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

A. B. E. Brooks[1][*], D. Humphreys[1,3][*], V. Singh[1], A.C. Davidson[1], S. D. Arden[2], F. Buss[2][$], V. Koronakis[1][$]

[1]Department of Pathology, University of Cambridge, Cambridge, United Kingdom, [2]Cambridge Institute for Medical Research, University of Cambridge, Cambridge, United Kingdom. [3]Department of Biomedical Science, University of Sheffield, Sheffield, United Kingdom

To establish infections, *Salmonella* injects virulence effectors that hijack the host actin cytoskeleton and phosphoinositide signalling 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 polymerisation 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 localisation to invasion foci and SopE-mediated activation of PAK recruited MYO6 to actin-rich membranes. We uncover that the virulence effector SopB requires MYO6 to regulate the localisation 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.

type 3 secretion system | motor protein | Rho GTPase

Introduction

Mammalian cells employs Rho GTPases Rac1, Cdc42 and RhoA as master regulators of the actin cytoskeleton to coordinate the formation of actin rich structures at the plasma membrane such as lamellipodia and filopodia (Sit & Manser 2011). 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 (Sit & Manser 2011). Rho effectors also include p21-activated kinase (PAK) that controls actin filament turnover by regulating the activity of actin-binding proteins (Edwards et al. 1999). PAK has also been shown to phosphorylate MYO6, a unique member of the myosin superfamily that moves towards the minus end of actin filaments (Wells et al. 1999; Buss et al. 1998). MYO6 has important roles in the endocytic pathway and in regulation of plasma membrane dynamics and membrane ruffle formation (Chibalina et al. 2010; Tumbarello et al. 2013).

To establish infections, the intestinal pathogen *Salmonella* Typhimurium subverts the actin cytoskeleton by injecting a cocktail of virulence effector proteins into host epithelial cells to facilitate uptake by macropinocytosis (Hardt et al. 1998; Patel & Galán 2006; Zhou et al. 2001; Humphreys et al. 2012; Friebel et al. 2001). Of particular importance is the virulence effector SopE, a Rac1 and Cdc42 GEF that is required for generating membrane ruffles and bacterial macropinocytosis (Misselwitz et al. 2011; Hardt et al. 1998). Indeed, deletion of SopE reduces *Salmonella* invasion by 60%, and in the absence of SopE, together with known *Salmonella* Cdc42 activators SopE2 and SopB, the pathogen cannot activate Rho GTPases nor invade host cells (Zhou et al. 2001; Patel & Galán 2006). There are likely a number

of Rho GTPase effectors hijacked by *Salmonella* but their identity, and how they interact with each other, and how they contribute to *Salmonella* invasion remain unresolved. As SopE activates both Rac1 and Cdc42, we set out to reconstitute SopE signalling at model membranes and identify the components of the Rho cytoskeleton network hijacked by *Salmonella*.

Results

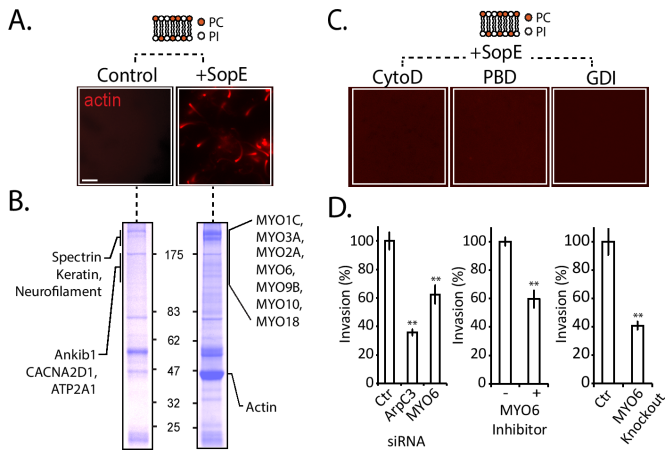### Identification of the cytoskeleton protein network recruited via SopE

