Inhibition of the PtdIns(5) kinase PIKfyve disrupts intracellular replication of *Salmonella*

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3-phosphorylated phosphoinositides (3-PtdIns) orchestrate endocytic trafficking pathways exploited by intracellular pathogens such as *Salmonella* to gain entry into the cell. To infect the host, *Salmonellae* subvert its normal macropinocytotic activity, manipulating the process to generate an intracellular replicative niche. Disruption of the PtdIns(5) kinase, PIKfyve, be it by interfering mutant, siRNA-mediated knockdown or pharmacological means, inhibits the intracellular replication of *Salmonella enterica* serovar *typhimurium* in epithelial cells. Monitoring the dynamics of macropinocytosis by time-lapse 3D (4D) videomicroscopy revealed a new and essential role for PI(3,5)P₂ in macropinosome-late endosome/lysosome fusion, which is distinct from that of the small GTPase Rab7. This PI(3,5)P₂-dependent step is required for the proper maturation of the *Salmonella*-containing vacuole (SCV) through the formation of *Salmonella*-induced filaments (SIFs) and for the engagement of the *Salmonella* pathogenicity island 2-encoded type 3 secretion system (SPI2-T3SS). Finally, although inhibition of PIKfyve in macrophages did inhibit *Salmonella* replication, it also appears to disrupt the macrophage's bactericidal response.

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Introduction

Salmonella are intracellular pathogens responsible for a variety of enteric diseases in all vertebrates. In humans, *Salmonella* are the causative agent of typhoid fever, paratyphoid fever and, in the case of *Salmonella enterica* serovar

typhimurium (*S. typhimurium*), human gastroenteritis (Haraga *et al*, 2008). Following ingestion, *Salmonella* navigates the acidic milieu of the stomach before invading the non-phagocytic epithelial cells of the intestinal wall, microfold (M) cells and CD18-expressing macrophages (Kohbata *et al*, 1986; Jones *et al*, 1994; Vazquez-Torres *et al*, 1999). In non-phagocytic cells this uptake is mediated by bacterial effector proteins that are translocated directly into the host via specialised apparatuses called type 3 secretion systems (T3SSs) (Hansen-Wester and Hensel, 2001). The secreted effectors manipulate the host's cytoskeletal elements and membrane trafficking pathways, ultimately initiating an actin-mediated endocytic process called macropinocytosis, through which *Salmonella* enters the cell (Francis *et al*, 1993). Once intracellular, the pathogen delivers a second suite of effectors, via the *Salmonella* pathogenicity island 2-encoded type 3 secretion system (SPI2-T3SS) (Deiwick *et al*, 1998), that alter the host's endolysosomal system and lipid metabolism, thereby creating a replicative niche called a *Salmonella*-containing vacuole (SCV) (Haraga *et al*, 2008). Shortly after its formation, this vacuole associates with the organelles of the endosomal system, acquiring markers such as EEA1 and transferrin receptor (TfR) (Steele-Mortimer *et al*, 1999). The later stages of SCV maturation are characterised by loss of these markers, acquisition of late endosomal markers (Garcia-del Portillo and Finlay, 1995) and the directed fusion of late endosomal membranes to the SCV, forming extensive tubular protrusions called *Salmonella*-induced filaments (SIFs) (Garcia-del Portillo *et al*, 1993). This process is known to be dependent upon the late endosomal small GTPase Rab7 and is essential for intracellular replication of the pathogen (Meresse *et al*, 1999). The invasion and intracellular survival of *Salmonella* therefore involves an elaborate suite of molecular interactions between host and pathogen that are all orchestrated by the pathogen (Haraga *et al*, 2008).

It is perhaps not surprising that the phosphoinositides are specifically targeted by *Salmonellae* as they are recognised to be key regulators of macropinocytosis (Araki *et al*, 1996; Norris *et al*, 1998; Dukes *et al*, 2006; Mallo *et al*, 2008). Derived from the reversible phosphorylation of phosphatidylinositol, the phosphoinositides (PtdIns) are an important class of membrane lipids that serve as both signalling molecules and localisation cues for a range of proteins in mammalian cells (Vicinanza *et al*, 2008). Concentrated at the cytosolic surface of the lipid bilayer, the PtdIns may be singly or multiply phosphorylated on the 3'-OH, 4'-OH or 5'-OH positions of the inositol ring, thereby giving rise to seven distinct PtdIns-species whose localisation and metabolism is restricted to discrete regions within the cell. Within the endolysosomal system, PtdIns(3,4,5)P₃ is recognised to be pivotal to the coordination of actin-polymerisation and signalling events at the plasma membrane, whereas PtdIns(3)P and PtdIns(3,5)P₂ are predominantly intracellular, regulating membrane trafficking events (Vicinanza *et al*, 2008).

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The role that PtdIns(3,5)P₂ and the kinase which generates it, PIKfyve, has in endocytosis is currently controversial. PIKfyve associates with PtdIns(3)P through an amino-terminal FYVE finger domain, synthesising PtdIns(3,5)P₂ and PtdIns(5)P through its carboxyl-terminal catalytic domain (Shisheva, 2008). In addition to its lipid kinase activity, PIKfyve also presents protein kinase activity for several substrates *in vitro* (Shisheva, 2008). As such, PIKfyve activity is likely to be intrinsic to a variety of cellular processes. With reference to endocytosis specifically, disruption of the yeast equivalent, Fab1p, leads to defects in the transport of proteins to the vacuole via the multivesicular body (MVB), an enlargement of the vacuole that fails to acidify and an inability to grow at high temperature (Cooke *et al*, 1998). Similarly, siRNA-mediated knockdown of PIKfyve and overexpression of a catalytically inactive PIKfyve (PIKfyve(K1831E)) construct in mammalian cells, leads to swollen endocytic compartments that resemble late endosomes (Ikononov *et al*, 2001, 2003; Rutherford *et al*, 2006).

Here we reveal a new role for PIKfyve in the fusion of macropinosomes with organelles of the late endosomal/lysosomal system. This process is required for SCV maturation as inhibition of PIKfyve activity, be it with dominant interfering mutants, RNAi-mediated knockdown or the small molecule inhibitor YM201636, abrogates SIF formation, and ultimately intracellular replication of the pathogen.

Results

Inhibition of PIKfyve activity interferes with the replication and survival of intracellular *S. typhimurium*

Intracellular *Salmonella* alter the lipid and protein composition of the encompassing macropinosome to generate a replicative niche (Haraga *et al*, 2008). Formation of this replicative niche shares many properties with normal endosomal maturation, including a dependence upon the 3-PtdIns (Araki *et al*, 1996; Norris *et al*, 1998; Dukes *et al*, 2006; Mallo *et al*, 2008).

