recruited into membrane ruffles (Fig. S2*B*).

MYO6 Accumulates PI(3)P at *Salmonella*-Associated Macropinocytic Cups. Having established that MYO6 is recruited into membrane ruffles, we next investigated the mechanism by which MYO6 facilitates pathogen invasion. Although the loss of MYO6 reduced the formation of pathogen-induced membrane ruffle (∼65% in the control relative to ∼45% in MYO6-depleted cells),

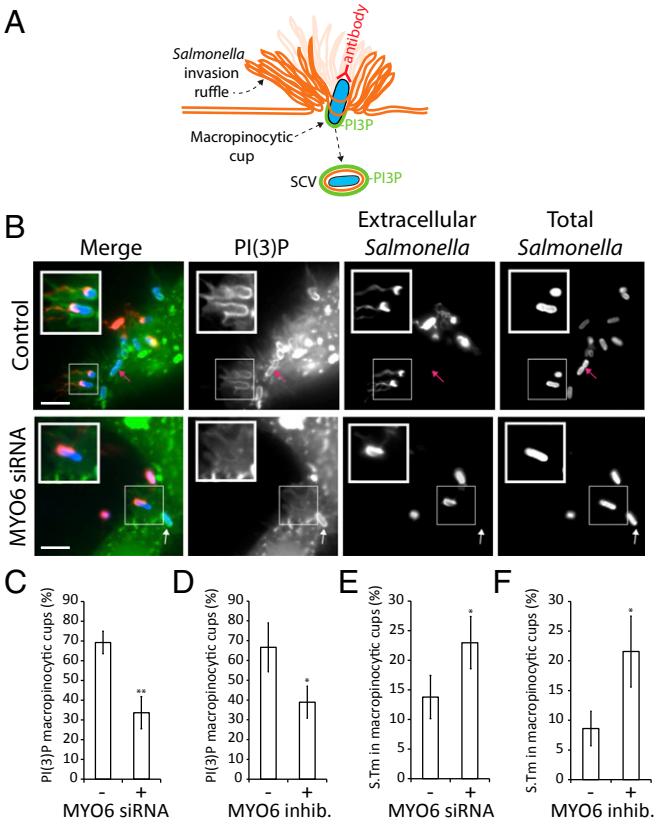

Fig. 3. MYO6 regulation of the *Salmonella* macropinocytic cup. (*A*) Cartoon depicting the macropinocytic cup and SCV labeling for the experiment shown in *B*. (*B*) Localization of PI(3)P at *Salmonella* macropinocytic cups. Cells were transfected with nontargeting siRNA (Control) or MYO6 siRNA before transfection with GFP-p40-PX [PI(3)P, green] and infection with Alexa-Fluor 350-labeled wild-type *Salmonella* (Total *Salmonella*, blue). Extracellular *Salmonella* (red) were visualized by staining nonpermeabilized cells with anti-*Salmonella* antibodies. (Scale bars: 5 μm.) *Insets* 2× magnify *Salmonella* macropinocytic cups. Arrows indicate intracellular SCVs. (*C* and *D*) The number of PI(3)P-rich macropinocytic cups was quantified from the experiment shown in *B* using MYO6 sRNA (*C*) and in cells treated with the small molecule inhibitor of MYO6 (MyoVI inhib) (*D*). (*E* and *F*) The number of *Salmonella* (S.Tm)-associated macropinocytic cups relative to the total number of colonizing bacteria was quantified from the experiments in *C* and *D*, respectively. Error bars represent ± SEM; $*P < 0.05$; $**P < 0.01$.

