PIKfyve/Fab1 is required for efficient V-ATPase delivery to phagosomes, phagosomal killing, and restriction of *Legionella* infection

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Abstract

By engulfing potentially harmful microbes, professional phagocytes are continually at risk from intracellular pathogens. To avoid becoming infected, the host must kill pathogens within the phagosome before they can escape or establish a survival niche. Here, we analyse the role of the phosphoinositide (PI) 5-kinase PIKfyve in phagosome maturation and killing, using the amoeba and model phagocyte *Dictyostelium discoideum*.

By binding to phosphatidylinositol-3-phosphate (PI3P) and phosphorylating it to PI(3,5)P_2, PIKfyve plays important, but poorly understood, roles in vesicular trafficking. Here we show that PIKfyve activity is essential during early phagosome maturation. Disruption of *PIKfyve* inhibited delivery of the vacuolar V-ATPase, dramatically reduced the ability of *Dictyostelium* cells to acidify newly-formed phagosomes. Consequently, *PIKfyve*-null cells were unable to digest phagosomal contents and generate an effective antimicrobial environment. PI(3,5)P_2 is therefore essential for phagocytes to efficiently kill captured bacteria and we demonstrate that cells lacking *PIKfyve* are more susceptible to infection by the intracellular pathogen *Legionella pneumophila.* We conclude that PIKfyve-catalysed PI(3,5)P_2 production plays a crucial and general role in ensuring early phagosomal maturation, thus protecting host cells from diverse pathogenic microbes.

Author summary

Cells that capture or eat bacteria must swiftly kill them to prevent potential pathogens surviving long enough to escape and establish an infection. This is achieved by the rapid delivery of components that produce an antimicrobial environment in the phagosome, the compartment containing the captured microbe. This is essential both for the function of immune cells and for amoebae that feed on bacteria in their environment. Here, we identify a new signalling pathway that regulates the delivery of antimicrobial components to the phagosome, and show that bacteria survive over three times as long within the host if this pathway is disabled. We show that this is of general importance for killing a wide range of pathogenic and non-pathogenic bacteria, and that it is physiologically important for cells to prevent infection by the opportunistic human pathogen *Legionella*.

Introduction

It is essential for professional phagocytes to kill their prey rapidly and efficiently to prevent the establishment of infections and disease. Multiple mechanisms are employed to achieve this and once phagosomes have been internalised they quickly become acidified and acquire reactive oxygen species, antimicrobial peptides and acid hydrolases. The timely and regulated delivery of these components is vital to protect the host from intracellular pathogens, but is incompletely understood.

After the phagosome is internalised, specific effector proteins are recruited to its cytoplasmic surface by interacting with several inositol phospholipids (PIPs) that play important roles in regulating vesicle trafficking and controlling maturation. The effectors of each PIP regulate particular aspects of compartment identity, membrane trafficking and endosomal maturation [1, 2]. Phosphatidylinositol-3-phosphate (PI(3)P), made by the class III PI 3-kinase VPS34, is one of the first PIPs to accumulate on vesicles after endocytosis, and it recruits proteins containing FYVE (Fab1, YOTB, Vac1 and EEA1) and PX domains – including, respectively, the characteristic early endosome markers EEA1 and Hrs, and sorting nexins [3, 4]. Also recruited to early endosomes by its FYVE domain is PIKfyve (known as Fab1 in yeast) [5], a phosphoinositide 5-kinase that phosphorylates PI(3)P to phosphatidylinositol-3,5-bisphosphate ($PI(3,5)P_2$) [6-10]. The roles of PI(3)P are well explored, but the formation of $PI(3,5)P_2$ and the identities and functions of its various effector proteins are less well understood [11-13]. $PI(3,5)P_2$ is thought to accumulate predominantly on late endosomes, and disruption of PIKfyve activity leads to multiple endocytic defects, including gross endosomal enlargement and accumulation of autophagosomes [14-19]. Recent research has begun to reveal important physiological roles of PIKfyve in a range of cell types and animals although mechanistic details remain sparse [20-24].

Like classical endocytosis, phagosome maturation is regulated by PIPs [25]. Phagosomes accumulate PI(3)P immediately after closure, and this is required for their subsequent maturation [26-28]. The recent identification of several PIKfyve inhibitors, including apilimod and YM201636 [29, 30], has allowed researchers to demonstrate the importance of PI(3)P to $PI(3,5)P_2$ conversion for phagosomal maturation in macrophages [31, 32]. However, there are conflicting reports on the roles of PIKfyve during key maturation steps such as acidification and degradation of bacteria, and its functional role remains subject to much debate.

To understand the function and physiological significance of PIKfyve, we have investigated its role in phagosome maturation and pathogen killing in the model organism *Dictyostelium discoideum,* a soil-dwelling amoeba and professional phagocyte that feeds on bacteria. *Dictyostelium* PIPs are unusual, with the lipid chain joined to the *sn*-1-position of the glycerol backbone by an ether, rather than

ester, linkage: these PIPs should correctly be named as derivatives of plasmanylinositol rather than phosphatidylinositol [33]. This however, appears to make no difference to downstream functions, which are dictated by interactions with the inositol polyphosphate headgroup. *Dictyostelium* has thus been an effective model for analysis of phosphoinositide signalling [34-37]. For convenience, both the mammalian and *Dictyostelium* inositol phospholipids are referees to as PIPs hereafter.

We find that genetic or pharmacological disruption of PIKfyve activity in *Dictyostelium* leads to a swollen endosomal phenotype reminiscent of defects in macrophages, and we provide a detailed analysis of phagosome maturation. We show that at least some of the defects in PIKfyve-deficient phagosome maturation are due to reduced recruitment of the proton-pumping vacuolar (V-ATPase). Furthermore, we provide the first demonstration that PIKfyve is required for the efficient killing of phagocytosed bacteria and for restricting the intracellular growth of the pathogen *Legionella pneumophila.*

Results

PIKfyve- null cells have swollen endosomes

The *Dictyostelium* genome contains a single orthologue of *PIKfyve* (*PIP5K3*). Like the mammalian and yeast proteins, *Dictyostelium* PIKfyve contains an N-terminal FYVE domain, a CCT (chaperonin Cpn60/TCP1)-like chaperone domain, a PIKfyve-unique cysteine/histidine-rich domain and a C-terminal PIP kinase domain [6]. In order to investigate the role of $PI(3,5)P_2$ in *Dictyostelium* we disrupted the *PIKfyve* gene by inserting a blasticidin resistance cassette and deleting a portion of the central PIKfyve-unique region (Supplementary Figure 1). To control for differences in genetic backgrounds, mutants were generated in both Ax3 and Ax2 axenic strains.

While the unusual ether-linked chemistry of the *Dictyostelium* inositol phospholipids prevented direct measurement of $PI(3,5)P_2$ loss by either the standard method of methanolysis followed by HPLC of deacylation products or mass spectrometry (P.T. Hawkins, personal communication), we found that each mutant strain was highly vacuolated (Figure 1A and B), resembling the swollen vesicle phenotype observed upon *PIKfyve* knockdown or inhibition in mammalian cells, *C. elegans*, *S. cerevisiae* and *D. melanogaster* [9, 14, 19, 38]. Identical results were also obtained with *PIKfyve*- cells generated in an Ax2 background and this effect was phenocopied by incubation with the PIKfyve-specific inhibitor apilimod [29], confirming that this phenotype was due to deficient $PI(3,5)P_2$ synthesis (Figure 1C).

When amoebae were hypotonically stressed in phosphate buffer, we observed a sustained increase in vacuolation for at least 5 hours. However, after 24 hours, when the cells became polarized (indicating the onset of starvation-induced development), *PIKfyve*- mutants became indistinguishable from the random integrant and parental controls (Figure 1B). This is most likely due to the well-documented suppression of fluid-phase uptake that occurs when *Dictyostelium* cells enter starvation-induced development [39, 40]. Consistent with this, *PIKfyve* null cells had no observable delay or other defects in development, and formed morphologically normal fruiting bodies with viable spores (Supplementary figure 2). Disruption of $PI(3,5)P_2$ synthesis therefore leads to endocytic defects, but it is not required for *Dictyostelium* development.

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observable delay or other defects in development, and formed morphologically normal fruiting bodies with viable spores (Supplementary Figure 2). We conclude that loss of PIKfyve activity therefore leads to endocytic defects, but it is not required for *Dictyostelium* development.

PIKfyve is important for phagocytic growth but not uptake

Laboratory strains of *Dictyostelium* can obtain nutrients either by macropinocytosis of liquid (axenic) medium or by phagocytosis of bacteria. Whilst *PIKfyve*- null cells had normal rates of endocytosis and exocytosis, axenic growth was slower than for wild-type cells, with mutants doubling every 16 hours compared to 10 hours for the controls (Figure 2A-C)..

In contrast, growth on bacteria was more strongly affected, and *PIKfyve* null cells formed much smaller colonies on lawns of avirulent *Klebsiella pneumoniae* (Figure 2D). To confirm this defect and test its generality, we screened *PIKfyve*-null cells for growth on a palette of diverse Gram-positive and Gram-negative bacteria, including both pathogenic and non-pathogenic strains. PIKfyve was required for efficient growth in all cases, indicating a general role for $PI(3,5)P_2$ synthesis for phagocytic growth (Figure 2E and F).

