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Modulation of host phosphatidylinositol phosphates by salmonella effector protein SOPB

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MODULATION OF HOST PHOSPHATIDYLINOSITOL PHOSPHATES BY SALMONELLA EFFECTOR PROTEIN SOPB

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For my family

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LIST OF ABBREVIATIONS

PI3K – Phosphatidylinositol-3-Kinase

PtdIns(3)P – Phosphatidylinositol-3-phosphate

PtdIns – phosphatidyinositol

PtdIns(3,4)P_2 – phosphatidylinositol-3-4-bisphosphate

PtdIns(3,4,5)P_3 – phosphatidylinositol-3-4-5- trisphosphate

PtdIns(3,5)P_2 – phosphatidylinositol -3-5-bisphosphate

EHEC – Enterohemorrhagic *Escherichia coli*

EPEC – Enteropathogenic *Escherichia coli*

WT – Wild type

T3SS – Type three secretion system

GEF – Guanine nucleotide exchange factor

GAP – GTPase activating protein

SPI – *Salmonella* pathogenicity island

GFP – Green fluorescent protein

RFP – Red fluorescent protein

siRNA – small interference RNA

shRNA – small hairpin RNA

GST - Glutathione S- Transferase

SCV – *Salmonella* containing vacuole

ABSTRACT

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Salmonella spp. are gram negative bacteria capable of infecting a number of eukaryotic hosts. In humans, *Salmonella* infection can range anywhere from acute gastroenteritis to typhoid fever which can oftentimes be fatal. *Salmonella* are facultative intracellular pathogens that have acquired the ability to enter non-phagocytic cells such as those lining the intestinal epithelium. Uptake into epithelial cells is mediated by the *Salmonella* pathogenicity island 1 (SPI1) encoded type III secretion system (T3SS), a needle-like complex composed of over 20 proteins that translocates effector proteins directly into the host cell cytosol. *Salmonella* possess a second type III secretion system encoded on *Salmonella* pathogenicity island 2 (SPI2) that secretes effector proteins involved in *Salmonella* containing vacuole (SCV) formation, maturation, and intracellular survival.

Entrance into epithelial cells is modulated by effector proteins SopE, SopE2 and SopB. SopE and SopE2 having 69% sequence identity are known to be guanine nucleotide exchange factors (GEFs). SopE is a GEF capable of activating both Rac1 and Cdc42 while SopE2 activates Cdc42 only. SopB is a known phosphatidylinositol phosphate phosphatase possessing both 4- and 5-phosphatase activities promoting membrane ruffling and invasion. The mechanisms through which SopB utilizes its phosphatase activities to mediate membrane ruffling and invasion are still unclear.

Previous research has demonstrated that SopB is responsible for the production of PtdIns(3)P at the *Salmonella* induced ruffles, but the mechanism through which SopB acts is still not understood. This work will directly link the phosphatase activity of SopB, demonstrating the requirement of both the 4- phosphatase and 5-phosphatase activities, to ruffle formation and invasion. We found that the 5-phosphatase activity is responsible for generating PtdIns(3,4)P_2 which recruits host SNX9, a protein involved in actin modulation to the plasma membrane. The 4-phosphatase activity of SopB is solely responsible for the hydrolysis of host PtdIns(3,4)P_2 into PtdIns(3)P which accumulates around the ruffles and becomes incorporated into the forming SCV membrane. Either activity alone does not result in ruffling or invasion, but when acting in conjunction with one another, the 4-phosphatase and 5-phosphatase activities of SopB lead to SNX9-mediated ruffling and *Salmonella* invasion.

CHAPTER 1. INTRODUCTION

1.1 *Salmonella enterica*

Salmonella spp. are gram negative facultative intracellular pathogens capable of causing disease ranging from mild diarrhea to typhoid fever. There are an estimated 1 billion cases of non-typhoidal *Salmonella* every year causing up to 155,000 deaths. 1.4 million of those total *Salmonella* cases occur in the U.S. alone (Majowicz et al., 2010). There are over 2,600 known serovars (a distinction based upon the cell surface antigen composition) of *Salmonella*, many of which can reside in a wide variety of species including mammalian, avian, and reptilian hosts. From these thousands of serovars only a few are known to actually cause disease in mammalian hosts. The most common disease-causing serovars to date are Typhimurium and Enteriditis (Brenner et al., 2000; Malik-Kale et al., 2011). Typhimurium and Enteriditis are causative agents of *Salmonella* gastroenteritis which can be defined as inflammation of the stomach and small and large intestines. The source of infection for *S. typhimurium* is most commonly poultry and cattle infected with the bacterium. Interestingly while Typhimurium infection is not species specific, *Salmonella enterica* serovar Typhi is extremely species specific and known only to infect human hosts. This serovar is the causative agent of the systemic disease Typhoid fever killing up to 200,000 infected people every year worldwide (Malik-Kale et al., 2011; Spano et al., 2011). The species specificity has recently been attributed to the T3SS effector protein GtgE. This effector present in *Salmonella typhimurium* and absent from *Salmonella typhi* is responsible for the degradation of host Rab32. It is speculated that a genetic polymorphism in Rab32 confers the necessity of the *gtgE* gene for typhi to survive in non-human hosts (Spano and Galan, 2012).

1.2 *Salmonella* Type III Secretion System and Effector Proteins

The hallmark of *Salmonella* infection is the ability of this pathogen to invade non-phagocytic cells such as those lining the intestinal epithelium. *Salmonella* have devised a unique mechanism to enter into epithelial cells driven by the *Salmonella* pathogenicity island 1 (SP1) encoded type III secretion system (T3SS). The T3SS is a needle like apparatus composed of over 20 proteins that spans from the bacterial cell into the host cell (Hernandez et al., 2004; Kubori et al., 1998; Patel and Galán, 2005). The purpose of the T3SS is to translocate bacterial proteins, termed effector proteins, through the expanse of the apparatus - from the bacterial cell cytosol into the host cell cytosol. The SPI1 encoded T3SS is a contact-dependent apparatus utilized by *Salmonella,* translocating effector proteins responsible for bacterial entry into the host cell. Over 10 known effector proteins are translocated into the host cell via the SPI1 encoded T3SS (Winnen et al., 2008).

In order for *Salmonella* to gain entry into host epithelial cells, the bacterium must perform an orchestrated remodeling of the actin cytoskeleton. This remodeling facilitates the formation of "*Salmonella*-induced membrane ruffles" which are accumulations of actin that surround and engulf invading bacteria. The process of bacterial internalization closely mimics the host process of macropinocytosis whereby host cells form membrane ruffles in order take in small particles or fluids from the external environment in response to stimuli. The actin remodeling orchestrated by *Salmonella* allowing for its subsequent uptake involves the coordinated effort of effector proteins SipC, SipA, SopB, SptP, SopE, and SopE2. Two bacterial effectors SipC and SipA are able to directly modulate actin dynamics. SipC is a multifunctional protein required not only for the efficient translocation of effector proteins, but also for nucleating and bundling F-actin. SipC is essential for bacterial entry into the host cell and directly nucleates actin polymerization inducing cytoskeletal rearrangements allowing for ruffle formation. Alternately, *Salmonella* effector protein SipA, though not required for entry into the host cell, binds F-actin preventing filament depolymerization and lowering the critical G-actin concentration needed for polymerization. The activity of SipA works in conjunction with SipC, to enhance the

efficacy of SipC by stabilizing F-actin. (Chang et al., 2005; McGhie et al., 2001; Myeni and Zhou, 2010; Zhou et al., 1999). In addition to directly modulating F-actin nucleation and polymerization, a number of effectors are known to indirectly promote F-actin polymerization triggering the host cytoskeletal reorganization. SopE and its homologue SopE2 sharing 69% identity and are guanine nucleotide exchange factors (GEFs) activating the small RhoGTPases Cdc42 and Rac1. SopE activates both Rac1 and Cdc42, while SopE2 activates only Cdc42 (Friebel et al., 2001; Patel and Galan, 2006). The activation of these small GTPases is known to induce cytoskeletal rearrangements by promoting actin polymerization (Patel and Galan, 2006). The effector protein SopB alternatively is a phosphatidylinositol phosphate phosphatase capable of dephosphorylating both the 4 and 5 positions of phosphatidylinositol phosphates perturbing the phosphatidylinositol balance to modulate the actin cytoskeleton. The 4- phosphatase activity of SopB is also responsible for the activation of SH3-containing guanine nucleotide exchange factor (SGEF), the exchange factor for RhoG. RhoG is capable of mediating actin cytoskeletal rearrangements and is involved in the host process, macropinocytosis. Thus far the exact host phosphatidylinositol substrates or products of SopB involved in actin cytoskeletal rearrangements have not been identified. (McGhie et al., 2004; Patel and Galan, 2006; Zhou et al., 2001). In contrast to the effectors that promote actin cytoskeletal reorganization is the effector SptP that acts to reverse their effects and bring the host cell back to its normal state. SptP is a GTPase-activating protein (GAP) for Rac-1 and Cdc42. During normal *Salmonella* infection, ruffling begins just minutes after the bacterium touches the cell surface and by 80 minutes after infection, the host cell begins to regain its normal pre-infection appearance utilizing the GAP activity of SptP (Fu and Galán, 1999). All the bacterial effectors involved in facilitating the entrance of *Salmonella* into the host cell work in conjunction with one another in a temporal fashion (Cain et al., 2008; Fu and Galán, 1999).

1.3 Mechanism of Ruffle Formation

1.3.1 Membrane ruffling

Actin cytoskeletal rearrangements are characteristic of motile cells and are crucial for growth, development, and signal transduction. The main structures involved in cell mobility are lamellipodia, filopodia and ruffles. Lamellipodia and filopodia differ from membrane ruffles in that they maintain the ability to adhere to the extracellular matrix while membrane ruffles cannot adhere and retract toward the cell. It is the retraction towards the cell that causes the characteristic ruffle structure. Additionally, the ADF/cofilin, actin binding proteins that disassemble actin, found in membrane ruffles are often phosphorylated (inactive) indicating lack of turnover and no disassembly of actin, while in lamellipodia and filopodia actin is constantly assembled and disassembled (Borm et al., 2005). Filopodia, lamellipodia and ruffles do have many aspects in common. All structures are formed in response to external signals such as growth factors (GF). These signal initiate cascades many of which lead to the activation of the Rho-family GTPases Rac1 and Cdc42 enabling actin assembly. (Hall, 1998; Ridley et al., 1992). Rac1 and Cdc42 are known to activate N-WASP which is directly linked to actin by its ability to activate the Arp2/3 complex known to nucleate actin. Rac1 and Cdc42 act in a synergistic manner to enhance the nucleation of actin and create membrane ruffles (Kurokawa et al., 2004).

1.3.2 *Salmonella*-induced membrane ruffles

Membrane ruffles are commonly associated with enhanced pinocytosis, the uptake of fluids from the extracellular environment (Doughman et al., 2003; Welliver et al., 2011). It is no wonder that *Salmonella* would hijack the host machinery involved in regulating actin polymerization promoting membrane ruffle formation to gain entry into the host cell. Unlike the ruffles formed as a result of external stimuli such as GF, *Salmonella*-induced ruffles are localized to the site of *Salmonella* invasion rather than involve the entirety of the cell. Each ruffle formed during *Salmonella* infection is directly associated with at least one bacterium. The bacterium, being greater than 1μm, gives rise to ruffles around 5-10 μm capable of surrounding

the invading bacterium. Studies estimate the maximum number of ruffles in HeLa cells occurs 20 minutes after the addition of bacteria, while as soon as 1 hour after infection the fewest ruffles were seen. *Salmonella* induced membrane ruffles are incredibly dynamic and consist of aggregations of polymerized actin, α actinin, tropomyosin, and tubulin above and beside the invading bacterium. Crucial to the formation of these structures that surround the invading bacterium, just like the GF stimulated structures, is the process of actin polymerization. A number of studies have demonstrated the importance of actin polymerization for *Salmonella* induced ruffling and subsequent invasion by treating HeLa cells with cytochalasin D, an inhibitor of actin polymerization. Without treatment there are few cell-associated bacteria that have not invaded at 20 minutes, indicating that bacterial entry is quick once the bacteria contact the host cell. Alternatively, after treatment with cytochalasin D, all the bacteria viewed at 20 minutes post infection were on the cell surface unable to gain entrance into the host cell (Finlay et al., 1991; Francis et al., 1993; Garcia-del Portillo and Finlay, 1994; Garcia-del Portillo et al., 1994; Jones et al., 1993).

1.4 Host Phosphatidylinositol Phosphates

In addition to the RhoGTPases Rac1 and Cdc42, phosphatidylinositol phosphates are also responsible for regulating the actin cytoskeleton. Phosphoinositides are phosphorylated derivatives of phosphatidylinositol (PtdIns) and as a group they have major roles in membrane traffic, regulation of the cytoskeleton, as well as nuclear events. Phosphatidylinositols are composed of a glycerol backbone with two fatty acid chains, and an inositol ring that can be reversibly phosphorylated at positions 3, 4, and/or 5 resulting in the generation of seven different phosphorylated species PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3 . The main precursor, PtdIns, is generated in the endoplasmic reticulum while its phosphorylated species are mainly concentrated at the cytosolic surface of membranes within the cell. PtdIns is commonly transported to these other membranes (where it is phosphorylated) by vesicular transport or transfer proteins (Lev, 2012).

The most prevalent phosphoinositides in the host are PtdIns(4)P and PtdIns(4,5)P_2 found predominantly the Golgi complex and the plasma membrane respectively. PtdIns(4)P although predominantly found the Golgi complex, is also found at the plasma membrane. It was originally thought that PtdIns(4)P was present at the plasma membrane to serve as the precursor for PtdIns(4,5)P_2, but recently it has been shown to induce the curvature of membranes (Furse et al., 2012). PtdIns(4,5)P_2 is involved in recruiting a number of proteins to the plasma membrane facilitating not only F-actin polymerization, but also the severing of phagocytic cups during phagocytosis. Several other phosphoinositides are present at low amounts in the resting cell, but upon stimulation by growth factors increase. PtdIns(3,4,5)P_3 for example, increases at the plasma membrane when cells are stimulated with growth factors. PtdIns(3,4,5)P_3 is an important signaling molecule responsible for recruiting actin modulating proteins to the plasma membrane. The seven phosphorylated species of PtdIns all have a specific cellular distribution and due to the reversible nature of the phosphorylation events many locations have more than one species present at a given time. The main purpose of these dynamic cellular components is to recruit an extremely diverse number of proteins. Their dynamic nature allows phosphoinositides to have the ability to recruit specific proteins to specific membranes at specific times which regulates signaling within the cell. Phosphoinositides are able to recruit and bind proteins through a number of domains including, but not limited to, FYVE, PX, PH, ENTH, ANTH, Tubby, and FERM domains. These domains target specific phosphorylation sites, some with high specificity (Lemmon, 2003).

1.4.1 Involvement of phosphatidylinositol phosphates in membrane dynamics

Because of their dynamic nature and ability to recruit a variety of proteins to their membrane locations, phosphoinositides are integral players in membrane dynamics. The most notable phosphoinositide responsible for membrane dynamics is PtdIns(4,5)P_2 which is also the most abundant phosphoinositide in the plasma membrane. PtdIns(4,5)P_2 is capable of binding the RhoGTPase, Cdc42 which

activates N-WASP. The activation of N-WASP causes a conformational change allowing for the binding and activation of the Arp2/3 complex that is directly involved in F-actin nucleation. In addition to the involvement of PtdIns(4,5)P_2 in binding N-WASP and activating Arp2/3, a number of other N-WASP activators such as Toca-1 are also recruited to the plasma membrane by PtdIns(4,5)P_2 as a way to amplify the signal of PtdIns(4,5)P_2 (Ho et al., 2004; Sechi and Wehland, 2000; Tomasevic et al., 2007; Yin and Janmey, 2003). Although a minor part of the plasma membrane in resting cells, upon stimulation by growth factors or activated Rac1, PtdIns(3,4,5)P_3 becomes abundant at the membrane. PtdIns(3,4,5)P_3 has been shown to recruit and activate WAVE2, a WASP family protein. WAVE2 recruitment and activation by this phosphoinositide is sufficient to produce lamellipodium at the leading edge necessary for cell migration and requires no Rac1 to do so (Oikawa et al., 2004; Yin and Janmey, 2003). In addition to PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 another phospoinositide, PtdIns(3,4)P_2 has more recently been linked to the recruitment of a number of actin modulating proteins to the plasma membrane where it resides. PtdIns(3,4)P_2 is responsible for recruiting TAPP1, TAPP2, Tsk4, Tks5, Bam32 and SNX9 to the membrane. These proteins all have a role in modulating the actin cytoskeleton. Both Tks4 and Tks5 are involved in podosome formation. Podosomes are the structures involved in the motility of mainly cancerous cells, but can also be found in other motile cell types including macrophages (Buschman et al., 2009). Similar to lamellipodia, filopodia, and ruffles, podosomes are structures of actin that require WASP and the Arp2/3 complex. In contrast, these structures are circular in nature and degrade the extracellular matrix (Akisaka et al., 2008; Buschman et al., 2009). TAPP1 and TAPP2 are specifically recruited to the plasma membrane by PtdIns(3,4)P_2. These proteins reside in actin rich structures and TAPP1 has been implicated in regulating actin mediated ruffling (Hogan et al., 2004). TAPP1 was shown to block circular dorsal ruffles, but not peripheral ruffles. After recruitment these two proteins seem to have regulatory roles in the formation of actin-rich structures (Hogan et al., 2004; Marshall et al., 2002). Bam32 is a B-cell adaptor molecule that upon phosphorylation, is able to activate Rac1. Rac1 activation by Bam32 results in an increase of F-actin rich membrane ruffles (Allam et al., 2004).

Lastly, the PtdIns(3,4)P_2 binding protein SNX9 is recruited to the plasma membrane where it is involved in the process of endocytosis. Recently SNX9 activity was linked to activating N-WASP resulting in actin polymerization and ruffle formation (Yarar et al., 2007). A number of actin-binding proteins also contain phosphoinositide-binding domains and the binding to specific phosphoinositides functions to regulate the activity of the proteins. The actin-depolymerization factor (ADF)/cofilin which functions to regulate actin dynamics by severing actin filaments as well as disassembling actin filaments is able to bind PtdIns(4,5)P_2, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 with high affinity. Studies suggest that binding to PtdIns(4,5)P_2 abrogates the ability of ADF/cofilin to depolymerize actin filaments. α-actinin, a protein involved in bundling and crosslinking actin filaments can bind both PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. It appears as though binding to the phosphoinositides disrupts and inhibits the bundling activity of α-actinin (Saarikangas et al., 2010).

1.4.2 Dynamic cycling of phosphatidylinositol species

The dynamic nature of phosphatidylinositol phosphates within the cell and especially on the plasma membrane has always been an area of great interest. In many cases cycling can be described as a timed production of one species and degradation of that same species that allows for certain events to occur properly. One well studied example is the requirement of PtdIns(4,5)P_2 for phagocytosis. One study performed an in-depth analysis of the lipid species at the site of phagocytosis. It was found that a local and rapid increase of PtdIns(4,5)P_2 at the site of phagocytosis was required to recruit actin. It is the conversion of this phosphatidylinositol phosphate into PtdIns(3,4,5)P_3 that allows for further cytoskeletal remodeling and the closure of the phagocytic cup (Botelho et al., 2000; Fairn et al., 2009; Szymanska et al., 2008). Additionally studies on cell migration have focused on the rapid phosphatidylinositol cycling at the leading edge of the plasma membrane. The study showed that although the composition in a motile cell at the front and the rear only differ two-fold, the turnover rate in the front is much higher than that of the rear which may provide the cell with polarization and a means for motility (Nishioka et al., 2008).

1.5 Modulation of Host Phosphatidylinositol Phosphates by bacterial Pathogens

A number of bacterial pathogens have devised unique mechanisms to modulate the production and turnover of host phosphatidylinositol phosphate phosphatases and kinases as a way to control the levels of phosphatidylinositol phosphates in specific locations of the cell. *Yersinia* species, *Listeria monocytogenes, Shigella flexneri, Mycobacterium tuberculosis,* and *Salmonella eterica* all have ways of subverting the phosphatidylinositol pathways for their own invasion or survival. Successful infection of *Yersinia pseudotuberculosis* a gram negative pathogen that replicates in local lymph nodes requires that the bacterium gain entry into the host cell. *Yersinia* invasion is a Rac1 dependent process requiring binding of the bacterial invasion protein to β1 integrin receptors. Additionally, the host kinase phosphoinositol-4-phosphate-5-kinase (PIP5K) is recruited to the phagocytic cup where it produces PtdIns(4,5)P_2. PtdIns(4,5)P_2 was shown to be essential to *Yersinia* infection as depletion of this phosphoinositide lowered bacterial uptake. It's been proposed that *Yersinia* locally increases PtdIns(4,5)P_2 levels either to recruit and orient a number of actin binding proteins or to act as a precursor to PtdIns(3,4,5)P_3, another phosphoinositide involved in promoting actin polymerization for its invasion (Wong and Isberg, 2003). *Listeria monocytogenes* is gram positive and the causative agent of listeriosis, a common food borne illness. It's widely accepted that *Listeria* gain entrance into the host by utilizing its invasion proteins, InlA and InlB. These two proteins facilitate entrance by interacting with host receptors E-cadherin (binds InlA) and Met (binds InlB). It has also been shown that host kinase PI(3)K is required for invasion into host cells, as after treatment with specific PI(3)K inhibitor LY294002 bacterial invasion is blocked. InlB was later shown to be an agonist of host PI(3)PK and also to phosphorylate a number of adaptor proteins such as Gab1, Shc, and Cbl which are able to translocate the class I PI(3)K to the plasma membrane. Products of PI(3)K such as PtdIns(3,4,5)P_3 are known to cause actin cytoskeletal rearrangements and aid in uptake of bacteria into the host cell (Ireton et al., 1999; Sidhu et al., 2005). *Shigella flexneri* is a gram negative pathogen that gains entrance into the host cell by translocating effector proteins via its Type III secretion system that modulate host

proteins. *Shigella* effector protein IpgD, the homologue to SopB in *Salmonella* is a phosphatidylinositol 4-phosphatase that acts specifically on dephosphorylating PtdIns(4,5)P_2 to PtdIns(5)P. IpgD has a role in filopodia extension and ruffle formation which allows for sufficient entry into the host cell. It is thought that IpgD causes a local detachment of the plasma membrane from the actin cytoskeleton by decreasing the level of PtdIns(4,5)P_2 and therefore changing the composition of proteins that anchor to the plasma membrane (Niebuhr et al., 2002; Niebuhr et al., 2000). *Mycobacterium tuberculosis*, the cause of tuberculosis which is a severe pulmonary disease, causes disease mainly by surviving within the tubercle bacillus in macrophages. *M. tuberculosis* differs from the previous pathogens in that it blocks the activation of PI(3)K. The recruitment of hVPS34, a PI(3)K to phagosomes is known to require calmodulin (CaM) and calmodulin-dependent kinase II (CaMKII). *M. tuberculosis* was shown to block CaM and CaMKII, using a component of its cell wall - mannose-capped lipoarabinomannan (ManLAM) that is able to inhibit Ca^{2+} levels in effect block the production of PtdIns(3)P and recruitment of EEA1 to the phagosome. Lack of PtdIns(3)P and EEA1 blocked phagosomal maturation. (Chua and Deretic, 2004; Fratti et al., 2001). Another bacterial pathogen that utilizes host phosphatidylinositols to gain entrance into and to promote survival inside the host is *Salmonella enterica.* *Salmonella* translocates an effector protein, SopB, into the host cell cytosol that acts a 4- and 5- phosphatidylinositol phosphate phosphatase. SopB, similar to its homologue IpgD in *Shigella flexneri*, has been shown to act upon PtdIns(4,5)P2 *in vivo.* Unlike IpgD, the dephosphorylation of PtdIns(4,5)P_2 produces PtdIns(4)P and has been linked to efficient fission of the phagosome from the plasma membrane (Hernandez et al., 2004; Marcus et al., 2002; Terebiznik et al., 2002). The phosphatase activity of SopB has also been linked to the recruitment of hVPS34 to the *Salmonella* containing vacuole (SCV) where it produces PtdIns(3)P and aids in vacuolar maturation (Mallo et al., 2008). Many pathogenic bacteria both gram positive and gram negative are able to modulate the production and turnover of host phosphatidylinositols for their own entrance into and survival within the host. Whether they have developed proteins to act directly on the phosphatidylinositols or

they are able to interfere in pathways that produce or hydrolyze these moieties is specific for each bacterium.

1.6 *Salmonella* Effector Protein SopB

Salmonella outer protein B, SopB, was first characterized in 1997 and attributed with fluid secretion and the inflammatory responses common during *Salmonella* infection. During the characterization of this protein the deletion strain was shown to have an invasion rate similar to that of wild type in cultured epithelial cells. (Galyov et al., 1997). Additional work at the time, identified sequence similarity to host 4-phosphatase and 5-phosphatases in the SopB protein with SopB containing a conserved CX_5R motif with a catalytic cysteine at C460 required for the 4-phosphatase activity. Homology to host 5-phosphatase Synaptojanin helped identify the catalytic K530 residue that confers the 5-phosphatase activity of SopB (Marcus et al., 2001; Norris et al., 1998). SopB is a *Salmonella* pathogenicity island 1 (SPI1) Type III secreted effector protein that along with other SPI effectors is targeted to the plasma membrane after translocation into the host cell. This is an interesting finding because most of the proteins included SopB contain no membrane-targeting domains (Cain et al., 2004).

1.6.1 Involvement in ruffle formation

A more in-depth look at the possible role of SopB in *Salmonella* invasion found that the T3SS effector protein SopE was masking the invasive role of SopB during *Salmonella* infection. Single deletions of either the *sopB* or *sopE* genes caused a modest defect in ruffle formation and invasion while the double deletion *sopBsopE* strain was abrogated in ruffle formation and invasion. This early work provided evidence for the role of SopB in invasion and ruffle formation. Additionally it linked the phosphatase activity of SopB to the ruffling and invasion by demonstrating that ectopic expression of the $SopB^{C460S}$ point mutation in the *sopE* background strain was not able to rescue the ruffling or invasion phenotype as the wild-type did (Zhou et al., 2001). Another SopB-dependent phenotype attributed to the phosphatase activity of

SopB is the production of PtdIns(3)P at the *Salmonella* induced membrane ruffles (Dai et al., 2007; Hernandez et al., 2004; Pattni et al., 2001). It was speculated that the production of PtdIns(3)P a phosphatidylinositol phosphate involved in membrane trafficking and vesicle fusion was important for proper phagocytosis and SCV formation. There is also evidence that PtdIns(3)P is involved in the recruitment of host protein VAMP8 to the *Salmonella* ruffles. VAMP8 is a v-SNARE protein involved in the homotypic fusion of early endosomes and late endosomes predicted to promote proper bacterial phagocytosis. Additionally, siRNA-mediated knock-down of VAMP8 led to a decrease in *Salmonella* invasion similar to that of the $sopB^{C460S}$ mutant strain supporting the role of VAMP8 in phagocytosis (Dai et al., 2007; Wang et al., 2004).

SopB is also involved in the activation of Rho-family GTPases upon translocation into the host cell. SopB was shown to bind Cdc42 via its N-terminal CRIB-like motif (Burkinshaw et al., 2012). The activation of Cdc42 was not required for proper *Salmonella* invasion, suggesting that SopB may activate another host component involved in cytoskeletal rearrangements. During wild-type infection it was found that SopB is able to activate an endogenous exchange factor, SGEF that activates RhoG, a Rho-family GTPase implicated in ruffle formation and micropinocytosis. SopB is able to activate RhoG independent of SopE and SopE2 to modulate the actin cytoskeleton and induce invasion (Patel and Galan, 2006; Wennerberg et al., 2002).

1.6.2 Role in Salmonella containing vacuole

SopB is also implicated in the maturation of the *Salmonella* containing vacuole (SCV) by promoting the production of PtdIns(3)P on the SCV surface. This is an important phenomenon because PtdIns(3)P is a host phosphatidylinositol vital in vesicle trafficking and fusion. In the absence of SopB and subsequently PtdIns(3)P, the SCV was not able to fuse empty vesicles prohibiting the proper maturation and formation of a spacious SCV for *Salmonella* to replicate in and reside. It was speculated that one reason PtdIns(3)P was produced at the plasma membrane ruffles so it could efficiently become incorporated into the SCV (Hernandez et al., 2004).

Additionally, work in 2008 identified the mechanism by which SopB promotes PtdIns(3)P production on the SCV. The work demonstrated that SopB recruits Rab5 to the surface of the SCV via its phosphatase activity. Rab5 then recruits Vps34 a host PI(3)K to the SCV where Vps34 produces PtdIns(3)P (Mallo et al., 2008). Further work on SopB and the SCV demonstrated the importance of host protein Myosin II for SCV positioning and stability. Myosin II is an actin based motor that is able to bind actin to promote motility. SopB was sufficient to cause phosphorylation of Myosin II allowing for its activation and was therefore involved in the Myosin II regulated SCV position and stability (Wasylnka et al., 2008).Via its phosphatase activity, SopB plays an integral part in the formation and maturation of the SCV. SopB is responsible for generating PtdIns(3)P on the SCV membrane which allows for the fusion to smaller vesicles creating a spacious place for the bacteria to replicate. Once the spacious vacuole is formed SopB is able to activate Myosin II to stabilize and localize the SCV.

1.6.3 Localization and ubiquitination

Upon translocation into the host cell, SopB, along with the other SPI-1 effectors involved in membrane reorganization is targeted to the plasma membrane where it can presumably perform its activities. Many bacterial effectors are degraded shortly after entry into the host cell, while SopB persists for many hours. SopB was discovered to become ubiquitinated, which is a post translational modification that usually results in targeting the protein to the proteasome for degradation, upon entry into the host cell. (Knodler et al., 2009; Patel et al., 2009; Rogers et al., 2008). Studies revealed that SopB ubiquitination is involved in localizing SopB from the plasma membrane to the SCV following bacterial invasion. The mutant deficient in ubiquitination persists at the plasma membrane and leads to enhanced Akt activation (an activity dependent upon the phosphatase activity of SopB that occurs at the plasma membrane). Additionally, the ubiqutination mutant of SopB does not localize to the SCV and renders the strain defective for intracellular replication. The ubiquitination of SopB therefore, does not affect the catalytic activity or stability of

SopB but rather effects the localization of SopB and activities that SopB performs after it is localized to the SCV (Knodler et al., 2009; Patel et al., 2009).

1.7 Introduction to thesis study

Salmonella is a sophisticated bacterium capable of modulating the actin cytoskeleton of non-phagocytic cells to gain entry into these cells. A number of SPI 1 secreted bacterial effectors, namely SipA, SipC, SopE, and SopE2 have direct and well characterized roles in actin modulation and ruffle formation. SipA and SipC both bind to actin to induce their activities while SopE and SopE2 are GEFs that activate Rac1 and Cdc42 which are Rho-family GTPases that are capable of modulating the actin cytoskeleton. SopB is a phosphatidylinositol phosphate phosphatase whose phosphatase activity is required to not only activate Cdc42, but also the Rho-family GTPase RhoG. RhoG activation was linked to SopB-dependent ruffle formation and invasion. Another unique phenotype requiring the phosphatase activity of SopB is the production of PtdIns(3)P at the *Salmonella*-induced membrane ruffles. PtdIns(3)P is responsible for recruiting host v-SNARE protein VAMP8 to the membrane which is required for efficient bacterial invasion. Neither PtdIns(3)P nor VAMP8 has any demonstrated role in promoting SopB-mediated ruffle formation. The phosphatase activity of SopB is required to promote ruffling and thus far no product or substrate of SopB has been implicated in ruffle formation. Additionally, the role SopB plays in the production of PtdIns(3)P at the membrane ruffles is unknown. This study seeks to understand how SopB promotes the production of PtdIns(3)P at the membrane ruffles and also how any products or substrates of SopB may contribute to SopB-mediated ruffle formation and subsequent bacterial invasion.

Chapter 2 of this dissertation will focus on the involvement of any host proteins in the production of PtdIns(3)P at the membrane ruffles. This was an important step towards determining which phosphatase activity of SopB either 4- or 5- was actually responsible for generating PtdIns(3)P. We identified that host PtdIns(3,4,5)P_3 5-phosphatase SKIP which generates PtdIns(3,4)$_2$ was involved in the SopB-mediated production of PtdIns(3)P. This provided a great hint that PtdIns(3,4)P_2 was likely

hydrolyzed by SopB to produce PtdIns(3)P. In chapter 3 we were able to delineate the two phosphatase activities of SopB providing evidence that the 4-phosphatase activity, utilizing host substrate PtdIns(3,4)P_2, was sufficient to produce PtdIns(3)P at the membrane ruffles. We also provided evidence that this phosphatase activity alone was not capable of producing membrane ruffles and that the 5-phosphatase activity of SopB was indispensable for ruffle formation. We speculate that the 5-phosphatase activity of SopB is required for hydrolyzing host PtdIns(3,4,5)P_3 into PtdIns(3,4)P_2 which we have demonstrated is responsible for the recruitment of host SNX9 to the plasma membrane.

No previous study has investigated the potential role of the 5-phosphatase activity of SopB in SopB-mediated ruffle formation and invasion. This study provides evidence that both the 4- and 5-phosphatase activities of SopB are required to produce a dynamic turnover of phosphatidylinositols at the plasma membrane allowing for the production and hydrolysis of PtdIns(3,4)P_2 into PtdIns(3)P. PtdIns(3,4)P_2 is responsible for recruiting host SNX9 to the membrane, a protein that is involved in endocytosis and actin cytoskeletal rearrangements by activating N-WASP. Additionally this phosphatidylinositol phosphate is hydrolyzed by SopB into PtdIns(3)P which is required for the recruitment of VAMP8 to the membrane ruffles and the proper phagocytosis of *Salmonella* into the host cell. PtdIns(3)P is also easily incorporated into the SCV membrane where it is crucial to the formation of a spacious vacuole by fusion with smaller vacuoles. It is a coordinated effort creating the production and turnover of PtdIns(3,4)P_2 that allows for SopB-mediated ruffling and invasion.

