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The Phosphoinositides: Key Regulators of *Salmonella* Containing Vacuole (SCV) Trafficking and Identity

M.C. Kerr, N.A. Castro, S. Karunaratne and R.D. Teasdale
*Institute for Molecular Bioscience, The University of Queensland
 Australia*

1. Introduction

Derived from the base of membrane ruffles in response to growth factor stimulation (Haigler, McKanna et al., 1979), the macropinosome is a large (diameters $>0.2\mu\text{m}$) phase-bright endocytic organelle that is readily labelled with fluid-phase markers. It is the primary means by which macrophages sample their immediate environment for antigens, is essential for proper renal function, is intrinsically linked to cellular migration and has a major role in the down-regulation of signalling from cell surface receptors (Kerr and Teasdale, 2009; Swanson and Watts, 1995). To promote invasion and survival, *Salmonella* subverts the host cell's normal macropinocytic machinery to gain entry into the non-phagocytic epithelial cells of the intestinal wall. Upon binding to the host cell surface the pathogen utilises a specialised apparatus called the type III secretion system (T3SS) to deliver a suite of bacterial virulence proteins directly into the host cell's cytoplasm. *Salmonellae* encode two distinct T3SSs within *Salmonella* Pathogenicity islands 1 and 2 (SPI1 and SPI2) that function at discrete stages of the infection. Whilst SPI1-T3SS is predominantly active on contact with the host cell's surface and serves to translocate virulence proteins across the plasma membrane, driving cytoskeletal rearrangements and signalling events that promote the uptake of the pathogen, SPI2-T3SS is active within intracellular compartments during the later stages of infection to generate a replicative niche (Haraga, Ohlson et al., 2008). As the environment of the encompassing macropinosome, also called the *Salmonella* Containing Vacuole (SCV) acidifies and matures, losing markers of the early endosomal system like transferrin receptor, EEA1 and Rab5, *Salmonellae* undergo extensive bacterial surface remodelling and expression and assembly of SPI2-T3SS is induced. The SPI2-T3SS enables the translocation of virulence factors across the SCV membrane into the host cell's cytoplasm. These virulence factors initiate a dramatic alteration in the host cell's vesicular trafficking pathways leading to the accumulation of late endosomal markers like Rab7 and LAMP1 and 2 on the SCV and the formation of long filamentous membrane structures. These *Salmonella*-induced filaments (SIFs) originate from the SCV, are LAMP1-positive, and function to increase the size of the SCV in a specific and directional fashion to accommodate bacterial replication during systemic infection as well as provide nutrients to the isolated pathogen (Garcia-del

Portillo and Finlay, 1995; Garcia-del Portillo, Zwick et al., 1993a, b; Haraga, Ohlson et al., 2008). What is clear from the literature is that this entire process reflects a carefully choreographed interaction between the bacterial virulence factors and the molecular machinery of the host cell.

2. Phosphoinositides and their effectors, tightly controlled regulators

Phosphoinositides (PI), the phosphorylated derivatives of the lipid phosphatidylinositol (PtdIns), can be singly or multiply phosphorylated on the 3', 4', and 5' position of the inositol headgroup to generate 7 distinct PI isoforms (Vicinanza, D'Angelo et al., 2008). Reversibly phosphorylated in a tightly regulated fashion by phosphatases and kinases that are heterogeneously localised within the cell, the PIs are consequently enriched on the cytosolic face of distinct intracellular membranes (Di Paolo and De Camilli, 2006). For example, the most abundant PIs, PI(4,5)P₂ and PI(4)P, are each constitutively present in the cytosolic leaflet of the plasma membrane and Golgi apparatus respectively whilst the 3-phosphorylated PIs, PI(3)P, PI(3,4)P₂ and PI(3,5)P₂ are found distributed throughout the endolysosomal system. The relative amounts of the PIs also vary dramatically between and within cells. Virtually undetectable in quiescent cells, PI(3,4,5)P₃ levels rapidly spike upon stimulation and during specialised membrane trafficking events through the coordinated and regulated activity of class Ia PI3-kinase phosphorylating the 3' position of PI(4,5)P₂ (Vanhaesebroeck, Leever et al., 2001).