To identify the membrane-associated cytoskeleton network of proteins targeted by SopE, we first reconstituted SopE-mediated actin filament polymerisation at immobilised phospholipid membrane bilayers in optimised cell-free brain extract, as previously described (Humphreys et al. 2012). Silica microspheres were coated with a phospholipid bilayer composed of equal concentrations of phosphatidylcholine and phosphatidylinositol (PC:PI), which have been previously used to reconstitute Rho-driven actin assembly (Rohatgi et al. 1999; Koronakis et al. 2011). When the microspheres were incubated in extract the control PC:PI beads were unable to polymerise actin (**Fig 1A**). In contrast, when the extract was supplemented with purified recombinant SopE, actin filaments were immediately assembled on the membrane surface that propelled the beads through the extract via actin comet tail formation (**Fig 1A**). Inhibiting actin polymerisation (Cytochalasin D) or the Rho GTPases Rac1 and Cdc42 (PBD domain of PAK or GDI, which binds and sequesters these Rho GTPases) (Koronakis

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Significance

Salmonella causes many different diseases including gastroenteritis and typhoid fever. For infection to take place, *Salmonella* has to 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.

Reserved for Publication Footnotes

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-labelled actin in the presence or absence (Control) of 1 μM SopE. Scale bars 5μm. **(B)** SDS-PAGE analysis and Coomassie blue staining of proteins recruited by the beads shown in (A). SopE-recruited myosins annotated right with proteins of equivalent molecular weight recruited by the control shown left. Molecular weight markers shown in kDa **(C)** Experiment performed as (A) in extract containing inhibitors of actin polymerisation (Cytochalasin D, 5 μM), or Rho GTPase signalling (PBD or GDI, 1 μM). Scale bars 5μm. **(D)** Salmonella invasion into HeLa cells where MYO6 was either depleted by siRNA, inhibited by TIP or knocked out by CRISPR/CAS9-based engineering. Error bars represent ±SEM, ** P<0.01.

Fig. 2. – SopE manipulation of MYO6 and PAK. **(A)** PC:PI beads incubated in cell extract in the absence (–) or presence of SopE (+) were analysed by immunoblotting with indicated antibodies. **(B)** Localisation of GFP-MYO6 (green) in HeLa cells infected with wild type or ΔsopE Salmonella. Bacteria were visualised using Salmonella antibodies (red) and actin filaments with Alexa-Fluor 350-conjugated phalloidin (blue). Insets magnify Salmonella-induced ruffles. Scale bars 5μm. **(C)** Quantification of MYO6 localisation to membrane ruffles. Experiment as (B) using wild type, ΔsopE, ΔsopE2, ΔsopE/E2 and ΔsopB Salmonella. Error bars represent ±SEM ** P<0.01. **(D)** SopE recruitment of MYO6 in the presence (+) or absence (-) of inhibitors of actin polymerisation (CytoD) and Rho GTPase signalling (PBD). Experiment performed as (A). **(E)** SopE recruitment of MYO6 in the presence (+) or absence (-) of the PAK inhibitor (IPA-3). Experiment performed as (A). **(F)** Salmonella invasion into HeLa cells in the absence (control) or presence of TIP (MYO6 inhibitor), with (IPA-3) or without (Mock) the PAK inhibitor IPA-3 in combination. Error bars represent ±SEM ** P<0.01. NS is non-significant. **(G)** Localisation of GFP-MYO6 (T405A) or (T405E) phosphomimics to Salmonella-induced ruffles. Experiment performed as (B). Scale bars 5μm.

et al. 2011) abrogated actin assembly (**Fig 1C**). This confirmed that SopE-activation of Rho GTPases was necessary for actin comet tail formation.