CHAPTER 2. HOST PROTEINS INVOLVED IN SOPB-MEDIATED PTDINS(3)P PRODUCTION

2.1 Abstract

Salmonella gains entry into the host cell by injecting a number of effector proteins into the host cell cytosol. SopB is one of these effector proteins and has proven essential to the formation of *Salmonella*-induced ruffles and subsequent invasion into the host cell. Previous work has characterized the SopB-mediated increase of PtdIns(3)P at the *Salmonella*-induced membrane ruffles establishing that the phosphatase activity of SopB is responsible for its production. SopB contains both 4- and 5-phosphatidylinositol phosphate phosphatase activities but it is unclear which phosphatase activity of SopB is responsible for PtdIns(3)P production at the membrane ruffles. It is also unclear what the host substrate of SopB is, and what host proteins, if any are involved in PtdIns(3)P production. This chapter addresses the possible involvement of host proteins in the production of PtdIns(3)P at the *Salmonella* membrane ruffles. We demonstrate here that PtdIns(3)P continues to be produced at the *Salmonella*-induced membrane ruffles in the presence of the PI3K inhibitor, wortmannin indicating the recruitment and activation of PI3K is not required for PtdIns(3)P production. We also show here that host PtdIns(3,4,5)P_3 5-phosphatase SKIP and PtdIns 4-kinase PI4KIIβ are involved in the pathway of SopB-mediated PtdIns(3)P production indicating that the host substrate of SopB in PtdIns(3)P production is likely PtdIns(3,4)P_2 . Additionally we were able to establish a model in yeast that could be used to corroborate some of this data.

2.2 Introduction

Salmonella effector protein SopB is responsible for the localized increase of PtdInd(3)P at the membrane ruffles induced during wild-type *Salmonella* infection. The production or recruitment of PtdIns(3)P is dependent upon the phosphatase activity of SopB as shown by the abolishment of PtdIns(3)P enrichment at the ruffles in cells infected with the $sopB^{C460S}$ catalytically dead mutant strain (Dai et al., 2007; Hernandez et al., 2004; Pattni et al., 2001). During wild-type *Salmonella* infection, there is also a localized increase of other 3-phosphorylated species including PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 at the *Salmonella*-induced membrane ruffles. The increase of these 3-phosphorylated phosphatidylinositols could indicate that host PI3K may be involved in *Salmonella* invasion (Hernandez et al., 2004; Mallo et al., 2008).

PtdIns(3)P is an important lipid species involved in the generation and maturation of the *Salmonella* containing vacuole (SCV). The pathway of PtdIns(3)P production at the SCV following bacterial invasion has already been established and involves the host PI3K, Vps34. Upon ubiquitination, SopB is localized to the SCV where it is involved in the recruitment of host protein Rab5 which subsequently recruits host PI(3)K, Vps34 to the SCV which phosphorylates PtdIns producing PtdIns(3)P (Mallo et al., 2008). The production of PtdIns(3)P on the SCV is crucial to the timing of the maturation of the SCV and intracellular growth of the bacteria (Hernandez et al., 2004). It is still unclear though, how SopB produces or recruits PtdIns(3)P to the *Salmonella*-induced membrane ruffles and if this enrichment of PtdIns(3)P has a role in *Salmonella* invasion.

We were able to rule out the requirement of host PI3K in SopB-mediated PtdIns(3)P production at the membrane ruffles. Without the requirement of host PI3K it is most likely that SopB is utilizing its own phosphatase activity acting upon a host substrate to produce PtdIns(3)P. To gain further insight into this, a screening of host phosphatidylinositol phosphatases and kinases that could potentially be involved in this SopB-mediated pathway was performed. This screening could also provide clues into the substrate of SopB. We determined that PtdIns(3,4,5)P_3 5-phosphatase

SKIP and PtdIns 4-phosphatase PI4KIIβ are involved in the SopB-mediated production of PtdIns(3)P. We were also able to devise a yeast model that could potentially corroborate this eukaryotic cells data.

2.3 Materials and Methods

Strains and plasmid construction

Wild-type *Salmonella enterica* serovar Typhimurim (*S. typhimurium*) strain SL1344 (Hoiseth and Stocker, 1981) and its *sopB*^{C460S} (SB933), and *sopBsopE* (ZP15) (Higashide and Zhou, 2006) have been previously described.

Wild-type *Saccharomyces cerevisiae* strain W303 was described previously (Thomas and Rothstein, 1989).

SKIP-GFP (pZP2199) and PI4KIIβ-RFP (pZP2195) eukaryotic expression plasmids were created as follows. SKIP (MGC-862) and PI4KIIβ (MGC-34571) cDNA clones were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Both genes were amplified via polymerase chain reaction (PCR) and inserted into pEGFP-C1 or pERFP-C1 respectively. Yeast expression plasmids were created as follows: p426MET25- SopB (pZP3189) was created by inserting SopB into the BamHI/EagI sites of p426MET25. p426MET25-SopBC460S (pZP3213) was created similarly by inserting SopB^{C460S} into the BamHI/EagI sites of p426MET25. p414MET25-FYVE3- GFP (pZP3188) was created by subcloning the $(FYVE)_3$–EGFP described previously (Kanai et al., 2001) into p414MET25. The probe for PtdIns(3)P - p40PX-EGFP (pEX48), the probe for PtdIns(3,4,5)P_3 GRP-EGFP (pEX53), the probe for PtdIns(3,4)P_2 p47PX-EGFP (pZP1749), and the probe for PtdIns(3,4,5)P_3/ PtdIns(3,4)P_2 PH-Akt-EGFP (pEX52) were described previously (Kanai et al., 2001).

GFP-SidF (pEX300), GFP-SidF^{C645S}(pEX301), GFP-SidF(1-760) (pEX339), GFP-SidF^{C645S}(1-760)(pEX340) were a gift from Dr. Mao. SidF(1-760) and SidF^{C645S}(1-760) were cloned into pGEX-KG creating (pZP3284) and (pZP3285) respectively.

Cell culture and bacterial infection

Mammalian cell line (Thomas and Rothstein, 1989) HeLa (CCL-2; ATCC, Manassas, VA, USA) was cultured in Dulbebecco's modified Eagle's medium containing 10% fetal bovine serum. All cells were maintained at 37°C and 5% CO2. Bacterial strains were grown overnight at 37°C and subcultured (1:20) in LB supplemented with NaCl to .3M for three hours on a rotating wheel for low aeration. HeLa cells were infected at an MOI of 10 for 15 minutes for phosphatidylinositol detection assays and an MOI of 3 for 10 minutes for all invasion assays. Invasion rates were assessed by inside/outside differential staining. After infection, cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 20 minutes. Outside bacteria were then probed with rabbit anti-*Salmonella* O antigen group B (Difco) antibody. The cells were washed 3X with PBS and probed with anti-rabbit Texas Red conjugate (Molecular Probes). The cells were then permeabilized with .2% triton-X-100 for 10 minutes washed 3X and total *Salmonella* were then probed similarly with anti-*Salmonella* O antibody, and then probed with anti-rabbit AF488 conjugate (Molecular Probes). Outside bacteria were subtracted from the total for number of internalized bacteria for our invasion rate. At least 300 cells were counted in three separate experiments. The WT infected bacterial invasion was normalized to 100%.

Gene silencing, transfection and real time PCR

shRNA containing a pool of 3-5 target-specific lentiviral vector plasmids (encoding 19-25 nt) shRNAs targeting various host phosphatases and kinases (Complete list in Table 1) including SKIP-(SC-106937-SH) and PI4KIIβ- (SC-89203-SH) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Florescene-conjugated shRNA control (sc-36869) was purchased from Santa Cruz Biotechnology. Validated silencer select siRNA targeting SKIP (ID-s28638) and PI4KIIβ (ID – s30681) and control siRNA (4390843) were purchased from Ambion/life technologies. HeLa cells were treated with 1µg shRNA plasmid DNA or 5nM silencer select siRNA using TransIT-express transfection reagent (Mirus) for 48 hours. Following siRNA or shRNA knockdown, RNA was isolated from HeLa cell lysates using RNAqueous RNA isolation kit according to manufacturer's instructions (AM1912-Ambion, Austin, TX). cDNA was synthesized using High Capacity cDNA

Reverse Transcription Kit according to manufacturer's instructions (4368814 – Applied Biosystems, Foster City, CA).

Gene expression was analyzed using TaqMan Universal PCR master Mix (4304437 – Roche) and Taqman Expression Assays PI4KIIβ -(Hs00217854) and SKIP (Hs00213017) (Applied Biosystems). As an endogenous control we used Beta Actin (4333762T). Relative expression was assessed on a BioRad CFX96 Real Time System. Data represent three independent experiments and results are normalized against β-actin expression. Statistical analysis was performed using the student's *t*-test. An asterice indicates a p-value <.05.

Plasmid DNA transfection of lipid probes was performed using TransIT-express transfection reagent (Mirus) according to manufacturer's instructions for 24 hours.

Protein purification and Phosphoinositide phosphatase activity assay

GST-tagged recombinant WT SidF(1-760) and its derivative SidF^{C645S}(1-760) were expressed in Escherichia coli BL21 (DE3) The purification was performed 50mM Tris-HCl (pH 7.5) and 2mM DTT void of Phosphates using glutathione-Sepharose 4B (Amersham Biosciences, Piscataway, NJ,USA).

Phosphoinositide phosphatase activity was measured using the malachite green assay (Echelon Research Laboratories, Salt Lake City, UT, USA) as described in (Marcus et al., 2001). Briefly, recombinant purified protein (100 ng) was incubated with synthetic short chain (octanoyl) phosphoinositides (Echelon Research Laboratories, Salt Lake City, UT, USA) (50μM) as indicated. The reactions were stopped by addition of 80 μl malachite green reagent and absorbance measured at 620 nm. The assay provided linear detection in the range of up to 1000 pmol of Pi. Results represent an average of three separate reactions carried out for each protein and substrate.

Chemical inhibitors

HeLa cells were treated with 100nM wortmannin purchased from Sigma (W1628 – St. Louis, MO) for 1 hour followed by infection with WT *Salmonella* for 15 minutes in the presence of the inhibitor. HeLa cells treated with YM201636, purchased from Symansis Cell Signaling Science (SY-YM201636 - Timaru, New

Zealand) at 800nM for 1hour followed by infection with WT Salmonella for 15 minutes in the presence of the inhibitor.

Yeast strains and assays

Yeast strain W303 was used for all yeast experiments. Yeast morphology: Yeast strain expressing wild-type SopB on the p426MET25 vector, $SopB^{C460S}$ mutant, and the p426MET25 empty vector were grown overnight with and without .2mM Methionine. Cultures were centrifuged at low speed for 5 minutes and the pellet was resuspended in PBS. Live yeast cells were imaged on a 100X objective. Yeast actin staining: Yeast strains were grown overnight with and without the addition of .2mM methionine. 10% formaldehyde was added to the cultures and strains continued to shake for an additional 10 minutes. The cultures were centrifuged for 5 minutes and rinsed 2X with PBS. The yeast cells were then permeabilized with .2% TritonX100 for 15 minutes at room temperature and subsequently washed 2X with PBS. Rhodamine phalloidin was then added at a 1:10 dilution and incubated for 1 hr, after which the cells were washed again 3X with PBS and imaged under the 100X objective. FM4-64 vacuolar staining: Yeast strains were grown overnight with and without .2mM methionine. 5mL of overnight cultures were spun at low speed for 5 minutes and washed 2X with YPD at 4°C. Stock FM4-64 (1mg/mL in DMSO) was diluted 1:50 in YPD. About 50µL of diluted Fm4-64 was added to the culture at 4°C for 30 minutes. The cells were then washed 2X with cold YPD and resuspended to 1mL in YPD at 30°C for 30-40 minutes before the cells are washed, resuspended in media and visualized. PtdIns(3)P localization: Yeast strains co-expressing the GFP-tagged FYVE3 probe for PtdIns(3)P and either of wild-type SopB, $SopB^{C460S}$, and pMET426 empty vector were grown overnight. Adenine was added to decrease the signal of GFP. Yeast strains were spun down at low speed for 5 minutes and resuspended in PBS for live cell imaging.

2.4 Results

2.4.1 SopB-dependent PtdIns(3)P production at the *Salmonella*-induced membrane ruffles does not require host PI(3)K

Previous work has characterized the mechanism of SopB-mediated PtdIns(3)P production on the *Salmonella* SCVsurface. This mechanism requires the direct involvement of host PI3K, Vps34 (Mallo et al., 2008; Pattni et al., 2001). Evidence from both Pattni et al. and Dai et al. indicates that PtdIns(3)P production at the ruffles though, is not sensitive to wortmannin, the chemical inhibitor of PI3K (Mallo et al., 2008; Pattni et al., 2001). Because this result is critical for the rationale of this work, this study began by confirming PtdIns(3)P production at the *Salmonella* induced membrane ruffles is PI3K independent (Dai et al., 2007; Pattni et al., 2001). HeLa cells were transfected with the GFP-tagged probe for PtdIns(3)P – p40PX-EGFP (Kanai et al., 2001) for 24 hours and infected with WT or the *sopB*^{C460S} mutant strain for 15 minutes as indicated. In uninfected cells, the punctate signal characteristic of PtdIns(3)P on the early endosomes is clearly visible while in WT infected cells PtdIns(3)P is enriched on the *Salmonella* induced membrane ruffles as well as present on the early endosomes. This enrichment at the ruffles is absent from cells infected with the *sopB*^{C460S} mutant strain (Fig. 2-1A). To assess the requirement of PI3K, cells transfected with the PtdIns(3)P probe were subsequently treated with 100nM wortmannin for 1 hour followed by infection with WT *Salmonella* for 15 minutes in the presence of the inhibitor. In uninfected cells the inhibitor effectively abolished the punctate signal of PtdIns(3)P indicating that PtdIns(3)P is no longer being produced by PI3K at the early endosomes. During WT infection PtdIns(3)P signal is enriched at the *Salmonella*-induced ruffles despite the disappearance of the PtdIns(3)P puncta on early endosomes (Fig 2-1B). This data allowed us to confidently conclude that SopB must be utilizing a host substrate to produce PtdIns(3)P at the membrane ruffles and that this host substrate must be a 3-phosphorylated species since host PI(3)K is not required.

To determine if other 3-phosphorylated phosphatidylinositol phosphates are enriched at the plasma membrane in the presence of wortmannin and could

potentially be the substrate of SopB for PtdIns(3)P production, HeLa cells were transfected with the probes for host PtdIns(3,4)P2 , p47PX-EGFP or PtdIns(3,4,5)P3, GRP-EGFP –and PH-Akt-EGFP the probe for PtdIns(3,4)P2/PtdIns(3,4,5)P3. It has already been established that host PtdIns(3,4)P2 / PtdIns(3,4,5)P3 as detected by the probe PH-Akt-GFP is not sensitive to wortmannin at the Salmonella induced

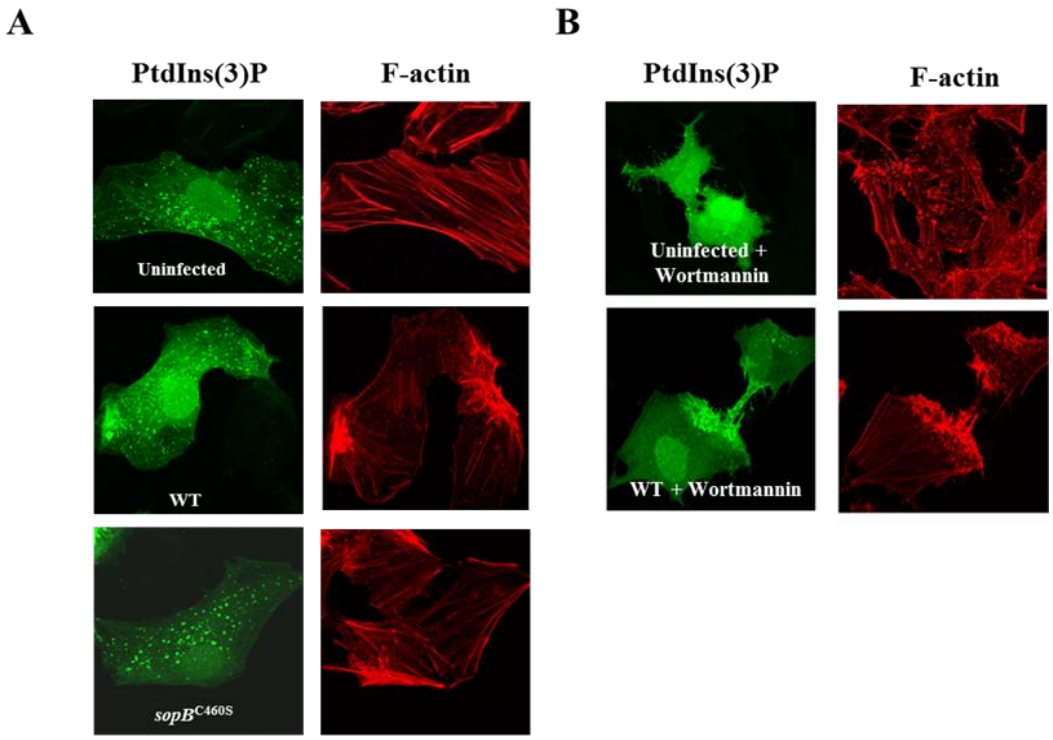

Fig. 2-1 SopB-dependent PtdIns(3)P production is PI(3)K independent. HeLa cells seeded on a 24-well plate were transfected with the probe for PtdIns(3)P – p40PX-EGFP (green) for 24 hours. (A) Following transfection cells were infected with WT or the sopBC460S mutant strain at an MOI of 10 for 15 minutes. (B) Following transfection, cells were treated with 100nM wortmannin for 1 hour, followed by infection with WT Salmonella at an MOI of 10 for 15 minutes. Ruffles were visualized by staining with Texas Red-conjugated Phalloidin (red). Images are a maximum intensity projection of Z-stacking images taken on a Zeiss LMS 700 confocal microscope.

ruffles, but either phosphatidylinositol phosphate individually was not investigated . HeLa cells were transfected with the indicated probe for 24 hours followed by infection with WT *Salmonella* for 15 minutes. PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 were recruited to the membrane ruffles during WT infection (Fig. 2-2 A). To

determine if these phosphatidylinositols are present and recruited to the ruffles in the absence of PI3K, the cells were treated with the PI3K inhibitor wortmannin. After transfection with any of the three probes for 24 hours and treatment with wortmannin for 1 hour followed by infection for 15 minutes with WT *Salmonella* we were able to confirm the previous PH-Akt-GFP probe result (both phosphatidylinositols were present at the ruffles) and also determine both individual phosphatidylinositols are enriched at the *Salmonella* induced membrane ruffles (Fig. 2-2B). This could indicate that either or both phosphatidylinositols are a substrate for SopB since they are present at the ruffles in the absence of PI3K (Hernandez et al., 2004; Mallo et al., 2008).

Unfortunately a probe specific for PtdIns(3,5)P_2 is not available. To elucidate the possible involvement of this phosphatidylinositol phosphate, cells were treated with the specific inhibitor of PtdIns(3)P 5-kinase (PIKfyve), YM201636. This small molecule inhibitor has been shown to deplete pools of PtdIns(3,5)P_2 in the cell (Jefferies et al., 2008). The involvement of PtdIns(3,5)P_2 in the fusion of the SCV with endosomes which is required for *Salmonella* replication was recently demonstrated. Its speculated that host protein PIKFyve phosphorylated PtdIns(3)P on the SCV producing PtdIns(3,5)P_2 promoting fusion and SCV maturation (Kerr et al., 2010). To determine if PtdIns(3,5)P_2 is a possible substrate of SopB, HeLa cells we transfected with the probe for PtdIns(3)P for 24 hours, then treated with 800nM YM201636 for 1hour and infected with WT *Salmonella* for 15 minutes. The production of PtdIns(3)P was assessed in the absence of PtdIns(3,5)P_2. The presence of PtdIns(3,5)P_2 was not crucial to PtdIns(3)P production during WT infection as PtdIns(3)P was still at the membrane ruffles after treatment with the inhibitor (Fig 2-3). The efficacy of the inhibitor was assessed by the appearance of large vesicles characteristic of PtdIns(3,5)P_2 depletion as is shown by the arrows in the enlarged DIC image in Fig 2-3. We can deduce that either PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 are the substrate for SopB, while PtdIns(3,5)P_2 does not have a significant role in the production of PtdIns(3)P during WT Salmonella infection.

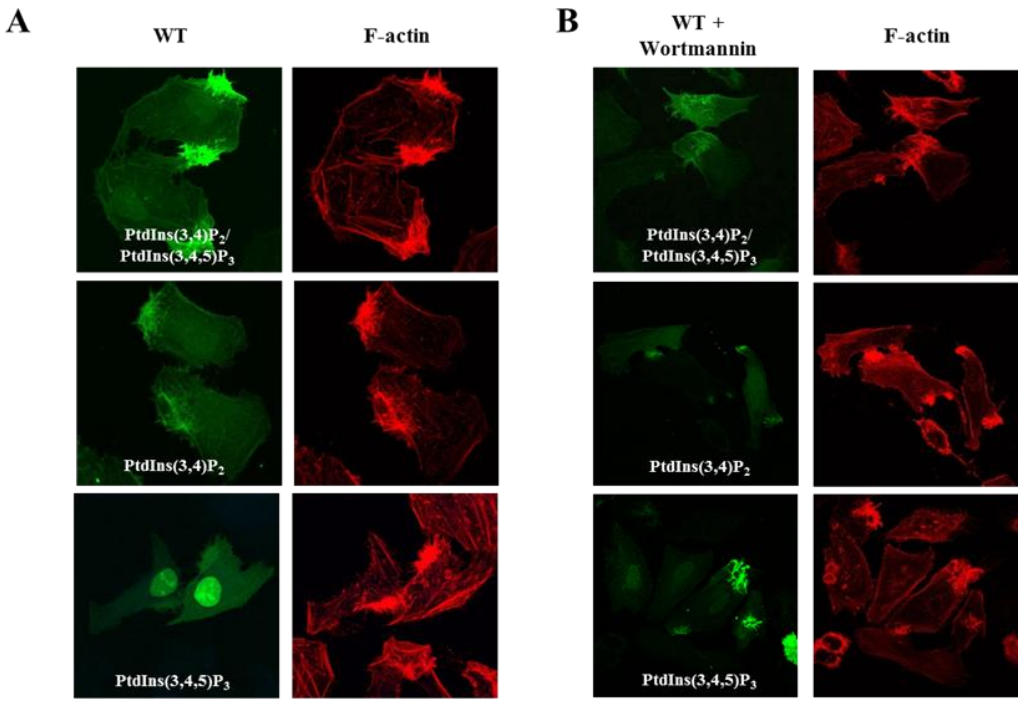

Fig. 2-2 PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 enrichment at the membrane ruffles is PI3K independent. HeLa cells were transfected with PH-Akt-EGFP the probe for PtdIns(3,4)P_2/ PtdIns(3,4,5)P_3, p47PX-EGFP the probe for PtdIns(3,4)P_2 or, GRP-EGFP the probe for PtdIns(3,4,5)P_3, for 24 hours (A) Following transfection the cells were infected with WT *Salmonella* at an MOI of 10 for 15 minutes. (B) Following transfection the cells were treated with 100nM wortmannin for 1hr and infected with WT *Salmonella* at an MOI of 10 for 15 minutes in the presence of the inhibitor. F-actin was visualized by staining with Texas Red conjugated Phalloidin (red). Images are a maximum intensity projection of Z-stacking images taken on a Zeiss LMS 700 confocal microscope.

2.4.2 Host proteins SKIP and PI4KIIβ are required for SopB-mediated PtdIns(3)P production.

We demonstrated that PtdIns(3)P production at the *Salmonella* ruffles was not dependent upon PI3K activity during WT *Salmonella* infection. We speculated that SopB is acting on host substrates PtdIns(3,4)P_2 and/or PtdIns(3,4,5)P_3 which are also enriched at the membrane ruffles in the absence of PI3K. To deduce which phosphatidylinositol could be the substrate of SopB we first sought to determine if any host phosphatases or kinases were involved in the SopB-mediated production

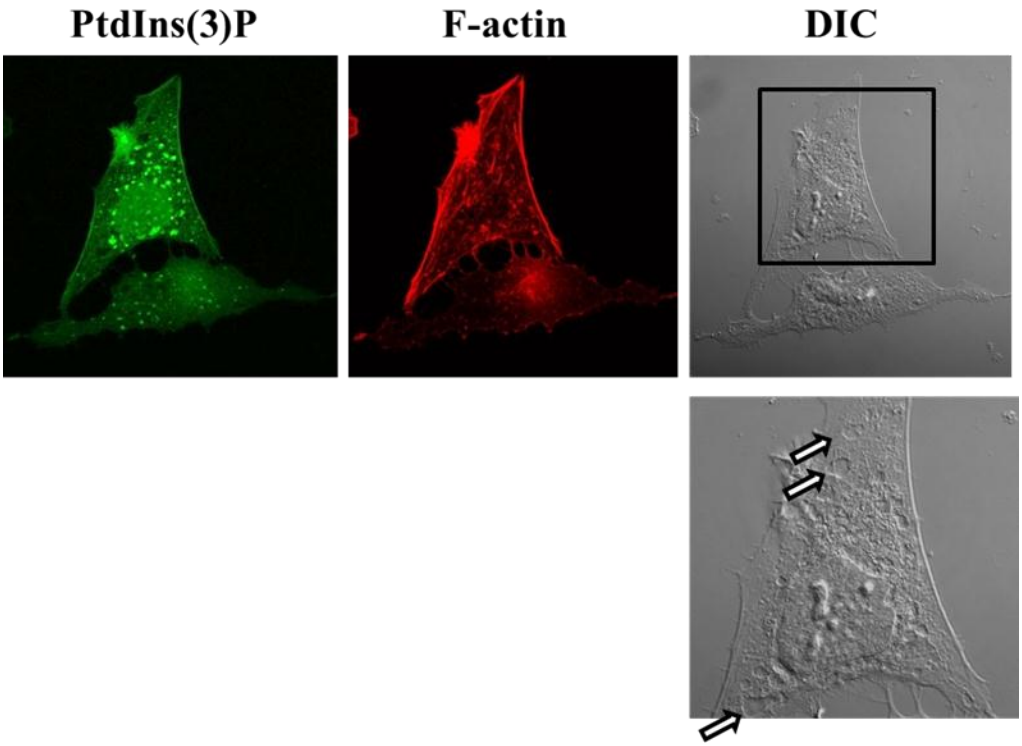

Fig. 2-3 PtdIns(3,5)P_2 is not required for SopB-mediated PtdIns(3)P production. HeLa cells were transfected with the probe for PtdIns(3)P – p40PX-EGFP (green) for 24 hours and treated with 800nM YM201636 for 1 hour. The cells were then infected with WT *Salmonella* at an MOI of 10 for 15 minutes. Ruffles are visualized by staining with Texas Red conjugated Phalloidin (red). The box around the DIC image represents the area which is magnified below. Arrows point to enlarged vesicles. All images were taken with a Zeiss LSM 700 confocal microscope.

of PtdIns(3)P. The involvement of PI(3)K and PIKFyve was already ruled out using the specific inhibitors wortmannin and YM201636 respectively. To examine the involvement of other host proteins we established a screening of host phosphatidylinositol phosphate phosphatases and kinases. shRNA constructs from Santa Cruz Biotechnology were used to knockdown the expression of the genes listed in Table 1. Each shRNA consists of a pool of 3-5 plasmids encoding target specific 19-25 nucleotides. To assess the possible involvement of these genes in SopB-mediated PtdIns(3)P production, HeLa cells were co-transfected with an shRNA construct, and p40PX-EGFP, the probe for PtdIns(3)P. The rationale is that if a kinase or phosphatase is involved in providing SopB with its substrate there would be no PtdIns(3)P production at the ruffles following WT infection. 48 hours following

co-transfection of the PtdIns(3)P probe and one of the shRNA constructs, cells were infected with WT *Salmonella* at an MOI of 10 for 15 minutes. *Salmonella*-induced ruffles that lacked PtdIns(3)P production at corresponding ruffles were investigated. We found two shRNA constructs capable of abolishing PtdIns(3)P production at the *Salmonella*- induced ruffles, one targeting SKIP and the other targeting PI4KIIβ (Fig. 2-4 A). SKIP is a PtdIns(3,4,5)P_3 5-phosphatase (Gurung et al., 2003; Ijuin et al., 2000) while PI4KIIβ is PtdIns 4-kinase (Jung et al., 2008; Pizarro-Cerda et al., 2007).

The role of host protein SKIP in SopB-mediated PtdIns(3)P production could indicate that PtdIns(3,4)P_2 is a possible substrate of SopB. SKIP is localized to the endoplasmic reticulum (ER) in resting cells and upon stimulation with growth factor translocates to the plasma membrane (Gurung et al., 2003). shRNA targeting this gene abolishes the conversion of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 during *Salmonella* infection. We had wanted to use the lipid probes to visualize the conversion of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2, but there are other host PtdIns(3,4,5)P_3 5-phosphatases, and the probes seem to be saturated at the membrane ruffles during *Salmonella* infection making any subtle changes impossible to detect. The potential role of PI4KIIβ in the pathway was a little less clear. PI4KIIβ mainly produces the precursor to PtdIns(4,5)P_2, PtdIns(4)P (Jung et al., 2008). It is possible that knockdown of PI4KIIβ was able to alter the phosphatidylinositol phosphate composition at the plasma membrane in a way that hindered PtdIns(3)P production. PtdIns(4,5)P_2 is the major phosphatidylinositol phosphate species at the plasma membrane and also the precursor of PtdIns(3,4,5)P_3. Any upset in phosphatidylinositol phosphatase balance at the plasma membrane could alter dynamic production and degradation of all other phosphatidylinositol phosphates. It is also possible that during WT *Salmonella* infection PI4KIIβ acts on the host substrate PtdIns(3)P to produce PtdIns(3,4)P_2. This seems a little counterintuitive, that PI4KIIβ would phosphorylate PtdIns(3)P into PtdIns(3,4)P_2 just so SopB could hydrolyze PtdIns(3,4)P_2, but this could indicate that the cycling of PtdIns(3,4)P_2 to PtdIns(3)P is an important aspect in *Salmonella* infection, something that will be investigated in a later chapter. The very precise lipid composition at the plasma membrane must be maintained and this kinase could be involved in maintaining the

balance, providing the substrate PtdIns(3,4)P_2 to SopB. Following our visual analysis of PtdIns(3)P production at the ruffles , we also performed an invasion assay following shRNA transfection to determine if the knockdown of these two genes had any effect on *Salmonella* invasion. We found that following WT infection, invasion levels for cells treated with both the SKIP and PI4KIIβ shRNA dropped to the invasion level of the *sopB*^{C460S} mutant strain (Fig 2-4C). This phenotype provided further evidence these shRNA constructs targeted genes involved in the SopB-mediated pathway.

Table 1. shRNA gene targets. Gene name and accession numbers of host phosphatases and kinases targeted by shRNA constructs obtained from Santa Cruz Biotechnology.

Gene Name	Accession No.
PI4KIIA	NM_018425
PI4KA	NM_002650
PI4K2B	NM_018323
PIP4K2B	NM_003559
PIK3R5	NM_014308
PIK3R3	NM_003629
PIK3CA	NM_006218
PIK3R1	NM_181504
PIK3R4	NM_014602
PIK3CB	NM_006219
PIK3CD	NM_005026
PIK3C2A	NM_002645
PIK3C2B	NM_002646
PIK3C3	NM_002647
PIP5KL1	NM_173492
PIP5K2C	NM_024779
PIP5K3	NM_152671
PIP5K1A	NM_003557
PIP5K1B	NM_003558
PIP5K1C	NM_012398
AKT1	NM_001014431
PAK3	NM_002578
CDC42BPA	NM_014826
EEA1	NM_003566
Rabenosyn-5	NM_022340
Fgd1	NM_04463
PTEN	NM_000314
MTM1	NM_000252
SKIP	NM_016532
PPAP2B	NM_003713
INPPL1	NM_001567
INPP5B	NM_005540

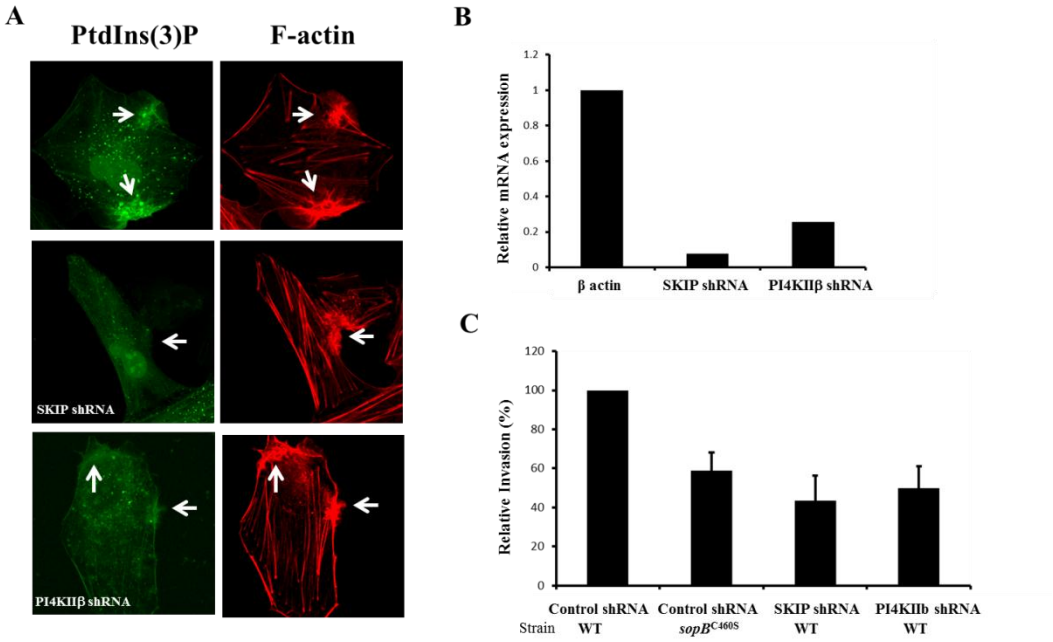

Fig. 2-4 SKIP and PI4KIIβ are involved in SopB-mediated PtdIns(3)P production. (A) HeLa cells were transfected with the probe for PtdIns(3)P- p40PX-EGFP or co-transfected with p40PX-EGFP and SKIP shRNA or PI4KIIβ shRNA as indicated for 48 hours. The cells were then infected with WT *Salmonella* at an MOI of 10 for 15 minutes. Ruffles were detected by staining with Texas Red conjugated Phalloidin (red). Arrows point at ruffles and corresponding PtdIns(3)P signal. (B) HeLa cells were transfected with the indicated shRNA for 48 hours. Relative mRNA expression was determined using β-actin control to detect shRNA knockdown efficiency. Results are normalized to β-actin expression. (C) HeLa cells were transfected with fluorescein conjugated control shRNA or co-transfected with GFP and the indicated shRNA for 48 hours. The cells were then infected with the indicated strain at an MOI of 3 for 10 minutes. Invasion rates were quantified by inside/outside differential staining and the invasion by WT *Salmonella* was normalized to 100%. The data is representative of 3 separate experiments with SD as shown

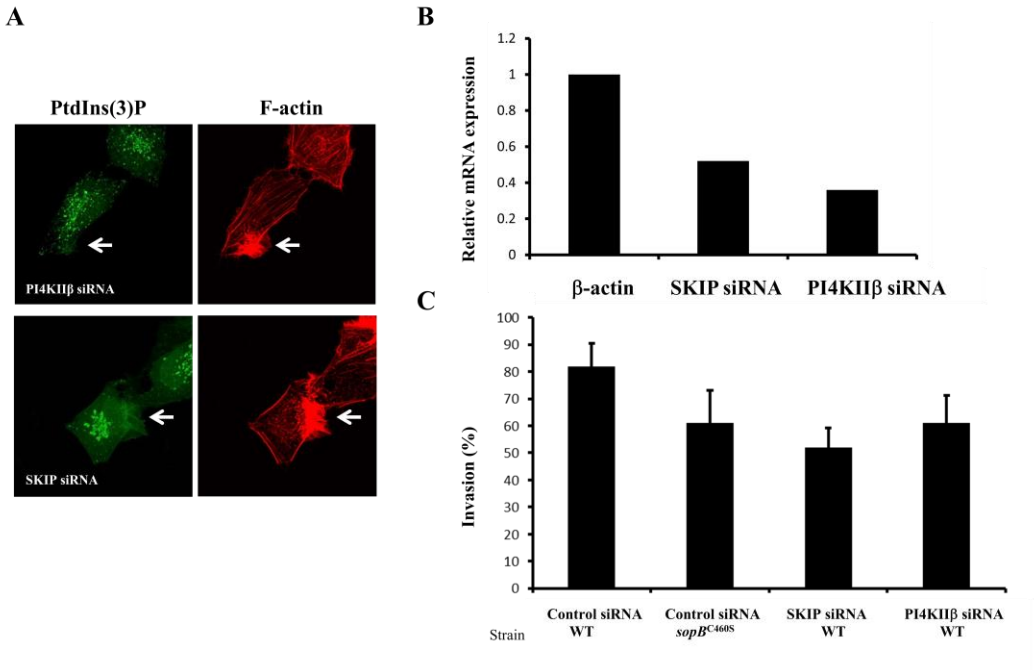

Fig. 2-5 siRNA knockdown of SKIP and PI4KIIβ interferes with SopB-mediated PtdIns(3)P production at the *Salmonella* ruffles and invasion. (A) HeLa cells were co-transfected with siRNA targeting the indicated gene and the probe for PtdIns(3)P- p40PX-EGFP for 48 hours. The cells were then infected with WT *Salmonella* at an MOI of 10 for 15 minutes. Ruffles were visualized by staining with Texas Red conjugated Phalloidin. Arrows point to ruffle and corresponding PtdIns(3)P signal. (B) HeLa cells were transfected with the indicated siRNA for 48 hours. Relative mRNA expression was determined using β-actin control to detect shRNA knockdown efficiency. Results are normalized to β-actin expression. C) HeLa cells were co-transfected with GFP and the indicated siRNA and infected with the indicated strain at an MOI of 3 for 10 minutes. Invasion was quantified by inside/outside differential staining. The data is representative of 3 separate experiments with SD as shown.