Remarkably complex, PI metabolism represents a delicate equilibrium balancing the relative abundance and position of these lipids within the cell. Briefly, PtdIns is converted to PI(3)P or PI(4)P on endosomes or the Golgi through the actions of vacuolar protein sorting (Vps) 34-p150 and PI(4)KII/ respectively. Additionally, conversion of PI(3,4,5)P₃ to PI(3)P on nascent endocytic compartments may be the consequence of the sequential dephosphorylation of PI(3,4,5)P₃ as catalysed by 4- and 5-phosphatases. Src homology 2 domain-containing inositol 5-phosphatase (SHIP) 1 and 2 are potential 5-phosphatase candidates, dephosphorylating PI(3,4,5)P₃ to PI(3,4)P₂ at the cell surface or on newly formed endocytic structures. Type I and II 4-phosphatases may then catalyse the conversion between PI(3,4)P₂ and PI(3)P (Krauss and Haucke, 2007). Alternatively PI(3,4,5)P₃ may simply be lost from the endocytic membrane and VPS34-p150, an effector of early endosomal Rab5, could drive the *de novo* synthesis of PI(3)P from PtdIns (see Figure 1) (Zerial and McBride, 2001).

Relatively high concentrations of PI(4,5)P₂ are constitutively maintained at the plasma membrane primarily through the actions of a diverse family of PI(4)P 5-kinases and the 3-phosphatases. In addition to being intrinsic to numerous signalling, cytoskeletal and endocytic events PI(4,5)P₂ also serves as a precursor to PI(4)P contributing to the pool found predominantly within the Golgi Apparatus and in secretory granules (Levine and Munro, 2002; Panaretou, Domin et al., 1997; Wang, Wang et al., 2003). The accumulation of PI(4)P within the Golgi reflects the presence of multiple PI(4)Ks and PI(4,5)P₂ phosphatases in conjunction with relatively low levels PI(4)P 5-kinase activity.

The most recently identified of the PIs, PI(3,5)P₂, is synthesised from PI(3)P by the PI(5) kinase, PIKfyve (Shisheva, 2008). Whilst the precise role PI(3,5)P₂ plays in the mammalian