Defective growth on bacteria could be due to disruption of either capture, killing or digestion. To measure if phagocytic uptake was affected by PIKfyve disruption, we monitored the ability of *Dictyostelium* cells to reduce the turbidity of an *E. coli* suspension over time (Figure 3A). No difference was observed in the ability of *PIKfyve* null cells to phagocytose bacteria compared to the parental strain. To confirm this, we also measured phagocytosis of fluorescent beads (Figure 3B) and GFP-expressing *Mycobacterium smegmatis* (Figure 3C) by flow cytometry and found no defect in uptake by *PIKfyve* null cells. As bacteria are efficiently captured, PIKfyve must therefore be important for phagosome processing.

PIKfyve does not regulate PI(3)P dynamics in *Dictyostelium*

PI(3)P is one of the best characterized early markers of maturing endosomes and phagosomes. In both mammalian macrophages and in *Dictyostelium.* Immediately following particle internalization, PI(3)P is generated on the phagosome by the class III PI 3-kinase VPS34 [26, 32, 41] and interacts with a number of important regulators of maturation such as Rab5 [42]. PIKfyve is both recruited by PI(3)P and phosphorylates it, forming $PI(3,5)P_2$. Absence of *PIKfyve* could perturb phagosome maturation by reducing PI(3)P consumption, by eliminating the actions of $PI(3,5)P_2$, or both. Indeed, studies in macrophages indicate that inhibition of PIKfyve can cause prolonged PI(3)P signalling [32].

We therefore investigated the contribution of PIKfyve to PI(3)P dynamics in *Dictyostelium*. PI(3)P can be visualized in cells using the well-characterised reporter GFP-2xFYVE [34, 43]. Expression of GFP-2xFYVE in control cells demonstrated PI(3)P is present on *Dictyostelium* phagosomes following engulfment, consistent with previous reports [41] (Figure 4A). However, we found defects in neither initial recruitment, nor dissociation of this reporter in *PIKfyve* mutants (Figure 4A and B). Therefore, PIKfyve activity does not significantly contribute to PI(3)P turnover in these cells. The defects in growth, and the swollen endosomes are thus due to lack of $PI(3,5)P_2$ synthesis rather than prolonged accumulation of PI(3)P.

PIKfyve deficient phagosomes have defective acidification and digestion

Next we investigated how absence of PIKfyve affects phagosomal maturation. One of the first stages of maturation is the acquisition of the proton-pumping V-ATPase, leading to rapid acidification [44]. The influence of PIKfyve on endosomal pH regulation, however, remains controversial. Whilst studies in *C. elegans,* plants and mammalian epithelial cells have shown that PIKfyve is required for efficient acidification [38, 45-48], others have shown that RAW 264.7 macrophages are still able to acidify their phagosomes to at least pH 5.5 when PIKfyve is inhibited [32].

We therefore assessed the ability of *Dictystelium PIKfyve*-null cells to acidify their phagosomes by measuring the relative fluorescence of beads labelled with both the pH-sensitive FITC and the pH-insensitive Alexa 594 succinimidyl ester [49]. The phagosomes of *PIKfyve*-null cells acidified much more slowly than wild-type cells and never achieved as low a pH as those of wild-type cells (Figure 4C).

Proper degradation of internalised material requires both acidification and the presence of proteases. We measured phagosomal proteolysis by following the increase in fluorescence due to the cleavage and unquenching of DQ-BSA coupled to beads [49] (Figure 4D). Strikingly, phagosomes of *PIKfyve*-null cells exhibited an almost complete loss of proteolytic activity, likely due to a combination of delayed delivery of hydrolytic enzymes and their lower activity in inadequately acidified phagosomes.

Phagosomal acidification is driven by the rapid recruitment and activity of the V-ATPase. The V-ATPase consists of V_0 (transmembrane) and V_1 (peripheral) sub-complexes. It has previously been shown that $PI(3,5)P_2$ can regulate V_0-V_1 assembly at the yeast vacuole allowing dynamic regulation of activity [50]. To differentiate between defective V-ATPase delivery and assembly we expressed GFP-fusions of both V_0 and V_1 subunits (GFP-VatM and VatB-GFP respectively) and observed their recruitment to nascent phagosomes by fluorescence microscopy [51]. Both proteins were expressed

equally in wild-type and mutant cells (Supplementary figure 3) and by observing phagocytosis of pH-sensitive pHrodo-labelled yeast we were also able to simultaneously monitor acidification.

Both GFP-VatM and VatB-GFP began accumulating on phagosomes immediately following internalisation both in *PIKfyve*$^{-}$ and control cells, but the rate of accumulation was substantially reduced in the mutants and only reached about half of the final levels observed in wild-type cells (Figure 5A-D). Defective acidification was again demonstrated by a reduced increase in pHrodo fluorescence (Figure 5E). It should be noted that expression of VatB-GFP (but not GFP-VatM) caused a partial, but dominant, inhibition of acidification in this assay (Supplementary Figure 3A). The observation that both V-ATPase components were equally affected indicates that PIKfyve is required for delivery of the entire V-ATPase to the phagosome, rather than specifically affecting V_0-V_1 association.

PIKfyve is essential for efficient killing of bacteria

Acidification and proteolysis are important mechanisms used by phagocytes to kill engulfed microbes. We therefore asked whether PIKfyve was physiologically important for killing. Bacterial death leads to membrane permeabilisation and intracellular acidification, so survival time within phagosomes can be inferred by observing the phagocytosis and subsequent quenching of GFP expressed by a non-pathogenic *Klebsiella pneumoniae* strain [52]. In this assay, the phagocytised bacteria survived for more than three times longer in *PIKfyve*$^{-}$ cells (median survival 12 min) than in wild-type cells (3.5 min) (Figure 6A and B). These benign bacteria did however eventually die in *PIKfyve* null cells, indicating either that the residual acidification is eventually sufficient, or other elements of the complex bacterial killing machinery remain functional in the *PIKfyve*$^{-}$ phagosomes.

PIKfyve activity restricts the persistence of *Legionella* infection

Many pathogenic bacteria infect host immune cells by manipulating phagosome maturation to establish a replication-permissive niche or to escape into the cytosol. To avoid such infection, host cells must kill such pathogens rapidly. PIKfyve may therefore be critical to protect host cells from infections.

Legionella pneumophila is a Gram-negative opportunistic human pathogen that normally lives in the environment by establishing replicative niches inside amoebae such as *Acanthamoeba.* This process can be replicated in the laboratory using *Dictyostelium* as an experimental host [53]. Following its phagocytosis, *Legionella* can disrupt normal phagosomal maturation and form a unique *Legionella*-containing vacuole (LCV). This requires the Icm/Dot (Intracellular multiplication/defective for

organelle trafficking) type IV secretion system that delivers a large number of bacterial effector proteins into the host (reviewed in [54]). These effectors modify many host signalling and trafficking pathways, one of which is to prevent the nascent *Legionella* phagosome from fusing with lysosomes [55].

Phosphoinositide signalling is heavily implicated in *Legionella* pathogenesis. *Legionella*-containing phagosomes rapidly accumulate PI(3)P. Its concentration then declines within 2 hours and PI(4)P accumulates [56], and some of the effectors introduced through the Icm/Dot system bind PI(3)P or PI(4)P [57-63]. The role of $PI(3,5)P_2$ in *Legionella* infection however is yet to be investigated. We therefore tested whether PIKfyve was beneficial, or detrimental for the host to control *Legionella* infection.

When we monitored the initial phase of infection, we found that both parental and *PIKfyve*$^-$ *Dictyostelium* phagocytised many more of the virulent wild-type *L. pneumophila* strain (JR32) than an avirulent strain that is defective in type IV secretion (*ΔicmT*) [64] (Figure 7A). These results are in agreement with the previous finding that expression of the Icm/Dot T4SS promotes uptake of *L. pneumophila* [65]. To compensate for this difference in bacterial uptake, subsequent experiments employed a multiplicity of infection (MOI) of 100 for the *ΔicmT* strain, and an MOI of 1 for the wild-type JR32.

We next investigated whether *L. pneumophila* continue to multiply in, or are killed by, their *Dictyostelium* hosts over several days. When viable bacteria released from the infected cells were measured, both parental and *PIKfyve*$^-$ *Dictyostelium* almost eliminated the burden of avirulent *ΔicmT* *Legionella* during the 6 days following infection (Fig 7B, note that the CFUs scales in Figs. 7B and 7C are logarithmic). This was confirmed by observing a similar decline in the number of viable intracellular bacteria that were released when the amoebae were lysed (Figure 7C).

However, the results with wild-type *Legionella* were very different: many more survived in *PIKfyve*$^-$ than in wild-type amoebae (Figure 7C) and were released into the medium in larger numbers (Figure 7B). Further confirmation of the enhanced survival of *Legionella* in *PIKfyve*$^-$ amoebae was obtained by flow cytometric analysis of the bacterial load within *Dictyostelium* cells infected with GFP-expressing *Legionella* (either wild-type or *ΔicmT*). The only *Dictyostelium* cells that accumulated substantial GFP fluorescent over several days were those infected by wild-type *Legionella*, and this happened sooner and to a greater degree in the *PIKfyve*-null cells (Figure 7D, central panels).