To confirm the knockdown efficiency conferred by the shRNA transfection, real time PCR was used. Unfortunately we were not able to confirm knockdown at the protein level by western blot analysis because we were not able to obtain adequate commercial antibodies against either protein. We therefore validated shRNA knockdown with mRNA expression in the shRNA treated cells (Fig. 2-4C).

For an independent assessment and confidence that the results were not due to off-target effects of the shRNA cells were transfected with siRNA from Ambion, targeting the same two genes (Fig. 2-5). We were able to see that cells transfected

with either PI4KIIβ siRNA or SKIP siRNA no longer produced PtdIns(3)P at the membrane ruffles following WT *Salmonella* infection (Fig. 2-5A). We also saw that the invasion rate of the siRNA treated cells infected with WT *Salmonella* were similar to the invasion rate of cells infected with the *sopB*^{C460S} mutant strain corroborating the results seen in cells treated with the shRNA constructs (Fig. 2-5C).

2.4.3 SKIP and PI4KIIβ overexpression and SopB-mediated ruffling

The knockdown of either SKIP or PI4KIIβ abolished SopB-mediated production of PtdIns(3)P and invasion into HeLa cells. The knockdown of these genes either blocked substrate production for SopB or perturbed the balance of phosphatidylinositol phosphates at the plasma membrane in such a way that the substrate could not be produced. This prompted us to investigate the overexpression of these proteins. To determine any role overexpression of these proteins could have and to further elucidate their role in the SopB-mediated PtdIns(3)P production pathway, HeLa cells were transfected with SKIP-GFP or PI4KIIβ-RFP constructs for 24 hours and infected with the *sopB*^{C460S} mutant strain for 15 minutes. Interestingly, we found that overexpressing both proteins rescued the invasion defect characteristic of the *sopB*^{C460S} infection (Fig. 2-6B). Both proteins were recruited to the *Salmonella* induced ruffles following infection with the mutant strain (Fig 2-6 A). The rescue of invasion in the SopB mutant by overexpressing these proteins could indicate that either of these proteins may be able to compensate for the loss of SopB and its 4-phosphatase or 5-phosphatase activity during infection. It is also possible that the products created by these proteins could be having some effect on the plasma membrane lipid composition which enhanced invasion.

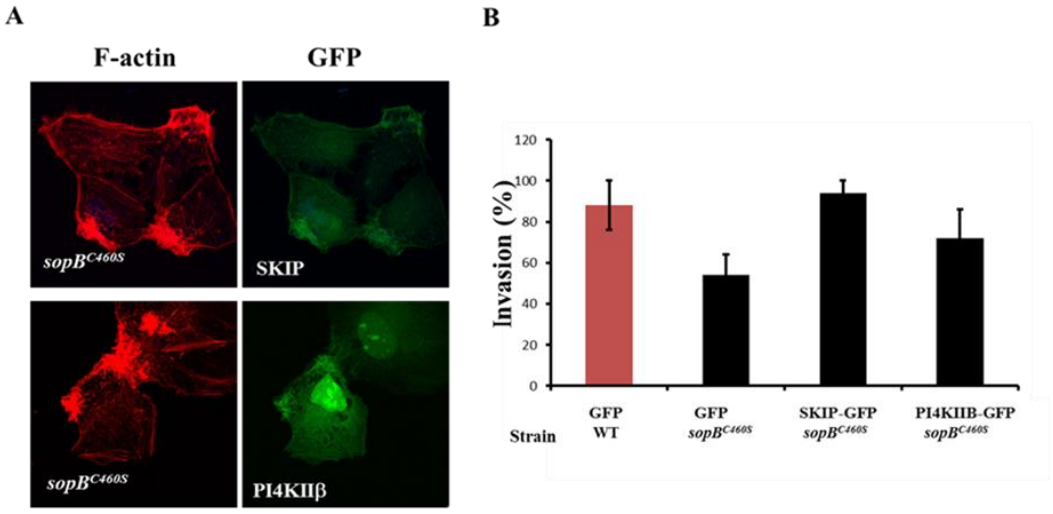

Fig. 2-6 SKIP and PI4KIIβ overexpression rescue the *sopB*^{C460S} invasion defect. (A) HeLa cells were transfected with either SKIP-EGFP or PI4KIIβ-RFP for 24 hours. Following transfection the cells were infected with *sopB*^{C460A} at an MOI of 10 for 15 minutes. Ruffles were visualized by staining with Texas Red conjugated Phalloidin. (B) HeLa cells were transfected with GFP control or SKIP-GFP or PI4KIIβ-RFP and infected with WT or the *sopB*^{C460S} strain as indicated. Invasion of transfected cells was quantified by inside/outside differential staining. The data is representative of 3 separate experiments with SD as shown.

There is a balance of phosphatidylinositol species that constantly cycle through a series of phosphorylation and dephosphorylation events at the plasma membrane and keeping this dynamic allows for efficient host processes such as phagocytosis and ruffle formation. It's possible the products of these proteins may have a role in altering the cytoskeletal structure of the plasma membrane during infection to enhance invasion, which is the phenotype seen in Fig 2-6B. To assess if SKIP and PI4KIIβ are able to compensate for SopB during invasion we overexpressed the proteins and infected with the *sopB-sopE-* strain for 15 minutes. The double deletion strain was used for this experiment because it is known that the

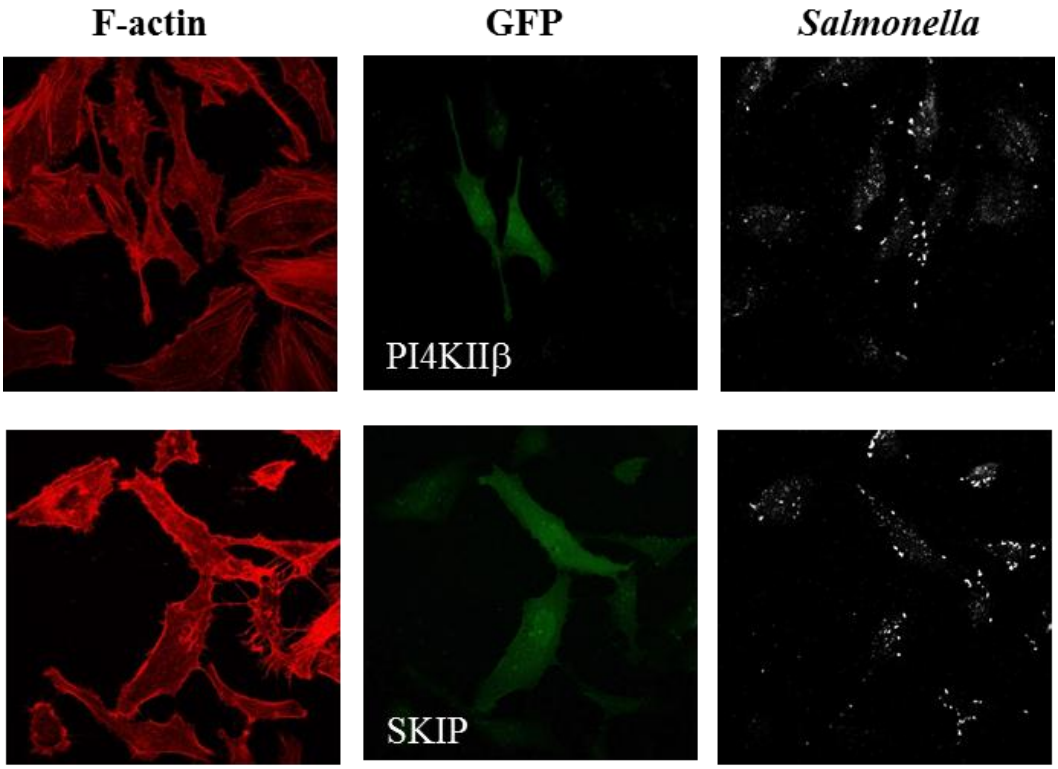

Fig. 2-7 SKIP or PI4KIIβ overexpression does not rescue the invasion phenotype of *sopB-sopE-* infected cells. HeLa cells were transfected with either SKIP-GFP or PI4KIIβ-GFP for 24 hours and infected with the invasion defective mutant strain *sopB-sopE-* at an MOI of 10 for 15 minutes. Ruffles were visualized by staining with Texas Red conjugated Phalloidin and *Salmonella* (white) was detected with rabbit anti-*Salmonella* anti-O antibody.

ruffling phenotype of SopB is often masked by SopE (Zhou et al., 2001). We found that overexpression of either protein was not sufficient to rescue the ruffling phenotype of the double deletion strain (Fig 2-7). This data is in contrast to that seen during the invasion of $sopB^{C460S}$ infected cells overexpressing the proteins. This does not allow us to make any solid conclusions, but rather indicates a number of possibilities. It is possible that the knockdown data regarding SKIP confirms the hypothesis that this gene is providing SopB with its substrate PtdIns(3,4)P_2 and without SKIP, there is no available substrate for SopB to hydrolyze to produce detectable PtdIns(3)P. It's also possible that the increased production of PtdIns(3,4)P_2 is important for some aspect of *Salmonella* invasion. The importance of PtdIns(3,4)P_2 could tie into the overexpression data seen in the $sopB^{C460S}$ infected

cells where the invasion defect of the SopB mutant was rescued. As seen previously, only in the presence of membrane alteration causing cell stimulation (such as that seen by the effector SopE) is SKIP recruited to the plasma membrane to produce PtdIns(3,4)P_2.In the absence of any membrane perturbations as seen with the *sopEsopB* double deletion infected cells, SKIP is probably localized to the cytosol or ER and is not active. We can speculate from these results that PtdIns(3,4)P_2 is an important aspect of invasion and is required for SopB-mediated PtdIns(3)P production.

Alternately PI4KIIβ provides the precursor for PtdIns(4,5)P_2. PtdIns(4,5)P_2 is an important component of the plasma membrane and anything that hinders its formation such as the knockdown of PI4KIIβ could have huge consequences on the dynamics of the cytoskeleton. PtdIns(4,5)P_2 recruits a number of actin binding proteins that directly affect the actin cytoskeleton. Additionally, PtdIns(4,5)P_2 at the plasma membrane is a precursor for PtdIns(3,4,5)P_3. Cells that lack PI4KIIβ, will display a decrease in PtdIns(3,4,5)P_3 and therefore less substrate for SKIP and less PtdIns(3,4)P_2. An increase in PtdIns(4,5)P_2 as seen in the overexpression data could alter the membrane during *Salmonella* infection in a way that is favorable for *Salmonella* invasion, but this must rely on ability of *Salmonella* to trigger the activation of Rac1 and Cdc42 by effector SopE. The product of PI4KIIβ, PI(4)P may only be converted to PtdIns(4,5)P_2 in cells that are stimulated and without stimulation, won't have any effect on membrane dynamics.

Recent work on the bacterium *Legionella pneumophila* characterized the effector protein SidF to be a PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 3-phosphatase(Hsu et al., 2012). The full length protein localizes to the ER, while the truncated 1-760 amino acids translocates to the cell periphery. To reduce PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 in the cell HeLa cells were transfected with GFP-SidF and infected with WT or *sopE*- for 10 minutes. The localization of the full length SidF is at the ER as expected in the uninfected cells, and the protein did not appear to get recruited to the *Salmonella* ruffles during WT or *sopE*- invasion as we had expected, this these are areas enriched in PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Fig. 2-8A). Additionally, in cells infected with the *sopE*- strain, there was no abolishment of ruffling, which indicates SopB is still

able to function and its substrate must still be present. Because the SidF truncation translocates to the plasma membrane and is still fully functional as a phosphatase (Fig. 2-8C), we wanted to determine if localized to the membrane, if this protein could affect *Salmonella* PtdIns(3)P production. HeLa cells were co-transfected with the truncated SidF1-760 and p40PX-RFP and infected with WT *Salmonella* for 10 minutes. Even though SidF is clearly present at the membrane ruffle, PtdIns(3)P is still produced a the membrane ruffle (Fig. 2-8B). We were not able to conclude that this protein was functional *in vivo* as during *Salmonella* infection, the probes for PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 are saturated and there was no detectible difference in the presence of truncated SidF. It's possible that this protein is not active, or does not compete effectively with SopB for substrate.

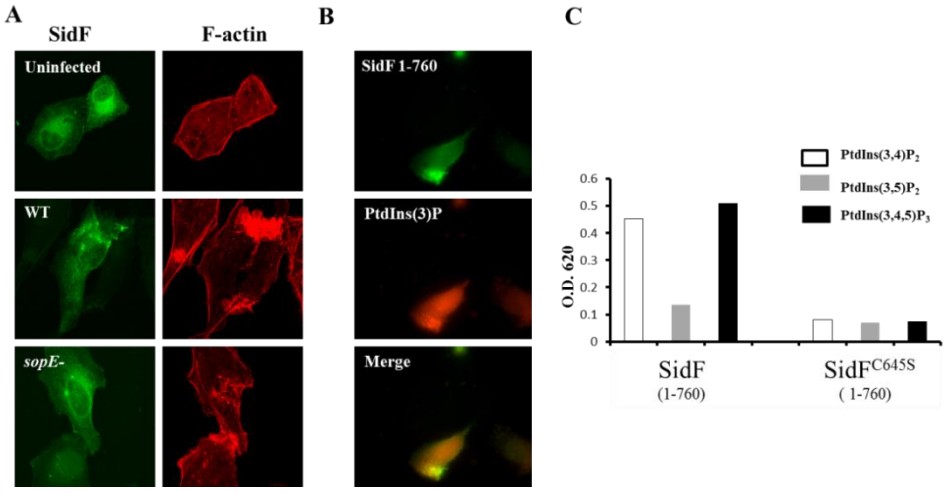

Fig. 2-8 *Legionella* effector SidF, does not affect *Salmonella* ruffling or PtdIns(3)P production. A) HeLa cells were transfected with GFP-SidF for 24 hours and infected with WT *Salmonella* or *sopE*- strain at an MOI of 10 for 10 minutes. Ruffles were visualized by staining for F-actin with Texas Red Phalloidin. B) HeLa cells were co-transfected with GFP-SidF1-760 and the probe for PtdIns(3)P- P40PX-RFP for 24 hours and infected with WT *Salmonella* at an MOI of 10 for 10 minutes. C) Quantification of phosphates released using the malachite green phosphatase assay.

2.4.4 Development of a yeast model to screen for proteins involved in SopB-mediated PtdIns(3)P production

At the same time the shRNA screening using mammalian epithelial cells was performed, we wanted an alternate method to screen the host phosphatases and kinases. Utilizing the yeast model would be an independent method to determine if any host proteins are involved in the SopB-mediated production of PtdIns(3)P. In order test the involvement of host phosphatases and kinases in this model we needed to establish a phenotype differentiating the WT SopB and the $SopB^{C460S}$ mutant expressing yeast cells. This will act as a baseline in the yeast model to monitor. We hoped to create a model for PtdIns(3)P distribution that mirrors that of the mammalian model where the $sopB^{C460S}$ infected cells had no PtdIns(3)P production at the ruffles compared to the WT infected. The yeast model would not be using infection conditions, and would only take into account the overall PtdIns(3)P signal. The *Saccharomyces cerevisiae* strain, W303 was used for our experiments to assess cell morphology, actin staining, PtdIns(3)P distribution, and vacuolar morphology in cells transformed with either empty vector – p426MET25, p426MET25 encoding WT SopB, or p426MET25-$SopB^{C460S}$. p426MET25 is a yeast vector containing a methionine repressible promoter that can regulate gene expression based upon the concentration of methionine used. This vector was chosen because SopB proved to be toxic to yeast and the addition of methionine to the samples reduced the expression of SopB and the toxicity to yeast cells transformed with the phosphatase (Mumberg et al., 1994). Yeast morphology and actin staining in yeast transformed with both the WT SopB and the mutant have been previously characterized and we verified these results independently (Aleman et al., 2005; Rodriguez-Escudero et al., 2006). We were able to confirm these results using the p426MET25 vector with the addition of .2mM methionine confirming the activity of SopB in the yeast model.

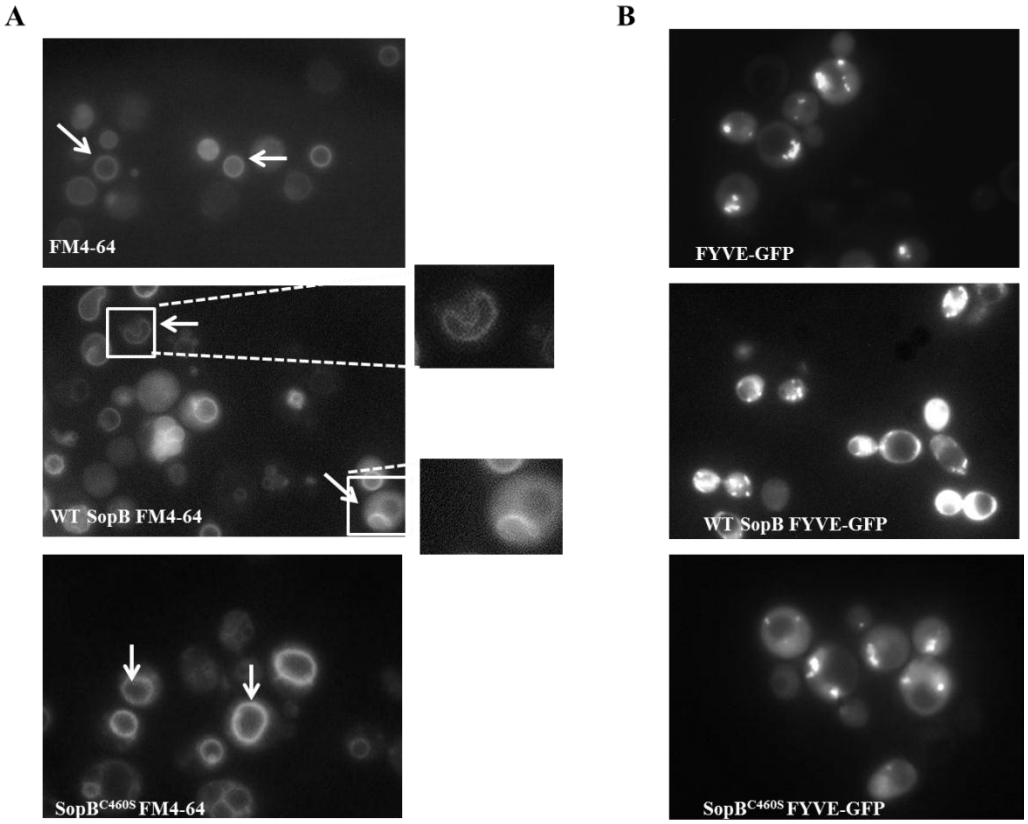

Fig 2-9 Development of a yeast model for SopB activity. (A) FM4-64 staining of yeast cells expressing WT SopB and SopB^{C460S}. Arrows point to vacuolar structures characteristic of those yeast cells. The vacuoles in WT SopB expressing cells are magnified for detail. (B) Yeast cells co-expressing the probe for PtdIns(3)P p414MET25-FYVE3-GFP and either WT SopB or SopB^{C460S} are grown overnight and visualized for GFP signal.

To analyze PtdIns(3)P distribution yeast were co-transformed with WT SopB or the SopB^{C460S} mutant along with the GFP-FYVE3-p414MET25, a probe that specifically targets PtdIns(3)P in the cells or GFP-FYVE3-p414MET25 alone. This probe has been published for use in the yeast system (Audhya et al., 2000) and PtdIns(3)P can be visualized in early endosomes in a punctate localization. The localization of PtdIns(3)P in the yeast expressing both the GFP probe and the SopB^{C460S} mutant had a punctate localization. We were able to see PtdIns(3)P at distinct puncta characteristic of PtdIns(3)P on early endosomes as seen in the cells not expressing SopB. (Fig 2-9 B) However, when expressing both WT SopB and GFP-FYVE, the signal of PtdIns(3)P was greatly enhanced compared to the strain expressing the mutant SopB (Fig. 2-9B) . It appeared as though there were more puncta and some of

the signal even appeared cytosolic. This phenotype resembles that of the eukaryotic cells infected with the WT *Salmonella* strain and the *sopB*^{C460S} strain where the former shows and obvious enrichment of PtdIns(3)P compared to the distribution of cells infected with the mutant strain. This could be a baseline phenotype in which to test the involvement of host proteins in SopB-mediated PtdIns(3)P production. (Audhya et al., 2000)

In addition, the vacuole morphology using the FM 4-64 dye to label yeast vacuole membranes was assessed. Previous literature has characterized the phenotype of the temperature sensitive yeast PI4K mutant *stt4*^{ts} as having a role in vacuolar structure. Additionally a mutant for the Fab1P which is a yeast 5-kinase, *Fab1*, alters the vacuolar morphology as well causing enlarged vacuoles that occupy the entire cell (Gary et al., 1998). These are important mutants to investigate since a mutation in the yeast 4-kinase or 5-kinase would potentially have a phenotype similar to that of WT SopB which has 4-phosphatase and 5-phosphtase activity. Researchers demonstrated that when treated with the vacuole membrane dye FM4-64, the *stt4*^{ts} yeast strain displayed a defect in vacuole structure where they appeared to fuse together or collapse. When we investigated vacuole morphology with yeast expressing WT SopB and SopB^{C460S}, we saw that the WT SopB was able to cause a similar vacuole collapse that the yeast 4-kinase mutation caused (Fig. 2-9A). This was another promising phenotype that was able to differentiate the WT SopB phenotype from that of the mutant which had round vesicles similar to control cells. We had hoped to use the yeast model to corroborate the data from the shRNA screening in mammalian cells, but this model did have limitations. We found that PtdIns(3,4,5)P_3 is not a component of the yeast strain we were using, and there is no SKIP homologue in yeast. This meant that we could not use this model to corroborate the role of a SKIP knockout or temperature sensitive mutant on PtdIns(3)P distribution when transformed with WT SopB. Given the phenotypic differences between SopB and the C460S mutant, this could potentially be a great model for future work.

2.5 Discussion

This chapter confirmed that the host PI(3)K is not required for the SopB-mediated production of PtdIns(3)P at the *Salmonella* induced membrane ruffles. This led to the hypothesis that SopB is acting upon a host substrate to produce PtdIns(3)P. It was important to confirm this result because of recent data from Mallo et al. implicating host Vsp34 a PI(3)K in the production of PtdIns(3)P at the SCV (Mallo et al., 2008). It was also crucial to investigate the wortmannin sensitivity of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 at the *Salmonella* ruffles to ensure they were potential host substrates. The presence of both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 at the ruffles in the absence of PI3K indicated that either phosphatidylinositol could be the substrate of SopB. As of yet, there is no specific probe to monitor the localization or signal of PtdIns(3,5)P_2 but we were able to rule out the involvement of this phosphatidylinositol by treating cells with the PIKFYVE inhibitor YM201636 (Jefferies et al., 2008; Kerr et al., 2010) which depletes the cell of PtdIns(3,5)P_2 by inhibiting the activity of the PtdIns(3)P 5-kinase. Our data demonstrates that even in the presence of this inhibitor, PtdIns(3)P was still produced at the ruffles during WT infection.

To pinpoint one or the other phosphatidylinositol phosphate as the substrate of SopB, or to provide further insight into the mechanism of SopB-mediated PtdIns(3)P production we conducted a screening of host phosphatases and kinases. This screening indicated the involvement of two host proteins in the SopB-mediated production of PtdIns(3)P. Host PtdIns(3,4,5)P_3 5-phosphatase SKIP (Gurung et al., 2003; Ijuin et al., 2000) when targeted via shRNA abolished the ability of SopB to produce PtdIns(3)P at the membrane ruffles. We hypothesized that SKIP could be providing SopB with the substrate PtdIns(3,4)P_2, that SopB would then hydrolyze to PtdIns(3)P. An invasion assay following transfection with the shRNA targeting SKIP revealed a decrease in invasion to the level of cells infected with the *sopB*^{C460S} mutant. This provides further evidence that SKIP could be providing SopB with its substrate. When SKIP was overexpressed and infected with the SopB mutant we found that SopB-mediated invasion was restored. This was an unexpected result and it led us to

speculate that the product of SKIP, PtdIns(3,4)P_2 could be important for invasion. In order to come to a solid conclusion about what is happening we would need to take a closer look at both the involvement of PtdIns(3,4)P_2 in *Salmonella* invasion, as well as the effect that SKIP overexpression has on the membrane structure and the process of phagocytosis leading to bacterial invasion. It has recently been shown that PtdIns(3,4)P_2 is able to recruit SNX9 to the membrane where it promotes F-actin nucleation (Yarar et al., 2008). This is one piece of evidence indicating that this product of SKIP could be involved in ruffle formation itself.

We speculated the lack of phenotype in cells overexpressing SKIP and infected with the *sopBsopE* strain, was due to an inactive SKIP protein. The cell must be stimulated before SKIP can translocate to the plasma membrane where it produces PtdIns(3,4)P_2. During WT *Salmonella* infection, PtdIns(3,4)P_2 levels increase at the membrane ruffles in a SopB-mediated manner. When SKIP was overexpressed in the *sopB* mutant strain, we presume this leads to the SopB-independent increase of PtdIns(3,4)P_2 which could compensate for the function of SopB possibly by continuing to recruit actin modulating proteins to the plasma membrane facilitating invasion.

The other host protein found to be involved in the SopB-mediated production of PtdIns(3)P was PI4KIIβ (Pizarro-Cerda et al., 2007). This is a 4-kinase that produces PtdIns(4)P which is the main precursor for PtdIns(4,5)P_2. PtdIns(4,5)P_2 is the main precursor for PtdIns(3,4,5)P_3 and it is possible that PI4KIIβ is providing SKIP with its substrate and in effect, SopB with its substrate. This kinase though, is most likely altering the process of phagocytosis. PtdIns(4,5)P_2 is the most abundant phosphatidylinositol at the plasma membrane and is known to be directly involved in ruffle formation, endocytosis, and phagocytosis. Because it forms the precursor for PtdIns(4,5)P_2, it is no wonder that the knockdown of PI4KIIβ has effects on SopB function and *Salmonella* invasion. PtdIns(4,5)P_2 production and subsequent hydrolysis has been analyzed with respect to *Salmonella* invasion previously (Terebiznik et al., 2002). The coordinated increase and decrease of this lipid is important for internalization of *Salmonella*. The overexpression of PI4KIIβ enhancing the invasion of the SopB point mutant, supports the idea that maintaining

levels of phosphatidylinositols at the plasma membrane is crucial to phagocytosis. Although the invasion levels in the *sopB* mutant increased, the quality and effects of overexpression on the size and maturation of the SCV was not investigated. It's possible that more bacteria were internalized, but that the SCV was altered in such a way that replication would be hindered later on in infection. The fact that PI4KIIβ was not able to rescue invasion in cells infected with the *sopBsopE* mutant, could once again that the cell may need to be stimulated in some respect for this proteins activity. (Botelho et al., 2000; Cain et al., 2008; Raucher et al., 2000; Sakisaka et al., 1997).

This data does lend us some insight into the mechanism of *Salmonella* invasion. We are able to speculate that SKIP is providing SopB with the substrate PtdIns(3,4)P_2 for subsequent PtdIns(3)P production and possibly the product PtdIns(3,4)P_2 is actually facilitating *Salmonella* invasion which could explain the overexpression data in the SopB mutant. All the overexpression data, though, could be explained by the drastic alteration of the phosphatidylinositol phosphate dynamics at the membrane. When these levels are perturbed, processes at the membrane such as phagocytosis can be altered. Further investigation into the effects SKIP and PI4KIIβ have on SCV generation and maturation would be useful in determining these conclusions. Overexpression of these phosphatases and kinases and their products could alter the composition of the forming SCV in a way that hinders maturation and later bacterial replication. Some early work investigated the importance of SopB hydrolyzing PtdIns(4,5)P_2 at the plasma membrane to aid in proper fission of the phagosome, but the effects of perturbing this phosphatidylinositol species later on in infection was not studied (Terebiznik et al., 2002).

Because the actin cytoskeleton is such a dynamic environment, we had hoped to corroborate this data using a yeast model for a clearer pathway. We were able to establish two distinct phenotypic differences between yeast expressing WT SopB and that expressing mutant SopB. A number of yeast libraries containing mutants of every single yeast gene are available and it would be interesting to see if WT SopB could have the PtdIns(3)P phenotype in a yeast strain mutated for SKIP. Unfortunately yeast do not contain PtdIns(3,4,5)P_3 and therefore have no SKIP

homologue. This model could still be used to other aspects as it clearly differentiates the phenotypes of either WT SopB or the mutant.

CHAPTER 3. SOPB REGULATES PHOSPHOINOSITIDE DYNAMICS TO PROMOTE BACTERIAL ENTRY

3.1 Abstract

Salmonella effector protein SopB is credited with a number of events required for *Salmonella* infection including SCV formation, maturation, membrane ruffling and invasion. SopB is known to possess both 4-phosphatase and 5-phosphatase activity and have a number of substrates *in vitro*. Previous work has demonstrated the involvement of the RhoGTPase RhoG in SopB-mediated invasion. So far the phosphatase activity of SopB which includes the products or the substrates has not been linked to the ability of SopB to promote ruffling and invasion. We will show here that SopB is able to alter the phosphoinositide composition at the plasma membrane allowing for ruffling and invasion required for efficient *Salmonella* infection.

3.2 Introduction

Salmonella spp. are facultative intracellular pathogens that have acquired the ability to enter non-phagocytic cells by modulating not only RhoGTPases which facilitate F-actin nucleation but also the phosphoinositide composition at the plasma membrane. Crucial to ruffle formation and subsequent invasion, are effectors SopB, SopE. Single deletions of these proteins lead to a modest decrease in ruffle formation while the *sopE-, sopB-* double deletion mutant drastically impairs ruffle formation as well as invasion into the host cell (Zhou et al., 2001). SopE is a known guanine nucleotide exchange factor for RhoGTPases CDC42 and Rac1 (Friebel et al., 2001), while SopB is a phosphatidylinositol phosphate phosphatase possessing 4-

phosphatase and 5-phosphatase activities(Marcus et al., 2001). *In vitro* studies have demonstrated that SopB has a number of substrates, while *in vivo* the substrate specificity of SopB is not well characterized. So far SopB was demonstrated to hydrolyze PtdIns(4,5)P_2. The deletion of SopB leads to enrichment of PtdIns(4,5)P2 at the ruffles and disruption of phagocytosis (Terebiznik et al., 2002).

SopB is responsible for the production of PtdIn(3)P at the *Salmonella*-induced membrane ruffles as well as on the surface of the SCV. The mechanism by which SopB induces the production of PtdIns(3)P on the SCV has been characterized. SopB recruits host protein Rab5 to the SCV where it acts as an effector for Vps34, a host PI3K. This is an indirect mechanism that requires the phosphatase activity of SopB (Mallo et al., 2008). The mechanism through which SopB produces PtdIns(3)P at the membrane ruffles is less clear. It's been demonstrated that the SopB-mediated production of PtdIns(3)P is wortmannin insensitive, in contrast to the production of PtdIns(3)P on the SCV. It has also been demonstrated that SopB produces PtdIns(3)P to recruit host protein VAMP8 to the ruffles aiding in phagocytosis (Dai et al., 2007; Pattni et al., 2001) (Pryor et al., 2004; Wang et al., 2004).

In addition to SopB mediated PtdIns(3)P production at the membrane, SopB has also been shown to mediate the increase of both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Dukes et al., 2006; Mallo et al., 2008). The role, if any, of these phosphoinositides in *Salmonella* infection has not been studied. This chapter investigates the mechanism through which SopB is able to produce PtdIns(3)P at the *Salmonella* induced membrane ruffles, and also goes a step further to implicate PtdIns(3,4)P_2 as an important product of SopB that recruits host protein SNX9 to the ruffles (Posor et al., 2013; Yarar et al., 2007). We also show that there is a dynamic balance of phosphoinositides at the plasma membrane and both the production and degradation of PtdIns(3,4)P_2 is required for sufficient ruffle formation and invasion.