To identify the putative SopE-signalling network, actin-based motility assays from **Fig 1A** were scaled up. Proteins recruited from the cytosol to PC:PI were separated by SDS-PAGE (**Fig 1B**) and identified by parallel mass spectrometry. Over 200 proteins were specifically recruited to PC:PI coated beads directly or indirectly via SopE signalling through Rho GTPases (**Supplemental Table 1**), including cellular effectors of Rac1 and Cdc42 that orchestrate actin filament polymerisation (Sit & Manser 2011), such as N-WASP and the WAVE complex (comprising Cyfip, Nap1, Abi, WAVE1, and homologues); formins (FMNL1, 2, and DIAPH2); PAK1 and 3; the BAR-protein FNBP1 and CEP4/BORG4, as well as its cognate septin binding partners (summarised in **Supplemental Table 2**). 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 7 myosin motor proteins that were absent from control PC:PI beads were recruited in a SopE-dependent manner (**Fig 1B, Supplemental Table 1 and Table 2**).

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 but also by regulating the architecture of the actin cytoskeleton and plasma membrane dynamics (Hartman & Spudich 2012). Given the importance of the myosin superfamily in these diverse cellular functions, we sought to address the role of myosin motors in Salmonella macropinocytosis.
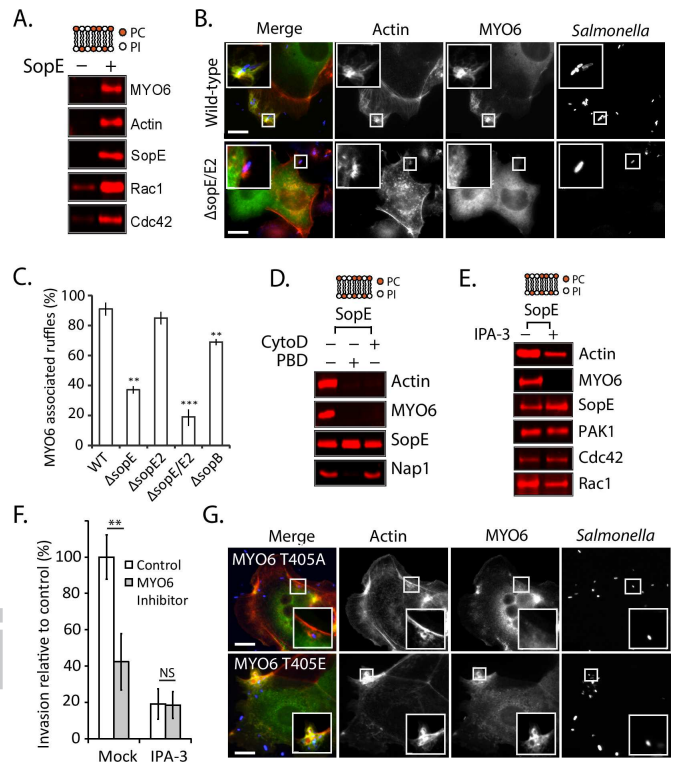
MYO6 facilitates Salmonella invasion

To investigate the importance of these myosin motors in SopE-induced actin filament remodelling, we transfected siRNAs targeting the 7 myosins listed in **Fig. 1B** and an additional 6 myosin family members expressed in HeLa cells (namely Myosins 1B, 1E, 1G, 2B, 2C, 5A, 5C) (Maliga et al. 2013) before assessing