252 Manipulation of PIKfyve-mediated signalling is therefore not required for *L. pneumophila* to pervert
253 phagosome maturation and replicate intracellularly. Rather, the role of PIKfyve in ensuring rapid
254 phagosomal acidification and digestion is crucial to prevent *Legionella*, and presumably other
255 pathogens, from surviving and establishing a permissive niche.

256

Discussion

In this study, we have characterised the role of PIKfyve during phagosome maturation using the model phagocyte *D. discoideum.* The roles of PI 3-kinases and PI(3)P signalling during phagosome formation and early maturation have been studied extensively, but the subsequent actions of PIKfvye and roles of $PI(3,5)P_2$ and PI(5)P are less well understood [2, 25]. In non-phagocytic cells such as fibroblasts and yeast, $PI(3,5)P_2$ production is important for endosomal fission and fragmentation of endolysosomal compartments [9, 17, 38, 46]. PIKfyve has also been shown to regulate macropinosome maturation, and be functionally important for intracellular replication of both the Vaccinia virus and *Salmonella* [47, 66, 67]. In this paper we show that PIKfyve is critical to ensure efficient phagosomal acidification and proteolysis, and demonstrate its physiological importance in the killing of bacteria and in suppression of intracellular pathogen survival.

Complex effects of PIKfyve inhibition on PIP-mediated signalling have hampered clear interpretation of PIKfyve function in some mammalian studies. For example, some studies report that disruption of PIKfyve prolonged PI(3)P-mediated signalling in addition to lack of $PI(3,5)P_2$ production [29, 31], making it difficult to determine which phosphoinositide change is responsible for the observed phenotypes. However, in agreement with other reports [17, 46], we found that deletion of PIKfyve had no impact on phagosomal PI(3)P dynamics in *Dictyostelium*. The observed defects in phagosome maturation appear therefore to be due to lack of $PI(3,5)P_2$ formation and not prolonged PI(3)P signalling in this system.

The role of PIKfyve in lysosomal acidification and degradation is currently disputed. Several studies which have measured vesicular pH at a single time point have shown that PIKfyve is required for acidification [9, 38, 46, 48], but others found that disruption of PIKfyve had little effect on phagosomal pH [32, 67, 68]. In contrast, we followed the temporal dynamics of V-ATPase delivery and of phagosomal acidification and proteolysis, and showed that V-ATPase delivery to PIKfyve-deficient phagosomes was substantially decreased and delayed, with consequent defects on initial acidification and proteolysis. $PI(3,5)P_2$ has also been proposed to regulate V-ATPase V_0-V_1 subcomplex association dynamically at the yeast vacuole [50], but we found no evidence for this during *Dictyostelium* phagosome maturation.

It is still not clear how PIKfyve-generated $PI(3,5)P_2$ regulates V-ATPase trafficking, and few $PI(3,5)P_2$ effectors are known. One of these is the lysosomal cation channel TRPML1/mucolipin, which is specifically activated by $PI(3,5)P_2$ [69]. This interaction was recently shown to underlie the role of PIKfyve in macropinosome fragmentation, although not acidification [67], and TRPML1 is also required for phagosome-lysosome fusion [70]. Additionally, $PI(3,5)P_2$ and TRPML1 have been

proposed to mediate interactions between lysosomes and microtubules [71]. Therefore PIKfyve may drive V-ATPase delivery to phagosomes by both microtubule-directed trafficking and by regulating fission. However the sole mucolipin orthologue in *Dictyostelium* is only recruited to phagosomes much later, during their post-lysosomal phase, and its disruption influences exocytosis rather than acidification [72].

Rapid phagosomal acidification and proteolysis is key for phagocytes to kill internalised bacteria. Many clinically relevant opportunistic pathogens, such as *L. pneumophila* [54, 73], *Burkholderia cenocepacia* [74] and *Cryptococcus neoformans* [75] have developed the ability to subvert normal phagosome maturation in order to maintain a permissive niche inside host phagocytes. This is likely to have evolved from interactions with their environmental predators such as amoebae [76-78].

L. pneumophila are phagocytosed in the lung by alveolar macrophages, and after internalisation employ effectors secreted via its type IV secretion system, some of which interfere with PI(3)P-signalling, to inhibit phagosome maturation [58, 79, 80]. We have shown that the lipid products of PIKfyve are not required for *L. pneumophila* to establish an intracellular replication niche. Rather, *L. pneumophila* survive much better in PIKfyve-deficient cells, suggesting that $PI(3,5)P_2$ helps *Dictyostelium* to eliminate, rather than harbour, *Legionella*. This is in contrast to the non-phagocytic invasion of epithelia that occurs during *Salmonella* infection when PIKfyve activity is necessary to promote the generation of a specialised survival niche within which the bacteria replicate [47]. *Salmonella* has therefore evolved a specialised role for the products of PIKfyve in generating a survival niche, whereas *Legionella* and other bacteria are suppressed by PIKfyve-driven rapid phagosomal maturation.

The molecular arms race between host and pathogens is complex and hugely important. The very early events of phagosome maturation are critical in this competition; host cells aim to kill their prey swiftly whilst pathogens try to survive long enough to escape. Although its molecular effectors remain unclear, PIKfyve and its products are crucial to tip the balance in favour of the host, providing a general mechanism to ensure efficient antimicrobial activity.

Materials and Methods

Cell strains and culture

Dictyostelium discoideum cells were grown in adherent culture in plastic Petri dishes in HL5 medium (Formedium) at 22°C. *PIKfyve* null mutants were generated in both Ax2 and Ax3 backgrounds, with appropriate wild-type controls used in each case. Cells were transformed by electroporation and transformants selected in 20 µg/ml hygromycin (Invitrogen) or 10 µg/ml G418 (Sigma). Apilimod was from United States Biological.

Growth in liquid culture was measured by seeding log phase cells in a 6 well plate and counting cells every 12 hours using a haemocytometer. Growth on bacteria was determined by plating ~10 *Dictyostelium* cells on SM agar plates (Formedium) spread with a lawn of *K. pneumophila*.

Plaque assays were performed as previously described [81]. Briefly, serial dilution of *Dictyostelium* cells (10-10^4) were placed on bacterial lawns and grown until visible colonies were obtained. The bacterial strains were kindly provided by Pierre Cosson and were: *K. pneumoniae* laboratory strain and 52145 isogenic mutant (Benghezal et al., 2006), the isogenic *P. aeruginosa* strains PT5 and PT531 (*rhlR-lasR* avirulent mutant) (Cosson et al., 2002), *E.coli* DH5α (Fisher Scientific), *E. coli* B/r (Gerisch, 1959), non-sporulating *B. subtilis* 36.1 (Ratner and Newell, 1978), and *M. luteus* (Wilczynska and Fisher, 1994). An avirulent strain of *K. pneumophila* was obtained from ATCC (Strain no. 51697).

The *Dictyostelium* development was performed by spreading 10^7 amoebae on nitrocellulose filters (47 mm Millipore) on top of absorbent discs pre-soaked in KK2 (0.1 M potassium phosphate pH 6.1) and images were taken at 20 hours [82].

Gene disruption and molecular biology

PIKfyve null cells in an Ax2 background were generated by gene disruption using homologous recombination. A blasticidin knockout cassette was made by amplifying a 5' flanking sequence of the *PIKfyve* gene (DDB_G0279149) (primers: fw- GGTAGATGTTTAGGTGGTGAAGT, rv-gatagctctgcctactgaagCGAGTGGTGGAATTCATAAAGG) and 3' flanking sequence (primers: fw-ctactggagtatccaagctgCCATTCAAGATAGACCAACCAATAG, rv- AGAATCAGAATAAACATCACCACC). These primers contained cross over sequences (in lower case) allowing a LoxP-flanked blasticidin resistance cassette (from pDM1079, a kind gift from Douwe Veltman) to be inserted between the two arms.

For *PIKfyve* gene disruption in an Ax3 background a knockout cassette was constructed in pBluescript by sequentially cloning fragment I (amplified by TAGTAGGAGCTCGGATCCGGTAGATGTTTAGGTGGTGAAGTTTTACCAAC and

TAGTAGTCTAGACGAGTGGTGGAATTCATAAAGGTACGTTCAT) and fragment II (amplified by TAGTAGAAGCTTCCATTCAAGATAGACCAACCAATAGTAGTCCTGC and TAGTAGGGTACCGGATCCCAGTGTGTAAATGAGAATCAGAATAAACATCACC). The blasticidin resistance gene was inserted between fragment I and II as a XbaI – HindIII fragment derived from pBSRδBam [83]. Both constructs were linearised, electroporated into cells and colonies were screened by PCR. GFP-2xFYVE was expressed using pJSK489 [34], GFP-PH_{CRAC} with pDM631 [84] and GFP-VatM and VatB-GFP with pMJC25 and pMJC31, respectively [85].

Microscopy and image analysis

Fluorescence microscopy was performed on a Perkin-Elmer Ultraview VoX spinning disk confocal microscope running on an Olympus 1x81 body with an UplanSApo 60x oil immersion objective (NA 1.4). Images were captured on a Hamamatsu C9100-50 EM-CCD camera using Volocity software by illuminating cells with 488 nm and 594 nm laser lines. Quantification was performed using Image J (https://imagej.nih.gov).

To image PI(3)P dynamics, cells were incubated in HL5 medium at 4 °C for 5 mins before addition of 10 µl of washed 3 µm latex beads (Sigma) and centrifugation at 280 x *g* for 10 seconds in glass-bottomed dishes (Mat-Tek). Dishes were removed from ice and incubated at room temperature for 5 mins before imaging. Images were taken every 30 s across 3 fields of view for up to 30 mins.