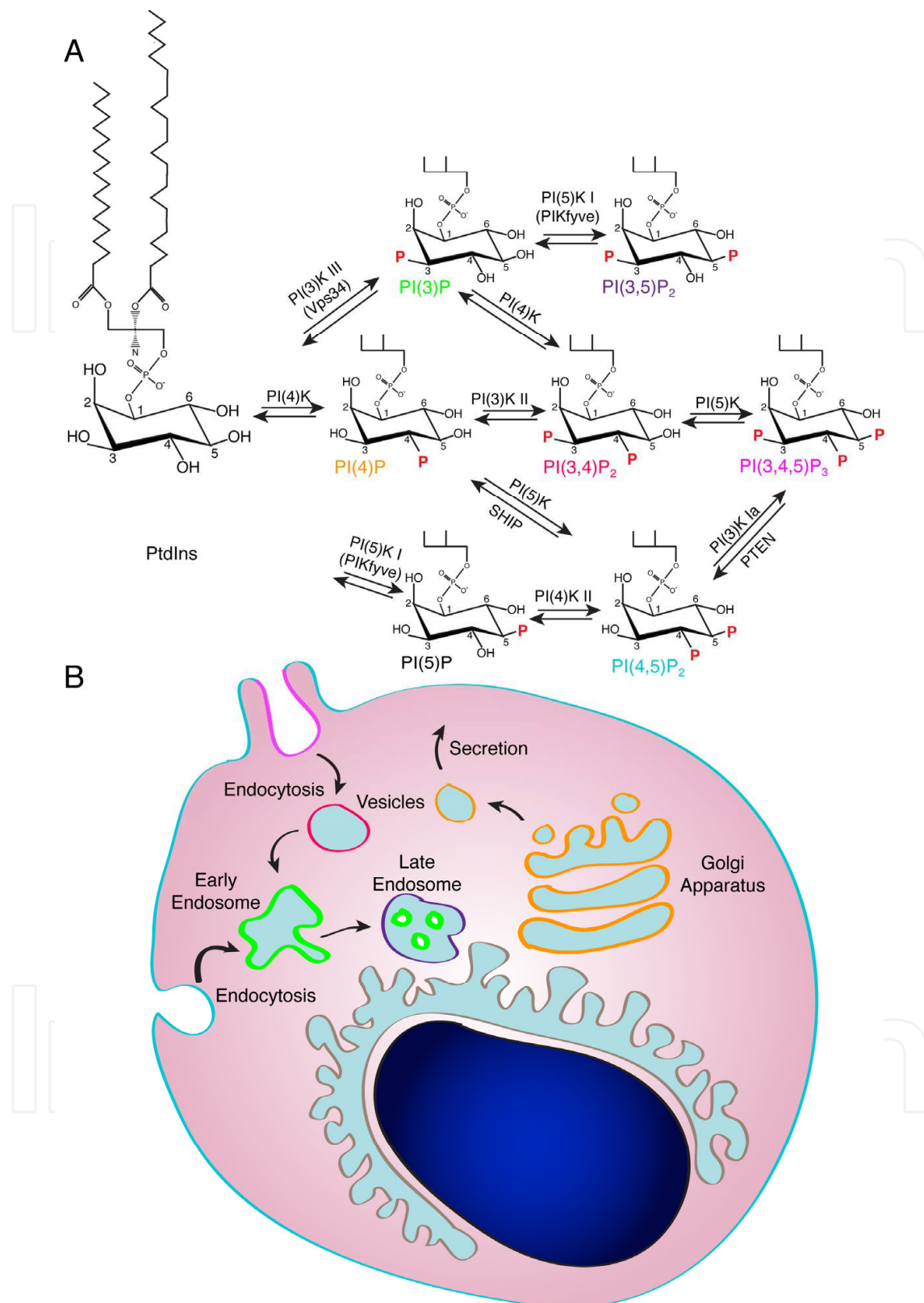


Fig. 1. PI metabolism and subcellular distribution. The phosphoinositides are phosphorylated derivatives of phosphatidylinositol. Their metabolism is regulated by kinases and phosphatases that are distributed heterogenously throughout the cell contributing to the accumulation of specific PIs in discrete intracellular membranes.

system is currently emergent, disruption of the yeast PIKfyve equivalent, Fab1p, results in a highly complex phenotype. The observation that a Δ FAB1 strain entirely devoid of PI(3,5)P₂ displays a dramatically enlarged vacuole that fails to acidify, partial defects in prevacuolar compartment (PVC; mammalian endosome equivalent) sorting, defective inheritance of vacuoles in daughter cells and a reduction in the number of intravacuolar vesicles seen by electron microscopy all suggest that PI(3,5)P₂ is integral to a number of processes essential for the maintenance of vacuolar/lysosomal system (Cooke, 2002; Cooke, Dove et al., 1998; Dove, McEwen et al., 1999; Dove, McEwen et al., 2002; Dove, Piper et al., 2004; Gary, Wurmser et al., 1998; Odorizzi, Babst et al., 1998; Yamamoto, DeWald et al., 1995). In agreement, we utilised time-lapse videomicroscopy to demonstrate that PIKfyve activity is essential for the fusion of macropinosomes with late endosomal/lysosomal membranes (Kerr, Wang et al., 2010).

The unique spatial and temporal distribution of the PIs provides the mechanism for the exquisite control with which this protein-lipid network regulates membrane trafficking and signalling events. Specifically, they serve as membrane anchors to recruit a suite of PI-binding molecules of diverse function through a variety of domains with differing PI-affinities and -specificities. The most thoroughly investigated of these, the *plekstrin homology* (PH) domain, is a ~120 residue motif found in 275 human proteins (DiNitto and Lambright, 2006; Lemmon and Ferguson, 2000). The relatively low sequence conservation between PH domains is reflected in the significant variation in specificity and affinity for individual PIs. For example, the PH domains of Grp1 and PLC δ 1 each bind PI(3,4,5)P₃ and PI(4,5)P₂ respectively, whilst those of DAPP1, PDK1 and PKB bind both PI(3,4)P₂ and PI(3,4,5)P₃ (Cronin, DiNitto et al., 2004; DiNitto and Lambright, 2006; Lemmon and Ferguson, 2000).

In contrast, the *FYVE* domain, named after the four cysteine-rich proteins in which it was first identified (Fab1, YOTB, Vac1, and EEA1) after, is a ~70 residue zinc-binding finger found in 28 human proteins that displays remarkably high affinity and specificity for PI(3)P. It serves to localise proteins with this domain predominantly to PI(3)P-enriched early endosomes, multivesicular bodies, phagosomes and macropinosomes (Cronin, DiNitto et al., 2004; DiNitto and Lambright, 2006; Lemmon and Ferguson, 2000). The afore-mentioned PI(5) kinase, PIKfyve, binds its substrate through this domain facilitating the turnover of PI(3)P to PI(3,5)P₂ on maturing endocytic organelles (Ikonov, Sbrissa et al., 2006; Rutherford, Traer et al., 2006; Shisheva, 2008).