Salmonella invasion (**Fig S1A**). 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 1B**). As MYO1C was recently reported to be required for Salmonella invasion (Brandstaetter et al. 2012), we further analysed 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 (Heissler et al. 2012), in CRISPR-Cas9 MYO6 knockout cells, and in siRNA-transfected MYO6-depleted cells (**Fig 1D**). Successful knockout and siRNA-mediated depletion of MYO6 were confirmed by immunoblotting (**Fig S1B, 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 recruitment of MYO6 as well as Cdc42, Rac1 and actin (**Fig 2A**), which was
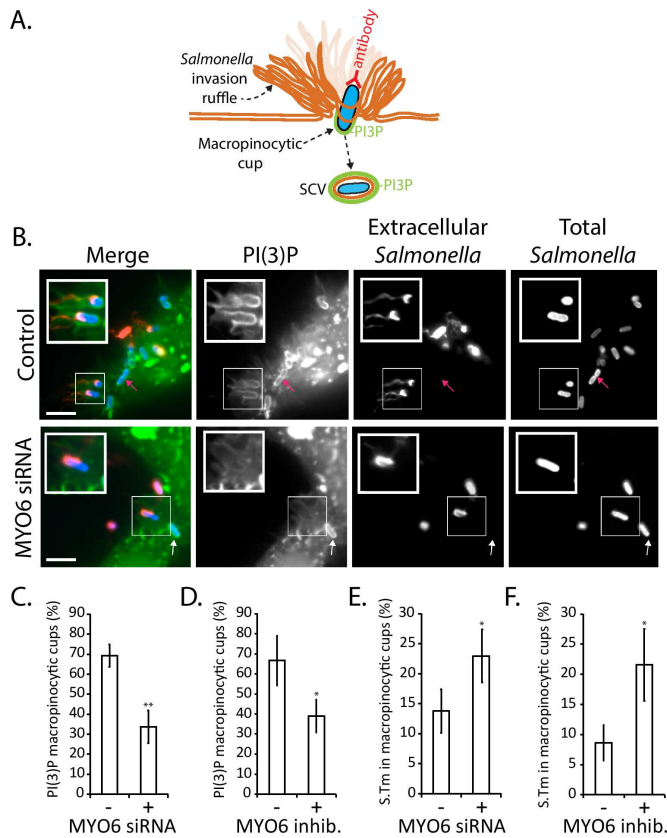
Fig. 3. – MYO6 regulation of the *Salmonella* macropinocytic cup.**(A)** Cartoon depicting macropinocytic cup and SCV labelling for experiment shown in (B). **(B)** Localisation of PI(3)P at *Salmonella* macropinocytic cups. Cells were transfected with non-targeting siRNA (Control) or MYO6 siRNA before transfection with GFP-p40-PX (PI3P - green) and infection with Alexa-Fluor 350-labelled wild type *Salmonella* (Total *Salmonella* – blue). Extracellular *Salmonella* were visualised by staining non-permeabilised cells with anti--*Salmonella* antibodies (Extracellular *Salmonella* – red). Insets magnify *Salmonella* macropinocytic cups. Arrows label intracellular SCVs. Scale bars 5μm.**(C)** The number of PI3P-rich macropinocytic cups were quantified from the experiment shown in (B) using MYO6 sRNA or in cells treated with the small molecule inhibitor (MyoVI inhib) of MYO6 inhibitor **(D)**. **(E, F)** The number of *Salmonella* (S.Tm) associated macropinocytic cups relative to the total number of colonising bacteria was quantified from the experiments in (C) and (D). Error bars represent ±SEM * P<0.05, ** P<0.01.