To determine whether PIKfyve has a role in *S. typhimurium* infection, A431 cells were transfected with mammalian expression constructs encoding wild-type PIKfyve fused to GFP, or the previously published PIKfyve catalytically inactive mutant (PIKfyve(K1831M)) fused to GFP (Osborne *et al*, 2008) for 16 h before infection with *S. typhimurium* wild-type strain SL1344 expressing mRFP (RFP-SL1344) for 10 min. The cells were then thoroughly washed before being cultured for a further 24 h in the presence of 50 µg ml⁻¹ gentamycin. The samples were then fixed, immunolabelled with a monoclonal antibody specific for LAMP1 followed by a fluorescent-conjugated secondary, counter-stained with DAPI and examined using confocal microscopy (see Figure 1A). Although infected cells expressing the wild-type construct presented large numbers of intracellular bacteria, which were often filamentous in nature (see Figure 1A), those transfected with the catalytically inactive mutant had markedly fewer intracellular bacteria and none with the filamentous morphology commonly observed at this stage of the infection. The filamentous morphology observed in untransfected cells, and those expressing PIKfyve-GFP has been used in the past as an indicator of bacterial health and replication (Marsman *et al*, 2004), thus suggesting that PIKfyve activity is required for replication of intracellular RFP-SL1344.

To confirm and extend upon this observation, siRNAs previously utilised to deplete cells of PIKfyve (Rutherford *et al*, 2006) and a regulatory subunit of the PI(3,5)P₂-synthesis machinery, Vac14 (Michell and Dove, 2009), were employed. A431 cells were transfected with Dharmacon siRNA duplexes, specific for PIKfyve, Vac14 or scrambled controls. As reported previously, 96 h post-transfection, extensive vacuolation of endosomal membranes was observed in the cells transfected with the PIKfyve or Vac14 RNAi duplexes (Rutherford *et al*, 2006). These swollen vacuoles were frequently LAMP1-positive (see Figure 1B). Quantitative RT-PCR of PIKfyve and Vac14 revealed an ~70% and ~90% decrease in the PIKfyve and Vac14 transcripts respectively when compared with scrambled controls (see Supplementary Figure 1). Similarly, western immunoblotting using antibodies against PIKfyve and Vac14 indicate a reduction in the levels of the endogenous protein in cell monolayers treated with siRNAs directed against PIKfyve and Vac14 respectively. At 72 h post-transfection, these cells were infected with RFP-SL1344 for 24 h as described earlier, fixed and counter-stained with monoclonal antibodies against LAMP1, appropriate secondary antibodies, DAPI and phalloidin conjugated to Alexa488 and examined by confocal microscopy. Consistent with the cells expressing the PIKfyve catalytically inactive mutant, those cells transfected with PIKfyve or Vac14 siRNAs, that had the swollen vacuolar phenotype, had significantly less intracellular bacteria than those transfected with the scrambled control siRNAs (see Figure 1B). This again suggests that PIKfyve activity is required for intracellular replication of *S. typhimurium*. This phenotype was observed with two independent siRNA duplexes targeting PIKfyve and three targeting Vac14 (data not shown).

Recently, a small molecule inhibitor of PIKfyve was published (Jefferies *et al*, 2008). A431 cells cultured in the presence of the PIKfyve-inhibitor, YM201636, or the equivalent volume of the carrier (DMSO) were infected for 24 h with RFP-SL1344, fixed, counterstained with monoclonal antibodies against LAMP1, DAPI and phalloidin conjugated to Alexa488 and examined using confocal microscopy as before. Although the control-infected cells presented extensive numbers of intracellular bacteria, often filamentous in nature (see Figure 1C), those cultured in the presence of YM201636 had dramatically fewer intracellular bacteria, and none with the filamentous morphology commonly observed at this stage of the infection. Although the specificity of this inhibitor may be broader than initially published (Jefferies *et al*, 2008; Ikononov *et al*, 2009) the use of three mechanistically independent strategies: dominant-negative interfering mutations; siRNA-mediated knockdown; and pharmacological inhibition, that each in turn produce consistent impact, demonstrate a potential role for PIKfyve in the intracellular replication of *S. typhimurium* in A431 cells (see Figure 1).

To gain further insight into this role the infection assay was repeated for a range of time-points post-infection (p.i.) in the presence of the pharmacological inhibitor, YM201636 (see Figure 2A). The RFP-fluorescence, and therefore the volume of bacterial material (from at least 150 infected cells from three independent experiments) was quantified as described in the Materials and methods section (see Figure 2B). Strikingly, although the relative RFP-fluorescence between the DMSO-treated and YM201636-treated samples is statistically

Figure 1 Perturbation of PIKfyve inhibits intracellular *Salmonella typhimurium* replication. (A) A431 cells transiently transfected with pPIKfyve-GFP or pPIKfyve(K1831M)-GFP. (B) A431 cells were treated with scrambled-, PIKfyve- and Vac14-siRNAs as described in the Materials and methods section for 72 h. (C) A431 cultured with 800 nM YM201636 or equivalent volumes of DMSO for 2 h. Cells were infected with RFP-SL1344 for 10 min, thoroughly washed and cultured for a further 24 h in the presence of 50 µg/ml gentamycin before being fixed, labelled with monoclonal antibodies against LAMP1 and counterstained with phalloidin conjugated to Alexa488 (B, C) and with DAPI (A–C). Maximum projections were generated from Z-stacks obtained by confocal microscopy as described in the Materials and methods section.

equivalent between 2 and 4 h p.i., at 8, 12 and 24 h p.i., significantly less RFP-fluorescence was observed in the YM201636-treated samples relative to DMSO-treated controls. To quantify the number of viable bacteria under the same

conditions, colony forming unit (CFU) assays were carried out (see Figure 2C). As with the fluorescence-based assay, little difference was observed between 2 and 4 h p.i., indicating that inhibition of PIKfyve activity had no apparent impact

Figure 2 The PIKfyve inhibitor, YM201636 blocks intracellular but not extracellular replication of *Salmonella typhimurium*. (A) A431 cultured with 800 nM YM201636 or equivalent volumes of DMSO for 2 h were infected with RFP-SL1344 for 10 min. The cells were thoroughly washed and cultured for a further 2, 4, 8, 12 or 24 h in the presence of 50 µg/ml gentamycin before being fixed and stained with phalloidin-conjugated to Alexa488 and DAPI. Maximum projections were generated from Z-stacks obtained by confocal microscopy as described in the Materials and methods section. (B) The relative area occupied by RFP-fluorescence was measured for infected cells using ImageJ and associated macros as described in the Materials and methods section. Error bars represent the standard error of the mean. $P < 0.005$. At least 150 cells were counted for each construct and the experiment repeated in triplicate. (C) Colony forming units were calculated as described in the Materials and methods section. (D) SL1344 were cultured in Luria-Bertani broth with 800 nM YM201636 or equivalent volumes of DMSO for the indicated times and OD₆₀₀ readings taken hourly.

upon the number of bacteria entering the cells. Again, between 8, 12 and 24 h p.i., the YM201636-treated samples presented significantly fewer colonies than those treated with DMSO, ultimately resulting in an almost six-fold reduction in the number of intracellular bacteria relative to controls 24 h

p.i. It should be noted that even in the presence of YM201636, there was some increase in colony numbers, indicating that the block in *S. typhimurium* replication was not complete. To verify that this apparent impact on intracellular bacterial viability reflects perturbation of the pathogen–host interaction,

growth curves of RFP-SL1344 broth cultures grown in the presence of 800 nM YM201636 or DMSO, independent of mammalian cells, were compared (see Figure 2D). No significant difference was observed under these conditions indicating that the inhibition did not reflect direct bactericidal activity of the compound, and suggests that it is the mammalian host cell that is specifically influenced. TUNEL assays were carried out to investigate any potential cytotoxicity exhibited by the compound after 24 h exposure (see Supplementary Figure 2). Even in infected cells no significant difference in DNA-fragmentation was observed between DMSO- and YM201636-treated cells indicating that exposure to YM201636 did not significantly induce apoptosis. Furthermore, apart from the induction of large vacuolar structures (see Figure 1), labelling of the nuclei and actin cytoskeleton indicated that these cells were intact and viable.