The PX domain is a ~130 residue motif named after the two phagocyte NADPH oxidase (*phox*) subunits in which it was first described (Ponting, 1996). Whilst PI(3)P appears to be the preferred target of most PX domain containing proteins (Seet and Hong, 2006), a variety of other specificities have been reported. Sorting Nexins (SNX) 9 and 18 both bind PI(4,5)P₂ where they appear to function in endocytic trafficking events at the plasma membrane and AP1-positive endosomal membranes respectively, whilst SNX1 is reported to bind both PI(3)P and PI(3,5)P₂ and is involved in retrograde trafficking events from early endosomes to the Golgi (Carlton, Bujny et al., 2004; Haberg, Lundmark et al., 2008; Shin, Ahn et al., 2008; Soulet, Yarar et al., 2005; Yarar, Waterman-Storer et al., 2007). Interestingly, the PX domain in isolation is often not sufficient to recruit these molecules to PI-enriched membranes.

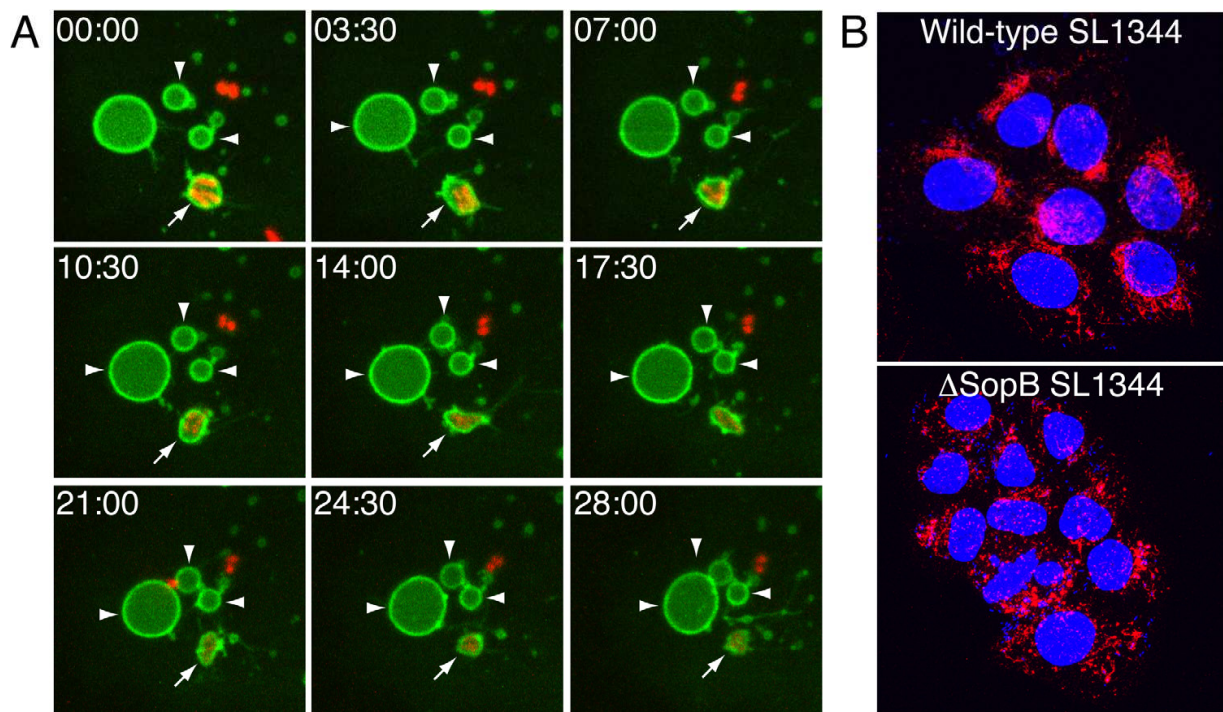
Removal of the membrane curvature sensing and bending Bin Amphiphysin Rvs (BAR) from sorting nexin 1 (SNX1) renders it cytosolic demonstrating the necessity for the *coincident detection* of both the specific PIs and the appropriately curved membranes for recruitment to endosomes (Carlton, Bujny et al., 2005).