V-ATPase recruitment and acidification was performed using *Saccharomyces cerevisiae* labelled with pHrodo red (Life Technologies) as per the manufacturers instructions. *Dictyostelium* cells in HL5 medium were incubated with $1x10^7$ yeast per 3 cm dish, and allowed to settle for 10 mins before the medium was removed and cells were gently compressed under a 1% agarose/HL5 disk. Images were taken every 10 s across 3 fields of view for up to 20 mins. Yeast particles were identified using the "analyse particles" plugin and mean fluorescence measured over time. V-ATPase recruitment was measured as the mean fluorescence within a 0.5 µm wide ring selection around the yeast. The signal was then normalised to the initial fluorescence after yeast internalisation for each cell.

Endocytosis and exocytosis

To measure endocytosis, *Dictyostelium* at 5×10^6 cells/ml were shaken at 180 rpm. for 15 mins in HL5 before addition of 100 mg/ml FITC dextran (molecular mass, 70 kDa; Sigma). At each time point 500 µl of cell suspension were added to 1 ml ice-cold KK2 on ice. Cells were pelleted at 800 x *g* for 2 mins and washed once in KK2. The pellet was lysed in 50 mM Na_2HPO_4 pH 9.3 0.2% Triton X-100 and measured in a fluorimeter. To measure exocytosis, cells were prepared as above and incubated in 2 mg/ml FITC-dextran overnight. Cells were pelleted, washed twice in HL5 and resuspended in HL5 at 5

× 10^6 cells/ml. 500 µl of cell suspension were taken for each time point and treated as described above.

Phagocytosis and phagosomal activity assays

Phagocytosis of *E. coli* was monitored by the decrease in turbidity of the bacterial suspension of over time as described [86]. An equal volume of 2 × 10^7 *Dictyostelium* cells was added to a bacterial suspension with an $OD_{600\,nm}$ of 0.8, shaking at 180 rpm, and the decrease in $OD_{600\,nm}$ was recorded over time. Phagocytosis of GFP-expressing *M. smegmatis* and 1 µm YG-carboxylated polystyrene beads (Polysciences Inc.) was previously described [49, 87]. 2 × 10^6 *Dictyostelium*/ml were shaken for 2 hours at 150 rpm. Either 1 µm beads (at a ratio of 200:1) or *M. smegmatis* (MOI 100) were added, 500 µl aliquots of cells were taken at each time point and fluorescence was measured by flow cytometry [49].

Phagosomal pH and proteolytic activity were measured by feeding cells either FITC/TRITC or DQgreen- BSA/Alexa 594-labelled 3 µm silica beads (Kisker Biotech) [49]. Briefly, cells were seeded in a 96 well plate before addition of beads, and fluorescence measured on a plate reader over time. pH values were determined by the ratio of FITC to TRITC fluorescence using a calibration curve, and relative proteolysis was normalised to Alexa594 fluorescence.

Bacteria killing assay

Killing of GFP-expressing *K. aerogenes* was measured as previously described [52]. Briefly, 10 µl of an overnight culture of bacteria in 280 µl HL5 was placed in a glass-bottomed dish and allowed to settle before careful addition of 1.5 ml of a *Dictyostelium* cell suspension at 1 × 10^6 cells/ml. Images were taken every 20 s for 40 min at 20x magnification. Survival time was determined by how long the GFP-fluorescence persisted after phagocytosis.

Western blotting

Ax2 or *PIKfyve* null cells expressing GFP-VatM or VatB-GFP were analysed by SDS-PAGE and Western blots using a rabbit anti-GFP primary antibody (gift from A. Peden) and a fluorescently conjugated anti-rabbit 800 secondary antibody, using standard techniques. The endogenous biotinylated mitochondrial protein MCCC1 was used as a loading control using Alexa680-conjugated Streptavadin (Life Technologies) [88].

Legionella infection assays

The following *L. pneumophila* Philadelphia-1 strains were used: virulent JR32 [89], the isogenic Δ*icmT* deletion mutant GS3011 lacking a functional Icm/Dot type 4 secretion system [64], and corresponding strains constitutively producing GFP [65]. *L. pneumophila* was grown for 3 d on

charcoal yeast extract (CYE) agar plates, buffered with *N*-(2-acetamido)-2-aminoethane-sulfonic acid (ACES) [90]. For infections, liquid cultures were inoculated in AYE medium at an OD_{600} of 0.1 and grown for 21 h at 37 °C (post-exponential growth phase). To maintain plasmids, chloramphenicol was added at 5 µg/ml.

Uptake by *D. discoideum*, intracellular replication or killing of GFP-producing *L. pneumophila* was analyzed by flow cytometry as described [62]. Exponentially growing amoebae were seeded onto a 24-well plate (5×10^5 cells/ml HL5 medium per well) and allowed to adhere for 1–2 h. *L. pneumophila* grown for 21 h in AYE medium was diluted in HL5 medium and used to infect the amoebae at an MOI of 100 or at the MOI indicated. The infection was synchronized by centrifugation (10 min, 880 *g*), infected cells were incubated at 25 °C and, 30 min post-infection, extracellular bacteria were removed by washing three to five times with SorC (2 mM Na_2HPO_4, 15 mM KH_2PO_4, 50 µM $CaCl_2$, pH 6.0). Infected amoebae were detached by vigorously pipetting, and 2×10^4 amoebae per sample were analyzed using a FACSCalibur flow cytometer (Becton Dickinson). The GFP fluorescence intensity falling into a *Dictyostelium* scatter gate was quantified using FlowJo software (Treestar, http://www.treestar.com).

Alternatively, intracellular replication of *L. pneumophila* in *D. discoideum* was quantified by determining colony forming units (CFU) in the supernatant as described [62, 91]. Exponentially growing amoebae were washed with SorC and resuspended in MB medium (7 g of yeast extract, 14 g of thiotone E peptone, 20 mM MES in 1 l of H_2O, pH 6.9). Amoebae (1×10^5 cells per well) were seeded onto a 96-well plate, allowed to adhere for 1–2 h, and infected at an MOI of 1 with *L. pneumophila* grown in AYE medium for 21 h and diluted in MB medium. The infection was synchronized by centrifugation, and the infected amoebae were incubated at 25 °C. At the time points indicated, the number of bacteria released into the supernatant was quantified by plating aliquots (10–20 µl) of appropriate dilutions on CYE plates. Intracellular bacteria were also quantified by counting CFU after lysis of the infected amoebae with saponin. At the time points indicated, MB medium was replaced by 100 µl of 0.8% saponin and incubated for 15 min. The infected cells were lysed by pipetting, and aliquots were plated.

Acknowledgments

The authors would like to thank Steve Dove for many helpful suggestions and comments, as well as Phill Hawkins for his endeavours to separate *Dictyostelium* PIP_2s.

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Figure Legends

Figure 1

Knockout or inhibition of PIKfyve leads to a swollen vesicle phenotype. (A) DIC images of Ax3, two independent *PIKfyve*-null clones and a random integrant in HL5 medium. Arrows indicate the enlarged vesicles. (B) Swollen vesicles in *PIKfyve*-null cells became more apparent after incubation for the time indicated in the low osmolarity starvation buffer (KK2), but were lost as cells entered differentiation. (C) Induction of swollen vesicles with 5 µM apilimod, a PIKfyve-specific inhibitor. Images taken in HL5 medium after 5 hours of treatment.

Figure 2

***PIKfyve* null cells have growth defects**. *PIKfyve*¯null cells had no defects in either (A) endocytosis or (B) exocytosis, as measured by uptake or loss of FITC dextran. Data shown are mean +/- SD. (C) Despite normal uptake, *PIKfyve*-null cells had a longer generation time than Ax3 cells when growing axenically in liquid culture, data shown are mean +/- SD. (D) *PIKfyve*-null cells grew markedly more slowly on lawns of *Klebsiella pneumophila* than either Ax3 or a random integrant. (E) Growth on bacteria is also defective for *PIKfyve*-null cells in the Ax2 genetic background and across multiple bacterial strains. Growth was assessed by plating serial dilutions of amoebae on lawns of different bacteria and dark patches indicate amoebae growth. Data for all bacteria are summarised in (F).

Figure 3

Phagocytic uptake is not dependent on PIKfyve. (A) There was no defect in the ability of *PIKfyve*-null cells to reduce the turbidity of a suspension of *E. coli* over time. (B) Uptake of 1 µm beads or (C) GFP-expressing *Mycobacterium smegmatis* was also not affected by absence of PIKfyve, as measured by flow cytometry. Data shown are means +/- SD.

Figure 4

PIKfyve is important for acidification and proteolysis. (A) PI(3)P dynamics are not altered by absence of PIKfyve. PI(3)P was monitored by visualising the recruitment of a GFP-2xFYVE probe to phagosomes following uptake of 3 µm beads (asterisks) by confocal time-lapse microscopy. (B) Time that GFP-2xFYVE stays associated with phagosomes following engulfment, indicating that PI(3)P removal is not PIKfyve-dependent. n indicates the total number of cells quantified in 3 independent experiments. (C) Phagosomal acidification and (D) proteolysis are severely disrupted in *PIKfyve* null cells, measured using 3 µm beads coupled to acidification or proteolysis reporters. Data shown are mean +/- SD.