Inhibition of PIKfyve activity therefore has a profound impact on the capacity of *S. typhimurium* to replicate in non-phagocytic cells. As *Salmonella* exploit macropinocytosis to gain entry into these cells, we have made use of cell-based models to examine the role of PIKfyve in macropinocytosis in an effort to reveal the mechanism by which this inhibition may occur.

Temporal recruitment of PIKfyve during macropinocytosis

Because of the highly tractable and dynamic nature of macropinocytosis, time-lapse videomicroscopy was employed to investigate the recruitment of the PI(3)P-binding $2 \times \text{FYVE}_{\text{Hrs}}$ (Gillooly *et al*, 2000) domain to newly formed macropinosomes (Figure 3). To monitor the fate of the macropinosomes post-formation, cells expressing GFP- $2 \times \text{FYVE}_{\text{Hrs}}$ were pulse-labelled with dextran-TR, and the dextran-filled macropinosomes monitored using 4D time-lapse videomicroscopy. Macropinosomes acquired and remained enriched for the PI(3)P-specific probe for 15–20 min before it rapidly dissociated from the organelle (see Figure 3A). The rapid dissociation of the GFP- $2 \times \text{FYVE}_{\text{Hrs}}$ probe from the macropinosome 15–20 min post-formation likely reflects the further phosphorylation of PI(3)P to PI(3,5)P₂ by PIKfyve (McEwen *et al*, 1999; Cooke, 2002; Jefferies *et al*, 2008; Shisheva, 2008). Indeed, treatment with YM201636 resulted in sustained accumulation of GFP- $2 \times \text{FYVE}_{\text{Hrs}}$ to the limiting membrane of the macropinosome supporting this notion (see Figure 3B). Although the direct accumulation of PI(3,5)P₂ on the macropinosome could not be monitored because of the absence of any PI(3,5)P₂-specific probes, PIKfyve-GFP was, however, readily observed to be recruited with a similar kinetic profile to that of the PI(3)P-specific probe GFP- $2 \times \text{FYVE}_{\text{Hrs}}$ (Figure 3A and C comparison). This is expected given that PIKfyve is itself recruited to PI(3)P-rich endosomal membranes by an amino-terminal PI(3)P-binding FYVE domain (Shisheva *et al*, 1999). It is interesting to note that unlike the $2 \times \text{FYVE}_{\text{Hrs}}$ probe, PIKfyve-GFP presented a somewhat discontinuous distribution on the limiting membrane of the macropinosome. In contrast to $2 \times \text{FYVE}_{\text{Hrs}}$, and PIKfyve-GFP, the catalytically inactive mutant (PIKfyve(K1831M)-GFP (Sbrissa *et al*, 2000; Osborne *et al*, 2008) was recruited uniformly, and remains resolutely associated with the developing macropinosome, because it is unable to phosphorylate PI(3)P (see Figure 3D). PIKfyve recruitment to macropinosomes is therefore tempo-

rally regulated with its association correlated with the presence of PI(3)P, and its dissociation requiring its catalytic activity.

PIKfyve regulates macropinosome late endosome/lysosome fusion

Given the recruitment of PIKfyve to the macropinosome (Figure 3C), we sought to define its role in the process. Using a macropinosome formation assay developed within the laboratory (Lim *et al*, 2008), we quantified the number of macropinosomes formed in the presence of DMSO or 800 nM YM201636 over a 5 min period (Figure 4A). No significant difference was observed in the number of macropinosomes formed under these conditions. This observation indicated that the impact PIKfyve had on the macropinosome was post formation. We next investigated the requirement of PIKfyve for the late stages of macropinosome maturation, at which time macropinosomes fuse with late endosomes and lysosomes (Lim *et al*, 2008). To do so, we adapted a dextran content mixing assay described by Bright *et al* (2005). Newly formed dextran-TR positive macropinosomes in cells expressing PIKfyve-GFP rapidly acquired content from the late endosomal/lysosomal system as monitored by the delivery of dextran-647 (Figure 4B). Over a period of 20–25 min, numerous smaller dextran-647-positive late endosomes/lysosomes were observed fusing directly with the macropinosomes. Concordant with this, PIKfyve-GFP was transiently recruited to the macropinosome before fusion with late endosomes/lysosomes as described earlier (Figure 3C). Conversely, in cells expressing the catalytically inactive PIKfyve(K1831M)-GFP construct, no content mixing between macropinosomes and late endosomes/lysosomes was observed. Quantification of at least 50 cells expressing PIKfyve-GFP using this strategy revealed ~90% of macropinosomes had fused with late endosomes/lysosomes within a 30 min period, whereas this was observed for only ~10% of macropinosomes in cells expressing the catalytically inactive mutant (Figure 4C). However, it should be noted that macropinosomes were observed to fuse homotypically with one another in the presence of the PIKfyve(K1831M)-GFP construct (see Supplementary Figure 3), indicating that the block in fusion was restricted to that occurring between macropinosomes and organelles of the late endocytic pathway. The content-mixing experiment was repeated in the presence of 800 nM YM201636. As with PIKfyve(K1831M)-GFP, macropinosomes cultured in the presence of YM201636 did not exchange contents with the late endosomes/lysosomes, whereas those treated with the carrier, DMSO, did so within 30 min of formation (Figure 4C). It is important to note that neither YM201636 treatment nor expression of the PIKfyve(K1831M)-GFP construct had an appreciable effect upon the amount of dextran uptake throughout the assay, as measured by total fluorescent intensity relative to control cells (data not shown). Inhibition of PIKfyve activity by two independent mechanisms therefore indicates that the enzyme activity is essential for the fusion of macropinosomes with the late endosome/lysosomal system. Expression of the Akt protein phosphorylation-deficient mutant, PIKfyve(S318A)-GFP (Berwick *et al*, 2004), did not prevent fusion with the late endosomes/lysosomes (data not shown), suggesting it is the lipid kinase activity of PIKfyve that is required for the late-stage maturation of macropinosomes. It should be noted

Figure 3 PI(3)P is transiently associated with the macropinosome due to PIKfyve activity. FlpIn HEK293 cells were transiently transfected with (A, B) pEGFP-2 × FYVE_{Hrs}, (C) pPIKfyve-GFP or (D) pPIKfyve(K1831M)-GFP cultured in the presence of 100 µg/ml dextran-TR (MW 10 000) for 4 min and newly formed dextran-TR-positive macropinosomes imaged in 4D as described in the Materials and methods section. (B) Samples were pretreated with 800 nM YM201636 for 2 h and the experiment run in the continued presence of this concentration of the pharmacological agent. Presented is the average fluorescent intensity of each GFP probe throughout the dextran-positive macropinosome over time. Total duration and interval were as indicated. Three independent replicates are presented. (E) Example frames of each condition is presented as a montage. Note: the presented example is represented by the blue line in each graph.

that, unlike the catalytically inactive mutant, expression of the Akt protein phosphorylation mutant had no apparent impact on the intracellular replication of *Salmonella* (data not shown).