consistent with Rho-mediated actin comet tail formation (**Fig 1C**). To investigate whether SopE regulated MYO6 localisation to actin filaments at the pathogen entry site during invasion, HeLa cells expressing GFP-tagged MYO6 were infected with either wild-type *Salmonella* or the isogenic Δ*sopE* mutant (**Fig 2B**). Both wild-type and Δ*sopE Salmonella* induced actin-rich membrane ruffles at pathogen foci (insets), which was expected given the role for multiple SPI-1 effectors in cytoskeleton remodelling (Cossart et al. 2004). In wild-type infected cells, robust MYO6 localisation was observed at ~90% of pathogen-induced ruffles (**Fig 2C**). This was not the case when cells were infected with Δ*sopE*, where MYO6 was absent from the majority of ruffles (exemplified in **Fig 2B**) with only ~35% showing co-localisation with MYO6 (**Fig 2C**). 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 localisation to ruffles (Friebel et al. 2001) (Patel & Galán 2006). Cells infected with Δ*sopE2* showed no reduction in MYO6 recruitment, presumably due to the presence of SopE. Indeed, upon infection with the double mutant Δ*sopE/E2*, MYO6 localisation at pathogen-induced ruffles was reduced to ~20% whilst this was reduced to
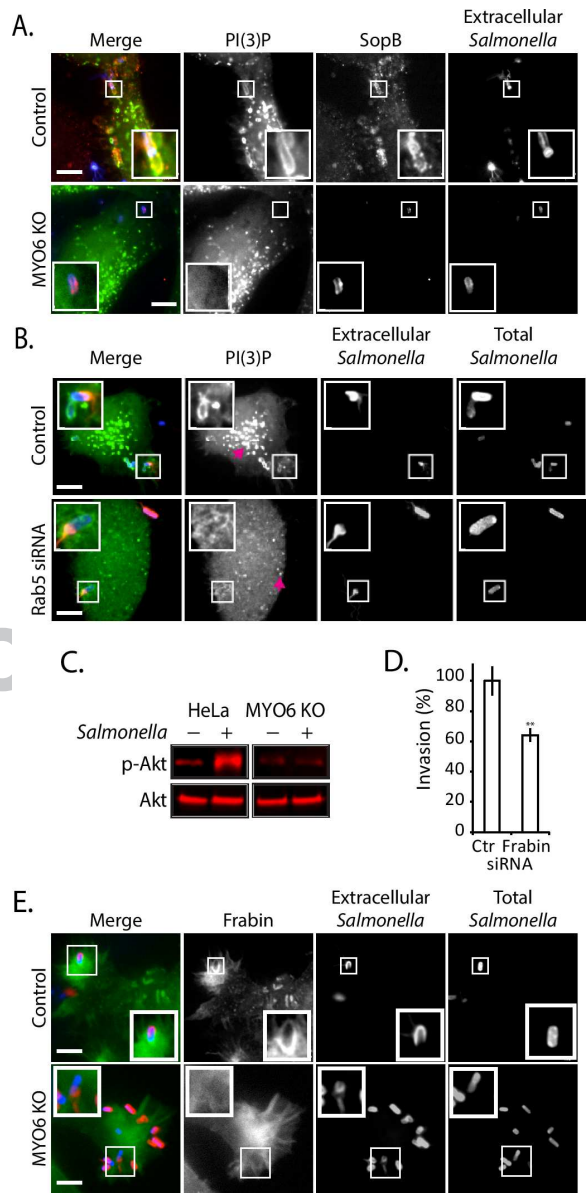
Fig. 4. – MYO6 regulation of Akt signalling and recruitment of frabin.**(A)** Localisation of SopB in control or MYO6 knockout HeLa cells expressing GFP-p40-PX (PI3P - green) were infected with *Salmonella* encoding FLAG tagged SopB. Non-permeabilised cells were labelled with and anti-*Salmonella* antibodies (blue) before permeabilisation and labelling with anti-FLAG antibodies (red). Insets magnify macropinocytic cup. Scale bars 5μm.**(B)** Localisation 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 (PI3P - green) and infection with Alexa-Fluor 350-labelled *Salmonella* (Total *Salmonella* – blue). Extracellular *Salmonella* were visualised by staining non-permeabilised cells with anti--*Salmonella* antibodies (Extracellular *Salmonella* – red). Insets magnify *Salmonella* macropinocytic cups. Arrows label PI3P-rich endosomes. Scale bars 5μm.**(C)** Control and MYO6 knockout HeLas were either non-infected (-) or infected (+) with *Salmonella* then whole cell lysates 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)** Localisation of CFP-Frabin during *Salmonella* invasion into control and MYO6 knockout cells. Experiment performed as (B). Insets magnify *Salmonella* macropinocytic cups. Scale bars 5μm.