3.3 Materials and Methods

Bacterial strains and plasmid construction

Wild-type *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) strain SL1344 (Hoiseth and Stocker, 1981) and its *sopB*^{C460S} (SB933), *sopB::aphT* (SB924) (Zhou et al., 2001), *sopBsopE* (ZP15), s*opB*^{C460S}*sopE* (ZP16) (Higashide and Zhou, 2006), and *sopE* (ZP189) (Hardt et al., 1998) mutant derivatives have been previously described. A mutant of SopB defective in its 5-phosphatase catalytic activity *sopB*^{K530A} (ZP471) was constructed by mutating the lysine at position 530 to an alanine using the QuikChange site-directed mutagenesis kit (Stratagene). The mutated gene was then subcloned into the BamHI site on a vector containing the in frame deletion of sopB- 500bp upstream and 500bp downstream in pSB890 (pZP153). pZP153 was introduced into the *Salmonella* chromosome by homologous recombination as described previously (Kaniga et al., 1994). The mutated gene was similarly introduced into the chromosome of ZP479 to create *sopB*^{K530A}*sopE* (ZP472). *sopEsopB::aphT* (ZP479) was constructed by cloning the Kanamycin resistance gene AphT into the BamHI site on (pZP152). The resulting pZP3186 was then subcloned into pSB890 and introduced into the chromosome of ZP189 by double homologous recombination creating ZP479.

Construction of pZP3179 used as the backbone for the *Salmonella* chromosomal insertion mutants was constructed in a stepwise manner. The promoter of SopB 500bp upstream of SopB and its translocation sequence (aa 1-100) were cloned into pSKII using SmaI-KpnI creating pZP3177. The downstream flanking 500bp of SopB was cloned into pDsRed using KpnI - NotI resulting in pZP3178. The SopB promoter and translocation sequence from pZP3177 were then subcloned into pZP3178 using KpnI-BamHI creating pZP3179. The SopB chromosomal substitution strain *sopB*::*ipgD* (ZP473) was created as follows. The SopB homologue IpgD was amplified from the *Shigella flexneri* strain M90T by PCR. The product was then cloned into the KpnI restriction site of pZP3179. The resulting pZP3180 plasmid using restriction sites EcoRV-NotI was subcloned into the SmaI-NotI sites of R6K-derived suicide vector pSB890 creating pZP3181 and introduced into the

chromosome of SB924 by double homologous recombination. Similarly *sopEsopB::ipgD* (ZP474) was created by introducing pZP3181 into the chromosome of ZP479. SKIP (MGC-862) and Inpp4B (MHS1768-99238348) cDNA clones were obtained from ATCC and Open Biosystems respectively. SKIP and Inpp4B were amplified by PCR and cloned into the KpnI site of pZP3179 creating pZP3184 and pZP3182 respectively. pZP3184 and pZP3182 were then subcloned using the restriction sites EcoRV-NotI into the SmaI-NotI sites of pSB890 to create pZP3185 and pZP3183 respectively. pZP3185 was then introduced into the chromosome of SB924 and ZP479 to create *sopB::SKIP* (ZP477) and *sopEsopB::SKIP* (ZP478) respectively. Similarly pZP3183 was introduced into the chromosome of SB924 and ZP479 by double homologous recombination to create *sopB::inpp4B* (ZP475) and *sopEsopB::inpp4B* (ZP476). To create the double insertion strains *sopEsopB::SKIP ipgD* (ZP484) and *sopEsopB::SKIP inpp4B* (ZP485), IpgD and Inpp4B were amplified and cloned using SacII-KpnI into the same sites of pZP3179 creating pZP3189 and pZP3188 respectively. SKIP was then amplified and cloned into the KpnI site of pZP3189 and pZP3188 to create pZP3190 and pZP3191 respectively. The resulting plasmids pZP3190 and pZP91 were subcloned using EcoRV-NotI into the SmaI-NotI sites of the suicide vector pSB890 creating pZP3193 and pZP3192. pZP3193 and pZP3192 were introduced into the chromosome of ZP189 as previously stated to create ZP484 and ZP485 respectively.

The probe for PtdIns(3)P p40PX-EGFP was described previously (Kanai et al., 2001). GFP-SNX9 and mCherry-SNX9 (RYK) were a gift from Dr. Schmid(Yarar et al., 2008).

His-tagged WT SopB(pZP3172) and its derivatives His-sopB^{C460S} (pZP3173) and His-sopB^{K530A} (pZP3174) were constructed as follows. WT SopB from pZP211 and *sopB^{C460S}* (pZP216) described previously (Zhou et al., 2001) were subcloned into the NotI-SalI sites or SacI, NotI sites of pET28b6HisA respectively. *sopB^{K530A}* (pZP2174) described previously (Perrett and Zhou, 2013)was subcloned into pET28b6HisA expression vector using NotI and SalI. Rab5 was cloned into YFP-C1 creating YFP-Rab5 (pZP889).

TAPP1, Tks4, and Bam32 clones were purchased from Thermo Scientific (MHS6278-202826789, MHS1010-74394, MHS1010-7429375) Tapp1 was cloned into RFP-C1 to create (pzP3275), Tks4 into GFP-C1 to create (pZP3274), and Bam32 into GFP-C1 to create (pZP3276). The phosphorylation deficient mutant of Bam32 was created by mutating the Tyrosine at position 139 to phenylalanine using the QuikChange site-directed mutagenesis kit (Stratagene) using the primers (#1907 and #1908) to create (pZP3277). The following oligo 5' CCGCGGGGAAGTCCAAGACAAAGTGTGTTATTATGTGAGCGGCCGC 3' 3' GCGGCCGCTCACATAATAACACACTTTGTCTTGGACTTCCCCGCGG 5' containing the CAAX motif was annealed and inserted into pEGFP-N1 to create (pZP3282) Inpp4B was subcloned from pZP3182 into CAAX-GFP-N1 to create (pZP3283). Inpp4B-CAAX was then subcloned from pZP3281 into pEYFP-C1 to create YFP-Inpp4B-CAAX (pZP3281).

The TEM1 fusion constructs used to monitor translocation of the exogenous phosphatases into the host cell were constructed as follows. The SopB gene was cloned into the EcoRI-BamHI sites of pM1644 previously described (Schlumberger et al., 2007) creating SopB-TEM1 (pZP3211). The pM1644 vector was modified by inserting an oligo containing the restriction sites SacII and EcoRV into the SmaI-EcoRI sites of pM1644 creating pZP3212. IpgD, Inpp4B, and SKIP were subcloned from pZP3180, pZP3182, and pZP3184 respectively into the NheI-SacII sites of pZP3212 creating IpgD-TEM1 (pZP3200), Inpp4B-TEM1 (pZP3201), and SKIP-TEM1(pZP3202). The first 100 amino acids of SopB containing the translocation sequence was then cloned into the NheI site of pZP3200, pZP3201, and pZP3202 creating pZP3206, pZP3207, and pZP3208 respectively.

Cell culture and bacterial infection

Mammalian cell line HeLa (CCL-2; ATCC, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. All cells were maintained at 37 °C and 5% CO2. *Salmonella* strains were routinely cultured in Luria-Bertani broth. For invasion experiments overnight cultures of *Salmonella* were diluted 1:30 in Luria-Broth supplemented with 0.3M NaCl and grown for 3 hours with low aeration on a rotating wheel. Following infection for 15 minutes, or as

indicated, ruffles were visualized using Texas Red conjugated Phalloidin for filamentous actin (Molecular Probes, Eugene, OR) and bacteria were visualized using rabbit anti-*Salmonella* O antigen group B (Difco) and a secondary anti-rabbit AF488 conjugate (Molecular Probes). Internalized bacteria were evaluated using the inside/outside differential staining assay as previously described (Chang et al., 2005) Images were taken on a Zeiss LSM 700 confocal microscope.

Protein purification and Phosphoinositide phosphatase activity assay

His-tagged recombinant WT SopB (pZP3172) protein and its derivatives $sopB^{C460S}$ (pZP3173) and $sopB^{K530A}$ (pZP3174) were expressed in Escherichia coli BL21 (DE3) using the pET expression system (EMD Biosciences, Madison, WI). The purification was performed solely in Tris buffered saline buffers void of Phosphates.

Phosphoinositide phosphatase activity was measured using the malachite green assay (Echelon Research Laboratories, Salt Lake City, UT, USA) as described in (Marcus et al., 2001). Briefly, recombinant purified protein (100 ng) was incubated with synthetic short chain (octanoyl) phosphoinositides (Echelon Research Laboratories, Salt Lake City, UT, USA) (50μM) as indicated. The reactions were stopped by addition of 80 μl malachite green reagent and absorbance measured at 620 nm. The assay provided linear detection in the range of up to 1000 pmol of Pi. Results represent an average of three separate reactions carried out for each protein and substrate.

Gene silencing by siRNA and transfection

SNX9 siRNA consisting of a pool of three to five target-specific 19-25 nt siRNAs was purchased from Santa Cruz biotechnology (sc-61597). HeLa cells were transfected for 48 and subsequently infected with the indicated strains for 15 minutes. Knockdown of SNX9 was confirmed via Western blot analysis using mouse monoclonal anti-SNX9 antibody from Abcam (ab118996) and mouse monoclonal anti-Actin antibody from Sigma (A3853-200UL). Cells were transfected with plasmids by using TransIT-express transfection reagent (Mirus) according to the manufacturer's instructions and infected 24 h after transfection. Statistical analysis was performed using a student *t*-test.

Immunoprecipitation

HeLa cells were seeded on a 6-well plate and transfected with GFP-Bam32 and the tyrosine mutant Y139F for 24 hours. HeLa cells were rinsed with PBS and then HBSS. The cells were infected at an MOI of 300 where indicated with either WT or the *sopB*^{C460} strain for 15 minutes. The cells monolayer was washed 2X with ice cold PBS and the cells were lysed with 500µL ice cold lysis buffer (20mM Tris-HCL, pH7.4, 150mMNaCl, 1% Triton X-100, 1mM EDTA, 1mM EGTA, 1mMPMSF, 1% Protease cocktail (Sigma P3840)). The lysates were then centrifuged at 10,000g for 10 minutes at 4◦C. Lysates were precleared with 20 µL protein A/G PLUS Agaraose (Santa Cruz Biotechnology) for 60 minutes on a rotator at 4◦C. The samples were spun at 1000g for 1 minute at 4◦C. 100uL of the supernatant was mixed with 2X gel loading buffer for the Pre-IP sample. 1uL of Rabbit anti-GFP antibody was added to the remaining supernatant and incubated for 60 minutes at 4C on the rotator. The immune complex was collected with 20uL Protein A/G PLUS-Agaraose overnight at 4C. The samples were spun at 1000g for 1 minutes at 4C. The pellet was washed 3X with ice-cold lysis buffer and once with ice cold lysis buffer without Triton-X100. 50uL of 1X gel loading buffer was added to washed pellet. After SDS PAGE and transfer onto nitrocellulose membrane, the blot was probed with mouse anti-4G10 and anti GFP antibodies.

Beta-lactamase assay

The Beta lactamase translocation assay described previously (Charpentier and Oswald, 2004) (Schlumberger et al., 2007) was performed to monitor protein translocation of the exogenous 5-phosphatase and 4-phosphatases into the host cell. HeLa cells were grown in a 96-well plate washed twice with Hank's balanced salt solution (HBSS, Gibco) and subsequently infected for 30 minutes with the indicated *S. Typhimurium* strain. Bacterial strains were subcultured (1:20) in LB supplemented with NaCl to .3M in the presence of .5mM IPTG prior to infection. The Beta-lactamase assay (Life Technologies, Madison, WI) was performed according to manufacturer's instructions. Following infection the CCF4-AM substrates (Invitrogen, Carlsbad, CA) were added to the wells for 1 hour. The percentage of infected cells was quantified by counting the number of cells emitting blue fluorescence.

3.4 Results

3.4.1 The 5-phosphatase activity of SopB is required for SopB-mediated ruffling and invasion

The 4-phosphatase activity of SopB is essential for ruffling and subsequent invasion of *Salmonella* into the host cell (Zhou et al., 2001). Previous research has already characterized the SopB point mutant at C460S. When a malachite green phosphatase assay was performed, this single point mutation was shown to render SopB catalytically inactive for all substrates *in vitro* (Marcus et al., 2001). Additional studies show that this point mutant no longer has the ability to cause SopB-mediated ruffling. In addition to the characterized 4-phosphatase activity, SopB also contains less characterized 5-phosphatase homology to host 5-phosphatase synaptojanin. Only *in vitro* studies with the 5-phosphatse point mutant at K530A have been performed. A malachite green assay demonstrated that this point mutant, like the SopB^{C460S} had no catalytic activity towards any of the substrates WT SopB is able to hydrolyze. No chromosomal K530A point mutant has been studied so in order to determine if the 5-phosphatase activity of SopB is involved in SopB-mediated ruffling and invasion, we created this mutant. We infected HeLa cells with the *sopB*^{K530A} mutant strain, WT *Salmonella* and the *sopB*^{C460S} mutant strain for 10 minutes to assess ruffling and invasion. We found that infection with the *sopB*^{K530A} mutant strain had an invasion rate similar to that of the *sopB*^{C460S} infection (Fig. 3-1A). This indicates that the 5-phosphatase activity of SopB has a role in invasion. It is well known in the

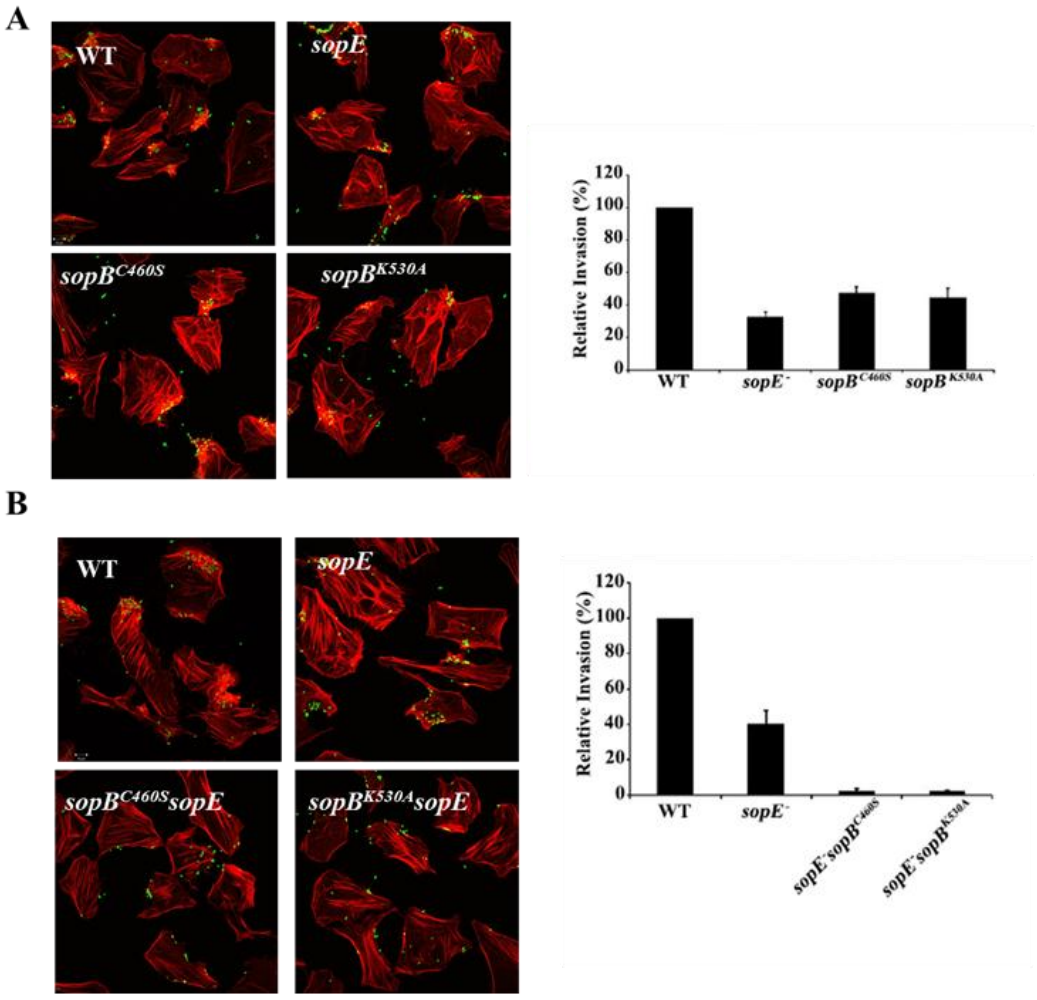

Fig. 3-1 The 5-phosphatase activity of SopB is required for SopB-mediated ruffling and invasion. (A & B) HeLa cells were infected with the indicated strain at an MOI of 10 for 10 minutes. Ruffles were visualized by staining for F-actin with Texas Red conjugated Phalloidin (red). *Salmonella* was visualized by staining with rabbit anti-*Salmonella* O antibody (green). Invasion was quantified by inside/outside differential staining following infection with the indicated strain at an MOI of 3 for 10 minutes WT infection was normalized to 100%. Data from three independent experiments is shown.

Salmonella field that the ruffling mediated by effector protein SopE can mask the effects of SopB in ruffling and invasion. To overcome these effects and see a more obvious ruffling phenotype we also made the point mutant in the *sopE*- background strain. As expected from the previous data, this strain did not cause any SopB-mediated ruffling or invasion after HeLa cells were infected for 10 minutes with the *sopB*^{K530A}*sopE*- strain (Fig 3-1B) (Higashide and Zhou, 2006; Zhou et al., 2001).

This phenotype mirrored that of the *sopB*^{C460S}*sopE*- strain that also lacked SopB-mediated invasion. This result indicated the involvement of the 5-phosphatase activity in SopB-mediated ruffling and invasion.

3.4.2 SopB^{K530A} retains 4-phosphatase activity *in vitro*

The SopB 5-phosphatase point mutant was previously reported to have no catalytic activity *in vitro* using the malachite green phosphatase assay (Marcus et al., 2001). To confirm that there was in fact no residual 4-phosphatase activity a malachite green phosphatase assay was performed using WT SopB, SopB^{C460S}, and the 5-phosphatase mutant SopB^{K530A}. PtdIns(3,4)P_2, PtdIns(3,4,5)P_3, and PtdIns(3,5)P_2 were used as the substrates for the assay. As previously reported, WT SopB hydrolyzed all three substrates while SopB^{C460S} had no activity towards any substrate. Interestingly in opposition to previously published data, we found that the 5-phosphatase mutant SopB^{K530A}, retained over 60% of its activity towards the substrate PtdIns(3,4)P_2 (Fig. 3-2). This was exciting data, as it further substantiates the involvement of the 5-phosphatase activity in SopB-mediated ruffling. This result also indicates that the 4- phosphatase activity alone is not sufficient for SopB-mediated ruffling and invasion, the K350A point mutant retains 4-phosphatase activity but is not able to promote ruffling in the *sopE*- background strain. All previous work on SopB-mediated ruffling and invasion thus far has focused on

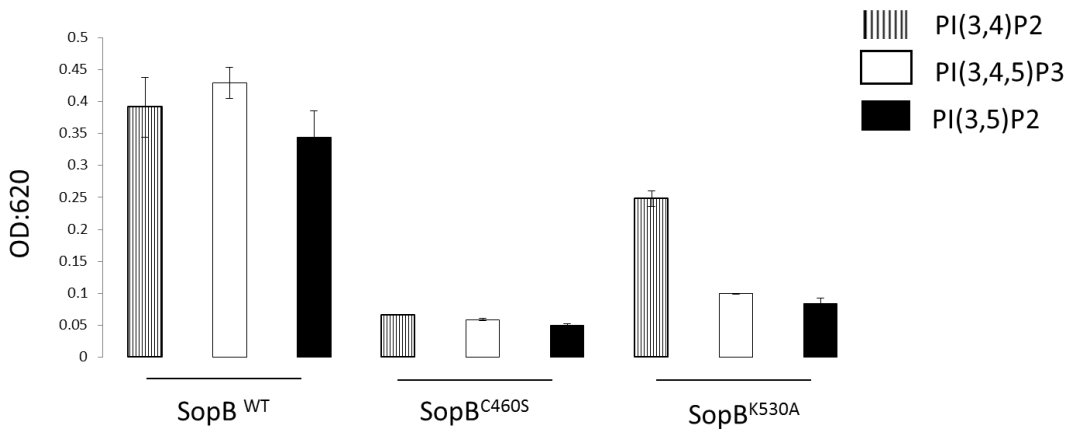

Fig. 3-2 SopBK530A retains 60% of its 4-phosphatase activity. A malachite green phosphatase activity assay was performed on purified SopB, $SopB^{C460A}$, and $SopB^{K530A}$. $PI(3,4)P_2$, $PI(3,4,5)P_3$, and $PI(3,5)P_2$ were used as substrates. Phosphatase release was measured after the addition of malachite green substrate and colormetrically detected at an absorbance of 620.

the 4-phosphatase activity of SopB (Hernandez et al., 2004; Mallo et al., 2008; Steele-Mortimer et al., 2000; Zhou et al., 2001), while this data suggests a role for the 5-phosphatase activity as well. To address the hypothesis that the 4-phosphatase activity alone is not sufficient to promote SopB-mediated ruffling and invasion, we swapped out *sopB* on the chromosome for well characterized IpgD and Inpp4B, 4-phosphatases and SKIP a 5-phosphatase. This experiment will allow us to see if the 4-phosphatase activity or 5-phospahtase activity alone is sufficient for SopB-mediated ruffling. This was a necessary step because we know that the 4-phosphatase mutant is also catalytically inactive for 5-phosphatase activity and we don't know if any other aspects of SopB, ubiquitination or Cdc42 binding are required for its activity in conjunction with its phosphatase activity.

3.4.3 4-phosphatase or 5-phosphatase activity alone is not sufficient to rescue SopB-mediated ruffling and invasion

In order to dissect the 4-phosphatase and 5-phosphatase activities of SopB we expressed exogenous 4-phosphatases or an exogenous 5-phosphatase on the *Salmonella* chromosome. We utilized the *sopB* homologue in *Shigella flexneri*, *ipgD*, and host Inpp4B as exogenous 4-phosphatases. IpgD is a known $PtdIns(4,5)P_2$ 4-

phosphatase (Niebuhr et al., 2002; Pendaries et al., 2006; Ramel et al., 2011), while Inpp4B is a known PtdIns(3,4)P_2 4-phosphatase (Fedele et al., 2010; Gewinner et al., 2009). We also utilized host SKIP a known PtdIns(3,4,5)P_3 5-phosphatase (Gurung et al., 2003; Ijuin et al., 2000). We translationally fused the first 100 amino acids of SopB – presumably containing the translocation signal to these genes and swapped out *sopB* for these genes on the *Salmonella* chromosome using homologous recombination (Fig. 3-3A). Additionally, the cognate promoter on the *Salmonella* chromosome will be used for expression. These insertion mutants will enable us to differentiate 4-phosphatase and 5-phosphatase activities, since it is unclear whether the C460S point mutant is able to retain 5-phosphatase activity *in vivo,* or if it is catalytically dead *in vivo* mimicking the *in vitro* phenotype (Marcus et al., 2001). To assess the role of these phosphatase activities in SopB-mediated ruffling and invasion, these insertions were made in the *sopE-* background strain. If they were able to promote ruffling in this background strain then we can therefore conclude which phosphatase activity is responsible for SopB-mediated ruffling. We found that none of the insertion mutants *sopE-sopB::ipgD, sopE-sopB::inpp4B, sopE-sopB::SKIP* were able to rescue the ruffling of the *sopBsopE* double deletion (Fig. 3-3B). Additionally these strains were not able to rescue the invasion defect in the *sopE-* background (Fig. 3-3C). To ensure these constructs were expressing and translocating at a rate similar to SopB, we checked the expression of the mutant strains and found that they were expressing to a comparable rate as SopB (Fig 3-4A). We fused the constructs from Fig 3-3A used to make the strains, to TEM and used a β-lactamase reporter assay to confirm the proteins could be translocated into the host cell at a rate similar to SopB. We confirmed the expression and translocation of the genes in these strains (Fig 3-4B,&C). This provided evidence for two possibilities. One, that there is some other aspect of the SopB protein (for example it's binding to Cdc42) responsible for SopB-mediated ruffling, or that either phosphatase activity alone is not sufficient for ruffling.

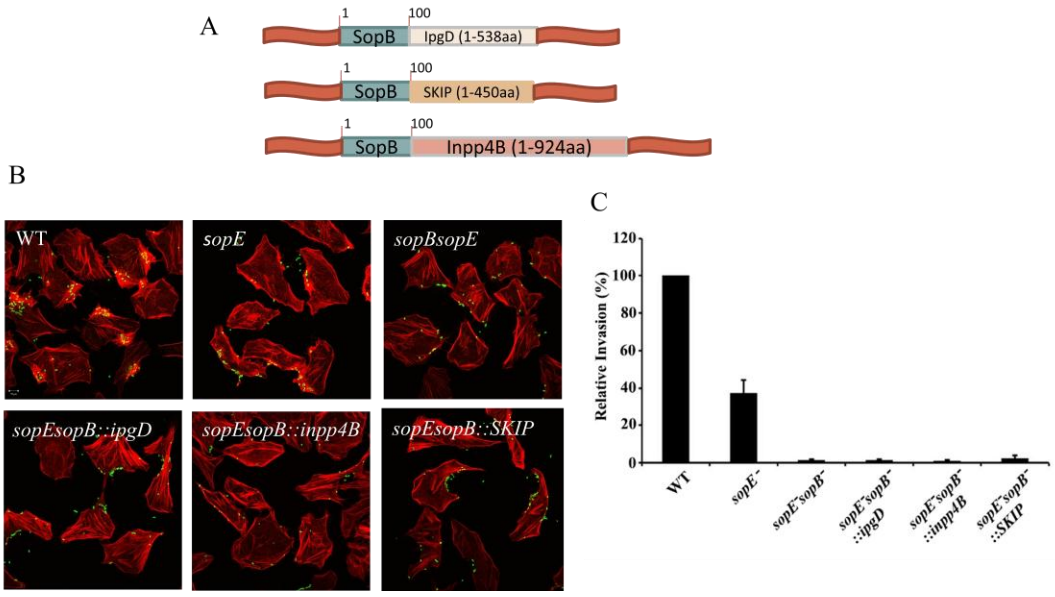

Fig. 3-3 4-phosphatase or 5-phosphatase activity alone is not sufficient to rescue the ruffling or invasion defect of the *sopB-sopE-* strain. (A) A schematic of the exogenous 4-phosphatases and 5-phosphatase fused to the first 100aa of SopB and inserted onto the chromosome. (B) HeLa cells were infected with the indicated strain at an MOI of 10 for 10 minutes. Following infection ruffles were assessed by staining for F-actin with Texas Red Phalloidin and *Salmonella* was visualized with staining with rabbit anti-*Salmonella* O antibody (green). (C) HeLa cells were infected with the indicated strain at an MOI of 3 for 10 minutes. Relative invasion was assessed by inside/outside differential staining. WT invasion was normalized to 100%. Data are from three independent experiments with SD shown.

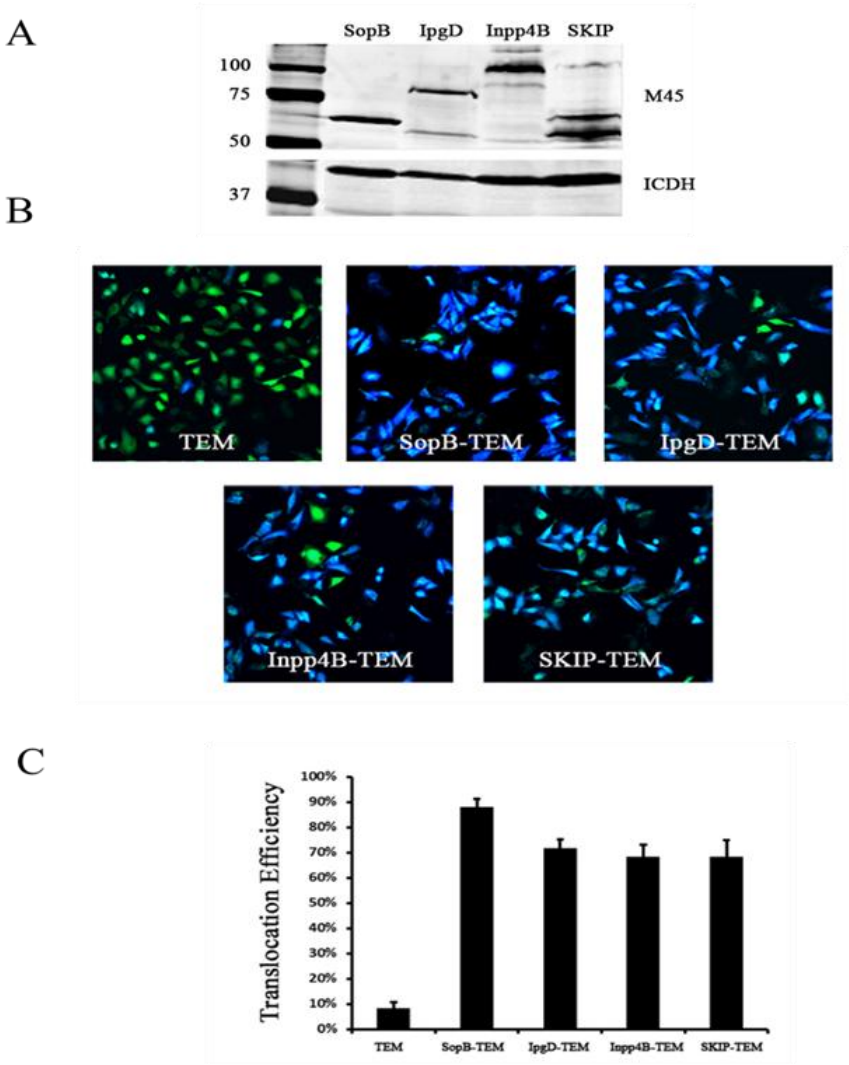

Fig. 3-4 Chromosomal mutant strains express and translocate similarly to SopB. (A) Mutant strains in the *sopE*- background were grown overnight and centrifuged at high speed. The pellet was lysed with SDS PAGE loading buffer and blotted with anti M45 antibody. anti IDCH was used as a loading control. (B) TEM fusions of either SopB or the first 100 amino acids of SopB fused to the indicated protein were expressed in *Salmonella.* HeLa cells were seeded on a 96 well plate. Strains were grown overnight and subsequently subcultured for 3 hours. Cells were infected at an MOI of 1 for 30 minutes. Images were taken and (C) translocation efficiency was determined 1 hour after CCF4-AM loading by counting cells emitting blue fluorescence signal.

3.4.4 *Salmonella* expressing both exogenous 4-phosphatase and 5-phosphatase is able to restore SopB-mediated ruffling and invasion

To explore the hypothesis that both phosphatase activities may be required for SopB-mediated ruffling and invasion, we sought to use a similar method as inserting the exogenous genes on the chromosome, this time creating a translational fusion of the first 100 amino acid residues of SopB to SKIP fused with either Inpp4B or IpgD to create *sopB::SKIP inpp4B* and *sopB::SKIP ipgD* (Fig. 3-5A). In order to detect ruffling or invasion, we created these strains in the *sopE*- background strain to produce, *sopE-sopB::SKIP inpp4B* and *sopE-::SKIP* ipgD. After invasion of HeLa cells with the phosphatase insertion strains in the *sopE*- background, for 10 minutes, we found that both strains were able to rescue SopB-mediated ruffling (Fig. 3-5B). We also performed and inside/outside differential staining and determined that invasion was restored to the same level as the *sopE*- strain indicating that SopB-mediated invasion only, was restored (Fig. 3-5C). This data indicates that both phosphatase activities of SopB are required for sufficient SopB-mediated ruffling and invasion. This data mimics past studies where the *sopEsopB*^{C460S} mutant infection can be restored with a plasmid expressing WT SopB, since WT SopB contains both activities. This also provides evidence that the 5-phosphatase activity of SopB does in fact have a role in SopB-mediated invasion. Another important SopB-dependent phenotype during *Salmonella* invasion is the production of PtdIns(3)P (Dai et al., 2007; Hernandez et al., 2004; Pattni et al., 2001). No work has investigated the possible involvement of the 5-phosphatase activity in the production of this phosphatidylinositol.

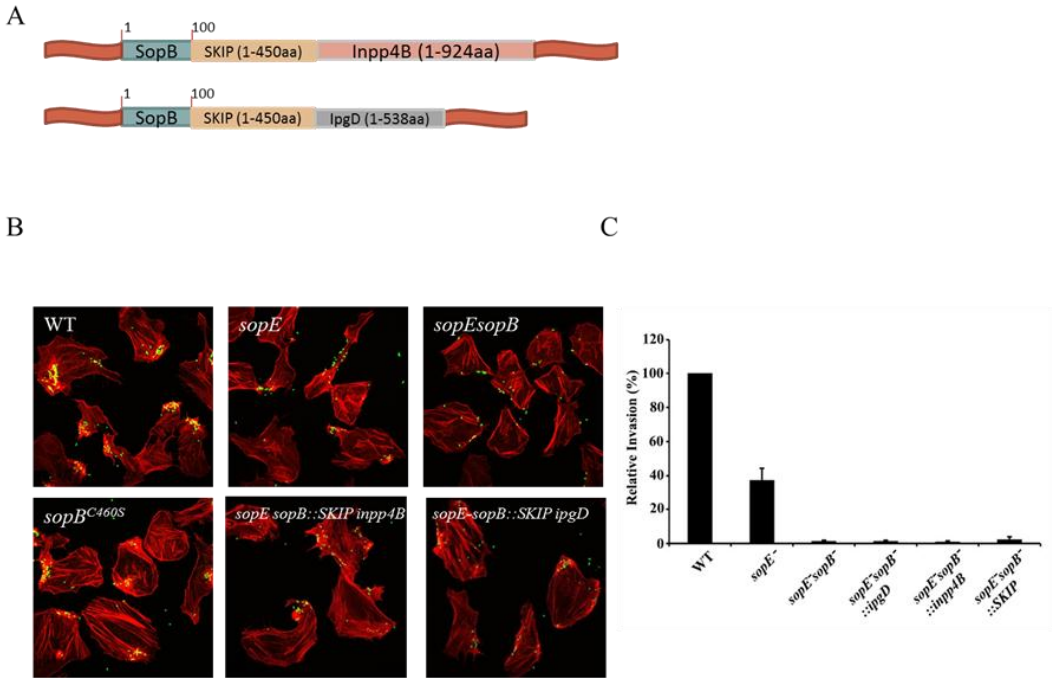

Fig. 3-5 4-phosphatase and 5-phosphatase activity in conjunction with one another are sufficient to restore SopB-mediated ruffling and invasion. (A) A schematic of the exogenous 4-phosphatases and 5-phosphatase fused to the first 100aa of SopB and inserted onto the chromosome. (B) HeLa cells were infected with the indicated strain at an MOI of 10 for 10 minutes. Following infection ruffles were assessed by staining for F-actin with Texas Red conjugated Phalloidin and *Salmonella* was visualized with staining with rabbit anti-*Salmonella* O antibody (green). (C) HeLa cells were infected with the indicated strain at an MOI of 3 for 10 minutes. Relative invasion was assessed by inside/outside differential staining. WT invasion was normalized to 100%. Data represent three independent experiments with SD shown.

