Regulation of Actin Dynamics during *Salmonella* Invasion

by

Dorothy Truong

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Molecular Genetics
University of Toronto

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Abstract

*Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) invasion begins with the translocation of bacterial virulence proteins (called effectors) into host cells via a type 3 secretion system (T3SS). These effectors serve to manipulate host cell machinery leading to the formation of a cell surface structure called an invasion ruffle that subsequently mediates bacterial internalization into the host cell. Following entry, the bacteria continues to manipulate host signalling factors to establish a replicative niche. There is still much to be learned about the host machinery that is exploited by *S*. Typhimurium during infection. In my thesis, I show that the formin, FHOD1 plays an important role in *S*. Typhimurium invasion. FHOD1 is phosphorylated during *S*. Typhimurium invasion, a process that requires the bacterial T3SS effectors SopB and SopE/E2, as well as host proteins RhoA and ROCKII. FHOD1 and Arp2/3 are both recruited to the same invasion ruffle, but contribute differently to its formation and also display different kinetics of recruitment.

The Rho GTPase family of proteins is comprised of 20 members. In the context of *S*. Typhimurium invasion, only Rac1, Cdc42, and RhoG have been extensively studied. A screen was conducted and revealed recruitment of RhoB, RhoD, RhoH, and RhoJ to the *S*. Typhimurium invasion site in a SopB-dependent manner. Regulation of Rho GTPases is
achieved via post-translational modifications such as palmitoylation. To this end, I demonstrated that palmitoylation of host proteins promotes *S. Typhimurium* invasion and PI(3,4,5)P₃ production at the invasion ruffle. Furthermore, the ability of SopB to recruit lipid rafts to the *S. Typhimurium* invasion site may allow for specific recruitment of palmitoylated Rho GTPases. Collectively, this data provides new insight on formation of the invasion ruffle and identifies new host factors that are exploited by *S. Typhimurium* to invade host cells.
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<th>Description</th>
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<tbody>
<tr>
<td>ABM</td>
<td>Actin based-motility</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>Actin-related protein 2/3 complex</td>
</tr>
<tr>
<td>CA</td>
<td>Constitutively active</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan fluorescent protein</td>
</tr>
<tr>
<td>DAD</td>
<td>Diaphanous Autoregulatory Domain</td>
</tr>
<tr>
<td>DID</td>
<td>Diaphanous Inhibitory Domain</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant negative</td>
</tr>
<tr>
<td>DRF</td>
<td>Diaphanous-Related Formin</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FH1</td>
<td>Formin homology 1</td>
</tr>
<tr>
<td>FH2</td>
<td>Formin homology 2</td>
</tr>
<tr>
<td>FH3</td>
<td>Formin homology 3</td>
</tr>
<tr>
<td>FHOD1</td>
<td>Formin homology 2 domain containing 1</td>
</tr>
<tr>
<td>FMNL1</td>
<td>Formin like 1</td>
</tr>
<tr>
<td>G-actin</td>
<td>Globular-actin</td>
</tr>
<tr>
<td>GAP</td>
<td>Rho GTPase activating protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GBD</td>
<td>GTPase binding domain</td>
</tr>
<tr>
<td>GDI</td>
<td>Rho guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GEF</td>
<td>Rho guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine-5’-triphosphatase</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
</tbody>
</table>
MβCD  Methyl-β-cyclodextrin
M cells  Microfold cells
MDCK  Madin-Darby canine kidney
mDia  Mammalian homolog of Drosophila diaphanous 2
MEF  Mouse embryonic fibroblast
mins  Minutes
MOI  Multiplicity of infection
mTOR  Mammalian target of rapamycin
NPF  Nucleating promoting factor
qPCR  Quantitative polymerase chain reaction
PAK  p21-activated kinase 1
PAT  Palmitoyl acyltransferase
PBD  p21-binding domain
PBS  Phosphate buffered saline
PDGF  Platelet-derived growth factor
PDK1  3’-phosphoinositide dependent kinase
PET  Palmitoylthioesterase
PH  Pleckstrin homology
p.i.  Post-infection
PI3K  Phosphoinositide-3 kinase
PI(3)P  Phosphatidylinositol-3-phosphate
PI(5)P  Phosphatidylinositol-5-phosphate
PI(4,5)P₂  Phosphatidylinositol-4,5-bisphosphate
PI(3,4,5)P₃  Phosphatidylinositol-3,4,5-triphosphate
PTEN  Phosphatase and tensin homologue deleted on chromosome 10
PVDF  Polyvinylidene fluoride
RBC  Red blood cell
Rho  Ras homologous
RNA  Ribonucleic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>SCV</td>
<td><em>Salmonella</em> containing vacuole</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SIM</td>
<td>Structured illumination microscopy</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Sop</td>
<td><em>Salmonella</em> outer protein</td>
</tr>
<tr>
<td>SPI</td>
<td><em>Salmonella</em> pathogenicity island</td>
</tr>
<tr>
<td>T3SS</td>
<td>Type 3 secretion system</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>VCA</td>
<td>Verprolin-homology, central and acidic domain</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASP family verprolin homologous protein</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
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</table>
Chapter One: Introduction