~70% in cells infected with Δ*sopB* (**Fig 2C**). 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 S1D**).

Rho GTPase subversion is a central virulence strategy of many bacterial pathogens. For example, *Shigella flexneri* also invade host cells using Rho GTPases that trigger membrane ruffling (Mounier et al. 1999), and indeed MYO6 was also present in *Shigella*-induced ruffles (**Fig S1E**). Since MYO6 binds actin-filaments, it remained possible that this motor is recruited to actin-filaments assembled at the plasma membrane independently of Rho signalling, e.g. to actin pedestals generated beneath the extracellular pathogen enteropathogenic *Escherechia coli* (EPEC) (Ben-Ami et al. 1998). When we examined MYO6 localisation in EPEC-infected host cells, MYO6 was absent from actin-pedestals (**Fig S1E**), as previously reported (Bonazzi et al. 2011). Taken together our data suggest that MYO6 specifically localises 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 localisation, we next addressed the mechanism by which MYO6 is recruited to actin-rich membrane ruffles. Inhibition of Rho GTPases (+PBD) or actin polymerisation (+CytoD) *in vitro* blocked SopE-dependent recruitment of MYO6 to the membrane (**Fig 2D**). 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 PBD indicating that MYO6 targeting is not solely regulated by RhoGTPases or membrane interactions, but also requires RhoGTPase induced actin filaments for recruitment.

Interestingly, our proteomics data also highlights the presence of the PAK family of Rac1 and Cdc42 effectors within the cytoskeleton network recruited by SopE (**Supplemental Table 1 and Table 2**). *Salmonella* is known to trigger activation of PAK (Chen et al. 1999) and PAK has also been reported to phosphorylate MYO6 in the motor domain (Buss et al. 1998). A potential phosphorylation site is the threonine in position 405 at a conserved site within the head domain of MYO6 (Bement & Mooseker 1995). To determine whether PAK regulates MYO6 recruitment, actin filament polymerisation was triggered at the membrane by SopE in extract supplemented with IPA-3 (**Fig 2E**), an inhibitor of PAK1-3 activation. IPA-3 completely ablated SopE-recruitment of MYO6 to membranes whilst still recruiting Rho GTPases and triggering actin polymerisation, thereby distinguishing MYO6 recruitment from actin filament formation. Thus, MYO6 recruitment required the combination of actin filaments and Rho GTPase activated PAK (**Fig 2D, 2E**). Furthermore, inhibiting PAK with IPA-3 in host cells reduced *Salmonella* invasion by ~80% (**Fig 2F**). Consistent with PAK regulation of MYO6, no further reduction in invasion was observed when inhibitors of PAK and MYO6 were used in combination (**Fig 2F**) 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 on its localisation to *Salmonella*-induced ruffles (**Fig 2G**). GFP-tagged MYO6 mutants mimicking the dephosphorylated (T405A) or phosphorylated (T405E) form were expressed in HeLa cells before *Salmonella* infection. Whilst MYO6 (T405E) was strongly recruited to *Salmonella*-induced ruffles, T405A mutant was not (**Fig 2G, S2A**) demonstrating that PAK-dependent phosphorylation in the motor domain may regulate MYO6 localisation to *Salmonella* invasion foci. Consistent with this, only the GFP-MYO6 head domain (residues 1-835) but not the tail domain alone was recruited into membrane ruffles (**Fig S2B**).

MYO6 accumulates PI(3)P at *Salmonella*-associated macropinocytic cups

Having established that MYO6 was recruited into membrane ruffles, we next investigated the mechanism by which MYO6 facilitates pathogen invasion. Although the loss of MYO6 reduced pathogen-induced membrane ruffle formation, indicating a potential role for MYO6 in regulating actin filament organisation (**Fig S2C**: ~65% in the control relative to ~45% in MYO6-depleted cells), *Salmonella*-induced ruffles were not completely inhibited in the absence of MYO6 (**Figs 2B, S2C**), suggesting that this myosin plays an additional role during pathogen uptake. Actin filament polymerisation at sites of *Salmonella* invasion leads to the formation of a macropinocytic cup, which can be visualised 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 (Mallo et al. 2008) (see **Fig S3A**).