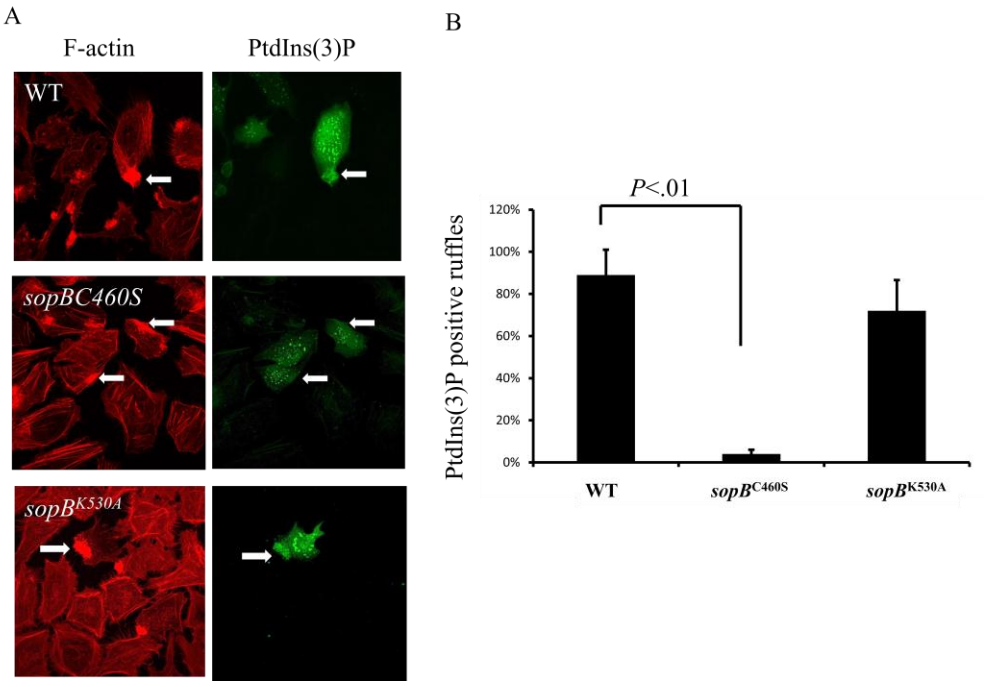

Fig. 3-6 The 5-phosphatase activity of SopB is not required for PtdIns(3)P production. (A) HeLa cells were transfected with the PtdIns(3)P probe - p40PX-EGFP for 24 hours and infected with the indicated strain at an MOI of 10 for 10 minutes. Ruffles were visualized by staining for F-actin with Texas Red conjugated Phalloidin. (B) The number of PtdIns(3)P positive ruffles were quantified 10 minutes after invasion. Data represents three independent experiments.

3.4.5 The 4-phosphtase activity of SopB is sufficient to produce PtdIns(3)P at the *Salmonella* induced membrane ruffles and recruit Rab5 to the SCV

To investigate the role the 5-phosphatase activity of SopB may have in the production of PtdIns(3)P at the membrane ruffles, we used the single point mutant strain *sopB*^{K530A}. To monitor PtdIns(3)P in the cells during infection, HeLa cells were transfected with the EGFP-tagged probe for PtdIns(3)P, p40-PX-EGFP. 24 hours following transfection, the cells were infected with WT, *sopB*^{C460S}, or *sopB*^{K530A} strains for 10 minutes. As expected, WT infection caused an abundant production of PtdIns(3)P at the ruffles, while infection with the *sopB*^{C460S} strain, lacked PtdIns(3)P at the ruffles. Infection with the 5-phosphatase mutant strain revealed a production of PtdIns(3)P at the membrane ruffles similar to the WT infected cells (Fig. 3-6A &B). This was an interesting result that indicated the 4-phosphatase activity of SopB is sufficient for PtdIns(3)P production at the membrane ruffles. We cannot rule out that

the 5-phosphatase activity may be involved, but we can conclude it is not required. This result was also interesting because this is the first evidence that the production of PtdIns(3)P and SopB-mediated ruffling are not linked. It was assumed that SopB was required to produce PtdIns(3)P in order to cause ruffling, and also if there was PtdIns(3)P production, there would be SopB-mediated ruffling. This is not the case, because the *sopB*^{K530A} strain produced PtdIns(3)P, but still had an invasion and ruffling defect in the *sopE*- background. Additionally, this result provided further evidence that SopB is utilizing PtdIns(3,4)P_2 as the substrate to produce PtdIns(3)P. The malachite green phosphatase activity alone exhibited activity towards the PtdIns(3,4)P_2 substrate *in vitro*.

To provide more convincing evidence that SopB is utilizing PtdIns(3,4)P_2 as its substrate, we wanted to examine the distribution of both species together during WT and *sopB*^{C460S} infection. HeLa cells were co-transfected for 24 hours with the RFP tagged probe for PtdIns(3)P p40PX-RFP and the probe for PtdIns(3,4)P_2, p47PX-EGFP. The transfected cells were then placed on a heated stage and infected at an MOI of 50. We took images for both channels every 10 seconds and fluorescent intensity was calculated over time. We found that during WT infection PtdIns(3)P and PtdIns(3,4)P_2 had a basal level intensity until ~34 minutes when the bacterium touched the cell surface triggering ruffling. Both signals at this point, increased with PtdIns(3)P increasing drastically, and PtdIns(3,4)P_2 increasing and tapering off shortly after. This tapering off is presumably due to PtdIns(3,4)P_2 conversion into PtdIns(3)P (Fig 3-7). This data provided any evidence that PtdIns(3,4)P_2 was being hydrolyzed into PtdIns(3)P based upon the pattern of signal intensity seen during WT infection. It also provided evidence that during infection with the *sopB*^{C460S} mutant there was no increase in PtdIns(3)P or PtdIns(3,4)P_2 at the ruffle as expected (Fig 3-7). This method of phosphatidylinositol detection was quite variable. We speculate this is due to the limitation of this system. We were only able to take images on a single plane and were not able to detect signal in the entire forming ruffle to try to minimize

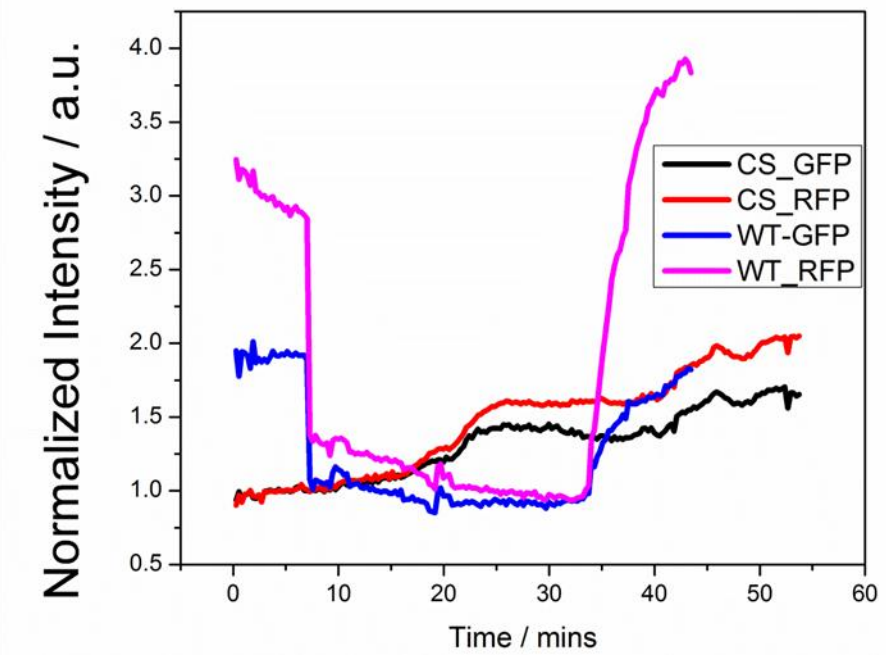

Fig 3-7 PtdIns(3)P and PtdIns(3,4)P_2 levels during WT and *sopB*^{C460S} infection. HeLa cells were co-transfected with the probes for PtdIns(3)P p40PX-RFP and PtdIns(3,4)P_2 p47PX-EGFP for 24 hours. Following transfection cells were placed on a heated stage and infected with either WT or sopB^{C460S} at an MOI of 50. Images were taken at 10 second intervals for each probe. Intensity at ruffles was analyzed over time for each probe for each infection condition. CS- *sopB*^{C460S}, GFP-PtdIns(3,4)P_2, WT- wild type infected, RFP-PtdIns(3)P.

bleaching of the signal.

To further investigate which phosphatase activity is required for PtdIns(3)P production, we transfected HeLa cells with the p40PX-EGFP probe for PtdIns(3)P detection and infected with *sopB::inpp4B*, *sopB::ipgD*, and *sopB::SKIP* to determine the role each phosphatase activity has individually. Following 10 minutes of infection we found that both 4-phosphatase mutants were able to rescue PtdIns(3)P production at the membrane ruffles, while the 5-phosphatase insertion could not (Fig 3-8A &B).

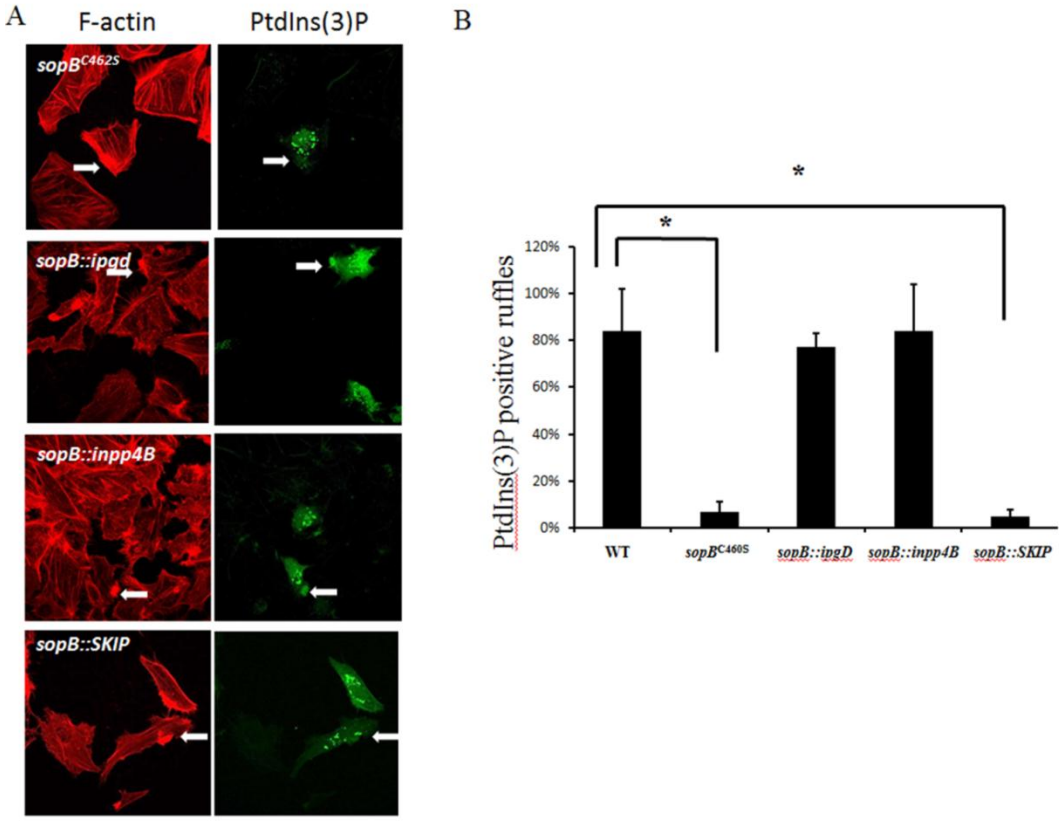

Fig 3-8 4-phosphatase activity is sufficient for PtdIns(3)P production at the membrane ruffles. (A) HeLa cells were transfected with the PtdIns(3)P probe p40PX-EGFP for 24 hours and infected with the indicated strain at an MOI of 10 for 10 minutes. Ruffles were visualized by staining for F-actin with Texas Red conjugated Phalloidin. (B) The number of PtdIns(3)P positive ruffles were quantified 10 minutes post infection. Data represents three independent experiments. Asterisks indicate a *P* value of <.005.

We were able to conclude that the 4-phosphatase activity is in fact sufficient to produce PtdIns(3)P at the membrane ruffles. We then sought to determine if the same is true on the about the SopB-mediated PtdIns(3)P production on the surface of the SCV. Previous work demonstrated the requirement of the 4-phosphatase activity of SopB for Rab5 recruitment the SCV following bacterial internalization. We transfected HeLa cells with YFP-Rab5 for 24 hours and subsequently infected the

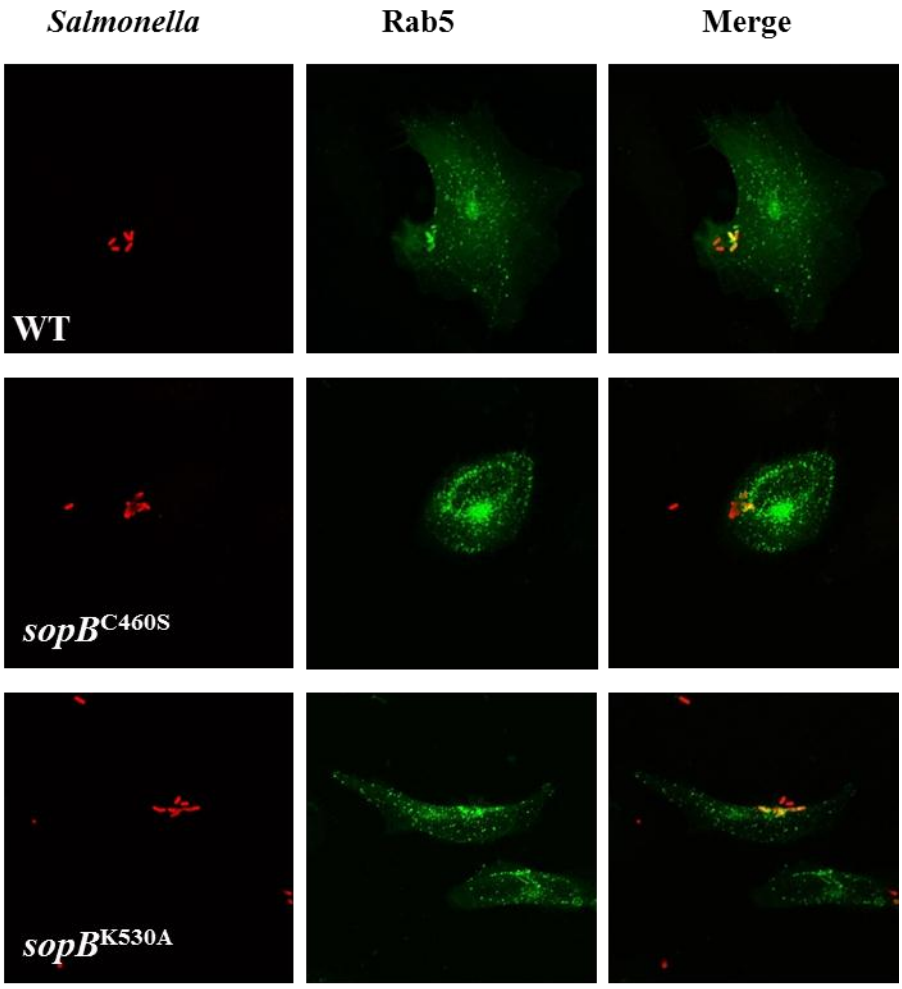

Fig 3-9 The 4-phosphatase activity of SopB is sufficient to recruit Rab5 to the SCV. HeLa cells were transfected with YFP-Rab5 for 24 hours and infected with the indicated strain at an MOI of 10 for 10 minutes. *Salmonella* was visualized by rabbit anti- Salmonella O antibody (red).

cells with WT, *sopB*^{C460S}, or *sopB*^{K530S} for 10 minutes. We found that only the *sopB*^{C460S} infected cells were unable to recruit Rab5 to the SCV surface. The *sopB*^{K530A} infected cells recruited Rab5 similar to WT infected cells (Fig 3-9). This data provides evidence that the 4-phosphatase activity of SopB is sufficient to not only hydrolyze the substrate PtdIns(3,4)P_2 into PtdIns(3)P at the membrane ruffles, but also recruit Rab5 to the SCV following bacterial internalization. Recruitment of Rab5 to the SCV is required to recruit host PI3K, Vps34 to the SCV to produce PtdIns(3)P. We are uncertain at this point why this protein would utilize two different mechanisms to produce PtdIns(3)P, but the SCV surface and the plasma membrane have very distinct compositions.

Since we have determined the 4-phosphatase activity of SopB is required for producing PtdIns(3)P at the membrane ruffles, but not sufficient for SopB-mediated ruffling and invasion, this led to the question of what role the 5-phosphatse activity of SopB could have in SopB-mediated ruffling and invasion. We know that both phosphatase activities are required, but as of yet we are uncertain what phosphatidylinositol phosphate the 5-phospahtase activity could be hydrolyzing. We hypothesize that the 5-phosphatase activity is responsible for hydrolyzing PtdIns(3,4,5)P_3. Unfortunately the probe for PtdIns(3,4,5)P_3 is not able to detect any changes in PtdIns(3,4,5)P_3, possibly because this phosphoinositide is present at such large amounts on the plasma membrane and the actin-rich ruffles. Previous work has demonstrated a SopB-dependent increase in PtdIns(3,4)P_2 which could be produced from the hydrolysis of PtdIns(3,4,5)P_3 at the 5- position. PtdIns(3,4)P_2 recruits a number of actin modulating proteins to the plasma membrane, and we wanted to investigate if any of these proteins are involved in SopB-mediated invasion.

3.4.6 Actin-modulating proteins recruited by PtdIns(3,4)P_2 during *Salmonella* infection

PtdIns(3,4)P_2 recruits a number of actin modulating proteins to the plasma membrane. To determine if any of these proteins were recruited to the PtdIns(3,4)P_2 present at the *Salmonella* ruffles, we investigated the localization of Tks4, TAPP1, Bam32, and SNX9 during WT and *sopB*^{C460S} infection to see if any of these proteins

are recruited in a SopB-dependent manner. We were interested in proteins recruited to the ruffles during WT infection, but absent from the ruffles during infection with the SopB mutant. Host protein Tks4 is required for podosome formation. Podosomes are actin rich structures on the plasma membrane involved in motility and invasion. This could potentially be a useful protein for *Salmonella* to modulate because of its ability to promote the formation of such actin rich structures. TAPP1 is known to accumulate within F-actin rich structures. Due to the presence of this protein in actin rich structures it is thought to modulate the actin cytoskeleton. It's been characterized as preventing the formation of certain ruffle structures such as circular ruffles, while still being present in peripheral ruffles. The exact role of TAPP1 in modulating these structures is still unclear. Bam32, is another protein recruited by PtdIns(3,4)P_2 to the plasma membrane. Bam32 was of particular interest because it is a PI3K adapter protein known to activate the RhoGTPase Rac1. Bam32 is directly involved in Rac1-mediated actin cytoskeletal rearrangements such as ruffle formation (Buschman et al., 2009; Hogan et al., 2004; Marshall et al., 2002). Lastly we wanted to investigate the role of SNX9, a protein recently demonstrated to activate N-WASP facilitating F-actin nucleation and ruffle formation (Soulet et al., 2005; Yarar et al., 2007). To determine a possible role of these proteins in SopB-mediated ruffling and invasion, we wanted to confirm whether or not they were recruited in a SopB-mediated manner. HeLa cells were transfected with GFP- Tks4 or RFP-TAPP1 for 24 hours. The cells were then infected with WT or *sopB*^{C460S} strains at an MOI of 10 for 15 minutes. We found that Tks4 was not recruited to the ruffles during the WT infection or *sopB*^{C460S} infection (Fig 3-10 A&B). Although Tks4 is known to be recruited by PtdIns(3,4)P_2 and involved in the production of actin rich structures, this result indicate that it is probably not part of the *Salmonella* mediated ruffle formation since it is absent from the ruffles. When we examined the recruitment of TAPP1 to the membrane ruffles and found it was recruited to the ruffles of both the WT and the *sopB*^{C460S} infected cells (Fig 3-10 A &B). We wanted to restrict our search to proteins recruited in a SopB-mediated manner, so we did not follow up with the possible involvement of this protein in *Salmonella* induced ruffle formation, although it is possible TAPP1 may have some role in ruffle formation or modulation that is not SopB-dependent.

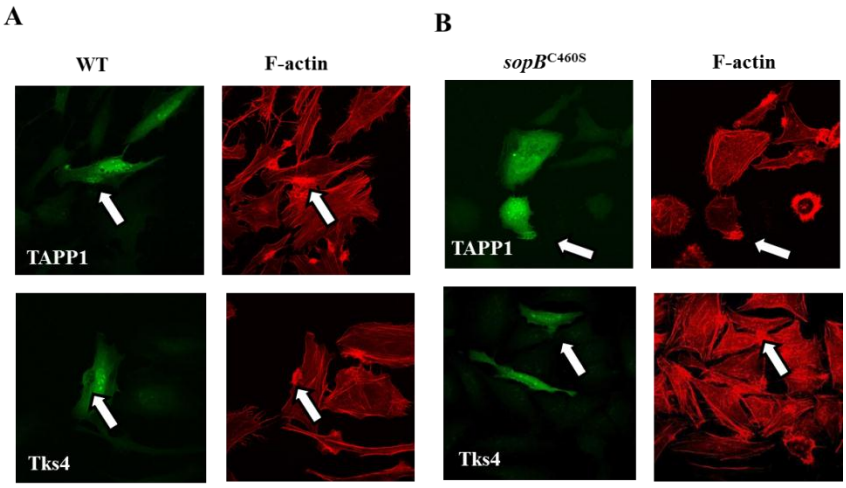

Fig 3-10 Host proteins TAPP1 and Tks4 are not recruited in a SopB-dependent manner. HeLa cells were transfected with RFP-TAPP1 or GFP-Tks4 for 24 hours. (A) following transfection the cells were infected with WT *Salmonella* at an MOI of 10 for 15 minutes. (B) following transfection the cells were infected with the *sopB*^{C460S} mutant strain at an MOI of 10 for 15 minutes. Ruffles were visualized with staining with Texas Red conjugated Phalloidin or AF488 conjugated Phalloidin. Arrows point at Ruffles and corresponding PtdIns(3)P signal. Pseudo colors were added on Zeiss software.

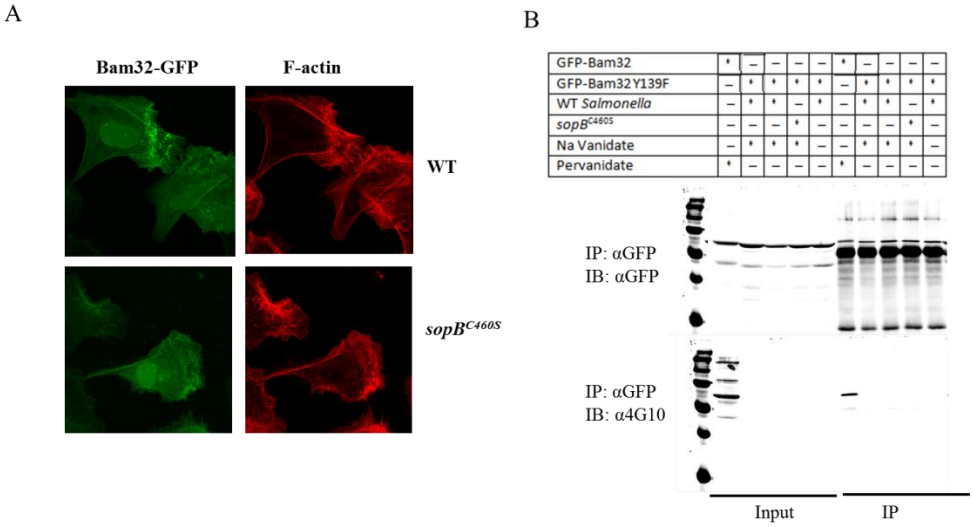

Fig. 3-11 Bam32 is not phosphorylated during WT *Salmonella* infection. (A) HeLa cells were transfected with Bam32-EGFP for 24 hours and infected with WT or the *sopB*^{C460S} mutant at an MOI of 10 for 10 minutes. Ruffles were visualized by staining for F-actin with Texas Red conjugated Phalloidin. (B) HeLa cells were transfected with WT Bam32-EGFP or Bam32Y139F for 24 hour. Following transfection the cells were treated and infected as indicated. Bam32 was precipitated with rabbit anti-GFP and probed for mouse anti 4G10.

3.4.7 Role of Bam32 in SopB-mediated ruffling and invasion

We then investigated the potential involvement of Bam32. We found that Bam32 is recruited to the *Salmonella* induced membrane ruffles during WT infection and is absent at the ruffles following infection with the SopB 4-phosphatase mutant (Fig 3-10 A). This indicated that SopB was able to produce the phosphatidylinositol responsible for recruiting Bam32 to the ruffles. Bam32 is a well characterized protein and literature states that for Bam32 to activate Rac1, it must first be phosphorylated at its tyrosine residue Y139(Allam and Marshall, 2005; Allam et al., 2004). To determine if Bam32 is phosphorylated and therefore activated during WT *Salmonella* infection we performed an immunoprecipitation in HeLa cells. HeLa cells were transfected with either the WT GFP-Bam32 or the Bam32 Y139F point mutant. Following 24 hours of transfection, the first lane, that was transfected with WT GFP-Bam32, was treated with 100μM pervanadate, a tyrosine phosphatase inhibitor and activator of tyrosine phosphorylation. All other lanes were treated with 1mM Sodium vanadate, a tyrosine phosphatase inhibitor for 30 minutes prior to infection. We infected the cells with WT *Salmonella* and the *sopB*^{C460S} mutant strain as indicated (Fig. 3-11 B). We immmunoprecipated the samples with anti-GFP at 1:100,000 dilution and probed the membrane for anti 4G10 at a 1:1000 dilution (an antibody that detects tyrosine phosphorylation). We found WT Bam32 was not phosphorylated during WT infection (Fig. 3-11B). This indicated that Bam32 was not activated at the ruffles during WT *Salmonella* infection.

3.4.8 SopB recruits SNX9 to the membrane ruffles

We next investigated the recruitment of SNX9 to the *Salmonella* ruffles. HeLa cells were infected with WT, *sopE-*,*sopB*^{C460S}, and *sopB*^{K530A} for 10 minutes. We found that SNX9 was recruited to the ruffles when infected with WT or the *sopE*-mutant, but was absent from the ruffles in the *sopB*^{C460S} and *sopB*^{K530A} mutant strains infection (Fig 3-12). This indicated that SNX9 was recruited in a SopB-dependent manner. This was taken one step further to determine if either the 4-phosphatase or 5-phosphatase activity alone was sufficient to produce PtdIns(3,4)P_2 and recruit SNX9

to the membrane. We infected HeLa cells with the single insertion mutants and probed for SNX9. We found that neither the 4-phosphatase or 5-phosphatase activity alone was sufficient to recruit SNX9 to the membrane (Fig. 3-13). To confirm that SNX9 was recruited to the ruffles by SopB-mediated PtdIns(3,4)P_2 production we obtained GFP-SNX9, as well as mCherry-SNX9 (RYK) from Dr. Sandra Schmid (Yarar et al., 2008). The RYK mutation of SNX9 is located in its PIP binding domain inhibiting the binding of SNX9 to PtdIns(3,4)P_2. HeLa cells were transfected with WT SNX9 as well as the RYK mutant and subsequently infected with WT and *sopE*-. We found that the GFP-SNX9 was recruited to the membrane ruffles just as endogenous SNX9, and the RYK mutant SNX9 was no longer recruited to the ruffles

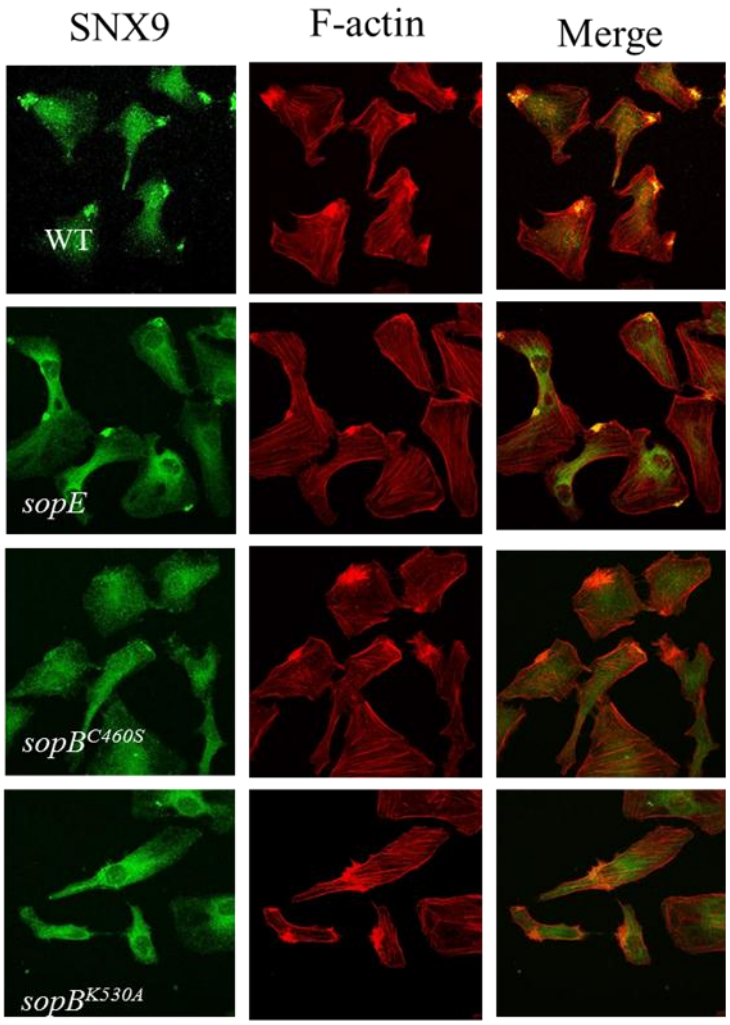

Fig 3-12 SNX9 is recruited to the *Salmonella* induced ruffles in a SopB-dependent manner. HeLa cells were infected with the indicated bacterial strain at an MOI of 10 for 10 minutes. SNX9 was detected using a monoclonal mouse anti SNX9 antibody (green). Ruffles were visualized by staining with Texas Red conjugated Phalloidin.

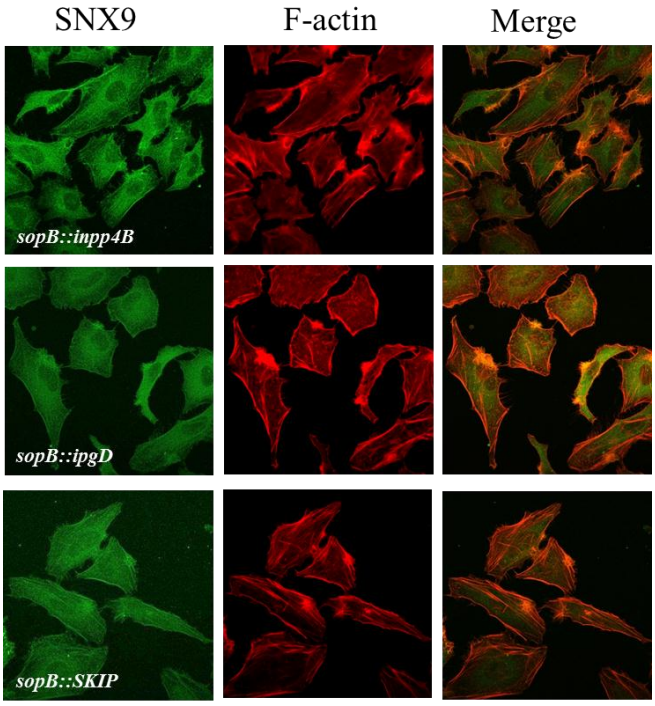

Fig. 3-13 4-phosphatase or 5-phosphatase activity is not sufficient to recruit SNX9 to the membrane. HeLa cells were infected with the indicated insertion mutant strain at an MOI of 10 for 10 minutes. Ruffles were visualized by staining with Texas Red conjugated phalloidin SNX9 distribution was monitored with mouse anti-SNX9 antibody (green).

of WT or the *sopE*- infected cells (Fig 3-14). This confirms that SNX9 is being recruited by the SopB-produced PtdIns(3,4)P_2. We also created a plasma membrane targeted Inpp4B (host PtdIns(3,4)P_2 4-phosphatase) construct by fusing a CAAX domain to its C-terminus, to deplete PtdIns(3,4)P_2 at the membrane. HeLa cells were transfected with YFP-Inpp4B-CAAX which should deplete PtdIns(3,4)P_2 on the membrane and subsequently infected with WT and the *sopE*- mutant. We found that no transfected cells had SNX9 present at the membrane ruffles (Fig 3-15). This data corroborates the SNX9 (RYK) recruitment data and further confirms that the SopB-mediated PtdIns(3,4)P_2 is required for SNX9 recruitment.