PIKfyve action during macropinosome late endosome/lysosome fusion is independent of Rab7

The recruitment of the small GTPase Rab7 is key to a number of early to late endosomal trafficking events and has

Figure 4 PIKfyve activity is required for macropinosome late endosome/lysosome fusion. (A) HEK-FlpIn cells cultured in the presence or 800 nM YM201636 or the equivalent volume of DMSO were pulsed-labelled with dextran (10 000MW) conjugated to tetramethylrhodamine for 5 min prior to fixation in 4% PFA. Macropinosomes, defined as dextran-positive structures $>0.5 \mu\text{m}$ in diameter, were counted using an automated image analysis protocol described previously (Lim *et al*, 2008). (B) To examine later stage macropinosome maturation, first the late endosomes and lysosomes of FlpIn HEK293 cells were fluorescently labelled by culturing the cells in the presence of 200 $\mu\text{g}/\text{ml}$ dextran-Alexa647 (MW 10 000) for 4 h before being thoroughly washed and further cultured in normal growth media for 16 h. Newly formed macropinosomes, labelled by culturing cells in the presence of 100 $\mu\text{g}/\text{ml}$ dextran-TR (MW 10 000) for 4 min, were imaged in 4D as described in the Materials and methods section. Presented is a montage of selected frames of macropinosomes in a cell transfected with pPIKfyve-GFP or pPIKfyve(K1831M)-GFP. Total duration and interval were as indicated. The total time taken to internalise dextran-TR (4 min) and initiate imaging is indicated in red. Movies can be viewed in Supplementary data. (C) To quantify the impact PIKfyve-GFP, PIKfyve(K1831M)-GFP, GFP-Rab7, GFP-Rab7(T22N), DMSO, YM201636, GFP-Rab7 + YM201636 and GFP-Rab7(Q67L) + YM201636 have upon macropinosome late endosome/lysosome fusion, >100 macropinosomes from at least 50 independent time-lapse videomicroscopy experiments were scored for content mixing. Presented is the proportion of macropinosomes that have acquired content from the late endosomes and lysosomes. Error bars represent the s.d. $P < 0.005$.

previously been implicated in intracellular *Salmonella* replication (Meresse *et al*, 1995, 1999). Similar to the catalytically inactive PIKfyve construct and YM201636 treatment,

macropinosomes in cells expressing a dominant negative Rab7 mutant (GFP-Rab7(T22N)) were unable to fuse with the late endosome/lysosome (Figure 4C). To investigate

whether the block in macropinosome maturation induced by PIKfyve(K1831M)-GFP reflects an inhibition in the recruitment of Rab7 to the maturing macropinosome, time-lapse videomicroscopy of cells expressing mRFP-Rab7 and PIKfyve(K1831M)-GFP was employed. Macropinosomes in cells expressing PIKfyve(K1831M)-GFP were readily observed to acquire increasing amounts of mRFP-Rab7 as they migrated centripetally into the cell (Supplementary Figure 4). Similarly, treatment with YM201636 has no apparent impact upon the recruitment of GFP-Rab7 to the maturing macropinosome (data not shown). Therefore inhibition of PIKfyve does not alter the recruitment of the Rab7 GTPase.

Although the recruitment of Rab7 to intracellular membranes is indicative of its activated state, we sought to verify whether this in the presence of wild type and catalytically inactive PIKfyve. As both PIKfyve and Rab7 activity are required for macropinosome late endosome/lysosome fusion, we examined whether the expression of the constitutively-activated mutant of Rab7 (GFP-Rab7(Q67L)) could rescue the block in fusion observed in the presence of YM201636. Similar to cells treated with 800 nM YM201636 alone, macropinosomes in cells expressing GFP-Rab7(Q67L) and treated with YM201636, were unable to exchange content with the late endosomes/lysosomes (see Figure 4C). When coupled with the observation that PIKfyve activity was not required for Rab7 recruitment, the failure of GFP-Rab7(Q67L) to rescue the YM201636 phenotype indicates that the mechanism by which PIKfyve coordinates macropinosome late endosome/lysosome fusion is not Rab7-dependent.

Inhibition of PIKfyve activity interferes with SCV maturation

Intracellular *Salmonella* deliver a number of effector proteins via the SPI2-T3SS, reviewed in detail by Haraga *et al* (2008), that direct the fusion between the SCV and the late endosomes/lysosomes of the host cell. In doing so, the SCV acquires markers of the late endosomal system and is expanded to form extensive LAMP1-positive SIFs (Haraga *et al*, 2008). As PIKfyve regulates macropinosome late endosome/lysosome fusion (see Figure 4), a process analogous to that which occurs during SCV maturation, we speculated that inhibition of PIKfyve activity might disrupt the fusion of the SCV with late endosomes and lysosomes, and ultimately the formation of SIFs.

To monitor the delivery of late endosomes and lysosomes to the SCV, and the impact YM201636 has on this process, these organelles were preloaded by culturing A431 cells in the presence of dextran conjugated to Alexa488 overnight, then chased in dextran-free media for 3 h. The cells were then treated with either DMSO or 800 nM YM201636 for 2 h before they were infected with RFP-SL1344 in the continued presence of the pharmacological agents as described previously. The samples were then fixed 8 h p.i., counterstained with DAPI, and examined by confocal microscopy (see Figure 5A). Consistent with previous reports (Drecktrah *et al*, 2007), dextran-488 was observed to colocalise with intracellular RFP-SL1344 (arrows) in cells treated with DMSO, frequently marking the lumen of the SCV (see Figure 5A inset). In contrast, no colocalisation was observed between intracellular RFP-SL1344 and late endosomes/lysosomes in cells treated YM201636 (arrows).

The failure of the SCV to exchange contents with the late endosomes and lysosomes suggests an upstream block in its maturation. Ordinarily, the SCV acquires markers of the late endosomal system, such as LAMP1, within 1 h p.i. To verify whether or not this occurs in the presence of YM201636, A431 cells were pretreated with 800 nM YM201636 or equivalent volumes of DMSO before infection with RFP-SL1344 for 1 h. The samples were then fixed, labelled with a monoclonal antibody against LAMP1, counterstained with DAPI and examined by confocal microscopy (see Figure 5B). Consistent with a block in maturation, unlike the control cells, there was little colocalisation between intracellular RFP-SL1344 and LAMP1 in cells treated with YM201636. Quantification of LAMP1-labelling of >150 SCVs from three biological replicates confirmed a significant perturbation in SCV maturation (see Figure 5C).