1.1 Overview

In this thesis I describe my studies into the interactions of a bacterial pathogen, *Salmonella enterica* serovar Typhimurium (S. Typhimurium) with cells of their mammalian host. I will focus on how these bacteria can alter host signal transduction cascades to control the actin cytoskeleton. To introduce these topics, I will begin with a background on regulation of the actin cytoskeleton in mammalian cells, followed by a discussion of pathogenic mechanisms used by S. Typhimurium to cause disease. Parts of this chapter contains excerpts that were reprinted with permission from *Bioessays*, 7: 687-96, Truong et al., Bacterial subversion of host cytoskeletal machinery: hijacking formins and the Arp2/3 complex, 2014.

1.2 The dynamic nature of actin filaments

The actin cytoskeleton is an essential component of the eukaryotic cell and is involved in many fundamental cellular functions such as phagocytosis, cytokinesis, and cell migration (Rotty et al., 2013). As such, it is important to tightly regulate the formation and dissolution of actin filaments. G-actin is a monomeric ATPase and is the most basic unit of actin filaments. When G-actin is in its ATP bound form, it can assemble into long polymers of actin filaments called F-actin, and upon ATP hydrolysis, G-actin dissociates from F-actin (Bugyi and Carlier, 2010). Actin monomers are added on the growing barbed end of the actin filament, and dissociate from the pointed end. The result of this process is actin treadmilling, whereby actin monomers assemble onto the barbed end and disassemble from the pointed end. F-actin filaments can coalesce to form lamellipodia (large and broad actin protrusion structures) or filopodia (finger-like projections extending from the surface of the cell). These F-actin structures can differentially contribute or cooperate in cellular functions such as cell migration
(Chhabra and Higgs, 2007). The regulation of these F-actin structures and the molecular machinery that contributes to their formation remains elusive.

The two ends of actin filaments differ in their structural and kinetic properties (Bugyi and Carlier, 2010). Actin filament elongation requires formation of an actin trimer, also known as a nucleus, which is thermodynamically unfavourable in vitro. This thermodynamic barrier can be overcome by actin nucleators such as the Arp2/3 complex, formins and Spire (Firat-Karalar and Welch, 2011; Rotty et al., 2013). In addition to the formation of actin filaments, there are many factors throughout the cell that can alter actin filaments by capping, crosslinking, severing, or bundling (Bugyi and Carlier, 2010). There is still much to be learned about the spatial and temporal regulation of these factors.

### 1.3 The Arp2/3 Complex: Understanding its function and regulation

The Arp2/3 complex is composed of 7 subunits (Machesky et al., 1994). Arp2 and Arp3 are the two largest subunits of the Arp2/3 complex and both belong to the family of actin-related proteins (Kelleher et al., 1995). Together, Arp2 and Arp3 structurally mimic a dimer of G-actin, allowing it to serve as a template to mediate new actin filament growth (Kelleher et al., 1995). The remaining 5 subunits (named ArpC1-ArpC5) are thought to aid in positioning and binding of the Arp2/3 complex to the mother filament (Rotty et al., 2013). Arp2/3-mediated filament elongation results in formation of Y-branched actin filaments (Rotty et al., 2013). Using a pre-existing filament as a scaffold, the Arp2/3 complex drives formation of a new daughter filament at a 70° angle (Amann and Pollard, 2001) to the pre-existing mother filament (Figure 1.1A).