We decided to examine whether MYO6 influenced the localisation of PI(3)P in the macropinocytic cup at invasion ruffles during pathogen uptake (**Fig 3**). As depicted in the cartoon (**Fig 3A**) and demonstrated experimentally (**Fig 3B**: control)**,** the PI3P-enriched macropinocytic cup formed a tight association with the invading pathogen in control cells, which was inaccessible to anti-*Salmonella* antibodies (**Fig 3B**: magnified 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*). This was in contrast to the intracellular bacteria within SCVs (pink arrows) that were completely surrounded by PI3P and completely inaccessible to antibodies (**Fig 3A, 3B**). To our surprise, we observed a striking reduction of PI(3)P at macropinocytic cups in MYO6 depleted cells (**Fig 3B**). PI(3)P was reduced from ~70% in the control to ~33% in MYO6 depleted cells (**Fig 3C: + MYO6 siRNA**) and in cells treated with the MYO6 inhibitor (**Fig 3D: + MYO6 inhibitor**). Intriguingly, PI(3)P still surrounded bacteria within intracellular SCVs (white arrows) (**Fig 3B**). Thus, MYO6 is required for the localisation of PI(3)P at the macropinocytic cup but not at the SCV, which, thus, reveals two distinct pools of the phosphoinositide PI(3)P associated with the pathogen.

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

MYO6 co-operates with SopB to mediate PI3-Kinase signalling

We next addressed how MYO6 facilitated localisation of PI(3)P at the macropinocytic cup. It is well established that the generation of PI(3)P by *Salmonella* is dependent upon the bacterial effector SopB, a phosphoinositide phosphatase with PI 4'- and 5'-phosphatase activity (Norris et al. 1998). 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, II) and PI(3)P (Class III) (Roppenser et al. 2013; Mallo et al. 2008). Consistent with this, *Salmonella* lacking SopB (Δ*sopB*) were unable to generate PI(3)P rich macropinocytic cups (**Fig S3B**). We reasoned that MYO6 might control SopB localisation, and thereby 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 4A**). In MYO6 knockout cells, SopB retained this localisation but the macropinocytic cup was devoid of PI(3)P (**Fig 4A**) showing that MYO6 is not required for SopB localisation 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 (Mallo et al. 2008). Consistent with Rab5-dependent endosomal PI(3)P formation, the number and size of PI3P-enriched endosomes was reduced in Rab5 depleted cells relative to control