Figure 5

PIKfyve is required for efficient V-ATPase recruitment. Recruitment of (A) the V-ATPase V_0 subunit GFP-VatM and (B) the V_1 peripheral subunit VatB-GFP to phagocytosed pHrodo-labelled yeast visualised by confocal time-lapse imaging. (C) Quantification of GFP-VatM recruitment over time, indicating reduced recruitment in *PIKfyve*-null mutants. (D) Quantification of VatB-GFP recruitment, following the same pattern. (E) Quantification of increase in yeast-associated pHrodo fluorescence, indicating acidification. The fluorescence increases significantly more in Ax2 cells than in *PIKfyve*-null cells. Data shown are mean +/- SEM, p values calculated by Student's t-test.

Figure 6

Bacterial survival is increased in *PIKfyve* null cells. (A) Kaplan-Meyer survival graph, with survival indicated by persistent fluorescence of phagocytosed GFP-expressing *Klebsiella pneumophila*. Bacteria survived for significantly longer in *PIKfyve*-null cells than in Ax2 ($p<0.0001$ as determined by Mantel-Cox test). 60 bacteria were followed across three independent experiments. (B) Stills from the widefield time-lapse movies quantified in (A). Arrows indicate phagocytosed bacteria

Figure 7

PIKfyve is required to suppress *Legionella* replication. (A) Uptake of GFP-producing wild-type (JR32) or avirulent (Δ*icmT*) *L. pneumophila* by *Dictyostelium* cells (MOI 100), analyzed by flow cytometry. Wild-type *L. pneumophila* show a higher infectivity than the avirulent strain both in Ax3 and in *PIKfyve*$^-$ *Dictyostelium*. (B) Intracellular growth and/or killing of *L. pneumophila* analyzed by colony forming units (CFU): *Dictyostelium* was infected with *L. pneumophila* wild-type (at an MOI of 1) or Δ*icmT* (MOI 100). In the course of multiple infection rounds (release and re-infection), more wild-type *L. pneumophila* accumulated in the medium surrounding *PIKfyve*$^-$ cells than wild-type. in contrast, Δ*icmT* mutant bacteria were killed by both *Dictyostelium* strains. (C) *Dictyostelium* were infected with *L. pneumophila* wild-type (MOI 1) or Δ*icmT* (MOI 100), lysed and intracellular CFU determined. Wild-type *L. pneumophila* grew significantly better in *PIKfyve*$^-$ cells than Ax3. (D) Analysis of intracellular growth of GFP-expressing *L. pneumophila* wild-type JR32 (MOI 1) or Δ*icmT* (MOI 100) by flow cytometry. Virulent *L. pneumophila* replicate more efficiently in *PIKfyve*$^-$ *Dictyostelium*, as indicated by the increasing proportions of amoebae containing high levels of GFP over time. Data represent means and standard deviation of duplicates (A; 10,000 counts each; n.s., not significant) or triplicates (B, C; **, $P < 0.01$) and are representative of 2-3 independent experiments.

Supplementary Figure 1

***PIKfyve* gene disruption.** (A) Schematic representation of *Dictyostelium* PIKfyve indicating the site where the blasticidin resistance cassette was inserted into the gene to generate knockouts.

Supplementary Figure 2

PIKfyve is not required for development. *PIKfyve*$^-$ cells formed (A) morphologically normal fruiting bodies as well as (B) normal spores.

Supplementary Figure 3

VatM expression has a dominant negative effect on acidification. (A) VatB-GFP expression in Ax2 and *PIKfyve*$^-$ cells suppressed acidification as measured by increase in pHrodo fluorescence, compared to the increase in fluorescence in cells expressing GFP-VatM. (B) Western blot of cells expressing VatB-GFP or GFP-VatM. There was no difference in expression levels between Ax2 and *PIKfyve*$^-$ cells for either reporter. However, VatB-GFP was expressed at a much higher level than GFP-VatM. Loading control is the mitochondrial protein MCCC1, recognised by Alexa680-conjugated streptavidin.