The Arp2/3 complex alone has low actin polymerization activity (Mullins et al., 1998) and requires activation by nucleation-promoting factors (NPF) (Goley et al., 2004; Rotty et al., 2013). NPFs are divided into two categories: type I NPFs and type II NPFs. Type I NPFs are characterized by the presence of a VCA domain which contains three conserved motifs:
verprolin-homology domain (V) (or otherwise known as WH2 domain), central domain (C), and acidic domain (A) (Stradal and Scita, 2006). The V domain aids in actin filament growth by recruiting G-actin to the growing or barbed end of the actin filament. The CA domain binds to the Arp2/3 complex (Padrick et al., 2011; Ti et al., 2011). Binding of the NPF C domain to Arp2/3 induces a conformational change leading to activation of the complex (Goley et al., 2004; Rodal et al., 2005). Wiskott-Aldrich syndrome protein (WASP) and neural WASP (N-WASP) are two well-studied type I NPFs. Under basal conditions, an intramolecular interaction between their Rho GTPase binding domain (GBD) and VCA domain maintains the NPF in an inactive state (Rotty et al., 2013). Activation is achieved upon Rho GTPase binding to the GBD, which disrupts the intramolecular interaction, allowing for the NPF to interact with the Arp2/3 complex (Rotty et al., 2013).

Type II NPFs such as cortactin, lack the V and C domains, and do not induce an activating conformational change in the Arp2/3 complex (Goley et al., 2004). Cortactin comprises of an N-terminus acidic domain allowing for Arp2/3 binding and tandem repeat domains allowing for binding to filamentous actin as opposed to G-actin (Weed et al., 2000; Wu and Parsons, 1993). The tandem repeat domains and acidic domain of cortactin are both needed to confer Arp2/3 activation, since expression of either domain alone is not sufficient to induce Arp2/3-mediated branch filaments (Weaver et al., 2001). Studies have alluded to synergistic Arp2/3 activation between both classes of NPFs (Helgeson and Nolen, 2013; Weaver et al., 2001). Recently, a model was presented whereby a complex of N-WASP (type I NPF), the Arp2/3 complex, and two actin monomers is needed to bind the pre-existing mother filament. However, upon removal of N-WASP by cortactin (type II NPF) (Helgeson and Nolen, 2013), activation of the Arp2/3 complex and filament elongation is accelerated. Additional studies are required to further understand the mechanism by which type I and type II NPFs synergistically induce Arp2/3 activation.
1.4 Formins: another family of actin nucleators mechanistically different from the Arp2/3 complex

Formins are a highly conserved family of cytoskeletal remodelling proteins named for the founding member of the family - the product of the mouse limb deformity gene, Formin1 (Fmn1) (Woychik et al., 1990). Formins are distinguished by the presence of two regions of homology designated formin homology 1 (FH1) and formin homology 2 (FH2) (Castrillon and Wasserman, 1994). There are fifteen formin family members encoded by the human genome and, based on their associated regulatory domains, these fifteen are divided into two major groups; the Diaphanous-Related Formins (DRFs) and the non-Diaphanous-Related Formins (Table 1.1) (Higgs, 2005; Schonichen and Geyer, 2010). The DRFs are distinguished by the presence of a GTPase Binding Domain (GBD) and Diaphanous Inhibitory Domain (DID) in the N-terminal half of the protein and a Diaphanous Autoregulatory Domain (DAD) in the C-terminal tail (Figure 1.1B). The non-DRFs differ from the DRFs in that they lack some or all of these regulatory motifs (Higgs, 2005; Schonichen and Geyer, 2010).