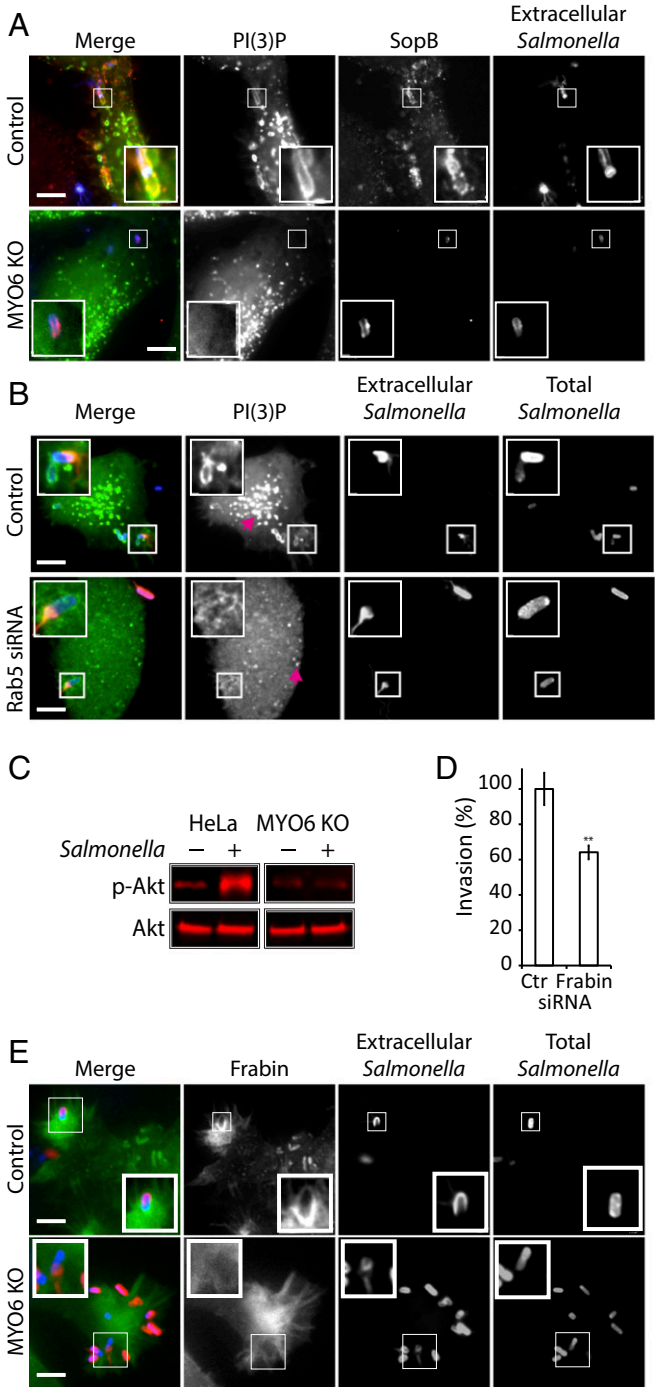

Fig. 4. MYO6 regulation of Akt signaling and recruitment of Frabin. (*A*) Localization of SopB in control or MYO6-knockout HeLa cells expressing GFP-p40-PX [PI(3)P, green] were infected with *Salmonella* encoding FLAG-tagged SopB. Nonpermeabilized cells were labeled with anti-*Salmonella* antibodies (blue) before permeabilization and labeling with anti-FLAG antibodies (red). (Scale bars: 5 μm.) *Insets* 3× magnify the macropinocytic cup. (*B*) Localization of PI(3)P at *Salmonella* macropinocytic cups in cells transfected with non-targeting siRNA (Control) or Rab5 siRNA before transfection with GFP-p40-PX [PI(3)P, green] and infection with Alexa-Fluor 350-labeled *Salmonella* (Total *Salmonella*, blue). Extracellular *Salmonella* were visualized by staining nonpermeabilized cells with anti-*Salmonella* antibodies (Extracellular *Salmonella*, red). (Scale bars: 5 μm.) *Insets* 3× magnify *Salmonella* macropinocytic cups. Arrows indicate PI(3)P-rich endosomes. (*C*) Control and MYO6-knockout HeLa cells were infected (+) or not (–) with *Salmonella*. Then whole-cell lysates were immunoblotted for Akt and phosphorylated Akt (p-Akt). (*D*) *Salmonella* invasion into HeLa cells where Frabin was depleted by siRNA transfection. Error bars represent ± SEM; $^{**}P < 0.01$. (*E*) Localization of CFP-Frabin during *Salmonella* invasion into control and MYO6-knockout cells. (Scale bars: 5 μm.) The experiment was performed as in *B*. *Insets* 2× magnify *Salmonella* macropinocytic cups.

indicating a potential role for MYO6 in regulating actin filament organization (Fig. S2*C*), *Salmonella*-induced ruffles were not completely inhibited in the absence of MYO6 (Fig. 2*B* and Fig. S2*C*), suggesting that this myosin plays an additional role during pathogen uptake. Actin filament polymerization at sites of *Salmonella* invasion leads to the formation of a macropinocytic cup, which can be visualized using a GFP-p40-PX domain fusion construct that binds to the phosphoinositide PI(3)P in the macropinocytic cup at the base of invasion ruffles (25) (Fig. S3*A*).