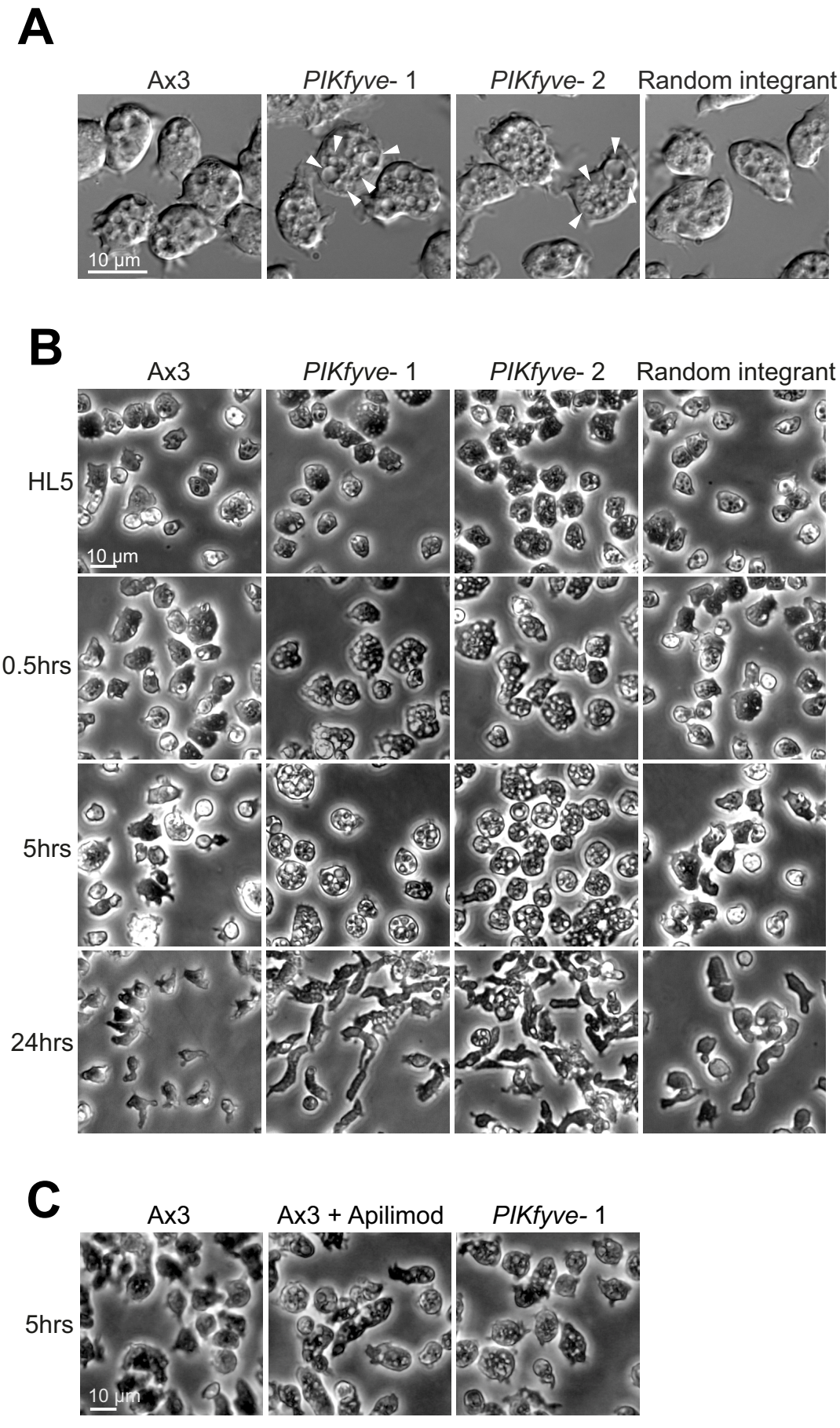

Figure 1: KO or inhibition of PIKfyve leads to a swollen vesicle phenotype

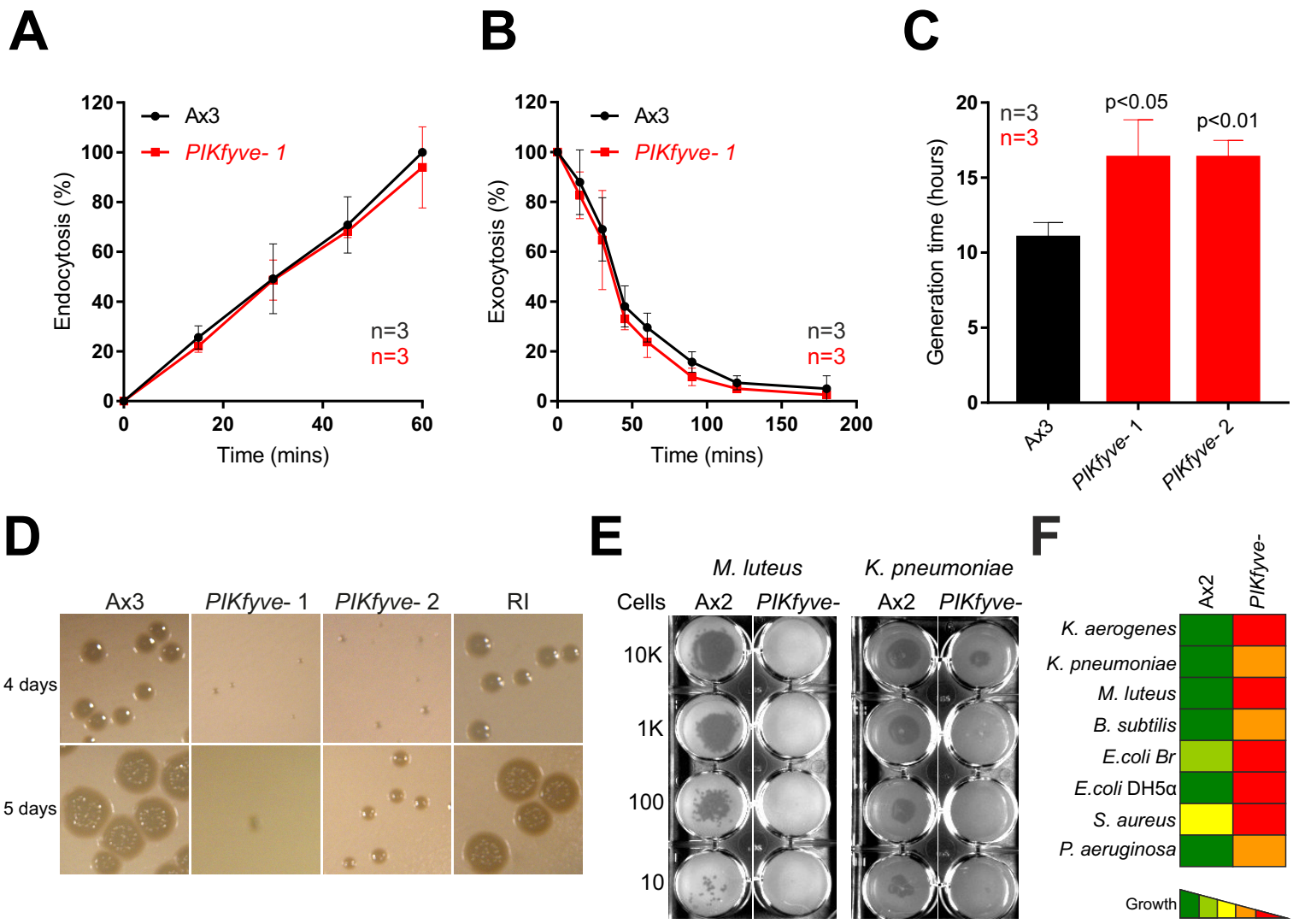

Figure 2: *PIKfyve*- cells have growth defects

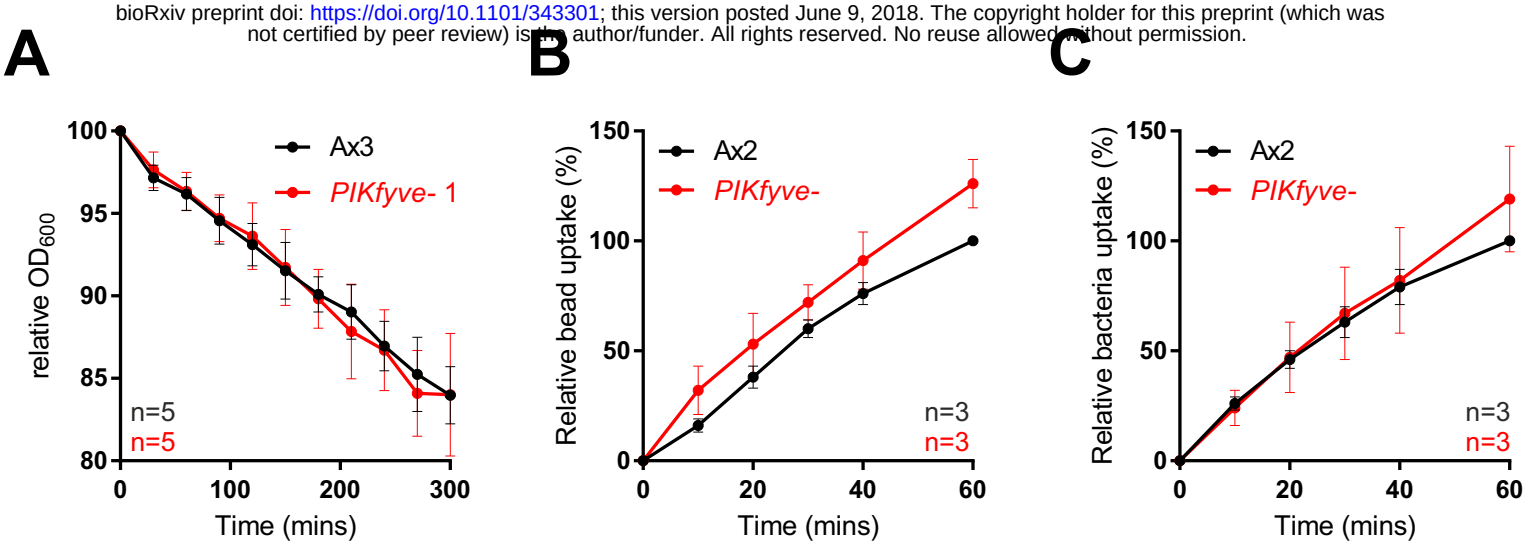

Figure 3: PIKfyve is not required for phagocytic uptake

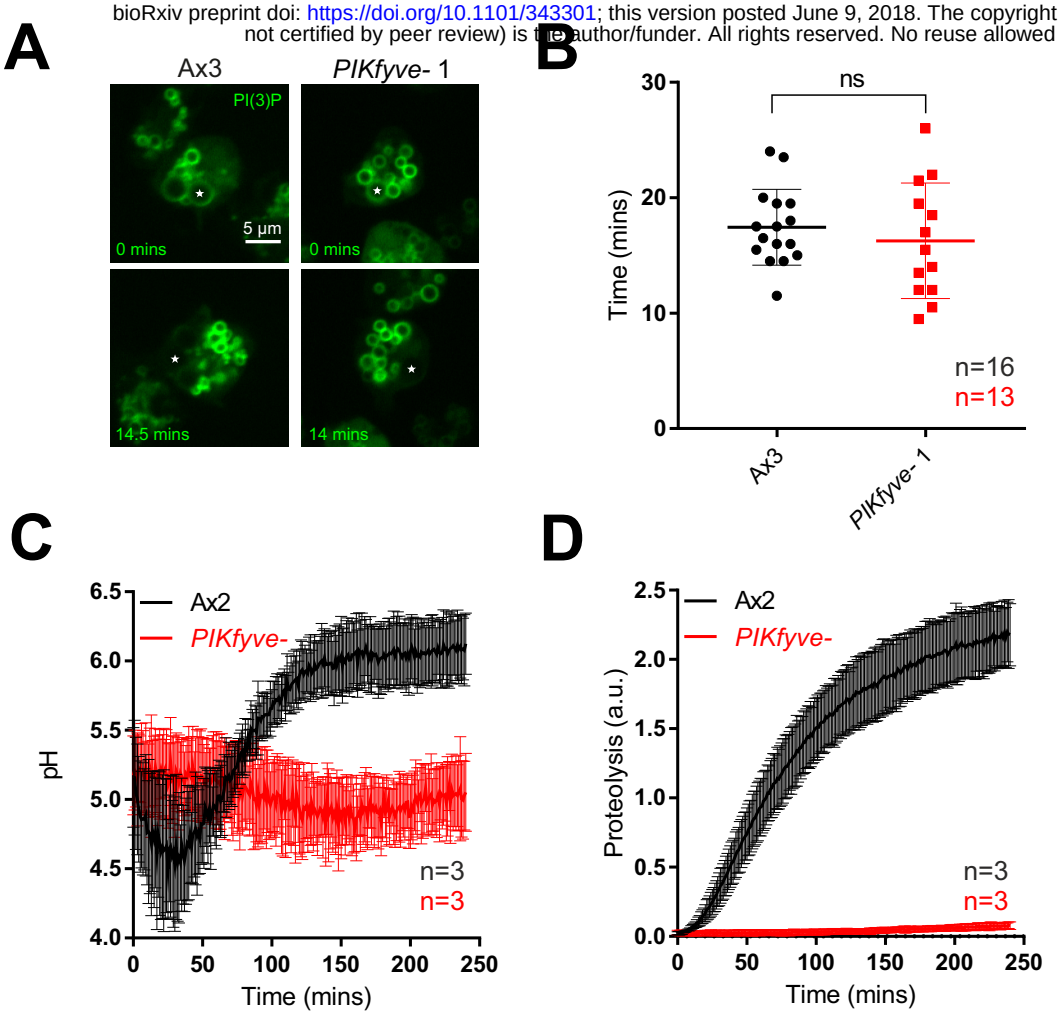