<table>
<thead>
<tr>
<th>DRFs</th>
<th>Non-DRFs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dia1</td>
<td>Fmn1</td>
</tr>
<tr>
<td>Dia2</td>
<td>Fmn2</td>
</tr>
<tr>
<td>Dia3</td>
<td>INF1</td>
</tr>
<tr>
<td>DAAM1</td>
<td>INF2</td>
</tr>
<tr>
<td>DAAM2</td>
<td>Delphilin</td>
</tr>
<tr>
<td>FMNL1</td>
<td></td>
</tr>
<tr>
<td>FMNL2</td>
<td></td>
</tr>
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<td>FMNL3</td>
<td></td>
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<tr>
<td>FHOD1</td>
<td></td>
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<tr>
<td>FHOD3</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1 The formin family of proteins are separated into the Diaphanous-related formins (DRFs) and the non-DRFs (Higgs, 2005; Schonichen and Geyer, 2010)
1.5 Formin mediated actin polymerization is dependent on its FH1 and FH2 domains

All formins catalyze the formation of long unbranched actin filaments, which is in contrast to the branched filaments catalyzed by the Arp2/3 complex. Formin-mediated actin polymerization is mediated through the action of the conserved FH1 and FH2 functional unit (Higgs, 2005). FH2 forms a donut-shaped head-to-tail dimer and FH2 dimerization is essential for the nucleation and elongation of actin filaments (Copeland et al., 2004; Lu et al., 2007; Shimada et al., 2004; Xu et al., 2004). Each half of the FH2 dimer contains two actin-binding sub-domains that contribute to actin polymerization and barbed end binding. Point mutations in the conserved isoleucine residue (I1431 in Bni1 and I649 in FMNL3) in the first half of the FH2 dimer ablates formin-mediated actin polymerization (Bartolini et al., 2008; Harris et al., 2010; Harris et al., 2006; Lu et al., 2007; Xu et al., 2004). The second contains a conserved lysine residue (K1601 in Bni1 and K800 in FMNL3), but is involved in activities other than actin nucleation and elongation (Copeland et al., 2004; Ishizaki et al., 2001; Scott et al., 2011; Thompson et al., 2013). For example, mutation of these conserved lysine residues in mDia1 prevented actin bundling, microtubule capture during cell motility and alignment of microtubules with actin (Daou et al., 2014; Ishizaki et al., 2001).

The FH2 dimer is thought to nucleate actin polymerization by stabilizing the rate-limiting formation of a G-actin dimer or trimer (Pring et al., 2003), although the exact mechanism is still not clearly defined (Thompson et al., 2013). Once polymerization has initiated, the FH2 dimer acts as a “leaky capper” that stays bound to the elongating barbed-end while still allowing the incorporation of G-actin subunits (Higashida et al., 2004; Kovar and Pollard, 2004; Mizuno et al., 2011; Pring et al., 2003; Romero et al., 2004; Zigmond et al., 2003). This leaky capping activity requires that the FH2 dimer must alternate between “open” and “closed” conformations. In the closed conformation, both halves of the dimer are in contact
with the barbed-end of F-actin (Pring et al., 2003). In the open conformation one half of the dimer releases the end of the filament to allow incorporation of a G-actin monomer and the process then repeats (Kovar and Pollard, 2004; Mizuno et al., 2011; Paul and Pollard, 2009; Shemesh et al., 2005; Thompson et al., 2013; Xu et al., 2004). This model predicts that FH2 must rotate relative to the actin filament as elongation proceeds and this rotation has been visualized directly in vitro (Kovar and Pollard, 2004; Mizuno et al., 2011). Barbed-end binding by FH2 also protects the actin filament from the termination of elongation by preventing binding of capping proteins (Harris et al., 2004; Romero et al., 2004; Suarez et al., 2015).