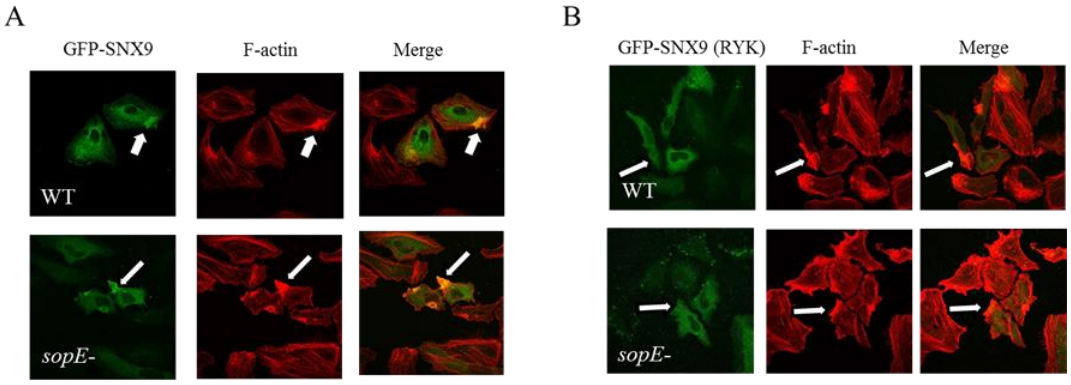

Fig. 3-14 The PIP binding domain is required for SNX9 recruitment to the membrane ruffles. HeLa cells were transfected with SNX9-EGFP or mCherry SNX9 RYK (green) for 24 hours. The cells were then infected with the indicated strain at an MOI of 10 for 10 minutes. Ruffles were visualized by staining with Texas Red conjugated phalloidin. Arrows point to *Salmonella* induced membrane ruffles and corresponding SNX9.

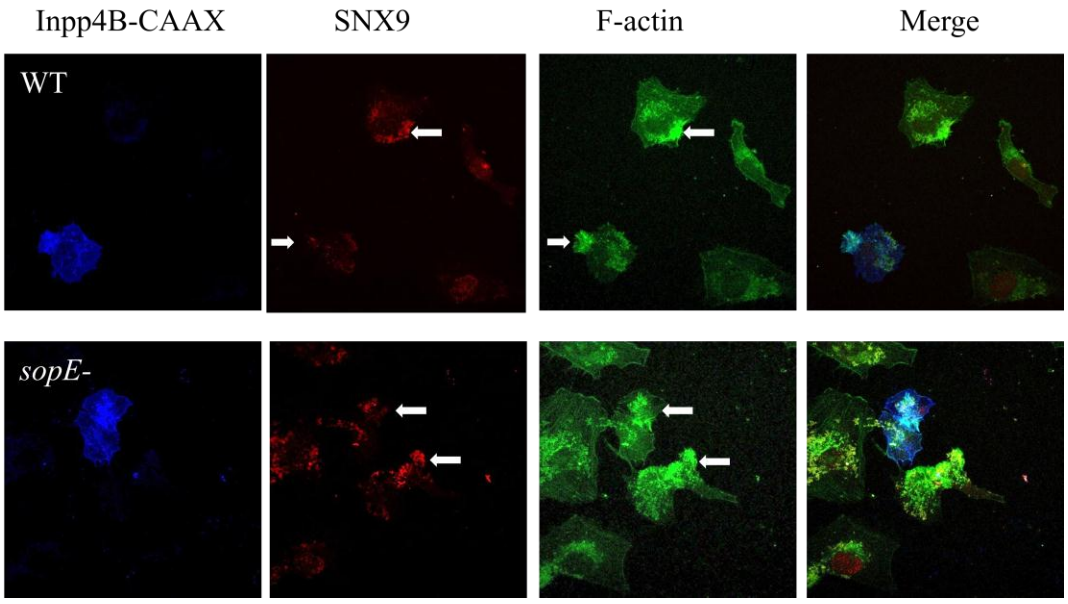

Fig 3-15 PtdIns(3,4)P_2 is required for SNX9 recruitment to the *Salmonella* induced membrane ruffles. HeLa cells were transfected with Inpp4B-CAAX-GFP (blue) for 24 hours then infected with WT or the *sopE* mutant strain at an MOI of 10 for 10 minutes. SNX9 distribution (red) was assessed by probing with mouse anti-SNX9, and ruffles were visualized by staining with Texas Red conjugated Phalloidin (green). Arrows point to ruffles with corresponding SNX9 recruitment. Pseudo coloring was done on Zeiss software.

3.4.9 SNX9 is required for sufficient *Salmonella* invasion and ruffling

Once we could conclude SNX9 was recruited in a SopB-dependent manner and the recruitment relied upon SopB-produced PtdIns(3,4)P_2, we wanted to determine if this protein was involved in ruffling and invasion. To assess the involvement of SNX9 in SopB-mediated ruffling, we purchased siRNA constructs against SNX9. The knockdown efficiency of SNX9 can be seen in Fig 3-15A via western blot analysis. Following 48 hour transfection for sufficient knockdown, we infected the HeLa cells with WT, *sopB*^{C460S},*sopB*^{K530A}, and *sopE*- strains. We calculated invasion efficiency by using the differential inside/outside staining. We found that following SNX9 knockdown the invasion of WT and *sopE*- strains decreased significantly P=.02 or P=.007 respectively (Fig 3-16B). The invasion of either SopB mutant remained the same which provides further evidence that this is a SopB-mediated process.

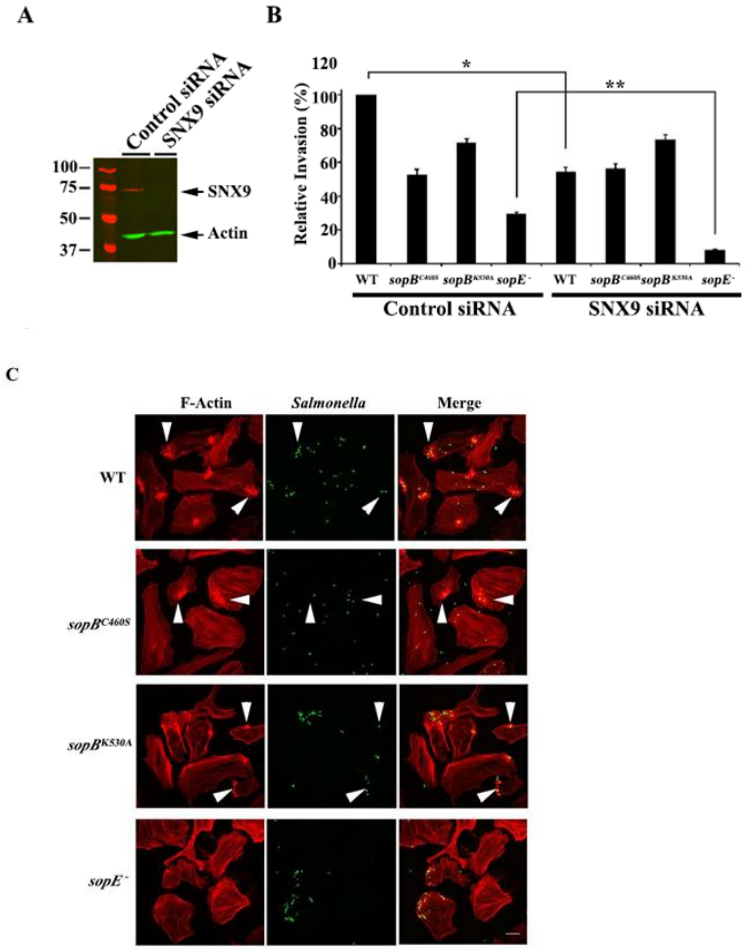

Fig 3-16 SNX9 is required for SopB-mediated invasion. (A) HeLa cells were seeded on a 6-well plate and transfected with control siRNA for siRNA targeting SNX9 for 48 hours. Following transfection cells were collected and subject to western blot analysis using mouse ant-SNX9 antibody. Anti actin was used as a loading control. (B) HeLa cells were transfected with control siRNA or siRNA targeting SNX9 for 48 hours. Following transfection cells were infected with the indicated strain for 10 minutes at an MOI of 3 and invasion rates were quantified by performing an inside/outside differential staining. Invasion of WT infected control cells was normalized to 100%. P-values were obtained by performing a paired Students *t*-test. * P<.05, **P<.001. C) Representative images of quantification in (B), Arrows point at ruffles with corresponding bacteria.

3.5 Discussion

This chapter focused on the SopB-mediated manipulation of host phosphatidylinositol phosphates for ruffle formation and invasion. A number of *Salmonella* effectors are involved in the entry of *Salmonella* into the host cell including SopE and SopB. SopE and its homologue SopE2 are GEFs that activate Rac1 and Cdc42 (Friebel et al., 2001; Hardt et al., 1998). SopB alternately is a phosphatidylinositol phosphate phosphatase capable of hydrolyzing a number of substrates at the 4- and 5-position *in vitro* (Marcus et al., 2001). Previous works on SopB have only characterized its 4-phosphatase activity during infection conditions meaning that all phenotypes of SopB, were therefore attributed to the 4-phosphatase activity.

One well studied phenotype of SopB is the production of PtdIns(3)P at the *Salmonella* induced membrane ruffles. The production of PtdIns(3)P at the ruffles is thought to be important for the recruitment of host v-SNARE protein VAMP8(Pryor et al., 2004; Ren et al., 2007). VAMP8 is required for proper phagocytosis of the bacteria into the host cell (Dai et al., 2007; Hernandez et al., 2004). To further investigate this phenotype and also attempt to elucidate the substrate of SopB required to produce PtdIns(3)P we expressed exogenous 4-phosphatase genes on the *Salmonella* chromosome, and deduced the 4-phosphatase activity of SopB was sufficient to produce PtdIns(3)P at the membrane ruffles during WT infection .We also determined that the 4-phosphatase activity alone was not sufficient to rescue SopB-mediated ruffling or invasion in the *sopE*- background. This was an important discovery because, all work up to this point had coupled the SopB-mediated PtdIns(3)P production to the ruffling activity of SopB, and this work delineates those two activities. This is not a surprise though, because PtdIns(3)P is required to recruit VAMP8 to the membrane and neither VAMP8 nor PtdIns(3)P have been demonstrated to cause ruffling or actin cytoskeletal modifications. This left further uncertainty as to what other aspect of SopB was required for ruffling, whether it be the 5-phosphatase activity or ubiquitination, or even the binding of SopB to Cdc42(Burkinshaw et al., 2012; Knodler et al., 2009; Patel et al., 2009).

The involvement of phosphatidylinositol phosphates in actin dynamics is well studied. PtdIn(4,5)P_2 is known to recruit actin-binding proteins to the plasma membrane and also itself activate N-WASP at the plasma membrane making this phosphatidylinositol indispensable for actin dynamics (Martin, 2001; Sechi and Wehland, 2000). During WT *Salmonella* infection, SopB dephosphorylates PtdIns(4,5)P_2 at the plasma membrane to facilitate proper fission of the phagocytic cup (Terebiznik et al., 2002). The hydrolysis of PtdIns(4,5)P_2 during WT infection though, was not attributed to the ruffling phenotype of SopB.

Another important aspect of phosphatidylinositol phosphates at the plasma membrane is their dynamic nature and the requirement of their turnover and cycling for certain processes (Fairn et al., 2009; Nishioka et al., 2008; Szymanska et al., 2008). This led to the hypothesis that both the 4 and 5-phosphatase activities of SopB may be involved and could be working off one another to modulate the phosphoinositide turnover involved in membrane ruffling. We found that the 5-phosphatase mutant of SopB retains 4-phosphatase activity for the host substrate PtdIns(3,4)P_2. Based upon this *in vitro* phosphatase assay data, we speculate that PtdIns(3,4)P_2 is the host phosphatidylinositol substrate for SopB to produce PtdIns(3)P. We also speculate that the 5-phosphatase activity of SopB is producing the SopB-dependent increase of PtdIns(3,4)P_2 by hydrolyzing PtdIns(3,4,5)P_3 thereby supplying the substrate for SopB to produce PtdIns(3)P. This could be the dynamic cycling that is required for SopB-mediated ruffle formation. We tested the hypothesis that both the 4-phosphatase and 5-phosphatase activities were required for SopB-mediated ruffling and invasion, by expressing both the exogenous 4-phosphatase and 5-phosphatase together on the *Salmonella* chromosome. Because the 4-phosphatase mutant on the *Salmonella* chromosome was abolished for both 4- and 5- phosphatase activities, this was a method to investigate the roles of the phosphatases. The two phosphatases were fused together, as well as translationally fused to the translocation signal of SopB. We found that in the *sopE*- background, the double insertion was able to rescue SopB-mediated ruffling and invasion. This result confirms that both phosphatase activities are required for SopB to promote ruffling and invasion.

We therefore sought to investigate how the 5-phosphatase activity may be involved. We speculated that the 5-phosphatase activity of SopB hydrolyzes PtdIns(3,4,5)P_3 to produce PtdIns(3,4)P_2. To determine if the production of PtdIns(3,4)P_2 and subsequent turnover (as indicated by the necessity of the 4-phosphatase activity) is responsible for recruiting proteins to the membrane that are involved in SopB-mediated ruffling and invasion, we investigated a number of known actin modulating proteins that are recruited to the membrane in a PtdIns(3,4)P_2 dependent manner. We investigated the recruitment of Tks4, TAPP1, Bam32 and SNX9. We found that SNX9 was recruited to the membrane in a SopB-dependent manner and its recruitment was dependent on the ability of SNX9 to bind PtdIns(3,4)P_2. SNX9 has been linked to the nucleation of F-actin, by directly activating N-WASP (Yarar et al., 2007). We found that during WT infection N-WASP and SNX9 were able to co-localize at the membrane ruffles. Further work needs to be done using various N-WASP dominant negative constructs or siRNA targeting N-WASP to determine if N-WASP activation is linked to the SNX9-dependent modulation of the actin cytoskeleton during infection. It's already been established that Arp2/3 activation is required for *Salmonella* invasion, so connecting N-WASP to the activation of Arp2/3 would be important for this study. There is a lot of conflicting data regarding whether or not N-WASP is actually involved in *Salmonella* infection, so this is something that would enhance the field a great deal and answer these questions (Criss and Casanova, 2003; Goley and Welch, 2006; Humphreys et al., 2013; Unsworth et al., 2004).

This work not only elucidated the substrate of SopB for the production of PtdIns(3)P, but also provides evidence that SopB modulates phosphatidylinositol phosphates at the plasma membrane promoting a state of phosphatidylinositol cycling. This cycling is required for the recruitment of SNX9 which is involved in modulating the actin cytoskeleton. It's interesting that *Salmonella* requires both the 4- and 5-phosphatase activities of SopB for SopB-mediated ruffling and subsequent invasion. The 4-phospahtase activity alone, although sufficient to dephosphorylate PtdIns(3,4)P_2 into PtdIns(3)P which recruits VAMP8 is not sufficient for ruffling. Similarly the 5-phosphatase activity alone is not sufficient to recruit SNX9 to the

membrane to induce ruffling. We speculate that the timing of these events is crucial to ruffle formation, but thus so we are not able to provide sufficient evidence that PtdIns(3,4)P_2 is hydrolyzed into PtdIns(3)P. We tried to use the specific lipid probes to answer this question, but there were serious pitfalls with the system and we were not able to get consistent results. It's important to the study, that the hydrolysis of PtdIns(3,4)P_2 into PtdIns(3)P be shown during the course of WT *Salmonella* infection. Once this system can be established it would be great to see how the SopB mutants are able to alter this cycling. The turnover of phosphatidylinositol phosphates are required for phagocytosis and cell migration, and *Salmonella* modulates these dynamics to promote ruffling and invasion into the host cell.

CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Research Summary

This dissertation examines the modulation of host phosphatidylinositol phosphates by the pathogen *Salmonella* and characterizes the role of *Salmonella* effector protein SopB as a phosphatidylinositol phosphate phosphatase in promoting ruffle formation and bacterial internalization.

SopB is able to hydrolyze a number of host phosphatidylinositol phosphates *in vitro*, but *in vivo*, while SopB has only been shown to hydrolyze PtdIns(4,5)P_2 (Marcus et al., 2001; Terebiznik et al., 2002). The hydrolysis of PtdIns(4,5,)P_2 proved crucial to proper closure of the phagocytic cup during phagocytosis but was not linked to membrane ruffle formation. SopB is also involved in the production of PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 at the *Salmonella* induced ruffles during WT infection. During infection with the *sopB*^{C460S} mutant strain which renders SopB catalytically inactive, none of these phosphatidylinositol phosphates increase at the membrane ruffles (Hernandez et al., 2004; Mallo et al., 2008).

This work confirmed that host PI3K is not required for SopB-mediated PtdIns(3)P production at the membrane ruffles, by treating cells with the PI3K inhibitor, wortmannin prior to infection. We found that although the puncta decorated with PtdIns(3)P disappear during treatment indicating the efficacy of wortmannin treatment, following WT infection, PtdIns(3)P was still produced at the membrane ruffles (Dai et al., 2007; Mallo et al., 2008; Pattni et al., 2001). This led us to the hypothesis that SopB, although only known to hydrolyze PtdIns(4,5)P_2 *in vivo*, must be hydrolyzing a host phosphatidylinositol phosphate to produce PtdIns(3)P at the membrane ruffles (Terebiznik et al., 2002). SopB possesses both 4-phosphatase and 5-phosphatase homology. In an attempt to narrow down or pinpoint a host

substrate, an shRNA screening of host phosphatidylinositol phosphatases and kinases was performed. We speculated that if the production of the substrate was inhibited, PtdIns(3)P would no longer be produced at the membrane ruffles during WT infection. We found that host PtdIns(3,4,5)P_3 5-phosphatase, SKIP and host PtdIns 4-kinase PI4KIIβ were involved in the SopB-mediated PtdIns(3)P production pathway.

The host PtdIns 4-kinase, PI4KIIβ, phosphorylates PtdIns forming PtdIns(4)P, the main precursor for PtdIns(4,5)P_2 in the cell (Jung et al., 2008). We speculate that the knockdown of this kinase caused an imbalance of PtdIns(4,5)P_2 at the membrane. PtdIns(4,5)P_2 is the most abundant phosphatidylinositol phosphate at the plasma membrane and is involved in a number of processes at the membrane such as phagocytosis, endocytosis, and actin polymerization (Raucher et al., 2000; Rohatgi et al., 2001; Sechi and Wehland, 2000). The knockdown of PI4KIIβ gene expression could lead to a lack/decrease of PtdIns(4,5)P_2 at the membrane and a lack of/decrease of the actin-binding proteins recruited by PtdIns(4,5)P_2. PtdIns(4,5)P_2 decrease could be responsible for the invasion defect seen in the cells transfected with the shRNA targeting PI4KIIβ. It's also possible that the knockdown of PI4KIIβ expression altered the plasma membrane in such a way that interfered in the production of PtdIns(3)P. PtdIns(4,5)P_2 is the precursor for PtdIns(3,4,5)P_3. PtdIns (3,4,5)P_3 is then hydrolyzed into PtdIns(3,4)P_2 and subsequently into PtdIns(3)P. A lack or decrease of one species at the plasma membrane could mean the production or availability of other species may be altered. Alternately an increase in PtdIns(4,5)P_2 at the plasma membrane from overexpressing PI4KIIβ could account for an increase in ruffling and phagocytosis, possibly by enhancing the recruitment of actin-binding proteins.

The host PtdIns(3,4,5)P_3 SKIP could be involved in the production of the substrate, PtdIns(3,4)P_2 (Gurung et al., 2003). Knockdown of SKIP expression leads to an increase of PtdIns(3,4,5)P_3, and a decrease in PtdIns(3,4)P_2 for SopB to hydrolyze as a substrate. We found that invasion levels of cells treated with shRNA targeting SKIP and infected with WT *Salmonella* were decreased to the level of the *sopB*^{C460S} infected cells. This would be the expected result if SKIP was required for producing the substrate for SopB.

We also found that overexpression of GFP-SKIP rescued the invasion defect of the *sopB*^{C460S} strain. When SKIP is overexpressed, there is enhanced production of PtdIns(3,4)P_2. This phosphatidylinositol phosphate has been linked with a number of actin modulating proteins and could itself be involved in actin dynamics which could explain why its increase could enhance invasion (Hogan et al., 2004; Posor et al., 2013). (Cain et al., 2008)

It is unclear why *Salmonella* would require a host 5-phosphatase for SopB – mediated ruffling and invasion when SopB already has 5-phosphatase activity. This prompted us to look into the previously uncharacterized 5-phosphatase activity of SopB. In order to address the possible role that the 5-phosphatase activity of SopB has in ruffling and invasion, we made a strain harboring the point mutation at K530A on the *Salmonella* chromosome. We found the invasion of HeLa cells with the *sopB*^{K530A} strain had an invasion defect similar to that of the *sopB*^{C460S} strain. We also made the point mutation in the *sopE*- background, and found that the 5-phosphatase activity of SopB was involved in SopB-mediated ruffling as well as invasion. This is the first work to describe a role for the 5-phosphatase activity of SopB.

We went on to characterize the 5-phosphatase point mutant *in vitro* which had previously been shown to lack activity, similar to the SopB^{C460S} point mutant. We found that SopB^{K530A} retained 60% of its activity against the substrate PtdIns(3,4)P_2. The 5-phosphatase point mutant did not have activity towards other substrates. This provided evidence that the SopB 5-phosphatase point mutant retained 4-phosphatase activity *in vitro*. This meant that the 4-phosphatase activity of SopB, alone, is not sufficient to promote SopB-mediated ruffling and invasion since the 5-phosphatase mutant strain was not able to promote SopB-mediated ruffling and invasion in the *sopE* background.

To provide further evidence supporting this hypothesis we made chromosomal insertion mutants where *sopB* was swapped for known 4-phosphatases, IpgD, or Inpp4B. IpgD is the *Shigella flexneri* homologue of SopB that is a well characterized PtdIns(4,5)P_2 4-phosphatase, while Inpp4B is a characterized mammalian PtdIns(3,4)P_2 4-phosphatase (Fedele et al., 2010; Gewinner et al., 2009; Niebuhr et al.,

2002). SopB was also swapped for host PtdIns(3,4,5)P_3, 5-phospahtase, SKIP(Ijuin et al., 2000). We found that expression of exogenous 4-phosphatase or 5-phosphatase on the chromosome of *Salmonella* was not sufficient to rescue ruffling or invasion in the *sopE*- background strain. We speculated that it's possible both the 4 and 5-phosphatase activities of SopB may be involved and that they may both be required to maintain the dynamic balance of phosphatidylinositols during WT infection. We then swapped *sopB* for either Inpp4B fused with SKIP or IpgD fused with SKIP. This would provide an artificial way to monitor whether the presence of both phosphatases could rescue the SopB-mediated ruffling and invasion in the *sopE*- background strain. We found that when both the 4-phosphatase and 5-phosphatse were present on the chromosome, that SopB-mediated ruffling and invasion could be restored.

Further characterization of the 5-phosphatase point mutation of SopB, led us to investigate the SopB-mediated production of PtdIns(3)P. We found that unlike cells infected with the *sopB*^{C460S} mutant strain, cells infected with *sopB*^{K530A} strain still produced PtdIns(3)P at the ruffles. This indicates that SopB was utilizing PtdIns(3,4)P_2 to produce PtdIns(3)P and the 4-phosphatase activity was sufficient for PtdIns(3)P production. To provide further evidence that the 4-phosphatase activity is sufficient for PtdIns(3)P production, we infected cells with the insertion mutants *sopB::inpp4B, sopB::ipgD,* and *sopB::SKIP.* We found that both 4-phosphatase insertion mutants were able to produce PtdIns(3)P at the ruffles while the 5-phosphatase insertion did not. This data confirms that PtdIns(3)P is produced solely by the 4-phosphatase activity of SopB. This lead to the question of what the 5-phosphatase activity could be doing to promote SopB-mediated ruffling and invasion. All the data indicates PtdIns(3,4)P_2 is the substrate for SopB to produce PtdIns(3)P, so we speculate that the 5-phosphatase activity is responsible for producing PtdIns(3,4)P_2.

We were not sure if the production of PtdIns(3,4)P_2 itself or the dynamic turnover of PtdIns(3,4)P_2 was involved in SopB-mediated ruffling and invasion. we investigated actin modulating proteins known to be recruited by PtdIns$(3,4)_2$ to the membrane. We looked at Tks4, required to form the actin rich podosomes at the plasma membrane, TAPP1, present in actin rich structures and known to modulate the

actin cytoskeleton, Bam32, a protein capable of activating Rac1 to promote actin cytoskeleton rearrangements, and SNX9, a protein involved in endocytosis known to activate N-WASP and promote F-actin nucleation (Allam and Marshall, 2005; Allam et al., 2004; Buschman et al., 2009; Hogan et al., 2004; Soulet et al., 2005; Yarar et al., 2007). We found that Tks4, although known to be recruited to the membrane by PtdIns(3,4)P_2, was not present at the *Salmonella* induced ruffles. We also found that TAPP1 was not recruited in a SopB-dependent manner. Bam32 and SNX9 were both recruited to the *Salmonella* ruffles only during WT infection and were absent in the ruffles of the *sopB*^{C460S} and *sopB*^{K530A} infected cells. Upon further investigation we found Bam32 was not phosphorylated and activated during WT infection. Alternately, we found that when SNX9 was knocked down via siRNA, there was an invasion defect in the WT infected and *sopE*- infected cells similar to the invasion defect of the *sopB*^{C460S} mutant. Upon further investigation we found that the RYK mutant of SNX9, which no longer binds PtdIns(3,4)P_2 is no longer recruited to the ruffles during WT infection. This confirmed that it is the SopB-mediated increase in PtdIns(3,4)P_2 that is recruited SNX9 to the ruffles. To further validate this result we depleted PtdIns(3,4)P_2 at the membrane by targeting Inpp4B, a host PtdIns(3,4)P_2, to the membrane by fusing it to a CAAX motif. Transfected cells did not have SNX9 recruited to the ruffles during WT or *sopE*- infection. Although SNX9 has been characterized to recruit and activate N-WASP at the plasma membrane to nucleate F-actin for endocytic events, more work needs to be done in order to confirm that N-WASP is required for the SNX9-mediated ruffling and invasion. We have shown that N-WASP and SNX9 co-localize at the *Salmonella* induced ruffles during WT infection. This only provides enough evidence to speculate that N-WASP may be involved in ruffle formation, but more experiments need to be done for more definitive conclusions regarding the role of SNX9 in ruffle formation and invasion.

We have data demonstrating the production of PtdIns(3)P in the 5-phosphatase mutant strain. But this same mutant cannot rescue ruffling in the *sopE*- background. This indicated that the production of PtdIns(3)P, although involved in ruffling formation and invasion, was not alone sufficient. We also have data indicating that the production of PtdIns(3,4)P_2 is responsible for recruiting the actin modulating

protein SNX9. This aspect alone is also not sufficient to promote SopB-mediated ruffling and invasion. We speculate that the dynamic cycling of PtdIns(3,4)P_2 to PtdIns(3)P is crucial to SopB-mediated ruffling and invasion. Both the 4-phosphatase and the 5-phosphatase activities are required for SopB-mediated ruffling and invasion. Without both activities there is only PtdIns(3)P production or PtdIns(3,4)P_2 production, and no SopB mediated ruffling and invasion. *Salmonella* has devised a mechanism in which it coordinates the production of PtdIns(3,4)P_2 and subsequent recruitment of SNX9 to its degradation into PtdIns(3)P and subsequent VAMP8 recruitment (Dai et al., 2007; Mallo et al., 2008). The exact temporal regulation of these events still needs to be evaluated, but it is clear that the dynamic cycling of PtdIns(3,4)P_2 to PtdIns(3)P is crucial to proper SopB-mediated ruffling and invasion.

4.2 Future Directions

SopB is a *Salmonella* effector protein responsible for a number of diverse roles including *Salmonella* invasion, SCV formation and maturation, inflammation, exocytosis, and apoptosis prevention. Much of the work published on SopB, attributes these phenotypes to the 4-phosphatase activity of SopB. Given that the C460S point mutant loses both 4-phosphatase and 5-phosphatase activities, it is possible that the 5-phosphatase activity of SopB is responsible for more than just PtdIns(3,4)P_2 production at the membrane and SNX9 recruitment. The K530A point mutant which retains 4-phosphatase activity could illuminate a number processes that have already been characterized. For example, we were able to deduce the 4-phosphatase activity of SopB is sufficient to recruit Rab5 to the SCV surface following *Salmonella* invasion, but a closer look into maturation of the SCV and intracellular replication during infection lacking the 5-phosphatase of SopB was not studied. This could lead to the better understanding of the mechanisms by which SopB enhances *Salmonella* pathogenicity.

In this study were not able to provide evidence that SopB was hydrolyzing PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2. We had hoped to demonstrate this data using the lipid probes. Our system of monitoring the appearance and disappearance of the

specific probes during a time lapse infection did not provide consistent results when monitoring the hydrolysis of PtdIns(3,4)P_2 to PtdIns(3)P. This process deserves more attention though, if we were able to overcome bleaching this could be a valuable tool.

It is also important to further explore the exact role of SNX9 in *Salmonella* infection. We speculate SNX9 is activating N-WASP to promote ruffling, but more in depth study into this mechanism needs to be performed. It's possible that SNX9 is acting through an alternate and previously undescribed pathway during *Salmonella* infection to promote ruffling.

CHAPTER 5. MISCELLANEOUS PROJECTS

5.1 EHEC Type III effector NleL is an E3 Ubiquitin Ligase that Modulates Pedestal Formation

5.1.1 Abstract

Infection with enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 causes hemorrhagic colitis which can result in hemolytic uremia, a syndrome that is potentially fatal. EHEC colonizes the intestinal mucosa where it utilizes its type III secretion system to secrete effector proteins promoting the formation of actin-rich pedestals. The two key effector proteins involved in pedestal formation are Tir and EspFu/TccP. Here we show that another type III secreted effector, Non-LEE-encoded Ligase (NleL) is involved in this process. We found that NleL modulates Tir-mediated pedestal formation. NleL is an E3 ubiquitin ligase that utilizes its E3 ligase activity, mediated by the conserved catalytic cysteine residue C753, for modulating pedestal formation. We found that the mutant deficient in ligase activity resulted in the formation of more pedestals compared to the wild-type strain. Additionally, we found that ectopically expressing wild-type EHEC *nleL* in the EPEC strain E2348/69 which normally lacks the *nleL* gene resulted in a decrease in pedestal formation while expressing the catalytically-deficient *nleL*(C753A) mutant did not. This work demonstrates that EHEC utilized the type III secreted E3 ubiquitin ligase activity of NleL to modulate Tir-mediated pedestal formation.

5.1.2 Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is an increasingly prominent cause of food-borne illness all over the world. Infection with EHEC O157:H7 is the cause of hemorrhagic colitis and can result in the potentially fatal

syndrome, hemolytic uremia (Garmendia et al., 2005; Kaper et al., 2004). Infection with EHEC results in the effacement of the brush border microvilli and intimate attachment of the bacterium to the plasma membrane allowing for the formation of characteristic actin-rich pedestals making this bacterium a member of the group of pathogens known as 'attaching and effacing' (A/E) pathogens (Frankel et al., 1998; Knutton et al., 1989; Knutton et al., 1987). Pedestal formation requires an intact type III secretion system (TTSS) allowing for the translocation of effector proteins Tir and EspFu/TccP. These two effector proteins are key players in pedestal formation.

Tir is a bacterial receptor secreted from the bacterium facilitating its binding to the protein intimin on the bacterial surface. Once bound to intimin a series of events including the subsequent binding of the N-terminus of Tir to host proteins, α-actinin, talin, vinculin and cortactin allows for the formation of pedestals (Goosney et al., 2000). Additionally, it has been documented that the C-terminus of Tir is involved in binding host protein Nck which is able to stimulate F-actin assembly (Campellone et al., 2002; Gruenheid et al., 2001). EspFu/TccP in contrast, is able to bind and activate N-WASP directly which is responsible for F-actin nucleation (Campellone et al., 2004b).

The reversible post-translational modification of proteins in which ubiquitin, a 76 amino acid polypeptide, is attached to the ε-amino group of lysines is a process called ubiquitination. This process involves the multi-enzyme cascade of E1, the ubiquitin activating enzymes, E2, the ubiquitin-conjugating enzymes and E3, the ubiquitin protein ligases. The specificity of the target protein is defined by E3 ubiquitin ligase activity (Pickart, 2001). E3 ubiquitin ligases contain a highly conserved cysteine residue located approximately 35 residues from the C-terminus (Schwarz et al., 1998).

Over 60 putative EHEC effectors were identified from a screening, identifying, EspX7 as a type III effector. Further work identified EspX7 as an E3 ubiquitin ligase with C753 being critical for its ligase activity. EspX7 was renamed NleL for (Non-LEE-encoded effector Ligase) in this study (Deng et al., 2004; Piscatelli et al., 2011).

5.1.3 Materials and Methods

Bacterial strains, plasmids, and mammalian cell lines

The Enterohemorrhagic Escherichia coli O17:H7 strain (ZP250) is a spontaneous naladixic acid mutant of the outbreak strain (RM1484). ZP250 was used as the parent strain to create the EHEC strain expressing the catalytically dead chromosomal mutant *nleL*^{C753A} (ZP254). This strain was generated with the help of an allelic exchange suicide vector using the plasmid encoding NleLC753A (pZP2111). The plasmid expressing wild-type EHEC NleL and its promoter sequence (pZP1666) or the catalytically dead mutant NleLC753A (pZP1667) were introduced into EPEC strain E2348/69.

HeLa cells from American Type Culture Collection (Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10%FBS. Cells were kept at 37°C with 5% CO2.

Bacterial infection and secretion assay

EHEC strains were grown overnight standing at 37°C in Luria Bertani (LB) broth supplemented with naladixic acid at 50µg/mL and used directly for infection. EPEC strains were grown overnight standing at 37°C in LB and subsequently subcultured (1:100) in HEPES-buffered DMEM until an OD at 600nm of .7. Cultures were then used to infect at an MOI of 100. HeLa cells were infected for 3 hours followed by an additional 3 hours in fresh media.

For secretion assay: bacterial strains were grown overnight in LB. The cultures were diluted 1:20 in M9 minimal media supplemented with .4% glucose, 44mM $NaHCO_{2,}$ .1% Casamino acids and 8mM $MgSO_4$ and grown standing at 37°C with 5% CO_2. The cultures were grown to an OD of .8. The cultures were then spun down and the supernatant was passed through a .2µm filter. The proteins in the supernatant were then precipitated with 10% TCA on ice for 1 hour and washed with ice cold acetone. The pellet was then lysed in 1X sample buffer.

Immunofluorescence microscopy

Infected HeLa cells were washed with PBS, fixed in 3% formaldehyde and permeabilized with 0.1% triton X-100. EHEC was detected with rabbit anti-O antibody (EHEC O157, Difco Laboratories, Detriot, MI) while EPEC was detected with rabbit anti-O antibody (EPEC O111, Denka Seiken CO, Tokyo, Japan) followed by Alexa Fluor 488 conjugated anti-rabbit secondary antibody. F-actin was visualized using Texas-red Phalloidin (1:300; Molecular probes, Carlband, CA). All images were obtained on a Zeiss LSM 700 confocal microscope.