Figure 4: PIKfyve is important for phagosome acidification and proteolysis

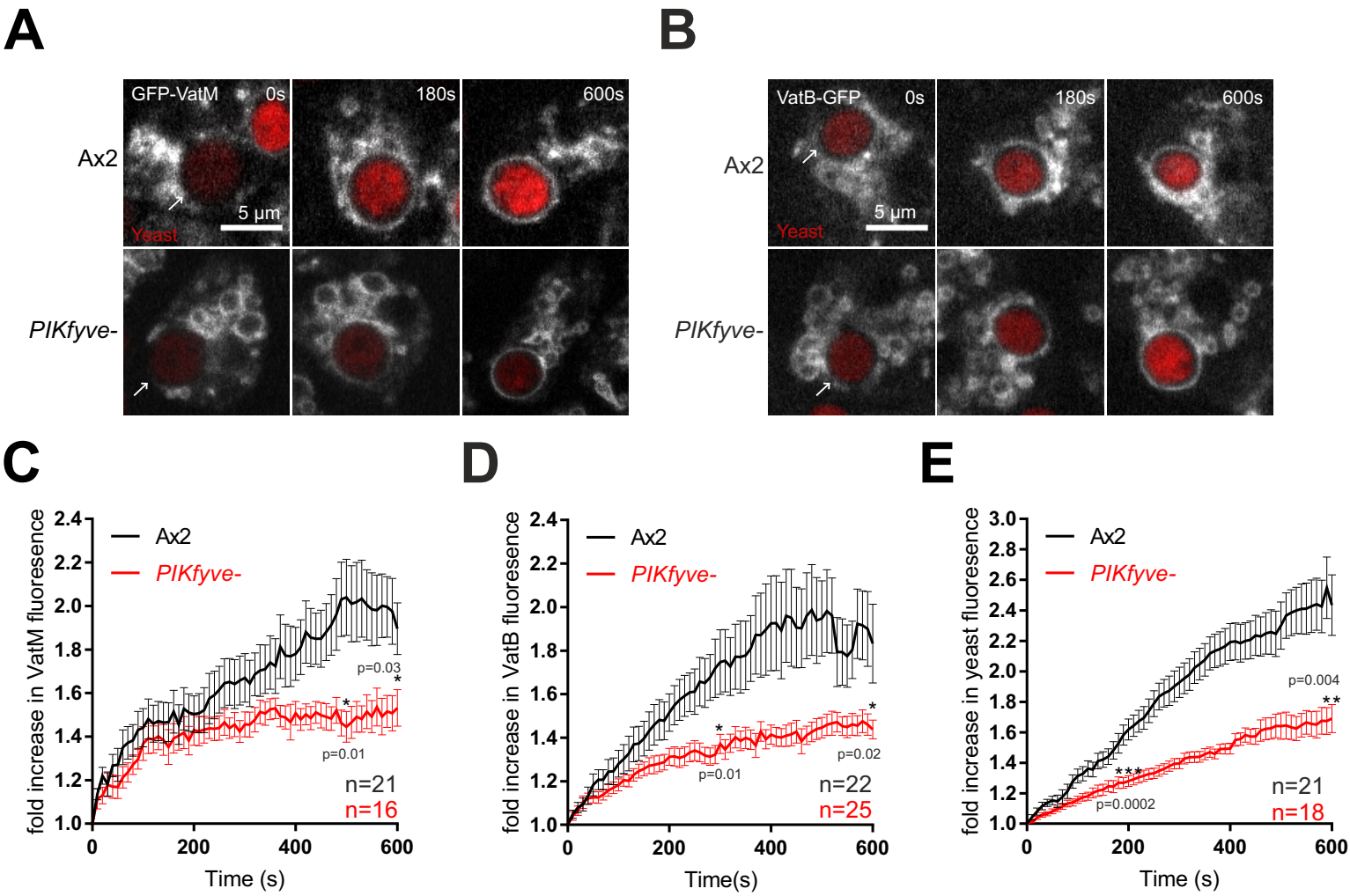

Figure 5: PIKfyve is required for efficient V-ATPase recruitment

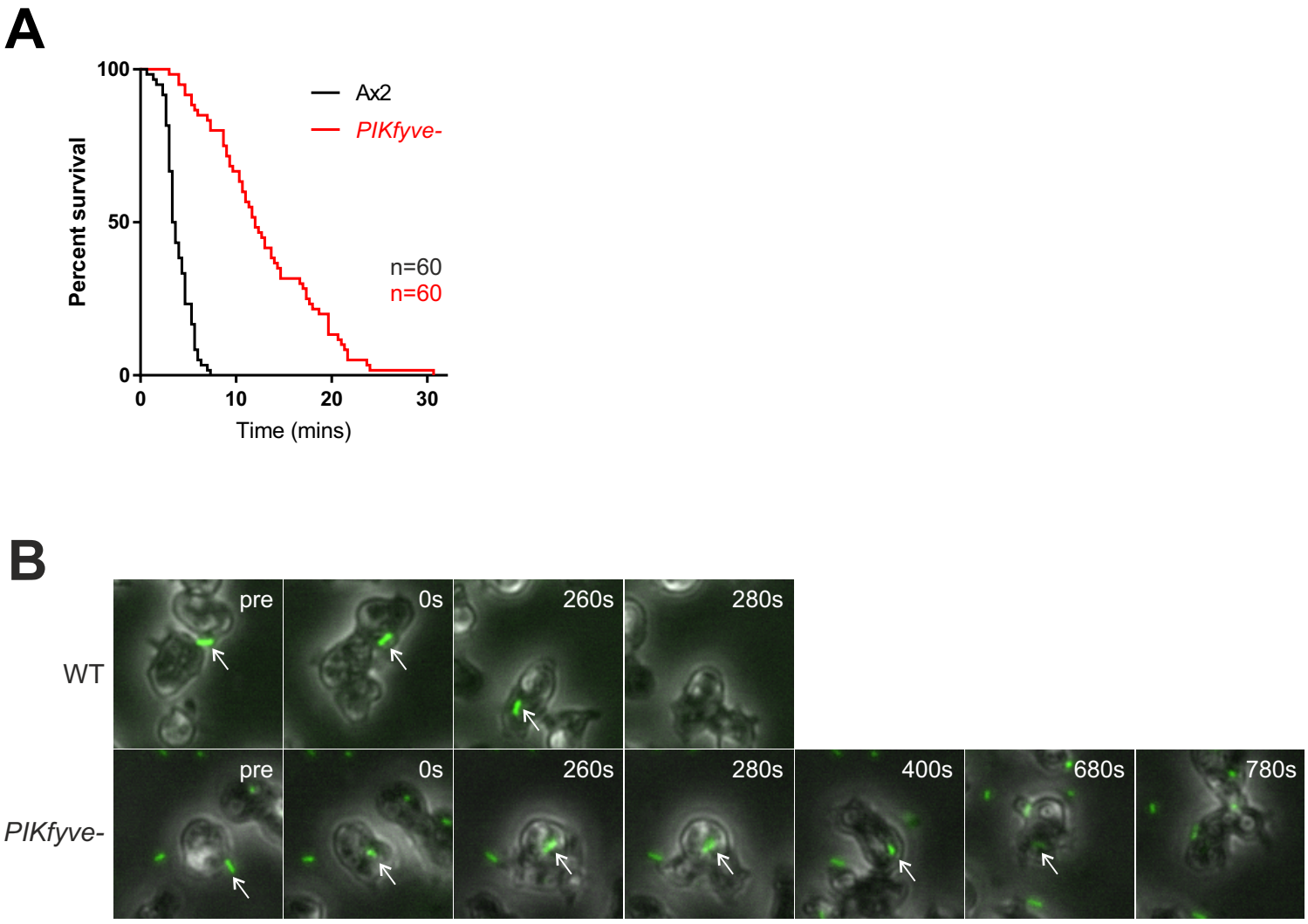

Figure 6: Bacterial survival is increased in *PIKfyve-* cells

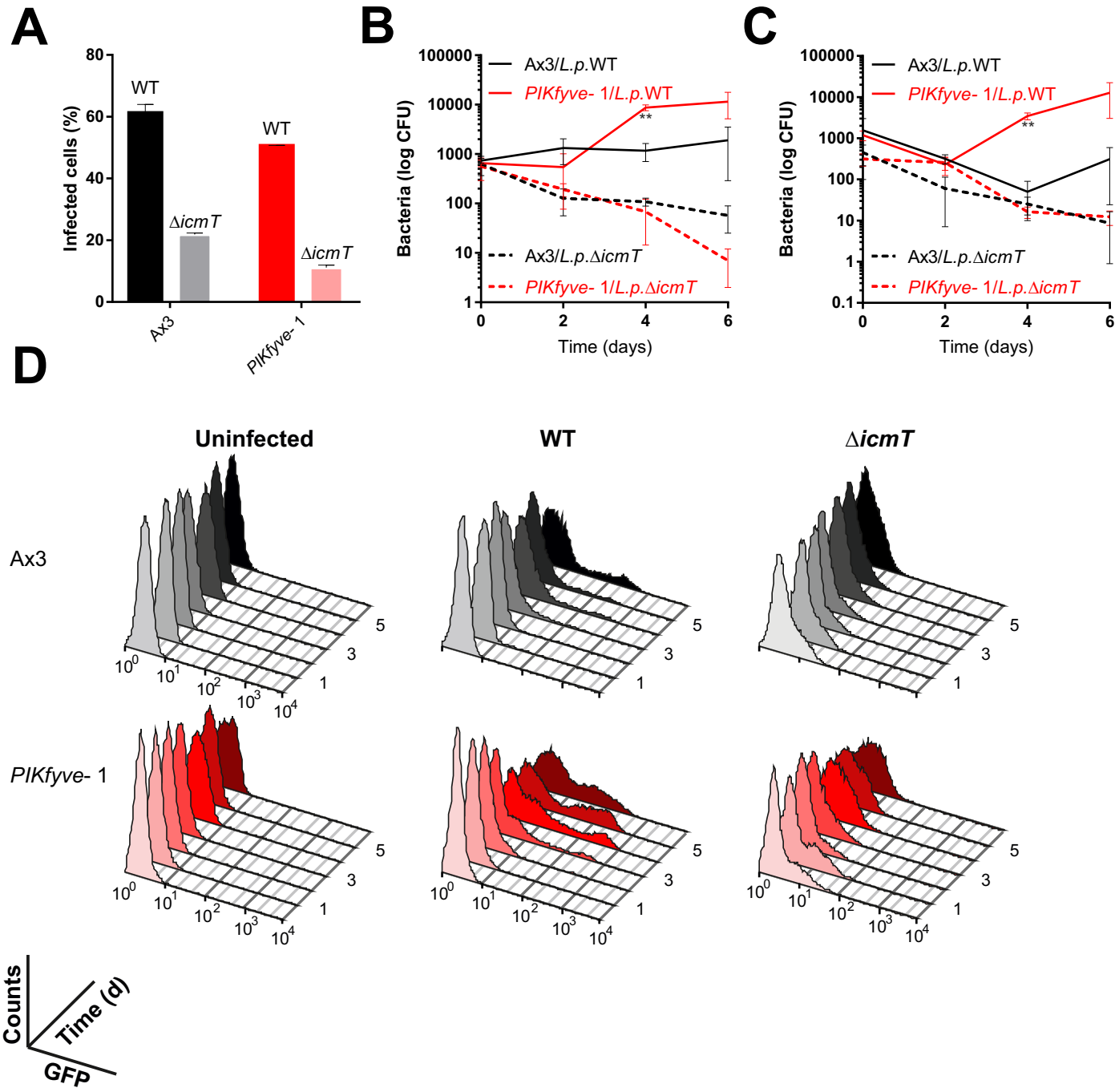

Figure 7: PIKfyve is required to suppress legionella replication