The isolated FH2 domain is able to nucleate polymerization from free G-actin in vitro, but in the cell G-actin is also found in a complex with the small actin-binding protein profilin (Pring et al., 2003; Romero et al., 2004). Utilization of profilin-actin by FH2, however, requires the action of two accessory domains: FH1 and the FH2 C-terminal tail. FH1 is rich in polyproline repeats and is a ligand for the small actin-binding protein profilin. As with the VCA domain of type I NPFs, the recruitment of profilin-actin complexes by FH1 is thought to feed these subunits to FH2 at the growing barbed-end. A similar role is also proposed for the C-terminal tail, a WH2-like motif followed by a short string of basic residues found in the region C-terminal to the FH2 domain in a number of formins (Gould et al., 2011; Heimsath and Higgs, 2012; Thompson et al., 2013). As with FH1, this motif is also thought to facilitate the use of profilin-actin by FH2 both for nucleation and elongation. The WH2-like motif also mediates F-actin binding and bundling as demonstrated in FMNL2 and FMNL3 whereby a point mutation in the WH2-like motif abolishes actin monomer binding (Heimsath and Higgs, 2012; Vaillant et al., 2008).

Separate from their effects on actin, FH1 and FH2 also play a role in governing the organization and stabilization of the microtubule network (Bartolini et al., 2012; Daou et al., 2014; Gaillard et al., 2011; Roth-Johnson et al., 2014; Thurston et al., 2012; Young et al., 2008).
Consistent with this observation, inhibition of formins with the FH2 targeting drug inhibitor, SMIFH2, resulted in loss of microtubules in maturing oocytes, alluding to a role for formins in microtubule maintenance (Kim et al., 2015).

1.6 Are all formins activated by the same mechanisms?

The mechanisms governing formin activity have been best described for the DRF subfamily. These proteins contain an N-terminal Diaphanous Inhibitory Domain (DID) that binds directly to the C-terminal Diaphanous Autoregulatory Domain (DAD) in an intramolecular regulatory complex that inhibits DRF function (Higgs, 2005; Schonichen and Geyer, 2010). In the autoinhibited conformation, DID is bound to DAD obscuring the actin binding surface of the FH2 domain. Activation occurs when the DID/DAD interaction is disrupted by binding of the active, cognate Rho GTPase to the N-terminal GBD (Figure 1.1B) (Higgs, 2005; Schonichen and Geyer, 2010). This induces a conformational change in DID that releases the autoinhibitory interaction and unmaskes the FH2 domain (Alberts, 2001; Copeland et al., 2007; Li and Higgs, 2003; Maiti et al., 2012; Nezami et al., 2010). This mechanism has been defined functionally both in vitro and in vivo and the molecular details have been confirmed through recent structural studies on the full-length mDia1 protein (Alberts, 2001; Copeland et al., 2004; Copeland et al., 2007; Li and Higgs, 2003; Maiti et al., 2012; Nezami et al., 2010).
Figure 1.1 Differences in actin polymerization by the Arp2/3 complex and formins. (A) In its inactive state, the Arp2/3 complex is not capable of binding pre-existing actin filaments. Binding and activation of the Arp2/3 complex by nucleation promoting factors, such as N-WASP, localizes the complex to pre-existing actin filaments. Actin polymerization then proceeds by recruitment of actin monomers to the growing (barbed) end of the filament. A new actin filament is created at a 70° angle to the pre-existing filament. (B) Under basal conditions, formins are maintained in an inactive state through an intramolecular interaction between its N and C terminus. Binding of an active Rho GTPase to the N terminus disrupts the intramolecular interaction allowing for formin-mediated actin elongation. Formins bind to the growing barbed end of actin filaments and mediate the formation of straight actin filaments. Adapted from Bioessays, 7: 687-96, Truong et al., Bacterial subversion of host cytoskeletal machinery: hijacking formins and the Arp2/3 complex, 2014.
The activity of the non-DRFs FMN1, Fmn2 and INF2 are also autoregulated through a similar, but distinct, mechanism (Kobielak et al., 2004; Quinlan et al., 2007; Ramabhadran et al., 2013). A notable exception is the novel formin INF1 which is constitutively active both in vivo and in vitro (Young et al., 2008). Surprisingly, studies of DRF auto-regulation have also shown that the binding of Rho-GTP to the GBD is not sufficient to fully relieve autoinhibition (Li and Higgs, 2003, 2005; Maiti et al., 2012; Nezami et al., 2006; Otomo et al., 2005; Rose et al., 2005), suggesting that additional factors must be at work to fully activate these proteins in vivo. Consistent with this hypothesis, mDia2 and FHOD1 autoinhibition is also directly regulated by Rho Kinase (ROCK)-induced phosphorylation (Gasteier et al., 2003; Staus et al., 2011; Takeya et al., 2008; Truong et al., 2013) and other formins are also kinase substrates (Cheng et al., 2011; Iskratsch et al., 2010; Wang et al., 2004). Additional mechanisms regulating formin activity in vivo include subcellular targeting (Copeland et al., 2007; Gorelič et al., 2011; Seth et al., 2006), lipid modification (Block et al., 2012; Han et al., 2009; Moriya et al., 2012), and direct activation by mechanical tension (Chan et al., 2010; Courtemanche et al., 2013; Higashida et al., 2013; Jegou et al., 2013).