To examine the impact YM201636 had on SIF formation, cells were pretreated with DMSO or YM201636 and infected with RFP-SL1344 for 8 h as described in the Materials and methods section. The cells were then fixed and immunolabelled with a monoclonal antibody specific for LAMP1, counterstained DAPI and examined using confocal microscopy (Figure 5D). Infected cells were then scored for the presence or absence of SIFs (arrows). Although ~75% of infected cells cultured in the presence of DMSO had induced SIFs, <~4% of those in cells cultured with YM201636 formed LAMP1-positive SIFs (see Figure 5E).

YM201636 perturbs SCV acidification and activation of SPI2

PipB is a SPI2-T3SS translocated effector and is localised to SIFs and the SCV during the later stages of infection (Knodler *et al*, 2002). To determine whether or not engagement of the SPI2-T3SS was disrupted by interfering with PIKfyve activity, A431 cells were cultured for 2 h with 800 nM YM201636 or equivalent volumes of DMSO before being infected with *S. typhimurium*-expressing PipB tagged with tandem haemagglutinin under the control of the PipB promoter (Knodler *et al*, 2002). The infected cells were cultured for 8 h in the presence of gentamycin before fixation and immunolabelling with an anti-HA antibody followed by the appropriate secondary antibody. Samples were counterstained with phalloidin conjugated to Alexa488 and DAPI before examination with a confocal scanning microscope. Although infected cells cultured in the presence of DMSO presented prominent HA-labelling on SCVs and SIFs, as well as smaller more peripheral puncta, those cells that were cultured with YM201636 had no apparent HA-labelling (Figure 6A). DAPI labelling confirmed the presence of intracellular *S. typhimurium* in both DMSO- and YM201636-treated samples. This was also confirmed by western blotting of the chaperone protein DnaK 8 h p.i. (see Figure 6B).

To verify that PipB-2 × HA was no longer being delivered in cells cultured with YM201636, a western immunoblot was conducted using samples treated in the same manner. Strikingly, although PipB-2 × HA was readily detected as a ~30 kDa band, 8 h p.i. in samples cultured with DMSO, significantly less was detected in samples cultured with YM201636 (see Figure 6B). As this may reflect an inability to translocate the effector into the host cells in appreciable amounts, the impact of YM201636 on the SPI2-T3SS system itself was examined. We focussed on SseA, which is a

Figure 5 YM201636 inhibits the maturation of the *Salmonella*-containing vacuole (SCV) and *Salmonella*-induced filaments (SIFs) during infection. (A) The late endosomes and lysosomes of A431 cells were fluorescently labelled by culturing the cells in the presence of 200 μ g/ml dextran–Alexa488 (MW 10 000) for 16 h before being thoroughly washed and further cultured in normal growth media for 4 h. The cells were cultured with 800 nM YM201636 or equivalent volumes of DMSO for 2 h and were then infected with RFP-SL1344 for 10 min. The cells were thoroughly washed and cultured for a further 8 h in the presence of 50 μ g/ml gentamycin before being fixed and counterstained DAPI. Maximum projections were generated from Z-stacks obtained by confocal microscopy as described in the Materials and methods section. (B, D) A431 cultured with 800 nM YM201636 or equivalent volumes of DMSO for 2 h were infected with RFP-SL1344 for 10 min. The cells were thoroughly washed and cultured for a further 1 h (B) or 8 h (D) in the presence of 50 μ g/ml gentamycin before being fixed, labelled with monoclonal antibodies against LAMP1 and counterstained and DAPI. Maximum projections were generated from Z-stacks obtained via confocal microscopy as described in the Materials and methods section. Arrows indicate SIFs (D). (C) The impact DMSO or YM201636 had on SCV maturation was quantified by counting the number of infected cells that displayed LAMP1-positive SCVs 1 h p.i. Presented is the proportion of SCVs that were LAMP1-positive. Error bars represent the s.d. $P < 0.005$. At least 100 transfected cells were counted for each treatment and the experiment repeated in triplicate. (E) The impact DMSO or YM201636 had upon SIF formation was quantified by counting the number of infected cells that displayed LAMP1-positive SIFs 8 h post-infection. Presented is the percentage of transfected cells that contained at least one SIF. Error bars represent the s. d. $P < 0.005$. At least 100 transfected cells were counted for each construct and the experiment repeated in triplicate.

Figure 6 YM201636 inhibits *Salmonella*-containing vacuole (SCV) acidification and activation of the SPI2 virulence locus. **(A)** A431 cells cultured with 800 nM YM201636 or equivalent volumes of DMSO for 2 h were infected with PipB-2 × HA SL1344 for 10 min. The cells were thoroughly washed and cultured for a further 8 h in the presence of 50 µg/ml gentamycin before being fixed and labelled with monoclonal antibodies against the HA-epitope. **(B)** A431 cells were cultured with 800 nM YM201636 or equivalent volumes of DMSO for 2 h and infected with PipB-2 × HA or SseA-2 × HA SL1344 for 2 or 8 h as indicated. Western immunoblotting was conducted as described. **(C)** A431 cells cultured in the presence of 1 µM LysoTracker Red with 800 nM YM201636 or equivalent volumes of DMSO for 2 h and were then infected GFP-SL1344 for 10 min. The cells were thoroughly washed and cultured in the continued presence of LysoTracker Red and YM201636 or DMSO and 50 µg/ml gentamycin for a further 90 min. Maximum projections of live infected cells were generated from Z-stacks obtained by confocal microscopy as described in the Materials and methods section. **(D)** A431s cultured in the presence of 800 nM YM201636 or the equivalent volume of DMSO were infected and cultured in gentamycin as described earlier for 24 h before being fixed and examined by resin electron microscopy as described in the Materials and methods section.

chaperone protein for SseB and SseD and is therefore required for the assembly of a functional SPI2-T3SS (Ruiz-Albert *et al*, 2003; Zurawski and Stein, 2003). A431 cells were cultured for 2 h with 800 nM YM201636 or equivalent volumes of DMSO before being infected with

S. typhimurium-expressing SseA tagged with tandem haemagglutinin under the control of the *sseA* promoter (Coombes *et al*, 2003). The infected cells were cultured for 8 h in the presence of gentamycin before preparation for western immunoblotting (see Materials and methods

section). SseA-2 × HA was readily detected as an ~13 kDa band in samples cultured in the presence of DMSO; however, as with PipB-2 × HA, significantly less was detected in samples cultured in the presence of YM201636, indicating that the SPI2 expression was not induced during infection in the presence of this compound. SPI2 expression is induced, at least in part, in response to the acidification of SCV (Alpuche Aranda *et al*, 1992; Rathman *et al*, 1996; Beuzon *et al*, 1999). Given the failure to induce SseA and PipB expression in the presence of YM201636, we speculated that perhaps inhibition of PIKfyve activity may be perturbing the acidification of SCV. To investigate this, A431 cells treated with YM201636 or

equivalent volumes of DMSO were cultured in the presence of 1 μm of LysoTracker Red for 2 h. They were then infected with GFP-SL1344 for 10 min, washed and cultured in the continued presence of LysoTracker Red and DMSO or YM201636 and gentamycin for a further 90 min. Strikingly, confocal imaging of live cells indicated that, although LysoTracker was readily detected within the lumen of SCVs in cells cultured with DMSO, those cultured with YM201636 were not labelled with the acidotropic dye (see Figure 6C). This indicates that inhibition of PIKfyve activity was indeed sufficient to inhibit SCV acidification, ultimately disrupting induction of SPI-2 virulence locus.