3. Phosphoinositides in *Salmonella* Infection

Early in the *Salmonella* invasion process cellular PI levels are reported to undergo rapid and dynamic shifts. HPLC analysis of lipids extracted from *Salmonella*-infected cells revealed remarkable elevation in the relative amounts of PI(4,5)P₂, PI(3,4)P₂ and PI(3,4,5)P₃ as well as more moderate elevation in PI(3)P when compared to control cells (Bakowski, Braun et al., 2010). Modulation of PI(4,5)P₂ and PI(3,4,5)P₂ serves to destabilise the cortical cytoskeleton leading to membrane ruffling, macropinosome formation and promoting bacterial uptake whilst the accumulation of PI(3)P on membrane ruffles and the nascent SCV promotes recruitment of the PI(3)P-binding r-SNARE VAMP8 which facilitates fusion events within the endosomal network (Dai, Zhang et al., 2007).

Shortly after formation, the enveloping macropinosome shrinks to form an adherent SCV around one or more *Salmonella*. This process is mediated, at least in part, by a suite of PI-binding effector molecules known as the sorting nexins (SNX). The SNXs represent a collection of approximately 50 human proteins with diverse domain architectures that are recruited to intracellular membranes via a common PX domain (Cullen, 2008). It was revealed that SNXs 1 and 5 form heterodimers upon the surface of maturing PI(3)P-rich macropinosomes via a conserved BAR domain within their carboxyl-terminus. This dimerisation leads to the formation of tubular carriers that retrieve large quantities of membrane from and the consequent condensation of the macropinosome as the organelle matures (Kerr, Lindsay et al., 2006). The transient nature of the SNX's recruitment to the SCV was demonstrated to reflect the subsequent phosphorylation of PI(3)P within the membrane of the macropinosome to PI(3,5)P₂ by the phosphatidylinositol 5-kinase, PIKfyve. This final PI transition is necessary for the ultimate fusion of macropinosomes with the late endosomal/lysosomal system (Kerr, Wang et al., 2010).

While the SCV appears to share properties in common with constitutive macropinosomes, including SNX-recruitment, the maturation process is significantly perturbed so that the SCV persists for hours. Hernandez *et al.* (2004) observed that unlike constitutive macropinosomes, SCVs containing wild-type *Salmonella* rapidly accumulate and maintain elevated levels of PI(3)P for up to 90mins (Hernandez, Hueffer et al., 2004). Bujny et al. demonstrated that one consequence of this was the elevated and sustained accumulation of SNX1 to the newly formed SCV. This in turn results in a grossly exaggerated tubulation event that leads to the rapid contraction of the enveloping SCV to form a tightly-wrapped adherent membrane around the bacterium (Bujny, Ewels et al., 2008). Time-lapse videomicroscopy elegantly demonstrates the precision with which the pathogen is able to manipulate its local environment as the tubulation and condensation of the SCV is not observed on the surrounding constitutive macropinosomes (see Figure 2). One might speculate that this embellished contraction may facilitate the eventual engagement of the SPI2-T3SS with the membrane of the SCV.



(A) A-431 cells transiently transfected with a mammalian expression construct encoding the high-affinity PI(3)P-probe 2xFYVE_{Hrs} fused with eGFP were infected with late-log phase wild-type *Salmonella* expressing RFP (RFP-SL1344) and imaged live using a Zeiss LSM 510 confocal scanning microscope. Initially spacious, the PI(3)P-rich SCV (arrows) is observed to undergo significant tubulation and in doing so condenses to form an adherent membrane around the bacteria (red). In contrast, surrounding constitutive macropinosomes (arrow heads) present limited tubulation and no condensation in the same time period. (B) This tubulation is mediated by SopB as evidenced by the more moderate tubulation observed in Δ SopB strain-infected cells when compared to those infected with wild-type *Salmonella*.

Fig. 2. SNX-mediated tubulation leads to dramatic condensation of the SCV.

3.1 SopB, a pleiotropic phosphoinositide phosphatase

This striking impact upon PI metabolism and SNX recruitment is achieved, at least in part, by directly modulating the PI-composition of the SCV through the delivery of virulence factors with PI-phosphatase activity. SopB (also called SigD) is a SPI-T3SS phosphoinositide phosphatase that has diverse influence upon the pathogenesis of *S. typhimurium*. It contributes to membrane sealing at the plasma membrane and actin-rearrangement through activation of SGEF (a guanine nucleotide exchange factor for RhoG), during bacterial invasion (Patel and Galan, 2006; Terebiznik, Vieira et al., 2002). It also inhibits induction of apoptosis through activation of Akt and promotes the early recruitment of Rab5 and its effector Vps34 to the SCV (Knodler, Finlay et al., 2005; Mallo, Espina et al., 2008; Steele-Mortimer, Knodler et al., 2000).