We decided to examine whether MYO6 influenced the localization of PI(3)P in the macropinocytic cup at invasion ruffles during pathogen uptake (Fig. 3). As depicted in the cartoon (Fig. 3*A*) and demonstrated experimentally (Fig. 3*B*), in control cells the PI(3)P-enriched macropinocytic cup formed a tight association with the invading pathogen, which was inaccessible to anti-*Salmonella* antibodies (Fig. 3*B*, *Inset*). Indeed, when *Salmonella* were in the macropinocytic cup, only the exposed tip of the pathogen and its flagella were accessible to antibodies (extracellular *Salmonella*). In contrast, the intracellular bacteria within *Salmonella*-containing vacuoles (SCVs) (pink arrows in Fig. 3*B*) were completely surrounded by PI(3)P and completely inaccessible to antibodies (Fig. 3 *A* and *B*). To our surprise, we observed a striking reduction of PI(3)P at macropinocytic cups in MYO6-depleted cells (Fig. 3*B*). PI(3)P was reduced from ∼70% in the control to ∼33% in MYO6-depleted cells (Fig. 3*C*, + MYO6 siRNA) and in cells treated with the MYO6 inhibitor (Fig. 3*D*, + MYO6 inhibitor). Intriguingly, PI(3)P still surrounded bacteria within intracellular SCVs (white arrows in Fig. 3*B*). Thus, MYO6 is required for the localization of PI(3)P at the macropinocytic cup but not at the SCV, thus revealing two distinct pools of phosphoinositide PI(3)P associated with the pathogen.

The reduction in PI(3)P levels either through MYO6 siRNA transfection or inhibitor treatment caused increased numbers of extracellular bacteria associated with macropinocytic cups at the cell surface (Fig. 3 *E* and *F*), suggesting a significant delay in pathogen uptake. In summary, our data suggest that MYO6 facilitates pathogen invasion by promoting membrane ruffling and by triggering localization of PI(3)P at the macropinocytic cup.

MYO6 Cooperates with SopB to Mediate PI3-Kinase Signaling. We next addressed how MYO6 facilitated localization of PI(3)P at the macropinocytic cup. It is well established that the generation of PI(3)P by *Salmonella* is dependent on the bacterial effector SopB, a phosphoinositide phosphatase with PI 4′- and 5′-phosphatase activity (26). SopB is thought to activate distinct classes of PI3-kinases (PI3K) responsible for generating PI(3,4,5)P3 and PI(3,4)P2 (e.g., class I and II) and PI(3)P (class III) (25, 27). Consistent with this activity, *Salmonella* lacking SopB (Δ*sopB*) were unable to generate PI(3)P-rich macropinocytic cups (Fig. S3*B*). We reasoned that MYO6 might control SopB localization and thereby the production of PI(3)P in the macropinocytic cup. In control cells, FLAG-tagged SopB was observed at the macropinocytic cup enriched in PI(3)P (Fig. 4*A*). In MYO6-knockout cells, SopB retained this localization, but the macropinocytic cup was devoid of PI(3)P (Fig. 4*A*), showing that MYO6 is not required for SopB localization but inhibits SopB-mediated production of PI(3)P at invasion foci through another mechanism. SopB is known to produce PI(3)P on SCVs via recruitment of Rab5 that activates the class III PI3K Vps34 (25). Consistent with Rab5-dependent endosomal PI(3)P formation, the number and size of PI(3)P-enriched endosomes was reduced in Rab5-depleted cells relative to control cells (Fig. 4*B*, arrows). However, PI(3)P

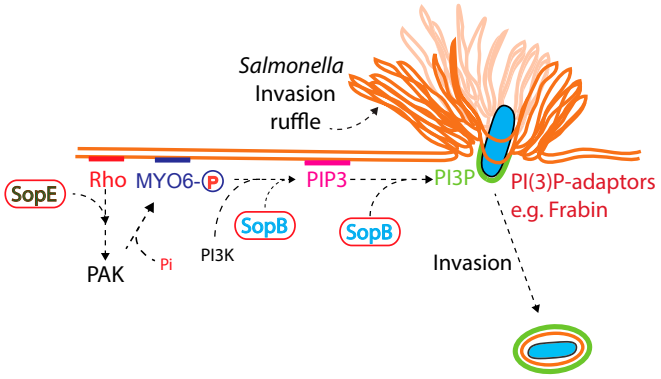

Fig. 5. Proposed model for *Salmonella* manipulation of MYO6 during invasion. *Salmonella* SopE activates Rho GTPases, which induce actin cytoskeleton reorganization and the recruitment of MYO6 to the membrane via PAK. SopB and MYO6 trigger PI3K signaling to generate PIP3. PIP3 acts as a substrate for SopB, which dephosphorylates PIP3 to generate PI(3)P at the macropinocytic cup of invading bacteria. PI(3)P recruits PI(3)P-binding proteins, such as Frabin, that promote *Salmonella* uptake.

was still present at the macropinocytic cup in Rab5-depleted cells (Fig. 4*B*). Indeed, we also found that Rab5-RFP localized to SCVs (Fig. S3*C*, white arrows), as previously observed (25), but was absent from actin-rich *Salmonella* invasion sites (Fig. S3*C*, pink arrow). These findings support our results shown in Fig. 3*B* and suggests that two distinct pools of PI(3)P are produced by *Salmonella*, namely a pool at the macropinocytic cup and a pool surrounding SCVs. Thus, PI(3)P is generated at the macropinocytic cup in a MYO6-dependent and Rab5-independent manner.