Figure 7 YM201636 inhibits intracellular replication of *Salmonella typhimurium* and macrophage bactericidal activity in bone marrow derived macrophages. (A) Bone marrow-derived macrophages (BMDMs) cultured with 800 nM YM201636 or equivalent volumes of DMSO for 2 h were infected with RFP-SL1344 for 10 min and cultured as indicated. Intracellular replication was monitored by measuring the relative area occupied by RFP-fluorescence in (B) infected cells and colony forming unit (C) assayed as described in Figure 2.

Figure 8 YM201636 treatment blocks bacterial degradation in macrophages. (A) Bone marrow-derived macrophages (BMDMs) cultured with 800 nM YM201636 or equivalent volumes of DMSO for 2 h were infected with RFP-SL1344 for 10 min. The cells were thoroughly washed and cultured for a 24 h in the presence of 50 μg/ml gentamycin before being fixed and stained with phalloidin-conjugated to Alexa488 and DAPI. Maximum projections were generated from Z-stacks obtained by confocal microscopy as described in the Materials and methods section. (B) BMDMs cultured in the presence of 800 nM YM201636 or the equivalent volume of DMSO were infected and cultured in gentamycin as described earlier for 24 h before being fixed and examined by resin electron microscopy as described in the Materials and methods section.

One possibility that may explain the absence of colocalisation between intracellular RFP-SL1344 and the transmembrane protein, LAMP1, SIF formation or SPI2-induction, is that the *Salmonella* may no longer be contained within the SCV following treatment with YM201636. To examine this possibility, A431 cells cultured in the presence of DMSO or 800 nM YM201636 were infected with RFP-SL1344 for 24 h, fixed, resin-embedded, sectioned and examined by electron microscopy (see Figure 6D). Consistent with the confocal data presented earlier (see Figure 1), *S. typhimurium* in DMSO-treated cells were frequently highly filamentous in nature and encapsulated within a tubular SCV. Strikingly, those found in YM201636-treated cells were less frequent, rod-shaped and resided within spacious vacuolar membranes. These vacuoles represent the swollen endosomal membranes described previously upon the application of YM201636 (Jefferies *et al*, 2008). Collectively, inhibition of PIKfyve activity interferes with SCV maturation as defined by SIF formation and through reduced SPI2 expression, but does not lead to outgrowth of the pathogen into the cytoplasm.

Inhibition of PIKfyve activity interferes with *Salmonella* replication in macrophages

Although *Salmonella* are capable of replicating within a range of different cell-types, during a systemic infection they are believed to primarily replicate within macrophages (Richter-Dahlfors *et al*, 1997). Primary murine bone marrow-derived macrophages (BMDMs) were treated with 800 nM YM201636 or the equivalent volume of the carrier (DMSO) for 2 h before

infection with RFP-SL1344 and confocal imaging as described in Figure 2 (see Figure 7A). As in A431 cells, between 2 h and 4 h p.i. little difference in the number and behaviour of the pathogen was observed. However, from 8, 12 and 24 h p.i., quantification of the RFP-fluorescence in infected cells revealed significantly fewer fluorescent bacteria within those cells treated with YM201636 when compared with those cultured in the presence of DMSO (see Figure 7B). This result is consistent with that observed in the epithelial cell line (Figure 2).

Analysis of *S. typhimurium* replication in macrophages by CFU assay, however, indicated that within the control cells, the number of viable *Salmonella* recovered at later time points decreased relative to the early time points (Figure 7C). This is consistent with the expected anti-bactericidal pathways present in macrophages. Indeed, microscopic investigation of DAPI-labelled RFP-SL1344 in BMDMs infected and treated with DMSO for 24 h revealed that in a significant proportion of the bacteria the DNA was condensed, a hallmark of bacterial degradation (see Figure 8A) (Meyer-Bahlburg *et al*, 2001). Examination by electron microscopy revealed that within infected cells treated with DMSO, the integrity of the bacteria appeared to be perturbed in a manner similar to that described previously (Meyer-Bahlburg *et al*, 2001) (see Figure 8B). In contrast, analysis of bacterial viability in YM201636-treated BMDMs by the CFU assay indicated that the number of viable *Salmonella* recovered increased over the time course (see Figure 7C). Those in cells treated with YM201636 had dispersed DNA throughout the

bacterium suggesting that they were intact and viable (Figure 8A). Examination of YM201636-treated cells by electron microscopy confirmed both a decrease in the total number of intracellular *S. typhimurium*, and the highly spacious nature of the SCV in cells cultured with YM201636. It should be noted that because of sectioning of the swollen vacuolar BMDMs, detection of the intracellular pathogen in these cells was difficult. We therefore conclude that, within macrophages, although inhibition of PIKfyve does interfere with the ability of *S. typhimurium* to replicate by preventing proper development of its replicative niche it also prevents the host from killing the internalised pathogens.

Discussion

Salmonella exploits macropinocytosis as a route of entry into non-phagocytic mammalian cells (Francis *et al*, 1993). Once within the cell they manipulate the encompassing macropinosome to generate a replicative niche that support its survival and growth (Haraga *et al*, 2008). The invasion and intracellular replication of *Salmonella* is a carefully choreographed interplay between the host cell's molecular machinery and secreted effectors of the pathogen (Haraga *et al*, 2008). We find that treatment with YM201636, a specific inhibitor of PIKfyve, which in itself does not inhibit bacterial growth, specifically inhibited intracellular replication, with no apparent impact on invasion of *S. typhimurium*. Similar results were observed when PIKfyve activity was perturbed by siRNA-mediated knockdown and overexpression of dominant-negative mutants. Ideally we would monitor the relative levels of PtdIns(3,5)P₂ within individual cells treated in this manner; however, in the absence of a specific probe to this lipid species we have demonstrated that perturbation of PIKfyve lipid-kinase activity by multiple strategies produces a consistent inhibition upon intracellular *Salmonella* replication.

Characterisation of the recruitment of PIKfyve to macropinosomes in the absence of the pathogen demonstrated that although it is recruited during the early stages of the macropinosome's maturation, its impact continues to the later stages of the process, during fusion between the macropinosome and late endosome/lysosome. Both overexpression of a catalytically inactive PIKfyve mutant and treatment with YM201636 were sufficient to inhibit the direct fusion of macropinosomes with organelles of the late endosomal/lysosomal system. Similarly, the interfering Rab7 mutant (GFP-Rab7T22N) blocked fusion of macropinosomes with the late endosomes and lysosomes. The recruitment of Rab7 to the maturing macropinosome is not, however, perturbed by the expression of the catalytically inactive PIKfyve construct, indicating that Rab7 recruitment does not require PIKfyve activity. Nor does constitutive activation of Rab7 rescue this phenotype, thus suggesting that the two function independently of one another.