Sharing similarity with mammalian PI 4- and 5-phosphatases, SopB is reported to hydrolyse a variety of PIs *in vitro*, including PI(3,4)P₂, PI(3,5)P₂ and PI3,4,5P₂, and more recently, PI(4,5)P₂ *in vivo* (Bakowski, Braun et al., 2010; Marcus, Wenk et al., 2001; Norris, Wilson et al., 1998). Given this apparent broad specificity and the capacity to promote Vps34 recruitment to, and presumably therefore *de novo* synthesis of PI(3)P on, the SCV, the wide ranging impact SopB has upon the infectious cycle of *Salmonella* is perhaps not surprising. Indeed, SopB was recently demonstrated to have profound affects upon the biophysical

properties of the SCV itself. Bakowski *et al.* (2010) The authors used an mRFP-tagged derivative of the K-ras tail with all the serine and threonine residues mutated to alanine and the lysine residues mutated to arginine so that its recruitment to intracellular membranes was only governed by surface potential and not phosphorylation or ubiquitination. They demonstrated that by reducing the levels of negatively-charged lipids like PI(4,5)P₂ and phosphatidylserine on the nascent SCV, SopB activity orchestrates the dissociation of a number of endocytic Rab proteins and inhibits fusion of the SCV with bactericidal lysosomal compartments (Bakowski, Braun *et al.*, 2010).

In addition to its lipid phosphatase activity, SopB was also recently revealed to interact with CDC42 suggesting an additional aspect to its contribution to the infectious process. Rodríguez-Escudero *et al.* (2011) demonstrated that a catalytically inactive SopB mutant can inhibit CDC42 but not Rac1 in a yeast model system. This interaction occurs independent of the activation state of CDC42. Interestingly, *Salmonella* strains harbouring SopB-mutations that render it unable to bind CDC42 presented similar invasion efficiencies when compared to the wild-type but reduced intracellular replication (Rodríguez-Escudero, Ferrer *et al.*, 2011; Rodríguez-Escudero, Rotger *et al.*, 2006). This indicates that the SopB-CDC42 association is pertinent to the intracellular adaptation of the pathogen rather than the actin-remodeling that occurs upon invasion as one might expect.

3.2 The sorting nexins coordinate *Salmonella* pathogenesis

By manipulating cellular PI metabolism, *Salmonella* effectively exaggerates and alters the function of the PI-effector molecules thereby creating a niche within the cell that supports replication and infection. In the context of SNX1, this is manifest in SopB-dependent over-recruitment and tubulation events leading to the accelerated condensation of the organelle and formation of the adherent SCV (See Figure 2). siRNA-mediated suppression of SNX1 is sufficient to inhibit SCV progression into the cell and had a moderate impact upon the intracellular replication of *Salmonella* (Bujny, Ewels *et al.*, 2008). Similarly, knockdown of SNX3, which constitutes little more than a PX domain and is found on tubular extensions of the SCV distinct from those of SNX1, inhibits SCV maturation and intracellular replication of *Salmonella* (Braun, Wong *et al.*, 2010). But what of the other PX proteins?

Wang *et al.* (2010) employed an ectopic screening strategy to demonstrate that a specific cohort of the SNXs, namely SNXs 1, 5, 9, 18 and 33, could significantly elevate the rate of macropinocytosis in cultured monolayers suggesting specific roles beyond the tubulation events described earlier (Wang, Kerr *et al.*, 2010). Perhaps these SNXs have unique roles to play in *Salmonella* pathogenicity? Towards this we have initiated a detailed examination of the recruitment of the SNXs to the nascent SCV. Immunofluorescent-labelling of infected cells transiently transfected with mammalian expression constructs encoding epitope-tagged SNXs and co-labelled with endogenous SNX1 revealed 19 SNXs recruited to the SCV within the first 30mins of infection (see Figure 3).