We reasoned that the plasma membrane pool of PI(3)P may derive from MYO6-dependent localization of PI(3,4,5)P3 and PI(3,4)P2 at invasion ruffles and that the plasma membrane pool in turn is dephosphorylated by SopB to generate PI(3)P at the macropinocytic cup. We thus investigated whether MYO6 promotes the localization of PI(3,4,5)P3 and PI(3,4)P2 at *Salmonella* invasion ruffles by examining the localization of the PH domain of Btk, which binds PI(3,4,5)P3, and that of TAPP1, which binds PI(3,4)P2 (*SI Experimental Procedures*). In control cells YFP-Btk and GFP-TAPP1 were enriched throughout the invasion ruffle, but this localization was markedly reduced in MYO6-depleted cells (Fig. S4 *A* and *B*, *Insets*). An established measure of downstream of PI3K activity is Akt phosphorylation, which is triggered during infection upon production of PI(3,4,5)P3 and PI(3,4)P2 (27, 28). To address whether MYO6 is required for the activation of PI3K, we examined Akt phosphorylation during infection of control and MYO6-depleted cells. *Salmonella* invasion stimulated Akt phosphorylation in control cells but not in MYO6-depleted cells (Fig. 4*C*). Together, these data demonstrate that SopB requires MYO6 to concentrate PI(3)P, PI(3,4)P2, and PI(3,4,5)P3 at invasion foci, leading to phosphorylation of Akt.

MYO6-Mediated PI(3)P Formation Recruits Frabin to Promote Invasion. We next examined the significance of PI(3)P at the macropinocytic cup. Previously, EPEC manipulation of phosphoinositides was shown to control the recruitment of PIP-binding proteins to the site of actin pedestals (29). Thus, we hypothesized that *Salmonella* targets MYO6 to generate PI(3)P-rich platforms to recruit specific PI(3)P-binding proteins that promote invasion. Upon closer inspection of the SopE-recruited cytoskeleton network, we noticed a PI(3)P-binding FYVE domain containing a protein called "Frabin" (FGD4; FYVE, RhoGEF, and PH domain-containing protein 4) (Tables S1 and S2). Indeed, we found that GFP-tagged Frabin colocalized with PI(3)P at the *Salmonella* entry site (Fig. S4*C*, pink arrow). Because MYO6 mediates PI(3)P localization at the macropinocytic cup (Fig. 3), we examined Frabin localization in infected MYO6-depleted cells (Fig. 4*E*). Frabin was enriched at the macropinocytic cup in *Salmonella*-infected control cells, but this recruitment was lost in MYO6-depleted cells, where Frabin was diffusely localized (Fig. 4*E*, *Insets*). Importantly, when we depleted host cells of Frabin by siRNA transfection, *Salmonella* invasion was reduced by ~40% (Fig. 4*D* and Fig. S4*D*). Together, the data indicate that SopB and MYO6 work in the same pathway to potentiate the recruitment of PI(3)P-binding proteins at the macropinocytic cup and thereby promote pathogen uptake.

Discussion

This study investigated how *Salmonella* hijacks the Rho GTPase networks to mediate uptake and establish intracellular infections. By reconstituting SopE-mediated Rho GTPase-driven actin polymerization, we identified a network of cytoskeleton proteins exploited by SopE that included members of the myosin motor protein family. Previous studies have shown that MYO1C and MYO2 are able to promote *Salmonella* invasion through lipid raft recycling (17) and through Arp2/3-independent actomyosin-mediated contractility (30), respectively. MYO2 also was found on actin filaments surrounding intracellular *Salmonella* within SCVs (31). It is becoming increasingly clear that MYO6 plays diverse roles in host–pathogen interactions. MYO6 was shown to defend host cells against intracellular *Salmonella* residing in the cytosol, revealing a role in xenophagy (32, 33). Moreover, MYO6 was found to promote the clathrin-dependent endocytosis of the intracellular pathogen *Listeria monocytogenes*, referred to as the "zipper mechanism" (19, 22). Our study shows that in host–pathogen interactions MYO6 facilitates *Salmonella* invasion by macropinocytosis through the trigger mechanism.