5.1.4 Results

5.1.4.1 E3 ligase activity of NleL down-regulates the EHEC pedestal formation

The defining factor of A/E pathogens is the production of actin-rich pedestals on the plasma membrane. The formation of these structures during EHEC infection begins when the effectors, Tir and EspFu are secreted from the bacterial cytosol by the TTSS. NleL is another secreted effector protein with an unknown role in pedestal formation. To assess any involvement of NleL in pedestal formation, an EHEC strain expressing the chromosomal catalytically dead mutant n*leL*^{C753A} was used to infect HeLa cells. Interestingly the number of pedestals formed in the HeLa cells infected with the mutant strain was significantly increased from the number of pedestal formed during wild-type EHEC infection (Fig 5-1 A). The phenotype of the n*leL*^{C753A} strain could be rescued by introducing the wild-type NleL expressing plasmid in the mutant strain. The number of cells with no pedestals decreased 2-fold in the *nleL*^{C753A} mutant strain infected cells compared to wild-type infected, while the number with more than 10-pedestals increased more than 2-fold in the mutant infected (Fig 5-1B).

We also found that the *nleL*^{C753A} mutation did not alter the expression or secretion of Tir. This result indicates that the E3 ubiquitin ligase activity of NleL is actually responsible for modulating Tir-mediated pedestal formation and this alteration does not involve the down regulation or blocking the secretion of Tir (Fig 5-2).

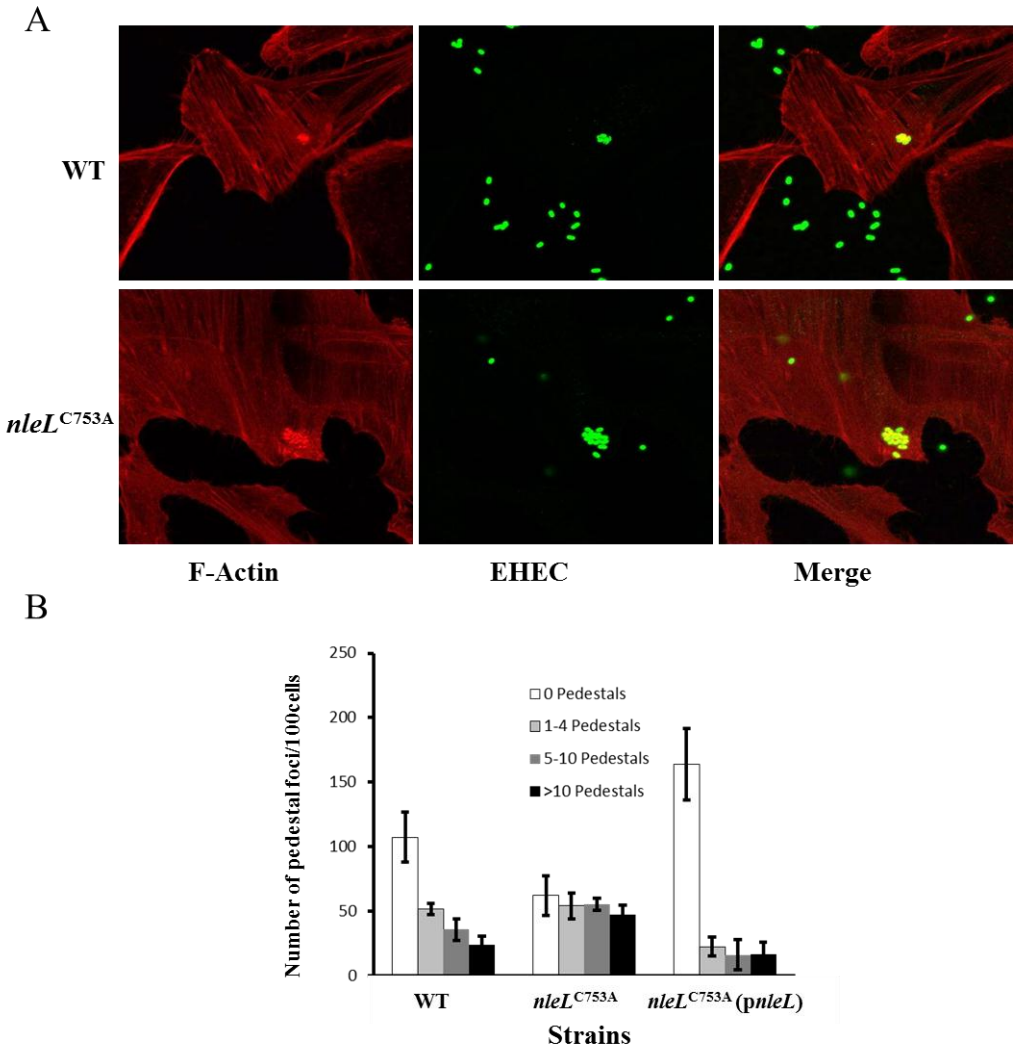

Fig. 5-1 The E3 Ligase activity of NleL modulates EHEC pedestal formation. (A) HeLa cells were infected with WT EHEC or the *nleL*^{C753A} strain at an MOI of 100 for 6 hours. EHEC were visualized by staining with anti-EHEC LPS antibody (green) and pedestals were assessed by staining for F-actin with texas Red conjugated Phalloidin. (red) (B) HeLa cells infected with WT EHEC, *nleL*^{C753A}, or *nleL*^{C753A} harboring a plasmid expressing WT NleL. The number of pedestals formed following an infection at an MOI of 100 for 6 hours were quantified and grouped as shown.

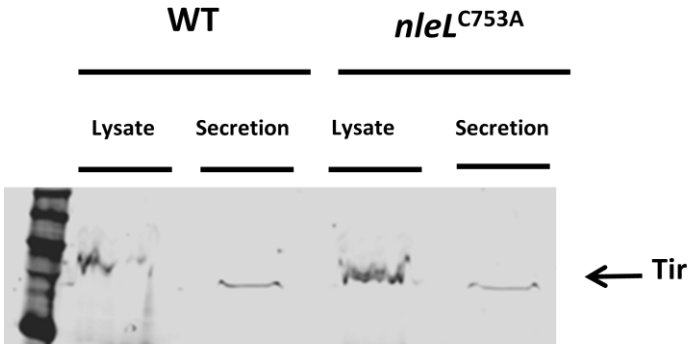

Fig. 5-2 The C753A point mutation does not affect Tir secretion. Expression and secretion of Tir in WT or the *nleL*^{C753A} mutant strain. For secretion bacterial strains were grown overnight in LB. The cultures were diluted 1:20 in M9 minimal media and grown standing at 37°C with 5% CO_2. The cultures were grown to an OD of .8 and secreted proteins were precipitated with 10% TCA before being resuspended in SDS PAGE loading buffer.

5.1.4.2 The E3 ligase activity of EHEC NleL down modulates EPEC pedestal formation

To further validate this result wild-type EHEC NleL was expressed in the Enteropathogenic *Escherichia coli* (EPEC) strain E2348/69. This strain naturally lacks the *nleL* homologue. EPEC, in general, is known to have more robust pedestal formation compared to wild-type EHEC infection. Unlike EHEC, EPEC does not encode EspFu, and forms pedestals via recruitment of host Nck to phosphorylated EPEC Tir (Gruenheid et al., 2001). EPEC Tir and EHEC Tir have 59% sequence similarity and we have shown that in EHEC the E3 ubiquitin ligase activity of NleL is able to modulate pedestal formation. Given the sequence similarity of the Tir genes in both EHEC and EPEC, wild-type EHEC NleL or the strain expressing the catalytically inactive mutant NleL^{C753A} were expreessed in the EPEC. The EPEC strain expressing wild-type EHEC NleL induced significantly less pedestals than the wild-type EPEC strain or the strain expressing the catalytically inactive mutant NleL^{C753A} (Fig 3-3 A&B). Because of the differenced in pedestal formation between the two strains this result could indicate that NleL may not affect a process specific to EspFu or Nck, but modulates a common step of both EHEC and EPEC pedestal formation (Campellone et al., 2002; Gruenheid et al., 2001).

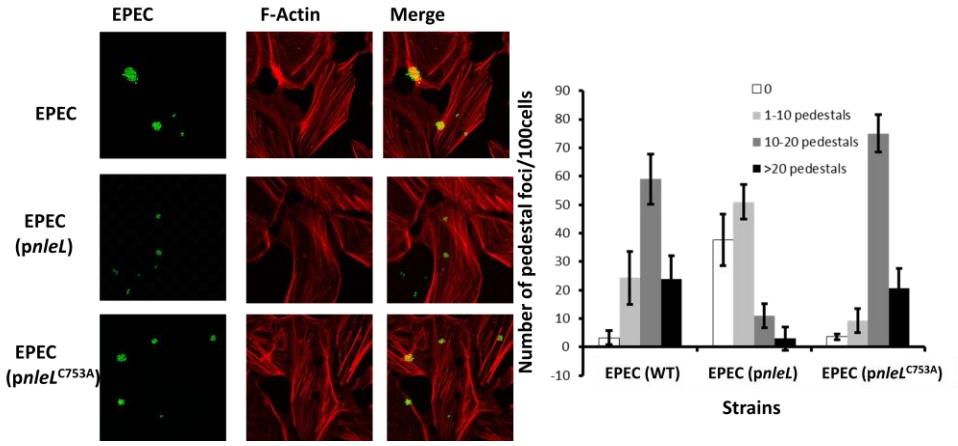

Fig. 5-3 The E3 ligase activity of NleL modulates EPEC pedestal formation. HeLa cells were infected with the indicated strain at an MOI of 100 for 4 hours. Bacteria were visualized by staining with anti-EPEC LPS antibody (green) and pedestals were visualized by staining for F-actin with Texas Red conjugated Phalloidin (red). The number of pedestals was quantified and grouped as shown. Analysis includes three independent experiments.

5.1.5 Discussion

Both EHEC and EPEC are A/E bacteria that utilize their TTSS to translocate bacterial effectors into the cytoplasm to promote the characteristic pedestal formation (Frankel et al., 1998; Knutton et al., 1987; Moon et al., 1983). The molecular events leading to pedestal formation differ between the two, but the end result is morphologically similar pedestals. The pathway leading to pedestal formation for both bacteria is fairly well characterized, while the modulation of this process is not well understood. This study has identified an E3 ubiquitin ligase, NleL, in EHEC that is capable of modulating the Tir-mediated pedestal formation. Loss of NleL activity leads to an increase in pedestal formation, while not altering the expression or secretion of Tir, an important effector in the production of pedestal formation. This could indicate that NleL does not exert its function until after the translocation of Tir. EPEC infection leads to more robust pedestal formation compared to EHEC infection. The mechanism of the pedestal formation differs between the two bacteria in that EPEC Tir is phosphorylated upon secretion and subsequently binds host protein Nck to stimulate Arp2/3 mediated actin polymerization (Campellone et al., 2006; Caron et al., 2006). Alternatively EHEC utilizes two effector proteins, Tir and EspFu. Tir interacts with a number of actin modulating proteins through its N-terminus during

EHEC infection while its C-terminus binds host protein Nck. EspFu which is not encoded in EPEC, binds and activates N-WASP during EHEC infection (Campellone et al., 2004a; Garmendia et al., 2004). Although these two mechanisms of pedestal formation differ, NleL is able to modulate pedestal formation in both bacteria, indicating there must be some common aspect that NleL is acting upon to modulated pedestal formation. The E3 ubiquitin ligase component of the ubiquitination process is responsible for substrate specificity. Although the exact substrate of NleL was not identified in this study, we can speculate that NleL must ubiquitinate either a bacterial or host factor involved in pedestal formation. This factor would then become limiting, or its localization altered in such a way that it could no longer contribute to pedestal formation. Further studies are needed to determine the substrate of NleL whether bacterial or host, to define the exact mechanism the E3 ligase activity of NleL ensues to modulate pedestal formation.

5.2 Inducible gene deletion

5.2.1 Abstract

Salmonella spp. contain two type III secretion systems encoded on *Salmonella* pathogenicity islands 1 and 2 (SPI1 and SPI2). It is well accepted that the bacterial effector proteins secreted by SPI1 are involved in gaining entrance into the host cell, while those translocated via SPI2 are involved in SCV formation and replication of the bacteria once inside the host cell. Several studies have demonstrated the involvement of the SPI1 secreted effector, SopB, in SCV maturation and *Salmonella* replication. Given the involvement of SopB in promoting sufficient invasion into the host cell, it is difficult to ensure some of these roles following invasion are carried out by SopB and not solely due to insufficient invasion that occurs when this gene is deleted. This work will attempt to devise a way to inducibly delete *sopB* after invasion to differentiate these two roles.

5.2.2 Introduction

Salmonella effector protein SopB is secreted and translocated in a SPI1 dependent manner. Effectors secreted in a SPI dependent manner are secreted once the bacterial cell contacts the host cell. SopB has been implicated in a number of mechanisms involving invasion of the bacterium into the host cell including phosphatidylinositol production and ruffle formation (Dai et al., 2007; Marcus et al., 2001; Terebiznik et al., 2002; Zhou et al., 2001). SopB is one of the key players along with SopE involved in causing rearrangements to the host actin cytoskeleton allowing *Salmonella* entrance into the host cell. The phosphatidylinositol phosphate phosphatase activity of SopB is crucial to this function. The mutation of the catalytic cysteine C460 abolishes all activity of SopB and causes a modest invasion defect (Marcus et al., 2001; Zhou et al., 2001). Interestingly, a number of events later on in the *Salmonella* life cycle have been attributed to SopB. SopB is said to be responsible for proper formation and maturation of the *Salmonella* containing vacuole (SCV) (Hernandez et al., 2004; Mallo et al., 2008). This phenotype could be due to the later activity of SopB, or due to improper invasion that occurs in the absence of SopB. When SopB is translocated into the host cell cytosol, it produces PtdIns(3)P at the *Salmonella* induced membrane ruffles. PtdIns(3)P is responsible for recruiting host v-SNARE proteins VAMP8 to the plasma membrane which is required for sufficient bacterial entry. PtdIns(3)P is also involved in endocytic vesicle fusion (Dai et al., 2007; Wang et al., 2004). It's been speculated that the purpose of PtdIns(3)P production at the membrane ruffles is to incorporate into the forming SCV membrane so the SCV and fuse small endosomes and become more spacious. Therefore, in a SopB deletion, PtdIns(3)P will not be present at the *Salmonella* induced ruffles and won't become incorporated into the forming SCV. This SCV will then be less spacious and prevent proper bacterial replication. It's possible that SopB does not have a defined role following invasion, but in fact affects later stages of the bacterial life cycle because of insufficient invasion and SCV formation. This work seeks to create a system in which *sopB* can be deleted from the bacterial chromosome in a temporal manner, allowing for proper invasion of *Salmonella* into the host cell followed by gene deletion. This would allow for a clearer view of the possible

involvement of SopB in later stages of *Salmonella* pathogenesis. This mechanism has been previously established in the bacterial pathogen *Legionella pneumophila* (Liu et al., 2008).

5.2.3 Materials and Methods

Bacterial strains and plasmid construction for gene 'knock-in'

Wild-type *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) strain SL1344 was previously described (Hoiseth and Stocker, 1981).

To create the floxed *sopB* strain, *sopB* flanked by two *lox*P sites, the two oligomers containing two directly repeated loxP sites separated by a SalI and NcoI site and BamHI overhangs

5'-
GGATCCATAACTTCGTATAGCATACATTATACGAAGTTATCAGGAGGAATTCCCATGGAA
GCTTGTCGACATAACTTCGTATAGCATACATTATACGAAGTTATGGATCC-3' were annealed. The oligomers were directly ligated into the BamHI site of pZP152 – a plasmid containing 500bp upstream and 500bp downstream SopB to create (pZP3278). Full length SopB was then inserted into the SalI, NcoI sites of pZP3278 to create (pZP3279). The 'floxed' *sopB* DNA fragment was subcloned from pZP3279 into the NotI, XbaI sites of the R6K-derived suicide vector pSB890 to create (pZP3280). pZP3280 was then introduced into the wild-type *Salmonella* chromosome by homologous recombination creating ZP480 creating the floxed *sopB* strain.

Plasmids for inducible expression of Cre

Five plasmids used for the inducible expression of Cre recombinase were a generous gift from the Luo lab and were described in detail previously (Liu et al., 2008). Briefly, the plasmids expressed Cre recombinase under the control of an isopropyl β-D-thiogalactoside (IPTG) inducible promoter, pZL638 and pZL639 were under looser control of expression while pZL680 and pZL681 were created for tighter control of expression. The final plasmid was a constitutively active Cre recombinase.

Gene Deletion and effector protein expression secretion

To evaluate gene deletion the Cre plasmids were individually expressed in ZP480 pZL638,639,680 and 681 were transformed into ZP480 and grown in LB

overnight at 37°C. They were then subcultured (1:20) in LB supplemented with .3M NaCl for 3 hours on a rotating wheel. The cultures were then induced for various time points with 1mM, .5mM, or 2mM IPTG as indicated. After induction they were centrifuged at high speed and washed 5X with PBS and plated. Colonies were picked to determine the knockout efficiency via PCR. To assess any effect the knock-on may have on the secretion of other bacterial effectors, we checked the expression and secretion of SipC, and SipB. Expression was assessed by growing the strain overnight in LB centrifuging the cultures and resuspending them in SDS PAGE loading buffer. Expression was detected via western blot using rabbit anti-SipC antibody and SipB using rabbit anti-SipB antibody described previously (Myeni et al., 2013). SopB was detected using anti SopB antibody. Secretion of SipC and SipB was obtained by growing the strains overnight in LB followed by subculturing (1:20) for 3 hours in LB supplemented with .3M NaCl. The cultures were then centrifuged and supernatant was removed and filtered in a .2micron filter to remove any bacteria. The protein was then precipitated with 10%TCA and incubated 30 min on ice. The samples were then centrifuged and washed 3X with ice cold acetone and resuspended in SDS PAGE loading buffer. The secreted proteins were then detected via western blot as previously described.

5.2.4 Results

5.2.4.1 Expression and secretion of TTSS effectors was not altered in floxed *sopB* strain

To investigate the specific role SopB may play following invasion into the host cell the Cre/*lox*P inducible gene deletion system described previously (Liu et al., 2008) was implemented for bacterial effector protein SopB. *sopB* was flanked by two *lox*P repeated (termed floxed) and inserted into the *Salmonella* chromosome via homologous recombination. Introducing a floxed *sopB* gene on the chromosome would enable the *sopB* gene to be deleted from the chromosome when an inducible Cre-recombinase was expressed. This system of temporal gene deletion has already been established in the bacterial pathogen *Legionella pneumophila*. Once the strain harboring the floxed *sopB* gene was created the expression and secretion of not only

SopB, but other important bacterial effectors SipC and SipB was confirmed to be at a similar level to the WT strain (Fig 5-4). This indicates that the Type III secretion system and the secretion and expression of important bacterial effectors were not altered by the floxed *sopB* insertion. WT, the in-frame deletion of *sopB* and the *invA*

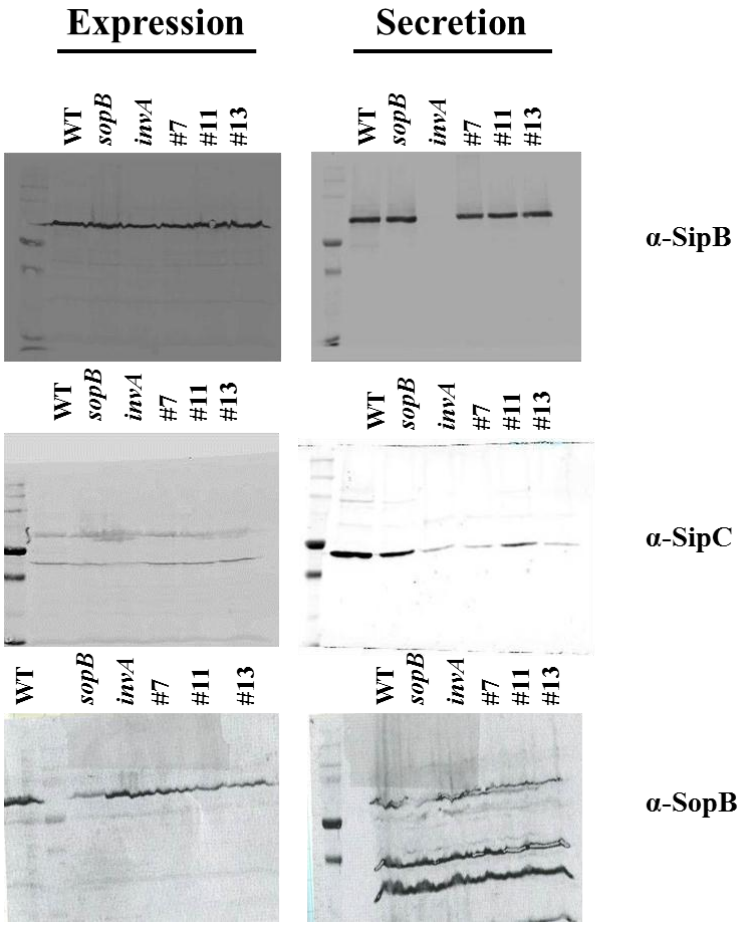

Fig. 5-4 The floxed mutants express and secrete the Type III effectors SopB, SipB, and SipC at similar levels to the WT. The indicated strain was grown overnight at 37˚C. For expression the cultures were centrifuged at high speed and the pellet was lysed with SDS PAGE loading buffer. For secretion the overnight cultures were subcultured for 3hrs at 37˚C on a rotating wheel for low rotation. The secreted proteins were precipitated with 10%TCA and resuspended in SDS PAGE loading buffer. Rabbit anti-SopB, anti-SipB, anti-SipC were used to detect the proteins.

Type III secretion system mutant were used as controls for expression and secretion. #7,#11, and #13 represent three separate floxed strains. Strain #13 was used for all subsequent studies.

5.2.5 SopB inducible deletion

Once the floxed strain was confirmed to have comparable proteins expression and secretion to the WT strain, the system was tested in the *Salmonella* strain. The more tightly regulated of the Cre plasmids, (pZL681) was expressed in the floxed strain. Cre recombinase was induced at 1mM IPTG for 0 min, 5-min, and 15min as indicated (Fig 5-5 A). Unfortunately under the control of the stringently controlled inducible promoter, there was no gene deletion at these time points (Fig. 5-5). This plasmid may have been too tightly controlled for this system, so we then expressed the less stringently controlled plasmids, pZL639 and pZL638 in the floxed strain. The plasmids were induced for 5 minutes and similar to the stringent plasmids also failed to delete *sopB* from the chromosome (Fig 5-5B). The induction time was then increased to 2.5 hours at 1mM IPTG which also resulted in no gene deletion. Finally, the concentration of IPTG for induction of the Cre recombinase was varied from 1mM to .5mM and 2mM with no visible deletion of *sopB* from the *salmonella* chromosome (Fig 5-5C). To overcome different induction methods, we then obtained a constitutively active Cre plasmid and expressed this in our *Salmonella* strain. Unfortunately there was still no detectable *sopB* deletion from the chromosome (Fig 5-5D). To determine if this was a problem with *Salmonella* or the system, the constitutively actin Cre plasmid and the floxed SopB plasmid were co-expressed in pSK. We were able to see deletion in this example (Fig 5-5E). It's possible that this system although functional, is not compatible with *Salmonella.* The plasmids used in this study were created for the bacterial pathogen *Legionella pneumophila* and it's possible that Cre recombinase should be expressed on a plasmid known to be compatible with *Salmonella,* such as an arabinose inducible plasmid.

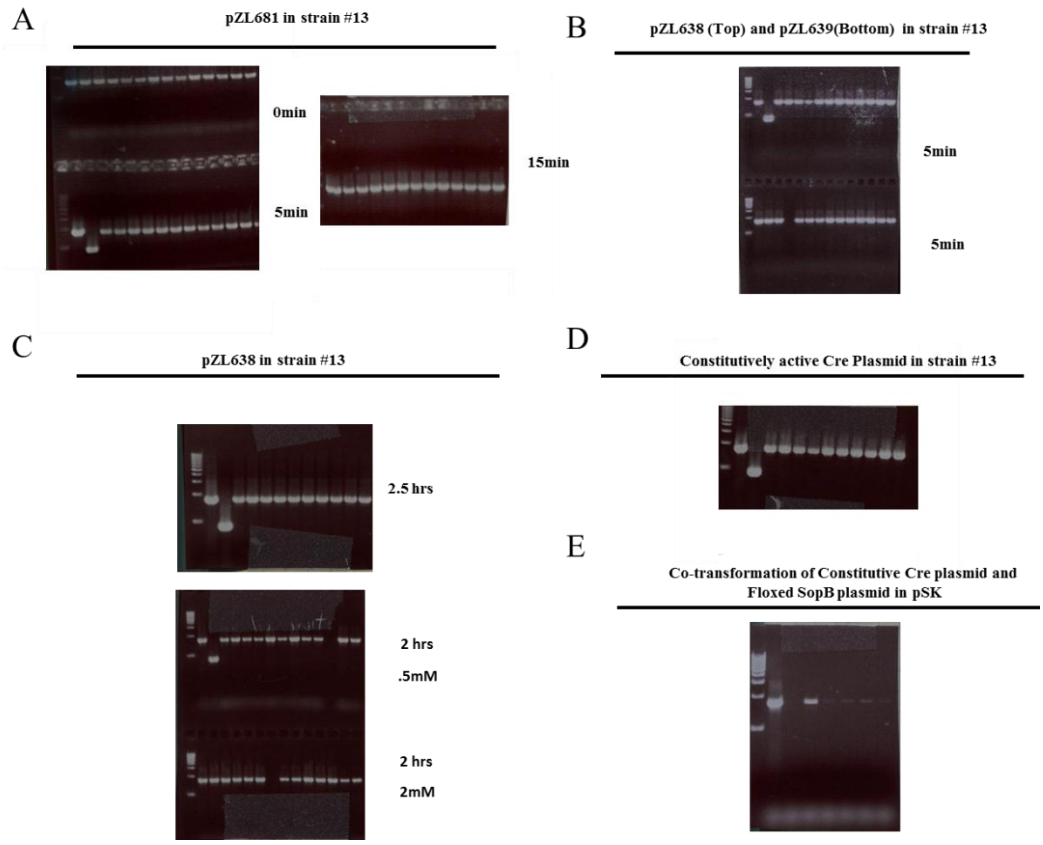

Fig. 5-5 Floxed SopB strain #13 expressing Cre recombinase to induce deletion of *sopB*. Floxed SopB strain #13 expressing the indicated Cre recombinase plasmid was grown overnight at 37°C then subcultured for 3 hours. Following induction as described, the cultures were centrifuged, washed, and diluted in PBS before being plated on Strep. Plates. Single colonies were picked from each condition and checked for deletion of *sopB* via PCR of the flanking region (lower band represents *sopB* deletion). (A) Cre expression was induced for 0min, 5min, and 15min as indicated with 1mM IPTG. (B) Cre expression was induced for 5min with 1mM IPTG. (C) Cre expression was induced for 2.5 hours with 1mM IPTG (top) or for 2hours with .5mM or 2mM as indicated. (D) The constitutively active Cre recombinase plasmid was expressed in the Floxed *sopB* strain. (E) The constitutively active Cre recombinase and the Floxed SopB plasmid were co-expressed in pSK.

5.2.6 Discussion

SopB is responsible for the ruffle formation and subsequent invasion of *Salmonella* into the host cell, as well as for producing PtdIns(3)P at the membrane ruffles (Dai et al., 2007; Hernandez et al., 2004; Zhou et al., 2001). SopB is also thought to be involved in SCV maturation and bacterial replication (Mallo et al., 2008;

Wasylnka et al., 2008). At this point it is difficult to determine if SopB is actively promoting the maturation following invasion or if it's fuction during invasion has lasting effects on SCV maturation. When *sopB* is deleted from the chromosome, there is a decrease in ruffling, invasion, and also an abolishment of PtdIns(3)P at the membrane ruffles. It is no surprise then, that when SopB is mutated or deleted the SCV's that form are not able to mature properly, something that has been linked to PtdIns(3)P. It is speculated that PtdIns(3)P is at the ruffles to incorporate into the SCV so that the SCV can fuse with nearby vesicles and become a spacious vacuole for the bacteria to multiply (Mallo et al., 2008). It's difficult to delineate the two activates and creating a system that could delete *sopB* from the chromosome after invasion could lend insight into this process. We had wanted to create a system in which minutes following bacterial invasion *sopB* could be deleted from the chromosome which would allow us to investigate whether the activities of SopB are in fact required for SCV maturation following proper invasion into the host cell. Unfortunately we were not able to create this system in the *Salmonella* model. This system worked in the bacterial pathogen *Legionella pneumophila* but was not successful in our model. We tested the efficacy of the floxed *sopB* co-expressed in *E. coli* with the constitutive Cre plasmid and found that fundamentally this system should work. Given the success of this system in other bacteria, it's possible that using alternate plasmids expressing the Cre recombinase such as an arabinose inducible plasmid known to express in *Salmonella* would be more efficient.

REFERENCES

REFERENCES

Akisaka, T., H. Yoshida, R. Suzuki, and K. Takama. 2008. Adhesion structures and their cytoskeleton-membrane interactions at podosomes of osteoclasts in culture. *Cell Tissue Res.* 331:625-641.

Aleman, A., I. Rodriguez-Escudero, G.V. Mallo, V.J. Cid, M. Molina, and R. Rotger. 2005. The amino-terminal non-catalytic region of *Salmonella typhimurium* SigD affects actin organization in yeast and mammalian cells. *Cell. Microbiol.* 7:1432-1446.

Allam, A., and A.J. Marshall. 2005. Role of the adaptor proteins Bam32, TAPP1 and TAPP2 in lymphocyte activation. *Immunol. Lett.* 97:7-17.

Allam, A., H. Niiro, E.A. Clark, and A.J. Marshall. 2004. The adaptor protein Bam32 regulates Rac1 activation and actin remodeling through a phosphorylation-dependent mechanism. *J. Biol. Chem.* 279:39775-39782.

Audhya, A., M. Foti, and S.D. Emr. 2000. Distinct roles for the yeast phosphatidylinositol 4-kinases, Stt4p and Pik1p, in secretion, cell growth, and organelle membrane dynamics. *Mol. Biol. Cell*. 11:2673-2689.

Borm, B., R.P. Requardt, V. Herzog, and G. Kirfel. 2005. Membrane ruffles in cell migration: indicators of inefficient lamellipodia adhesion and compartments of actin filament reorganization. *Exp. Cell Res.* 302:83-95.

Botelho, R.J., M. Teruel, R. Dierckman, R. Anderson, A. Wells, J.D. York, T. Meyer, and S. Grinstein. 2000. Localized biphasic changes in phosphatidylinositol-4,5-bisphosphate at sites of phagocytosis. *J. Cell Biol.* 151:1353-1368.

Brenner, F.W., R.G. Villar, F.J. Angulo, R. Tauxe, and B. Swaminathan. 2000. Salmonella nomenclature. *J. Clin. Microbiol.* 38:2465-2467.

Burkinshaw, B.J., G. Prehna, L.J. Worrall, and N.C. Strynadka. 2012. Structure of Salmonella effector protein SopB N-terminal domain in complex with host Rho GTPase Cdc42. *J. Biol. Chem.* 287:13348-13355.

Buschman, M.D., P.A. Bromann, P. Cejudo-Martin, F. Wen, I. Pass, and S.A. Courtneidge. 2009. The novel adaptor protein Tks4 (SH3PXD2B) is required for functional podosome formation. *Mol. Biol. Cell*. 20:1302-1311.

Cain, R.J., R.D. Hayward, and V. Koronakis. 2004. The target cell plasma membrane is a critical interface for *Salmonella* cell entry effector-host interplay. *Mol. Microbiol.* 54:887-904.

Cain, R.J., R.D. Hayward, and V. Koronakis. 2008. Deciphering interplay between Salmonella invasion effectors. *PLoS Pathog.* 4:e1000037.

Campellone, K.G., M.J. Brady, J.G. Alamares, D.C. Rowe, B.M. Skehan, D.J. Tipper, and J.M. Leong. 2006. Enterohaemorrhagic Escherichia coli Tir requires a C-terminal 12-residue peptide to initiate EspF-mediated actin assembly and harbours N-terminal sequences that influence pedestal length. *Cell. Microbiol.* 8:1488-1503.

Campellone, K.G., A. Giese, D.J. Tipper, and J.M. Leong. 2002. A tyrosine-phosphorylated 12-amino-acid sequence of enteropathogenic Escherichia coli Tir binds the host adaptor protein Nck and is required for Nck localization to actin pedestals. *Mol. Microbiol.* 43:1227-1241.

Campellone, K.G., S. Rankin, T. Pawson, M.W. Kirschner, D.J. Tipper, and J.M. Leong. 2004a. Clustering of Nck by a 12-residue Tir phosphopeptide is sufficient to trigger localized actin assembly. *J. Cell Biol.* 164:407-416.

Campellone, K.G., D. Robbins, and J.M. Leong. 2004b. EspFU is a translocated EHEC effector that interacts with Tir and N-WASP and promotes Nck-independent actin assembly. *Dev. Cell*. 7:217-228.

Caron, E., V.F. Crepin, N. Simpson, S. Knutton, J. Garmendia, and G. Frankel. 2006. Subversion of actin dynamics by EPEC and EHEC. *Curr. Opin. Microbiol.* 9:40-45.

Chang, J., J. Chen, and D. Zhou. 2005. Delineation and characterization of the actin nucleation and effector translocation activities of *Salmonella* SipC. *Mol. Microbiol.* 55:1379 - 1389.

Charpentier, X., and E. Oswald. 2004. Identification of the Secretion and Translocation Domain of the Enteropathogenic and Enterohemorrhagic *Escherichia coli* Effector Cif, Using TEM-1 {beta}-Lactamase as a New Fluorescence-Based Reporter. *J. Bacteriol.* 186:5486-5495.

Chua, J., and V. Deretic. 2004. Mycobacterium tuberculosis reprograms waves of phosphatidylinositol 3-phosphate on phagosomal organelles. *J. Biol. Chem.* 279:36982-36992.

Criss, A.K., and J.E. Casanova. 2003. Coordinate regulation of Salmonella enterica serovar Typhimurium invasion of epithelial cells by the Arp2/3 complex and Rho GTPases. *Infect. Immun.* 71:2885-2891.

Dai, S., Y. Zhang, T. Weimbs, M.B. Yaffe, and D. Zhou. 2007. Bacteria-generated PtdIns(3)P recruits VAMP8 to facilitate phagocytosis. *Traffic*. 8:1365-1374.

Deng, W., J.L. Puente, S. Gruenheid, Y. Li, B.A. Vallance, A. Vazquez, J. Barba, J.A. Ibarra, P. O'Donnell, P. Metalnikov, K. Ashman, S. Lee, D. Goode, T. Pawson, and B.B. Finlay. 2004. Dissecting virulence: systematic and functional analyses of a pathogenicity island. *Proc. Natl. Acad. Sci. U. S. A.* 101:3597-3602.