Interestingly, significant variation in the precise distribution of these SNXs on the early SCV was observed. Those most related to SNX1, and those previously demonstrated to have a role in endosomal trafficking, namely SNXs 2, 4, 5, 6, 7 and 8, were found on the aforementioned SNX1-labelled tubular extensions of the SCV. mPLD2, SNX12, SNX16, SNX21, SNX23 and SNX28 were found evenly associated with the tubules and the body of the SCV

proper whilst hPLD1, SNX10, SNX11, SNX15, SNX27 and p40phox were restricted to the body of the SCV only. The notable absence of SNX3 on the SCV perhaps reflects a difference in the temporal nature of the SNXs recruitment. Unlike SNX1, which is most evident on the SCV very early in the infection, SNX3 is recruited ~60mins into the infection indicating additional levels of complexity in the mechanism of recruitment within this PI-effector family (Braun, Wong et al., 2010). Indeed the PX-containing subunits of the NADPH oxidase complex represent a significant threat to intracellular *Salmonella*, providing the means to deliver a bactericidal oxidative burst to the SCV in macrophages. Virulent *Salmonella* strains avoid this through a SPI2-dependent mechanism that inhibits trafficking or targeting of NADPH oxidase-containing vesicles to the vicinity of the SCV (Vazquez-Torres, Xu et al., 2000). It will be interesting to see the likely diverse roles played by the SNXs during *Salmonella* pathogenicity emerge in the near future.