What is the molecular role of MYO6 during pathogen invasion? A long-standing mystery in *Salmonella* invasion is the mechanism by which SopB activates PI3Ks to generate the phosphoinositide PI(3,4,5)P3. Investigations are partly hampered by SopB's insensitivity to classical PI3K inhibitors such as wortmannin, which targets class I PI3K, and also by kinase redundancy, e.g., *Salmonella* exploits class II PI3K and inositol polyphosphate multikinase (27, 28). We found that MYO6 was critical to SopB-mediated activation of PI3K; however, the molecular role of MYO6 in this process remains to be established. MYO1D, MYO1E, and MYO1F are known to bind PI(3,4,5)P3 (34), but to our knowledge a myosin has not previously been implicated in PI3K activity. This study places MYO6 at the center of the mechanism by which SopB activates PI3K and generates phosphoinositides at the plasma membrane. Unraveling the PI3K–MYO6 mechanism will be a substantial focus of future studies for researchers studying a spectrum of MYO6-dependent functions in health and disease.

SopB is known to dephosphorylate PI(3,4,5)P3 to generate PI(3,4)P2 and PI(3)P (35). PI(3,4,5)P3 and PI(3,4)P2 are enriched throughout the ruffle (25), and PI(3,4)P2 was recently shown to recruit SNX9 to enhance invasion (36). In contrast, we found that PI(3)P was coincident with SopB localization at the macropinocytic cup and was enriched in a MYO6-dependent manner. We found that PI(3)P facilitated the recruitment of Frabin to promote pathogen invasion. Frabin comprises a PI(3)P-binding FYVE domain, a Cdc42 GEF, and an F-actin–binding domain. Frabin has been implicated in the invasion of the intracellular parasite *Cryptosporidium parvum* in a PI3K-dependent manner (37) and in the generation of filopodium-like microspikes (38). Even so, Frabin's role in the cell remains unclear, and no previous interaction with a bacterial pathogen has been reported. Understanding Frabin's involvement in *Salmonella* invasion will likely shed light on its role in the cell and in disease.

In summary, we reveal a mechanism by which SopE mediates MYO6 recruitment to the membrane via Rho GTPase activation of PAK. We identify MYO6 as a participant in phosphoinositide

distribution that acts with the *Salmonella* effector SopB to regulate lipid and protein composition of the macropinocytic cup during pathogen uptake. In doing so, we uncover a mechanism by which *Salmonella* effectors work in synergy to manipulate MYO6 and facilitate pathogen invasion (Fig. 5).

Experimental Procedures

***Salmonella* Strains and Invasion of HeLa Cells.** Wild-type, *S. enterica* serovar Typhimurium SL1344 (gift from Jean Guard-Petter, University of Georgia College of Veterinary Medicine, Athens, GA) and isogenic Δ*sopE*, Δ*sopE2*, Δ*sopE*/Δ*sopE2*, and Δ*sopB* strains were used as previously described (10, 39). To quantify invasion, *Salmonella* encoding pM975, which expresses GFP when bacteria are intracellular (12), were used to invade HeLa cells (15 min). Infected cells then were incubated for 90 min in fresh growth medium containing 50 μg/mL gentamicin to kill extracellular bacteria. Intracellular GFP^+ *Salmonella* were quantified microscopically. For fluorescence microscopy bacteria were visualized by labeling with Alexa Fluor 350 carboxylic acid succinimidyl ester (Life Technologies) or anti-*Salmonella* antibodies. When appropriate, cells were preincubated for 30 min and then for 15 min during *Salmonella* invasion with 40 μM MYO6 inhibitor Triiodolphenol (TIP; Sigma), 40 μM inhibitor of PAK activation-3 (IPA-3; Merck), 40 μM inhibitor of Rac1 (EHT1864; Merck), Cdc42 (ML141; Merck), or Rac1 and Cdc42 (AZA1; Merck).

Quantification of Macropinocytic Cups. HeLa cells were infected for 5 min with Alexa-Fluor 350-labeled *Salmonella* to mark total bacteria. Macropinocytic cups protected the penetrating tip of invading *Salmonella* from labeling with anti-*Salmonella* antibodies on nonpermeabilized cells. Macropinocytic cups thus were identified by anti-*Salmonella* antibody labeling of exposed extracellular portions of bacteria (e.g., the bacterial pole and/or flagella). When possible, PI(3)P accumulating at the base of invasion sites via peGFP-p40-PX expression also marked macropinocytic cups. The proportion of macropinocytic cups relative to the total number of colonizing *Salmonella* (i.e., surface-adherent and intracellular bacteria) was quantified.

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