cells (**Fig 4B:** arrows). However, PI(3)P was still present at the macropinocytic cup in Rab5 depleted cells (**Fig 4B**). Indeed, we also found that Rab5-RFP localised to SCVs (**Fig S3C: white arrows**), as previously observed (Mallo et al. 2008), yet Rab5-RFP was absent from actin-rich *Salmonella* invasion sites (**Fig S3C: pink arrow**). These findings support our results shown in **Fig 3B** 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, PI3P 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 localisation of PI(3,4,5)P3 and PI(3,4)P2 at invasion ruffles, which in turn is dephoshorylated by SopB to generate PI(3)P at the macropinocytic cup. We thus investigated whether MYO6 promotes localisation of PI(3,4,5)P3 and PI(3,4)P2 at *Salmonella* invasion ruffles by examining the localisation 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 localisation was markedly reduced in MYO6 depleted cells (**Fig S4A; S4B;** insets). An established measure 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 (Cooper et al. 2011; Roppenser et al. 2013). To address whether MYO6 is required for 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 4C**). 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, which leads 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 phospho-inositides was shown to control the recruitment of PIP-binding proteins to the site of actin-pedestals (Smith et al. 2010). Thus, we hypothesised that *Salmonella* targets MYO6 to generate PI(3)P rich platforms to recruit specific PI(3)P binding proteins, which promote invasion. Upon closer inspection of the SopE-recruited cytoskeleton network, we noticed a PI(3)P binding FYVE domain containing protein called Frabin (FGD4; FYVE, RhoGEF and PH domain-containing protein 4) (**Supplementary Table 1 and Table 2**). Indeed, we found that GFP-tagged Frabin co-localised with PI(3)P at the *Salmonella* entry site (**Fig S4C;** exemplified by pink arrow). As MYO6 mediates PI3P localisation at the macropinocytic cup (**Fig 3**), we examined Frabin localisation in infected MYO6 depleted cells (**Fig 4E**). 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 localised, as exemplified in the insets (**Fig 4E**). Importantly, when we depleted host cells of Frabin by siRNA transfection, *Salmonella* invasion was reduced by ∼40 % (**Fig 4D, S4D**). 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 to 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 polymerisation 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 (Brandstaetter et al. 2012) and through Arp2/3 independent actomyosin-mediated contractility (Hänisch et al.

2011), respectively. MYO2 was also found on actin filaments surrounding intracellular *Salmonella* within SCVs (Odendall et al. 2012). 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 (Tumbarello et al. 2015; Verlhac et al. 2015). Moreover, MYO6 was found to promote the clathrin-dependent endocytosis of the intracellular pathogen *Listeria monocytogenes*, referred to as the 'zipper' mechanism (Bonazzi et al. 2011; Cossart et al. 2004). Our study presents a new role for MYO6 in host-pathogen interactions as this myosin 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 insensitivity to classical PI3K inhibitors such as wortmannin that targets Class I PI3K, and also by kinase redundancy, e.g. *Salmonella* exploits Class II PI3K and inositol polyphosphate multikinase (Roppenser et al. 2013; Cooper et al. 2011). 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. While MYO1D, MYO1E and 1F are known to bind PI(3,4,5)P3 (Chen & Iijima 2012), to our knowledge, this represents the first time a myosin has been implicated in PI3K activity. In doing so, this study places MYO6 at the centre of the mechanism by which SopB activates PI3K and generates phosphoinositides at the plasma membrane. Unravelling 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 (Marcus et al., 2001; Norris et al., 1998). PI(3,4,5)P3 and PI(3,4)P2 are enriched throughout the ruffle (Mallo et al. 2008), and PI(3,4)P2 was recently shown to recruit SNX9 to enhance invasion (Piscatelli et al. 2016). In contrast, we found that PI(3)P was coincident with SopB localisation 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 a F-actin binding domain. Frabin has been implicated in the invasion of the intracellular parasite *Cryptosporidium parvum* in a PI3K dependent manner (Chen et al. 2004), and in the generation of filopodium-like microspikes (Ono et al. 2000). 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 novel player 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

cally. For fluorescence microscopy bacteria were visualised by labelling with Alexa Fluor 350 carboxylic acid succinimidyl ester (Life technologies) or anti-*Salmonella* antibodies. When appropriate, cells were preincubated for 30 mins and then during *Salmonella* invasion (i.e. 15 mins) 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 with Alexa-Fluor 350-labelled *Salmonella* (5 mins) to mark 'total bacteria'. Macropinocytic cups protected the penetrating tip of invading *Salmonella* from labelling with anti-*Salmonella* antibodies on non-permeabilised cells. Macropinocytic cups were thus identified by anti-*Salmonella* antibody labelling of exposed extracellular portions of bacteria (e.g. 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 was quantified relative to the total number of colonising *Salmonella* (i.e. surface adherent and intracellular bacteria).

Author contributions:

AB, DH, VS and SA performed experiments. AB, DH, FB, and VK wrote the paper.

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