The role that PIKfyve has in endocytosis is currently controversial because of the complex phenotype observed following its perturbation (Shisheva, 2008). In addition to its role in MVB formation, siRNA-mediated knockdown and application of the catalytically inactive mutant suggests a role for PIKfyve in the retrograde trafficking of the cation-independent mannose 6-phosphate receptor (CI-M6PR) and furin from early endosomes to the *trans*-Golgi network (TGN)

(Rutherford *et al*, 2006; Ikonov *et al*, 2008). Surprisingly, however, application of YM201636, has little effect on the retrograde trafficking of the CI-M6PR, suggesting that this might represent a terminal phenotype rather than a direct consequence of PIKfyve inhibition (Jefferies *et al*, 2008). Consistent with this, YM201636 did not perturb the tubulovesicular sorting nexin network previously observed to form from the limiting membrane of the macropinosome (Kerr *et al*, 2006) (data not shown). It is through this network that CI-M6PR is trafficked from early endocytic compartments to the TGN (Bujny *et al*, 2007). The capacity to monitor the behaviour of discrete organelles through time-lapse videomicroscopy has enabled us to definitively confirm a role for PIKfyve in the later stages of the endocytic itinerary, regulating fusion between maturing and late endosomal/lysosomal compartments. That is not to say, however, that PIKfyve may not also have had earlier roles. It is, after all, recruited rapidly following macropinosome formation.

During infection of non-phagocytic cells *Salmonella* manipulate the macropinocytic process to generate the SCV. To accommodate the expanding numbers of replicating bacteria, by necessity the SCV must be enlarged. Ordinarily this occurs by the directed fusion of late endosomes and lysosomes with the SCV to form tubular SIFs. In addition to expanding the compartment, SIFs may also serve to target nutrient-rich organelles to the SCV, thereby facilitating growth of the pathogen in an environment removed from the cytoplasmic host-defense machinery (Haraga *et al*, 2008). As it does in the absence of the pathogen, disruption of PIKfyve activity perturbs fusion of the SCV and the late endosomal/lysosomal compartments of the cell, thereby inhibiting SCV maturation (see Figure 5). This in turn inhibits replication as SIF formation is concomitant with *Salmonella* multiplication. *Salmonella* within epithelial cell-lines and macrophages cultured in the presence of YM201636 were both less numerous and did not have the distinctive filamentous morphology ordinarily observed. YM201636 treatment also resulted in reduced expression of the SPI2 proteins. As activation of this locus is sensitive to a reduction in the pH of the SCV, the likelihood is that acidification of the SCV is also blocked upon YM201636 treatment, an observation confirmed by labelling live-infected cells with the acidotropic dye LysoTracker Red. This is consistent with a failure to deliver acidic late endosomal and lysosomal contents to the SCV in the presence of YM201636. As disruption of PIKfyve activity blocks fusion of macropinosomes with late endosome/lysosomal compartments independent of *Salmonella*, the interference observed likely reflects a perturbation in membrane delivery as well as associated host proteins, rather than specific *Salmonella* effectors.

With the development of antibiotic resistant strains, new strategies to combat infection must be devised. By targeting cellular processes essential for bacterial replication, but not entirely essential for the more genetically diverse host cell, it is possible to circumvent the relatively rapid adaptability of the invading bacterium. Indeed, disruption of PIKfyve activity in infected cells may be a viable strategy to combat acute infection. The marked impact on both replication and SPI2 expression suggests that the *Salmonella* is stalled at a relatively early stage of intracellular infection. Intriguingly, YM201636 treatment also has a profound impact upon retroviral budding (Jefferies *et al*, 2008). Retroviruses usurp

the Endosomal Sorting Complex Required for Transport (ESCRT), which is normally involved in endosomal sorting and MVB formation, to facilitate budding of the enveloped virion from the limiting membrane of the cell (Martin-Serrano *et al*, 2003). Jefferies *et al* (2008) reported a dramatic reduction in the release of virus particles following treatment with YM201636 (Jefferies *et al*, 2008). It was observed that although the virus particles were predominantly associated with the intercellular spaces in the control samples, those treated with YM201636 were found almost exclusively within large electron-lucent vacuoles, indicating that the inhibition of PIKfyve activity blocks the formation and release of mature viral particles likely from the MVB. It is interesting to note that although the mechanisms of *Salmonella*- and retrovirus-pathogenesis are distinct, their dependence on the late endosomal system for the later stages of their infectious cycle is shared.

In conclusion, when studying pathogen–host interactions we are faced with the conundrum of understanding both organisms, as well as how they interact and influence one another. By first developing a clearer understanding of normal processes, such as the role key endocytic enzymes like PIKfyve have in macropinocytosis, we can then attempt to dissect the process in the presence of the pathogen, thereby defining those mechanisms involved in the host-pathogen association. Here we have defined a role for PIKfyve in macropinosome late endosome/lysosome fusion and demonstrated how this revelation can be exploited as a pharmacological target to inhibit intracellular *Salmonella* replication and pathogenesis.

Materials and methods

Constructs and reagents

pEGFP-2 × FYVE_{Hrs}, pPIKfyve-GFP, pPIKfyve(S318A)-GFP and pPIKfyve(K1831M)-GFP were as described previously (Kavran *et al*, 1998; Gillingham *et al*, 2000; Osborne *et al*, 2008). Monoclonal antibodies against LAMP1, the haemagglutinin epitope (16B12) were supplied by BD Biosciences and Abcam, respectively. Polyclonal antibodies against PIKfyve (H00200576-A01) and Vac14 (H00055697-B01P) were supplied by Sapphire Biosciences. Secondary antibodies, phalloidin-fluorescent conjugates, LysoTracker Red and dextran-fluorescent conjugates were purchased from Molecular Probes (Invitrogen). YM201636 was purchased from Symansis: Cell Signalling Science. *S. typhimurium* strains used include the wild-type strain SL1344 (Hoiseth and Stocker, 1981), and isogenic derivatives thereof, GFP-SL1344 (pFPV25.1) (Knodler *et al*, 2005), RFP-SL1344 (pBR-RFP.1) (Birmingham *et al*, 2006), Δ pipB (pACB C-2HA) (Knodler *et al*, 2002) and Δ seA (psseA:2HA) (Coombes *et al*, 2003).

Cell culture and transfection

Flp-In Hek293 (Invitrogen) and A431 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum and 2 mM L-glutamine (Invitrogen) in humidified air/atmosphere (10% CO₂) at 37°C. Cells were transfected with 4 µg of DNA using Lipofectamine 2000 as per manufacturer's instructions (Invitrogen). Primary BMDM cells were prepared and cultured as described previously (Pagan *et al*, 2003; Sester *et al*, 2006).

siRNA-mediated knockdown

A431 cells were treated with siRNA duplexes from Dharmacon using the HiPerfect Fast-forward protocol as per manufacturer's instructions (Qiagen). Cells were transfected with siRNA for 48 h before passaging and equal numbers reseeded for a second round of siRNA treatment for 48 h. Infections were conducted 72 h after the initial transfection the cells. Knockdown was gauged by the

presence of swollen vacuolar structures within the cytoplasm of the treated cells. Dharmacon PIKfyve siRNA duplexes used successfully were J-005058-13 and J-005058-14. Dharmacon Vac14 siRNA duplexes used successfully were J-015729-05, J-015729-07 and J-015729-08.