Doughman, R.L., A.J. Firestone, M.L. Wojtasiak, M.W. Bunce, and R.A. Anderson. 2003. Membrane ruffling requires coordination between type Ialpha phosphatidylinositol phosphate kinase and Rac signaling. *J. Biol. Chem.* 278:23036-23045.

Dukes, J.D., H. Lee, R. Hagen, B.J. Reaves, A.N. Layton, E.E. Galyov, and P. Whitley. 2006. The secreted *Salmonella dublin* phosphoinositide phosphatase, SopB, localizes to PtdIns(3)P-containing endosomes and perturbs normal endosome to lysosome trafficking. *Biochem. J.* 395:239-247.

Fairn, G.D., K. Ogata, R.J. Botelho, P.D. Stahl, R.A. Anderson, P. De Camilli, T. Meyer, S. Wodak, and S. Grinstein. 2009. An electrostatic switch displaces phosphatidylinositol phosphate kinases from the membrane during phagocytosis. *J. Cell Biol.* 187:701-714.

Fedele, C.G., L.M. Ooms, M. Ho, J. Vieusseux, S.A. O'Toole, E.K. Millar, E. Lopez-Knowles, A. Sriratana, R. Gurung, L. Baglietto, G.G. Giles, C.G. Bailey, J.E. Rasko, B.J. Shields, J.T. Price, P.W. Majerus, R.L. Sutherland, T. Tiganis, C.A. McLean, and C.A. Mitchell. 2010. Inositol polyphosphate 4-phosphatase II regulates PI3K/Akt signaling and is lost in human basal-like breast cancers. *Proc. Natl. Acad. Sci. U. S. A.* 107:22231-22236.

Finlay, B.B., S. Ruschkowski, and S. Dedhar. 1991. Cytoskeletal rearrangements accompanying *Salmonella* entry into epithelial cells. *J. Cell Sci.* 99:283-296.

Francis, C.L., T.A. Ryan, B.D. Jones, S.J. Smith, and S. Falkow. 1993. Ruffles induced by *Salmonella* and other stimuli direct macropinocytosis of bacteria. *Nature*. 364:639-642.

Frankel, G., A.D. Phillips, I. Rosenshine, G. Dougan, J.B. Kaper, and S. Knutton. 1998. Enteropathogenic and enterohaemorrhagic Escherichia coli: more subversive elements. *Mol. Microbiol.* 30:911-921.

Fratti, R.A., J.M. Backer, J. Gruenberg, S. Corvera, and V. Deretic. 2001. Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. *J. Cell Biol.* 154:631-644.

Friebel, A., H. Ilchmann, M. Aepfelbacher, K. Ehrbar, W. Machleidt, and W.D. Hardt. 2001. SopE and SopE2 from *Salmonella typhimurium* activate different sets of RhoGTPases of the host cell. *J. Biol. Chem.* 276:34035-34040.

Fu, Y., and J.E. Galán. 1999. A *Salmonella* protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. *Nature*. 401:293-297.

Furse, S., N.J. Brooks, A.M. Seddon, R. Woscholski, R.H. Templer, E.W. Tate, P.R.J. Gaffney, and O. Ces. 2012. Lipid membrane curvature induced by distearoyl phosphatidylinositol 4-phosphate. *Soft Matter*. 8:3090-3093.

Galyov, E.E., M.W. Wood, R. Rosqvist, P.B. Mullan, P.R. Watson, S. Hedges, and T.S. Wallis. 1997. A secreted effector protein of *Salmonella dublin* is translocated into eukaryotic cells and mediates inflammation and fluid secretion in infected ileal mucosa. *Mol. Microbiol.* 25:903-912.

Garcia-del Portillo, F., and B.B. Finlay. 1994. *Salmonella* invasion of nonphagocytic cells induces formation of macropinosomes in the host cell. *Infect. Immun.* 62:4641-4645.

Garcia-del Portillo, F., M.G. Pucciarelli, W.A. Jefferies, and B.B. Finlay. 1994. *Salmonella typhimurium* induces selective aggregation and internalization of host cell surface proteins during invasion of epithelial cells. *J. Cell Sci.* 107:2005-2020.

Garmendia, J., G. Frankel, and V.F. Crepin. 2005. Enteropathogenic and enterohemorrhagic Escherichia coli infections: translocation, translocation, translocation. *Infect. Immun.* 73:2573-2585.

Garmendia, J., A.D. Phillips, M.F. Carlier, Y. Chong, S. Schuller, O. Marches, S. Dahan, E. Oswald, R.K. Shaw, S. Knutton, and G. Frankel. 2004. TccP is an enterohaemorrhagic *Escherichia coli* O157:H7 type III effector protein that couples Tir to the actin-cytoskeleton. *Cell. Microbiol.* 6:1167-1183.

Gary, J.D., A.E. Wurmser, C.J. Bonangelino, L.S. Weisman, and S.D. Emr. 1998. Fab1p is essential for PtdIns(3)P 5-kinase activity and the maintenance of vacuolar size and membrane homeostasis. *J. Cell Biol.* 143:65-79.

Gewinner, C., Z.C. Wang, A. Richardson, J. Teruya-Feldstein, D. Etemadmoghadam, D. Bowtell, J. Barretina, W.M. Lin, L. Rameh, L. Salmena, P.P. Pandolfi, and L.C. Cantley. 2009. Evidence that inositol polyphosphate 4-phosphatase type II is a tumor suppressor that inhibits PI3K signaling. *Cancer cell*. 16:115-125.

Goley, E.D., and M.D. Welch. 2006. The ARP2/3 complex: an actin nucleator comes of age. *Nat. Rev. Mol. Cell Biol.* 7:713-726.

Goosney, D.L., S. Gruenheid, and B.B. Finlay. 2000. Gut feelings: enteropathogenic E. coli (EPEC) interactions with the host. *Annu. Rev. Cell Dev. Biol.* 16:173-189.

Gruenheid, S., R. DeVinney, F. Bladt, D. Goosney, S. Gelkop, G.D. Gish, T. Pawson, and B.B. Finlay. 2001. Enteropathogenic E. coli Tir binds Nck to initiate actin pedestal formation in host cells. *Nat. Cell Biol.* 3:856-859.

Gurung, R., A. Tan, L.M. Ooms, M.J. McGrath, R.D. Huysmans, A.D. Munday, M. Prescott, J.C. Whisstock, and C.A. Mitchell. 2003. Identification of a novel domain in two mammalian inositol-polyphosphate 5-phosphatases that mediates membrane ruffle localization. The inositol 5-phosphatase skip localizes to the endoplasmic reticulum and translocates to membrane ruffles following epidermal growth factor stimulation. *J. Biol. Chem.* 278:11376-11385.

Hall, A. 1998. Rho GTPases and the actin cytoskeleton. *Science*. 279:509-514.

Hardt, W.D., L.M. Chen, K.E. Schuebel, X.R. Bustelo, and J.E. Galán. 1998. *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell*. 93:815-826.

Hernandez, L.D., K. Hueffer, M.R. Wenk, and J.E. Galán. 2004. *Salmonella* modulates vesicular traffic by altering phosphoinositide metabolism. *Science*. 304:1805-1807.

Higashide, W., and D. Zhou. 2006. The first 45 amino acids of SopA are necessary for InvB binding and SPI-1 secretion. *J. Bacteriol.* 188:2411-2420.

Ho, H.Y., R. Rohatgi, A.M. Lebensohn, M. Le, J. Li, S.P. Gygi, and M.W. Kirschner. 2004. Toca-1 mediates Cdc42-dependent actin nucleation by activating the N-WASP-WIP complex. *Cell*. 118:203-216.

Hogan, A., Y. Yakubchyk, J. Chabot, C. Obagi, E. Daher, K. Maekawa, and S.H. Gee. 2004. The phosphoinositol 3,4-bisphosphate-binding protein TAPP1 interacts with syntrophins and regulates actin cytoskeletal organization. *J. Biol. Chem.* 279:53717-53724.

Hoiseth, S.K., and B.A. Stocker. 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature*. 291:238-239.

Hsu, F., W. Zhu, L. Brennan, L. Tao, Z.Q. Luo, and Y. Mao. 2012. Structural basis for substrate recognition by a unique Legionella phosphoinositide phosphatase. *Proc. Natl. Acad. Sci. U. S. A.* 109:13567-13572.

Humphreys, D., A.C. Davidson, P.J. Hume, L.E. Makin, and V. Koronakis. 2013. Arf6 coordinates actin assembly through the WAVE complex, a mechanism usurped by Salmonella to invade host cells. *Proc. Natl. Acad. Sci. U. S. A.* 110:16880-16885.

Ijuin, T., Y. Mochizuki, K. Fukami, M. Funaki, T. Asano, and T. Takenawa. 2000. Identification and characterization of a novel inositol polyphosphate 5-phosphatase. *J. Biol. Chem.* 275:10870-10875.

Ireton, K., B. Payrastre, and P. Cossart. 1999. The Listeria monocytogenes protein InlB is an agonist of mammalian phosphoinositide 3-kinase. *J. Biol. Chem.* 274:17025-17032.

Jefferies, H.B., F.T. Cooke, P. Jat, C. Boucheron, T. Koizumi, M. Hayakawa, H. Kaizawa, T. Ohishi, P. Workman, M.D. Waterfield, and P.J. Parker. 2008. A selective PIKfyve inhibitor blocks PtdIns(3,5)P(2) production and disrupts endomembrane transport and retroviral budding. *EMBO Rep*. 9:164-170.

Jones, B.D., H.F. Paterson, A. Hall, and S. Falkow. 1993. Salmonella typhimurium induces membrane ruffling by a growth factor-receptor-independent mechanism. *Proc. Natl. Acad. Sci. U. S. A.* 90:10390-10394.

Jung, G., J. Wang, P. Wlodarski, B. Barylko, D.D. Binns, H. Shu, H.L. Yin, and J.P. Albanesi. 2008. Molecular determinants of activation and membrane targeting of phosphoinositol 4-kinase IIbeta. *Biochem. J.* 409:501-509.

Kanai, F., H. Liu, S.J. Field, H. Akbary, T. Matsuo, G.E. Brown, L.C. Cantley, and M.B. Yaffe. 2001. The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. *Nat. Cell Biol.* 3:675-678.

Kaniga, K., J.C. Bossio, and J.E. Galán. 1994. The *Salmonella typhimurium* invasion genes *invF* and *invG* encode homologues of the AraC and PulD family of proteins. *Mol. Microbiol.* 13:555-568.

Kaper, J.B., J.P. Nataro, and H.L. Mobley. 2004. Pathogenic Escherichia coli. *Nat. Rev. Microbiol.* 2:123-140.

Kerr, M.C., J.T. Wang, N.A. Castro, N.A. Hamilton, L. Town, D.L. Brown, F.A. Meunier, N.F. Brown, J.L. Stow, and R.D. Teasdale. 2010. Inhibition of the PtdIns(5) kinase PIKfyve disrupts intracellular replication of *Salmonella*. *EMBO J.* 29:1331-1347.

Knodler, L.A., S. Winfree, D. Drecktrah, R. Ireland, and O. Steele-Mortimer. 2009. Ubiquitination of the bacterial inositol phosphatase, SopB, regulates its biological activity at the plasma membrane. *Cell. Microbiol.* 11:1652-1670.

Knutton, S., T. Baldwin, P.H. Williams, and A.S. McNeish. 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic Escherichia coli. *Infect. Immun.* 57:1290-1298.

Knutton, S., D.R. Lloyd, and A.S. McNeish. 1987. Adhesion of enteropathogenic Escherichia coli to human intestinal enterocytes and cultured human intestinal mucosa. *Infect. Immun.* 55:69-77.

Kubori, T., Y. Matsushima, D. Nakamura, J. Uralil, M. Lara-Tejero, A. Sukhan, J.E. Galán, and S.I. Aizawa. 1998. Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science*. 280:602-605.

Kurokawa, K., R.E. Itoh, H. Yoshizaki, Y.O. Nakamura, and M. Matsuda. 2004. Coactivation of Rac1 and Cdc42 at lamellipodia and membrane ruffles induced by epidermal growth factor. *Mol. Biol. Cell*. 15:1003-1010.

Lemmon, M.A. 2003. Phosphoinositide recognition domains. *Traffic*. 4:201-213.

Lev, S. 2012. Nonvesicular lipid transfer from the endoplasmic reticulum. *Cold Spring Harbor perspectives in biology*. 4.

Liu, Y., P. Gao, S. Banga, and Z.Q. Luo. 2008. An *in vivo* gene deletion system for determining temporal requirement of bacterial virulence factors. *Proc. Natl. Acad. Sci. U. S. A.* 105:9385-9390.

Majowicz, S.E., J. Musto, E. Scallan, F.J. Angulo, M. Kirk, S.J. O'Brien, T.F. Jones, A. Fazil, and R.M. Hoekstra. 2010. The global burden of nontyphoidal Salmonella gastroenteritis. *Clin. Infect. Dis.* 50:882-889.

Malik-Kale, P., C.E. Jolly, S. Lathrop, S. Winfree, C. Luterbach, and O. Steele-Mortimer. 2011. Salmonella - at home in the host cell. *Front. Microbiol.* 2:125.

Mallo, G.V., M. Espina, A.C. Smith, M.R. Terebiznik, A. Aleman, B.B. Finlay, L.E. Rameh, S. Grinstein, and J.H. Brumell. 2008. SopB promotes phosphatidylinositol 3-phosphate formation on Salmonella vacuoles by recruiting Rab5 and Vps34. *J. Cell Biol.* 182:741-752.

Marcus, S.L., L.A. Knodler, and B.B. Finlay. 2002. *Salmonella enterica* serovar Typhimurium effector SigD/SopB is membrane-associated and ubiquitinated inside host cells. *Cell. Microbiol.* 4:534-546.

Marcus, S.L., M.R. Wenk, O. Steele-Mortimer, and B.B. Finlay. 2001. A synaptojanin-homologous region of *Salmonella typhimurium* SigD is essential for inositol phosphatase activity and Akt activation. *FEBS Lett.* 494:201-207.

Marshall, A.J., A.K. Krahn, K. Ma, V. Duronio, and S. Hou. 2002. TAPP1 and TAPP2 are targets of phosphatidylinositol 3-kinase signaling in B cells: sustained plasma membrane recruitment triggered by the B-cell antigen receptor. *Mol. Cell. Biol.* 22:5479-5491.

Martin, T.F. 2001. PI(4,5)P(2) regulation of surface membrane traffic. *Curr. Opin. Cell Biol.* 13:493-499.

McGhie, E.J., R.D. Hayward, and V. Koronakis. 2001. Cooperation between actin-binding proteins of invasive *Salmonella*: SipA potentiates SipC nucleation and bundling of actin. *EMBO J.* 20:2131-2139.

McGhie, E.J., R.D. Hayward, and V. Koronakis. 2004. Control of actin turnover by a salmonella invasion protein. *Mol. Cell*. 13:497-510.

Moon, H.W., S.C. Whipp, R.A. Argenzio, M.M. Levine, and R.A. Giannella. 1983. Attaching and effacing activities of rabbit and human enteropathogenic Escherichia coli in pig and rabbit intestines. *Infect. Immun.* 41:1340-1351.

Mumberg, D., R. Muller, and M. Funk. 1994. Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res.* 22:5767-5768.

Myeni, S.K., L. Wang, and D. Zhou. 2013. SipB-SipC complex is essential for translocon formation. *PLoS ONE*. 8:e60499.

Myeni, S.K., and D. Zhou. 2010. The C terminus of SipC binds and bundles F-actin to promote Salmonella invasion. *J. Biol. Chem.* 285:13357-13363.

Niebuhr, K., S. Giuriato, T. Pedron, D.J. Philpott, F. Gaits, J. Sable, M.P. Sheetz, C. Parsot, P.J. Sansonetti, and B. Payrastre. 2002. Conversion of PtdIns(4,5)P(2) into PtdIns(5)P by the S.flexneri effector IpgD reorganizes host cell morphology. *EMBO J.* 21:5069-5078.

Niebuhr, K., N. Jouihri, A. Allaoui, P. Gounon, P.J. Sansonetti, and C. Parsot. 2000. IpgD, a protein secreted by the type III secretion machinery of Shigella flexneri, is chaperoned by IpgE and implicated in entry focus formation. *Mol. Microbiol.* 38:8-19.

Nishioka, T., K. Aoki, K. Hikake, H. Yoshizaki, E. Kiyokawa, and M. Matsuda. 2008. Rapid turnover rate of phosphoinositides at the front of migrating MDCK cells. *Mol. Biol. Cell*. 19:4213-4223.

Norris, F.A., M.P. Wilson, T.S. Wallis, E.E. Galyov, and P.W. Majerus. 1998. SopB, a protein required for virulence of *Salmonella dublin*, is an inositol phosphate phosphatase. *Proc. Natl. Acad. Sci. U. S. A.* 95:14057-14059.

Oikawa, T., H. Yamaguchi, T. Itoh, M. Kato, T. Ijuin, D. Yamazaki, S. Suetsugu, and T. Takenawa. 2004. PtdIns(3,4,5)P3 binding is necessary for WAVE2-induced formation of lamellipodia. *Nat. Cell Biol.* 6:420-426.

Patel, J.C., and J.E. Galan. 2006. Differential activation and function of Rho GTPases during Salmonella-host cell interactions. *J. Cell Biol.* 175:453-463.

Patel, J.C., and J.E. Galán. 2005. Manipulation of the host actin cytoskeleton by Salmonella--all in the name of entry. *Curr. Opin. Microbiol.* 8:10-15.

Patel, J.C., K. Hueffer, T.T. Lam, and J.E. Galan. 2009. Diversification of a Salmonella virulence protein function by ubiquitin-dependent differential localization. *Cell*. 137:283-294.

Pattni, K., M. Jepson, H. Stenmark, and G. Banting. 2001. A PtdIns(3)P-specific probe cycles on and off host cell membranes during Salmonella invasion of mammalian cells. *Curr. Biol.* 11:1636-1642.

Pendaries, C., H. Tronchere, L. Arbibe, J. Mounier, O. Gozani, L. Cantley, M.J. Fry, F. Gaits-Iacovoni, P.J. Sansonetti, and B. Payrastre. 2006. PtdIns(5)P activates the host cell PI3-kinase/Akt pathway during *Shigella flexneri* infection. *EMBO J.* 25:1024-1034.

Perrett, C.A., and D. Zhou. 2013. Salmonella type III effector SopB modulates host cell exocytosis. *Emerg Microbes Infect*. 2:e32.

Pickart, C.M. 2001. Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* 70:503-533.

Piscatelli, H., S.A. Kotkar, M.E. McBee, S. Muthupalani, D.B. Schauer, R.E. Mandrell, J.M. Leong, and D. Zhou. 2011. The EHEC type III effector NleL is an E3 ubiquitin ligase that modulates pedestal formation. *PLoS ONE*. 6:e19331.

Pizarro-Cerda, J., B. Payrastre, Y.J. Wang, E. Veiga, H.L. Yin, and P. Cossart. 2007. Type II phosphatidylinositol 4-kinases promote *Listeria monocytogenes* entry into target cells. *Cell. Microbiol.* 9:2381-2390.

Posor, Y., M. Eichhorn-Gruenig, D. Puchkov, J. Schoneberg, A. Ullrich, A. Lampe, R. Muller, S. Zarbakhsh, F. Gulluni, E. Hirsch, M. Krauss, C. Schultz, J. Schmoranzer, F. Noe, and V. Haucke. 2013. Spatiotemporal control of endocytosis by phosphatidylinositol-3,4-bisphosphate. *Nature*. 499:233-237.

Pryor, P.R., B.M. Mullock, N.A. Bright, M.R. Lindsay, S.R. Gray, S.C. Richardson, A. Stewart, D.E. James, R.C. Piper, and J.P. Luzio. 2004. Combinatorial SNARE complexes with VAMP7 or VAMP8 define different late endocytic fusion events. *EMBO Rep*. 5:590-595.

Ramel, D., F. Lagarrigue, V. Pons, J. Mounier, S. Dupuis-Coronas, G. Chicanne, P.J. Sansonetti, F. Gaits-Iacovoni, H. Tronchere, and B. Payrastre. 2011. Shigella flexneri infection generates the lipid PI5P to alter endocytosis and prevent termination of EGFR signaling. *Science signaling*. 4:ra61.

Raucher, D., T. Stauffer, W. Chen, K. Shen, S. Guo, J.D. York, M.P. Sheetz, and T. Meyer. 2000. Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion. *Cell*. 100:221-228.

Ren, Q., H.K. Barber, G.L. Crawford, Z.A. Karim, C. Zhao, W. Choi, C.C. Wang, W. Hong, and S.W. Whiteheart. 2007. Endobrevin/VAMP-8 is the primary v-SNARE for the platelet release reaction. *Mol. Biol. Cell*. 18:24-33.

Ridley, A.J., H.F. Paterson, C.L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*. 70:401-410.

Rodriguez-Escudero, I., R. Rotger, V.J. Cid, and M. Molina. 2006. Inhibition of Cdc42-dependent signalling in Saccharomyces cerevisiae by phosphatase-dead SigD/SopB from Salmonella typhimurium. *Microbiology*. 152:3437-3452.

Rogers, L.D., A.R. Kristensen, E.C. Boyle, D.P. Robinson, R.T. Ly, B.B. Finlay, and L.J. Foster. 2008. Identification of cognate host targets and specific ubiquitylation sites on the Salmonella SPI-1 effector SopB/SigD. *J Proteomics*. 71:97-108.

Rohatgi, R., P. Nollau, H.Y. Ho, M.W. Kirschner, and B.J. Mayer. 2001. Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. *J. Biol. Chem.* 276:26448-26452.

Saarikangas, J., H. Zhao, and P. Lappalainen. 2010. Regulation of the actin cytoskeleton-plasma membrane interplay by phosphoinositides. *Physiol. Rev.* 90:259-289.

Sakisaka, T., T. Itoh, K. Miura, and T. Takenawa. 1997. Phosphatidylinositol 4,5-bisphosphate phosphatase regulates the rearrangement of actin filaments. *Mol. Cell. Biol.* 17:3841-3849.

Schlumberger, M.C., R. Kappeli, M. Wetter, A.J. Muller, B. Misselwitz, S. Dilling, M. Kremer, and W.D. Hardt. 2007. Two newly identified SipA domains (F1, F2) steer effector protein localization and contribute to Salmonella host cell manipulation. *Mol. Microbiol.* 65:741-760.

Schwarz, S.E., J.L. Rosa, and M. Scheffner. 1998. Characterization of human hect domain family members and their interaction with UbcH5 and UbcH7. *J. Biol. Chem.* 273:12148-12154.

Sechi, A.S., and J. Wehland. 2000. The actin cytoskeleton and plasma membrane connection: PtdIns(4,5)P(2) influences cytoskeletal protein activity at the plasma membrane. *J. Cell Sci.* 113 Pt 21:3685-3695.

Sidhu, G., W. Li, N. Laryngakis, E. Bishai, T. Balla, and F. Southwick. 2005. Phosphoinositide 3-kinase is required for intracellular Listeria monocytogenes actin-based motility and filopod formation. *J. Biol. Chem.* 280:11379-11386.

Soulet, F., D. Yarar, M. Leonard, and S.L. Schmid. 2005. SNX9 regulates dynamin assembly and is required for efficient clathrin-mediated endocytosis. *Mol. Biol. Cell*. 16:2058-2067.

Spano, S., and J.E. Galan. 2012. A Rab32-dependent pathway contributes to Salmonella typhi host restriction. *Science*. 338:960-963.

Spano, S., X. Liu, and J.E. Galan. 2011. Proteolytic targeting of Rab29 by an effector protein distinguishes the intracellular compartments of human-adapted and broad-host Salmonella. *Proc. Natl. Acad. Sci. U. S. A.* 108:18418-18423.

Steele-Mortimer, O., L.A. Knodler, S.L. Marcus, M.P. Scheid, B. Goh, C.G. Pfeifer, V. Duronio, and B.B. Finlay. 2000. Activation of Akt/protein kinase B in epithelial cells by the *Salmonella typhimurium* effector sigD. *J. Biol. Chem.* 275:37718-37724.

Szymanska, E., A. Sobota, E. Czurylo, and K. Kwiatkowska. 2008. Expression of PI(4,5)P2-binding proteins lowers the PI(4,5)P2level and inhibits FcgammaRIIA-mediated cell spreading and phagocytosis. *Eur. J. Immunol.* 38:260-272.

Terebiznik, M.R., O.V. Vieira, S.L. Marcus, A. Slade, C.M. Yip, W.S. Trimble, T. Meyer, B.B. Finlay, and S. Grinstein. 2002. Elimination of host cell PtdIns(4,5)P(2) by bacterial SigD promotes membrane fission during invasion by *Salmonella*. *Nat. Cell Biol.* 4:766-773.

Thomas, B.J., and R. Rothstein. 1989. The genetic control of direct-repeat recombination in Saccharomyces: the effect of rad52 and rad1 on mitotic recombination at GAL10, a transcriptionally regulated gene. *Genetics*. 123:725-738.

Tomasevic, N., Z. Jia, A. Russell, T. Fujii, J.J. Hartman, S. Clancy, M. Wang, C. Beraud, K.W. Wood, and R. Sakowicz. 2007. Differential regulation of WASP and N-WASP by Cdc42, Rac1, Nck, and PI(4,5)P2. *Biochemistry*. 46:3494-3502.

Unsworth, K.E., M. Way, M. McNiven, L. Machesky, and D.W. Holden. 2004. Analysis of the mechanisms of *Salmonella*-induced actin assembly during invasion of host cells and intracellular replication. *Cell. Microbiol.* 6:1041-1055.

Wang, C.C., C.P. Ng, L. Lu, V. Atlashkin, W. Zhang, L.F. Seet, and W. Hong. 2004. A role of VAMP8/endobrevin in regulated exocytosis of pancreatic acinar cells. *Dev. Cell*. 7:359-371.

Wasylnka, J.A., M.A. Bakowski, J. Szeto, M.B. Ohlson, W.S. Trimble, S.I. Miller, and J.H. Brumell. 2008. Role for myosin II in regulating positioning of Salmonella-containing vacuoles and intracellular replication. *Infect. Immun.* 76:2722-2735.

Welliver, T.P., S.L. Chang, J.J. Linderman, and J.A. Swanson. 2011. Ruffles limit diffusion in the plasma membrane during macropinosome formation. *J. Cell Sci.* 124:4106-4114.

Wennerberg, K., S.M. Ellerbroek, R.Y. Liu, A.E. Karnoub, K. Burridge, and C.J. Der. 2002. RhoG signals in parallel with Rac1 and Cdc42. *J. Biol. Chem.* 277:47810-47817.

Winnen, B., M.C. Schlumberger, A. Sturm, K. Schupbach, S. Siebenmann, P. Jenny, and W.D. Hardt. 2008. Hierarchical effector protein transport by the Salmonella Typhimurium SPI-1 type III secretion system. *PLoS ONE*. 3:e2178.

Wong, K.W., and R.R. Isberg. 2003. Arf6 and phosphoinositol-4-phosphate-5-kinase activities permit bypass of the Rac1 requirement for beta1 integrin-mediated bacterial uptake. *J. Exp. Med.* 198:603-614.

Yarar, D., M.C. Surka, M.C. Leonard, and S.L. Schmid. 2008. SNX9 activities are regulated by multiple phosphoinositides through both PX and BAR domains. *Traffic*. 9:133-146.

Yarar, D., C.M. Waterman-Storer, and S.L. Schmid. 2007. SNX9 couples actin assembly to phosphoinositide signals and is required for membrane remodeling during endocytosis. *Dev. Cell*. 13:43-56.

Yin, H.L., and P.A. Janmey. 2003. Phosphoinositide regulation of the actin cytoskeleton. *Annu. Rev. Physiol.* 65:761-789.

Zhou, D., L.M. Chen, L. Hernandez, S.B. Shears, and J.E. Galán. 2001. A *Salmonella* inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. *Mol. Microbiol.* 39:248-259.

Zhou, D., M.S. Mooseker, and J.E. Galán. 1999. Role of the *S. typhimurium* actin-binding protein SipA in bacterial internalization. *Science*. 283:2092-2095.

APPENDICES

Appendix A Plasmid List

Plasmid Name	Description
pZP3172	His-SopB in pET28b
pZP3173	His-SopBC460S in pET28b
pZP3174	His-SopBK530A in pET28b
pZP3175	His-SopBC462A-K530A in pET28b
pZP3176	sopBK530A insertion in pSB890
pZP3177	SopB Upstream in pSK
pZP3178	SopB downstream in pDsRed
pZP3179	SopB Flank in pDsRed
pZP3180	SopB flank IpgD in pDsRed
pZP3181	IpgD insertion in pSB890
pZP3182	sopB flank Inpp4B in pDsRed
pZP3183	Inpp4B insertion in pSB890
pZP3184	SopB flank SKIP in pDsRed
pZP3185	SKIP insertion in pSB890
pZP3186	SKIP-CAAX
pZP3187	RhoG in RFP-C1
pZP3188	Fyve3-EGFP in p414MET25
pZP3189	SopB in p426MET25
pZP3190	SopB flank SKIP-IpgD in pDsRed
pZP3191	SopB flank SKIP-Inpp4B in pDsRed
pZP3198	SKIP-Inpp4B insertion in pSB890
pZP3199	SKIP-IpgD insertion in pSB890
pZP3200	IpgD-TEM in pZP3212
pZP3201	Inpp4B-TEM in pZP3212
pZP3202	SKIP-TEM in pZP3212
pZP3203	Inpp4B SKIP-TEM in pZP3212
pZP3204	IpgD SKIP-TEM in pZP3212
pZP3205	SopB 1-100 in pSK
pZP3206	Transloc. IpgD-TEM in pZP3212
pZP3207	Transloc. Inpp4B-TEM in pZP3212
pZP3208	Transloc. SKIP-TEM in pZP3212
pZP3209	Transloc. IpgD SKIP-TEM in pZP3212

pZP3210	Transloc. Inpp4B SKIP-TEM in pZP3212
pZP3211	SopB-TEM in pZP3212
pZP3212	pM1644 modified EcoRV and SacII
pZP3213	SopB C460S in p426MET25
pZP3278	SopB flank loxp oligo in pzp152
pZP3279	SopB Flank loxp-SopB in pZP152
pZP3280	loxp-SopB insertion in pSB890
pZP3281	Inpp4B-CAAX in YFP-C1
pZP3282	MyosinX in GFP-C1
pZP3283	Inpp4B-CAAX in EGFP-N1
pZP2196	MTM1 in RFP-C1
pZP3285	IpgD-CAAX
pZP3286	sidf1-760 in RFP
pZP3287	sidf1-760 cs in RFP
pZP3288	SidF WT in RFP
pZP3289	SidF cs in RFP
pZP3290	GRP1 in RFP-C1
pZP2199	SKIP in GFP
pZP2195	PI4KIIb in GFP
pZP3274	Tks4 in GFP
pZP3276	Bam32 in GFP
pZP3277	Bam32y139F in GFP
pZP3275	TAPP1 in RFP
pZP889	YFP-Rab5
pZP1749	p47PX in GFP
pZP2780	p47PX in RFP
pZP2111	NleL c/a
pZP1667	NleL c/s in pZP1137
pZP1666	NleL WT in pZP1137
pEX339	SidF1-760 in GFP
pEX340	SidF1-760 cs in GFP
pEX53	GRP1 in GFP
pEX52	pAkt in GFP
pEX46	FYVE3 in GFP
pEX244	TIR in HA
pEX338	RhoG
pEX339	Inpp4B
pEX336	Bam32
pEX335	Tks4
pEX0328	mCherry-SNX9

pEX0329	mCherry-SNX9 (RYK)
pEX0330	GFP-SNX9
pEX233	SipA-TEM1
pEX269	pZL638
pEX270	pZL639
pEX271	pZL680
pEX272	pZL681
pEX273	constitutive Cre recombinase
pEX274	p426 MET25
pEX276	Rabenosyn 5
pEX277	Tapp1
pEX278	MTM1
pEX279	PI3K
pEX280	PTEN
pEX300	SidfWT in GFP
pEX301	SidFcs in GFP
pEX48	p40PX in GFP
pEX247	p40PX in RFP

Appendix B Strain List

Strain	Genotype
SB330	WT
SB933	*sopB*^{C462S}
ZP189	*sopE-*
ZP016	*sopB*^{C462S} *sopE-*
ZP015	*sopB-sopE-*
SB924	*sopB::aphT*
ZP471	*sopB*^{K530A}
ZP472	*sopB*^{K530A} *sopE-*
ZP473	*SopB::ipgD*
ZP474	*SopB::ipgD, sopE-*
ZP475	*sopB::Inpp4B*
ZP476	*sopB::Inpp4B, sopE*
ZP477	*SopB::SKIP*
ZP478	*sopB::SKIP, sopE-*
ZP479	*sopE-sopB::aphT*
ZP480	floxed sopB #13
ZP484	*sopEsopB::SKIP ipgD*
ZP485	*sopEsopB::SKIP Inpp4B*
ZP495	floxed sopB #7
ZP496	floxed sopB #11
ZP250	EHEC
ZP254	*nleL*^{C753S}
EX099	EPEC
ZP508	*sopB::SKIP ipgD*
ZP509	*sopB::SKIP inpp4B*
ZP497	*sopB::Inpp4B*-m45
ZP498	*sopB::SKIP*-M45
ZP499	*sopB::ipgD*-M45

ZP500	*sopEsopB::Inpp4B*-m45
ZP501	*sopESopB::SKIP*-M45
ZP502	*sopESopB::ipgD*-M45
ZP503	*sopEsopB::SKIP ipgD*-M45
ZP504	*sopEsopB::SKIP inpp4B*-M45
ZP505	*sopB::SKIP ipgD*-M45
ZP506	*sopB::SKIP inpp4B* -M45

VITA

VITA

Heather Piscatelli was born and raised in the state of Rhode Island. She became interested in the study of Biology during her studies in high school and in 2003 she went on to major in Microbiology at Rutgers University in New Jersey. She was awarded the degree Bachelor of Arts in 2007 at which time she decided to further her studies in Microbiology by pursuing her Ph.D. in the Department of Biological Sciences at Purdue University. Her current research focuses on studying the host-pathogen interaction during *Salmonella* infection.

PUBLICATIONS

PUBLICATIONS

Piscatelli, H., S.A. Kotkar, M.E. McBee, S. Muthupalani, D.B. Schauer, R.E. Mandrell, J.M. Leong, and D. Zhou. 2011. The EHEC type III effector NleL is an E3 ubiquitin ligase that modulates pedestal formation. *PLoS ONE*. 6:e19331.

Piscatelli H, Zhou D. 2014. Dual phosphoinositide 4- and 5-phosphatases regulate phosphoinositide dynamics to promote bacterial entry. (Manuscript in preparation)