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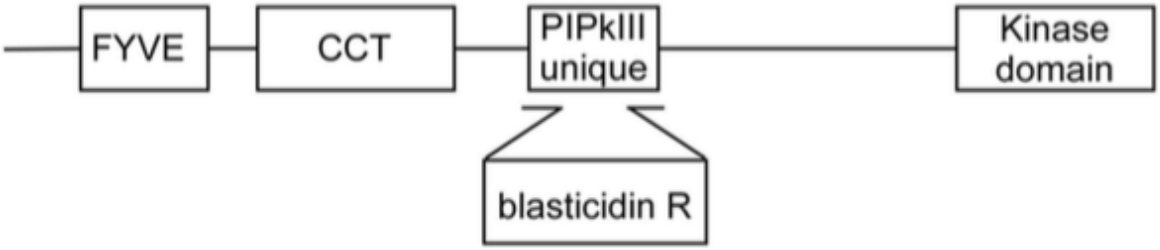
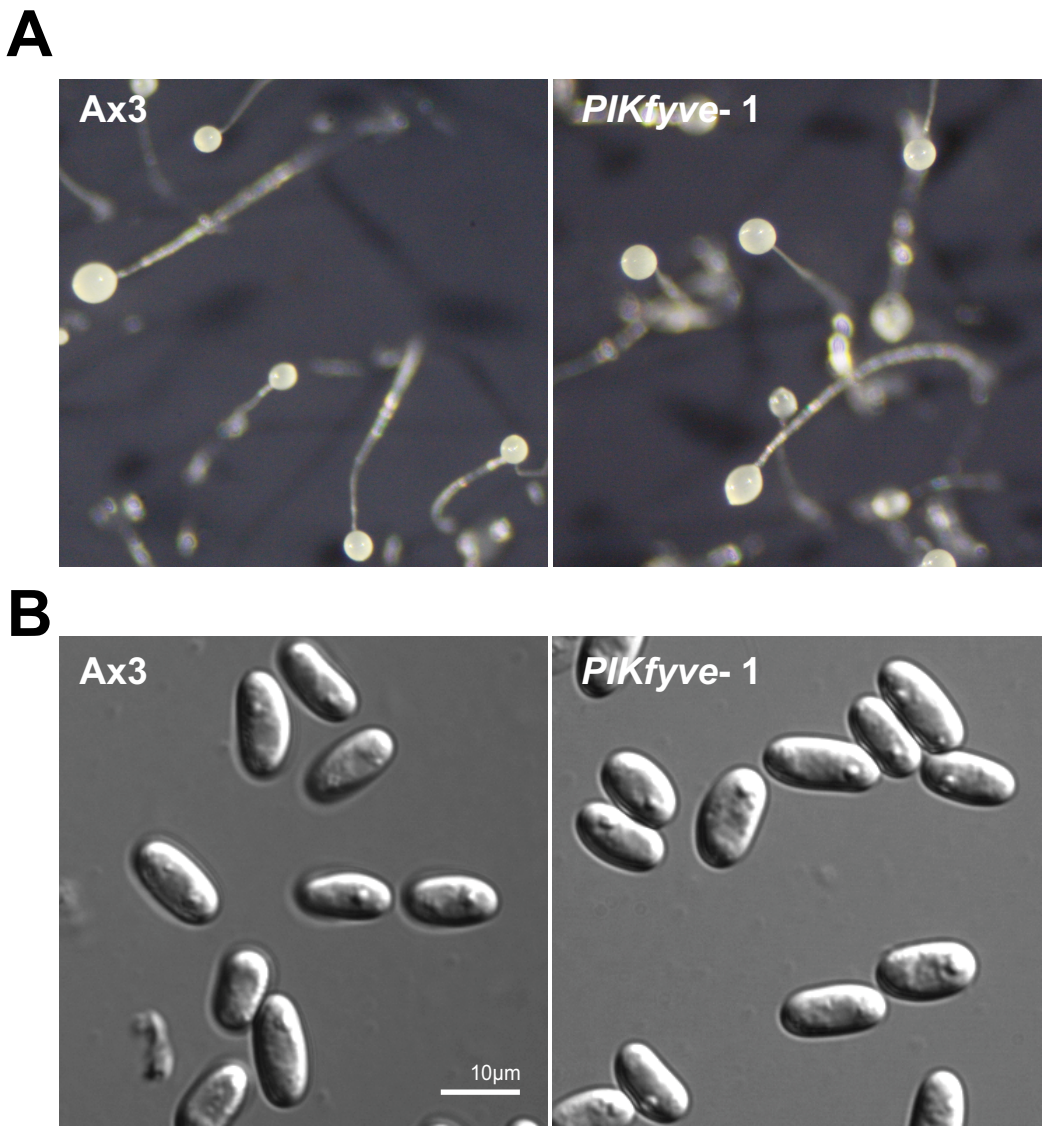
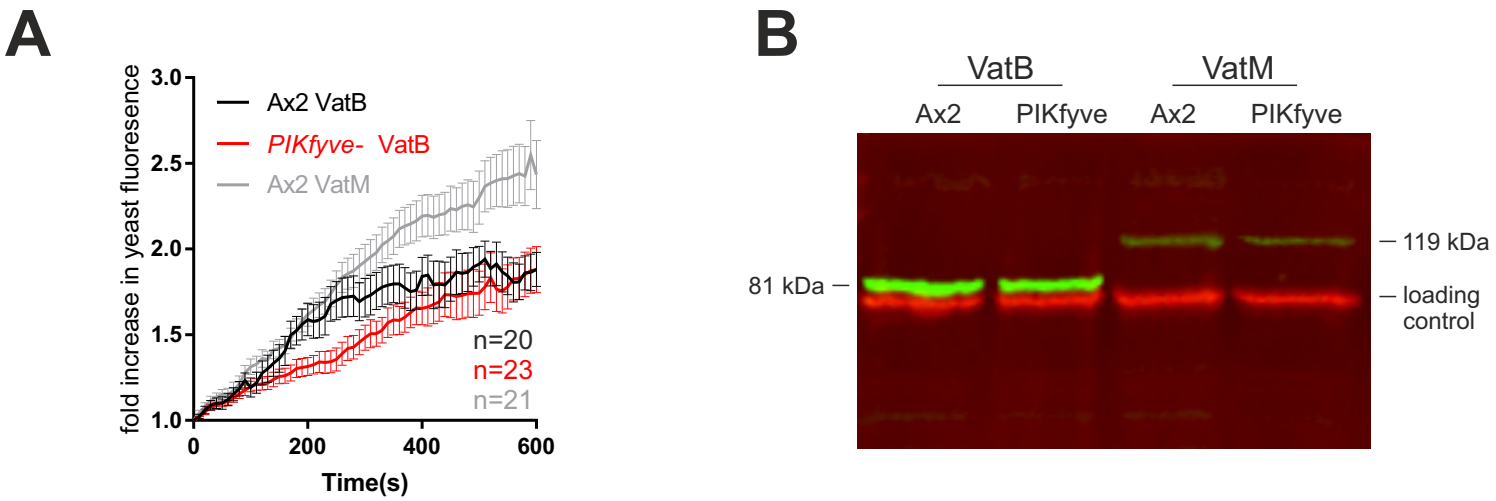


Supplementary figure 1: PIKfyve gene disruption



Supplementary figure 2: PIKfyve is not required for development



Supplementary figure 3: VatB expression has a dominant negative effect on acidification