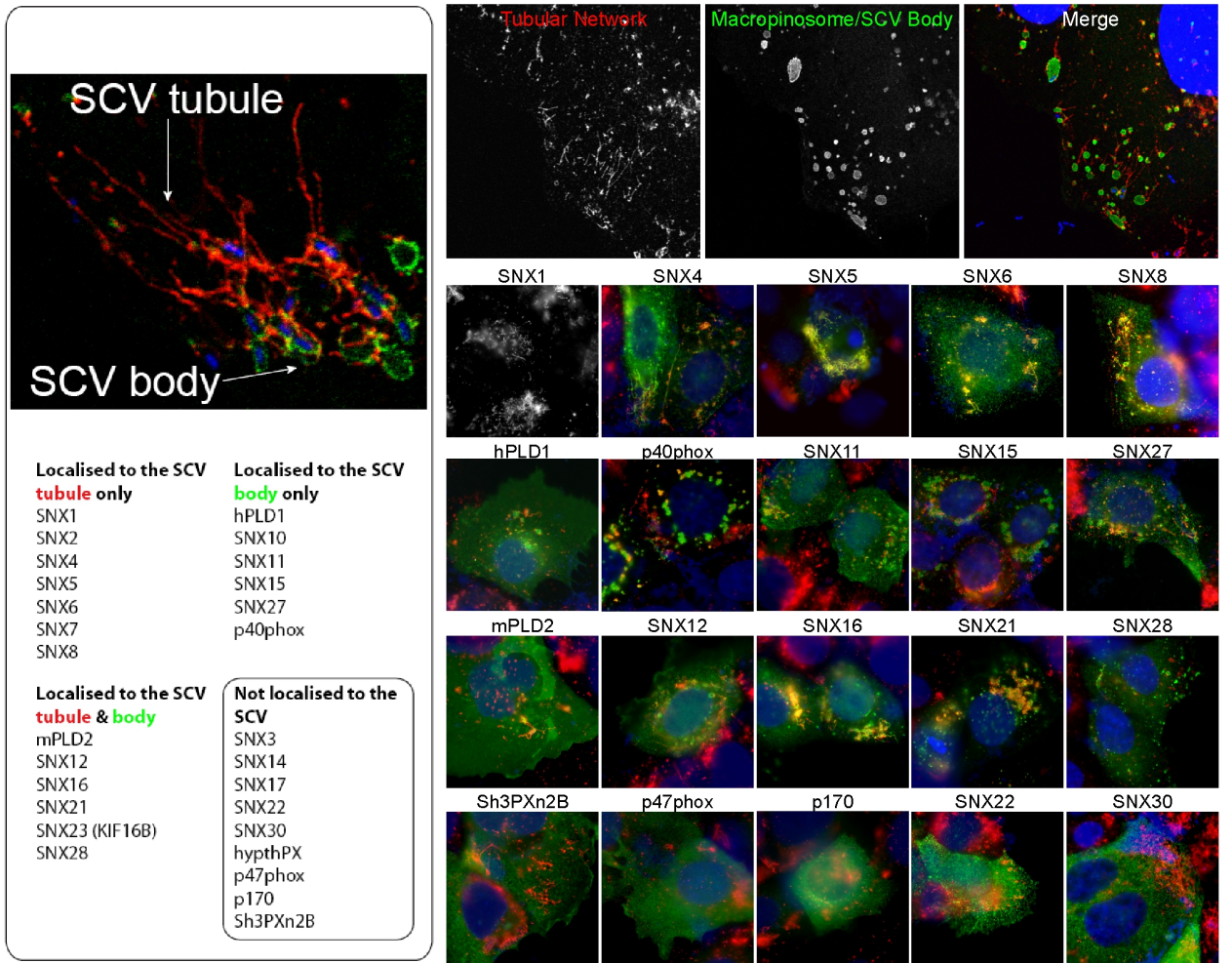


Fig. 3. *Salmonella* specifically and directly manipulates SNX recruitment to the nascent SCV. A-431 cells transiently transfected with mammalian expression constructs encoding myc-epitope tagged SNXs were infected with late-log phase *Salmonella* (SL1344) for 30mins, fixed with 4% PFA and labelled with an α -myc polyclonal antibody, a monoclonal antibody specific for SNX1, DAPI and appropriate secondary antibodies. Images were captured using a Zeiss LSM 510 confocal scanning microscope. Images were examined and the recruitment of the overexpressed SNX construct (green) was compared with that of endogenous SNX1 (red).

3.3 Targeting the phosphoinositides for therapeutic intervention

With the development of antibiotic resistance, new strategies to combat intracellular pathogens, like *Salmonella*, need to be developed. One emergent approach is to manipulate non-essential host cellular pathways required by the pathogen during its infectious cycle (Schwegmann and Brombacher, 2008). Given the frequent connections between the PIs, their effectors, and those of *Salmonella*, this protein-lipid network represents a potential opportunity for novel modes of intervention.

We recently demonstrated that disruption of PI(3,5)P₂-synthesis through perturbation of PIKfyve activity, be it by interfering mutant, siRNA-mediated knockdown or pharmacological means (YM201636), was led to a profound inhibition in the fusion of maturing macropinosomes with the late endosomal/lysosomal network. As mentioned earlier, these directed fusion events are necessary for the formation of SIFs during an infection with *S. typhimurium*. Remarkably, inhibition of PIKfyve was sufficient to halt SIF formation, SCV acidification, induction of the SPI2 Operon and ultimately intracellular replication of *Salmonella* whilst still maintaining the pathogen within an intracellular compartment (Kerr, Wang et al., 2010).

Of course a thorough understanding of the potential consequence of targeting PIs is required for such host-directed therapeutics to be effective. Inhibition of PI(3)P accumulation on the SCV with wortmannin is sufficient to halt recruitment of FYVE-domain containing molecules like EEA1 and even the SIF-marker LAMP1 but does not perturb intracellular replication of *Salmonella* (Scott, Cuellar-Mata et al., 2002). In fact, some have observed elevated rates of replication in host treated with wortmannin (Brumell, Tang et al., 2002). This is because, unlike those cultured in the presence of YM201626, the integrity of the SCV itself is disrupted releasing the bacteria into the cytosol, where they may freely replicate.

Aside from counteracting the evolution of bacterial antibiotic resistance, these *host-directed therapeutics* may provide broad-spectrum solutions to a variety of pathogens. Indeed, *Salmonella* is not unique in its partiality for targeting the PIs and associated molecules. *Yersinia* species activate PI(5) kinase to stimulate PtdIns(4,5)P₂ production at sites of bacterial invasion whilst *Listeria monocytogenes* and uropathogenic *E. coli* stimulate class I PI(3) kinase generating PtdIns(3,4,5)P₃ to promote uptake (Gavicherla, Ritchey et al., 2010; Ireton, 2007; Ireton, Payraastre et al., 1999; Martinez, Mulvey et al., 2000). *Mycobacterium tuberculosis* inhibits class III PI(3) kinase, Vps34, involved in the formation of PI(3)P effectively arresting phagosome maturation (Chua and Deretic, 2004). Thus by targeting the PIs, we may be able to specifically interfere with a variety of infections at different stages of there pathogenic cycle.

4. Conclusion

Despite comprising less than 1% of cellular lipids, the PIs and their effectors are key regulators of intra- and inter-cellular signalling, cell growth and survival, cytoskeletal dynamics and membrane trafficking pathways. It is perhaps not surprising that intracellular pathogens that exploit these processes directly target this remarkably complex protein-lipid network during the infectious process. Whilst the roles the PIs play directly and through their effectors like the sorting nexins during a *Salmonella* infection are only now coming to light, there is already strong evidence to consider them as viable therapeutic targets for intervention.

5. Acknowledgments

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