Dextran uptake

Macropinosomes were labelled with fluorescent dextran by culturing live cells in the presence of 100–200 µg/ml dextran (MW10 000) conjugated to tetramethylrhodamine or Alexa647 for 4–5 min before being thoroughly washed with excess media and either imaged live or fixed and prepared for further analysis. Late endosomes and lysosomes of Flp-In Hek293 were labelled by culturing the cells in media containing 100–200 µg/ml dextran (MW10 000) Alexa647 for 4 h before thoroughly washing cells in media and culturing for a further 16 h in the absence of dextran. Late endosomes and lysosomes of A431 cells were labelled by culturing the cells in media containing 100–200 µg/ml dextran (MW10 000) Alexa488 for 16 h before thoroughly washing cells in media and culturing for a further 3 h in the absence of dextran.

Time-lapse videomicroscopy

For live cell imaging, monolayers were cultured on 35 mm glass-bottom dishes coated with poly-L-lysine. Time-lapse videomicroscopy was carried out on individual live cells using a Zeiss LSM 510 meta confocal scanning microscope with a × 40 objective. GFP was excited with the 488-nm argon laser line, and confocal sections were collected using a 505–530-nm BP emission setting. mCherry was excited with the 543-nm krypton laser line, and confocal images were collected using a 560-nm LP emission setting. Where dextran-TR and dextran-Alexa647 were employed simultaneously, they were each excited with 543-nm and 647-nm laser lines and collected using 560–615-nm BP and 653–718-nm emission setting respectively. For 3D time-lapse (4D) imaging, confocal Z-stacks (0.98 µm) were sequentially captured and reconstructed using the LSM software (Zeiss). Post-capture image analysis and quantification was conducted using the LSM software (Zeiss) and ImageJ 1.37v (NIH) (Collins, 2007). To quantify the recruitment of the fluorescently tagged constructs to the developing macropinosome, the dextran-TR signal was utilised to generate a mask to isolate the region of interest. Briefly, in ImageJ the red channel was converted to grey scale, smoothed using a median filter ($r=1$), and auto-thresholded. To remove small non-vesicular regions, the Analyse Particles plugin was employed (minimum particle size 350 pixels) to generate a mask of the macropinosome. The green channel stack was then converted to grey scale and multiplied by the mask stack using the Calculator Plus ImageJ plugin. The multiplication was given by $I(x,y) = G(x,y) \times M(x,y)/255$, where $G(x,y)$ is the grey scaled green channel pixel intensity for coordinates (x,y) and $M(x,y)$ is the mask intensity (0 or 255) at those coordinates. The effect is to create an image stack for which the pixels outside the mask region are set to 0, whereas in the mask region they give the green channel intensities. A simple ImageJ plugin to calculate the average of the non-zero pixels in each image in the resulting stack was created and then used to quantitate the average fluorescent intensity in the vesicular region over time.

Quantitative RT-PCR

Quantitative RT-PCR was conducted as described previously (Town *et al*, 2009). Briefly, RNA from siRNA-treated cells was extracted using TriReagent according to the manufacturer's directions (Sigma). In all, 1 µg of total RNA was used to produce cDNA using oligodT primers and Superscript III (Invitrogen). Quantitative RT-PCR was conducted on two samples for each siRNA-treatment, with each sample analysed in triplicate. The housekeeping gene GAPDH was used as an internal control to calculate the Δ CT for each sample. PIKfyve and Vac14 expression was quantified using TaqMan gene expression assays as per the manufacturer's instructions (Applied Biosystems) using 384 well plates.

TUNEL assay

TUNEL assays were conducted using the Click-iT TUNEL Alexa Fluor 488 imaging assay (Invitrogen).

Macropinosome quantification

Maximum projection images were captured using a Zeiss LSM 510 Meta confocal laser scanning microscope using a × 40 objective

and the number of newly formed macropinosomes present per cell were counted using an automated image analysis protocol in ImageJ v1.40 (Collins, 2007). Briefly, the 'Subtract Background' functionality was executed, and images thresholded to a binary image. Tetramethylrhodamine-positive structures of at least 0.5 μm in diameter were quantified through the 'Analyze Particles' feature. No less than 1500 cells were analysed per condition.

Quantification of SIFs

Salmonella was grown in Luria-Bertani (LB) broth for 16 h at 37°C with shaking, then subcultured at a dilution of 1:25 in LB broth and incubated at 37°C with shaking for ~2.5 h. The bacterial inoculum was prepared by pelleting the bacteria at 10 000g in a microfuge, then resuspending the bacteria in PBS. The inoculum was diluted in DMEM supplemented with 10% (v/v) foetal bovine serum and 2 mM L-glutamine (Invitrogen) so that ~100 bacteria were added per cell. The transfected monolayers were infected for 10 min after which free bacteria were immediately removed by washing three times with normal growth media. The infected cells were further incubated for 30 min in growth media followed by growth media supplemented with 50 $\mu\text{g}/\text{ml}$ gentamycin (Sigma-Aldrich) for 8 h. The monolayers were then fixed and labelled with monoclonal antibodies raised against LAMP1. Maximum projections of transfected cells were generated using a Zeiss LSM 510 Meta confocal laser scanning microscope and the presence or absence of SIFs scored.

Quantification of *Salmonella* infection

Infection of cells with RFP-SL1344 was quantified using the CellProfiler software (Carpenter *et al*, 2006) as follows. Each image contained three channels: an actin marker to delineate cellular regions; a DAPI marker to delineate the nuclear regions; and RFP to mark the *Salmonella*. Each image was then segmented into individual cells utilising the actin and nuclei images as per the Speckle Counting and Human cytoplasm-nucleus translocation array examples from the CellProfiler package, with minor modifications to improve the results. The RFP image was then processed to select the regions containing the bacteria by applying a threshold of intensity 51 (8-bit images). Using the cell segmentation, the total number of cells and area in pixels of RFP per cell was recorded for each image.

CFU assay

A431 cells were cultured in the presence or 800 nM YM201636 or the equivalent volume of DMSO for 2 h before infection with late log

phase *Salmonella* for 10 min, after which free bacteria were immediately removed by washing three times with normal growth media. The infected cells were further incubated for 30 min in growth media with YM201636 or DMSO followed by growth media supplemented with YM201636 or DMSO and 50 $\mu\text{g}/\text{ml}$ gentamycin (Sigma-Aldrich) for 2–12 h. The cells were then lysed in 0.25% SDS 1 $\times$ PBS and a serial dilution of the resultant lysate was plated onto LB agar plates containing 100 $\mu\text{g}/\text{ml}$ ampicillin. The plates were incubated at 37°C overnight and the resultant colonies counted to calculate the colony forming potential of the samples.

Electron microscopy

Cells were fixed in 2.5% glutaraldehyde in 0.05M sodium cacodylate buffer and processed for resin electron microscopy as previously described (Leonard *et al*, 2003).

Western immunoblotting

Western immunoblotting was conducted as described previously (Merino-Trigo *et al*, 2004).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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