1.7 FHOD1

FHOD1 was first identified in a yeast-2-hybrid screen from a B-cell library that revealed FHOD1 as a binding partner of the acute myeloid transcription factor, AML-1B (Westendorf et al., 1999). The physiological relevance of this interaction however, remains elusive. FHOD1 is one of two members of the FHOD family of mammalian formins, with the other family member being FHOD3. Quantitative real-time PCR (qPCR) revealed that relative to other mammalian formins, FHOD1 is the most highly expressed, and its expression can be detected in cells of hematopoietic and non-hematopoietic lineages (Krainer et al., 2013). Immunostaining with
endogenous antibodies reveals cytoplasmic localization of FHOD1 under basal conditions (Westendorf et al., 1999).

The N-terminus of FHOD1 was resolved to 2.9 Å and comprises of a GBD, FH3, and helical domain (Schulte et al., 2008) (Figure 1.2). Structural analysis demonstrated low structural homology between the N-terminus of FHOD1 to the N-terminus of mDia1 (Schulte et al., 2008). This is consistent with observations that different regulatory mechanisms exist for mDia1 and FHOD1 (explained in more detail in Section 1.7.1).

1.7.1 Regulation of FHOD1

Similar to other formin family members, FHOD1 also contains a FH1 and FH2 domain and is maintained in an autoinhibited state via an intramolecular interaction between its N and C terminus. The N-terminus of FHOD1 also contains a Rho GTPase binding domain that binds to Rac1. For most DRFs, binding of a Rho GTPase to the GBD confers activation (Schonichen and Geyer, 2010). Interestingly, FHOD1 binds to Rac1 in a nucleotide-independent manner and expression of dominant negative Rac1 does not affect the biological activity of FHOD1, suggesting that other mechanisms contribute to FHOD1 regulation (Gasteier et al., 2003; Westendorf, 2001). Treatment of NIH3T3 cells with the Rho-associated kinase (ROCK) inhibitor, abolished the biological activity of active FHOD1, implying a contribution of ROCK in FHOD1 regulation (Gasteier et al., 2003). Consistent with this observation, phosphorylation of three conserved residues (S1131, S1137, and T1141) in the FHOD1 C-terminus by ROCK is sufficient to prevent binding between the N and C terminus of FHOD1 and induce formation of actin stress fibres (Takeya et al., 2008) (Figure 1.2). This suggests that phosphorylation of the C terminus of FHOD1 is capable of releasing it from an autoinhibited state and inducing FHOD1 activation.
While it is unclear whether Rho GTPase binding is sufficient to induce FHOD1 activation, it was suggested that Rho GTPases may dictate localization of FHOD1. The GBD of FHOD1 relocalizes from the cytoplasm to the plasma membrane upon co-expression with active GTPase variants of Rac1, Rac2, Rac3, RhoJ, Rnd1, Rnd2, or Rnd3 (Gasteier et al., 2003). Thus far, only direct interaction between Rac1 and FHOD1 has been demonstrated.

1.7.2 Insights into FHOD1 activity

Insights into FHOD1 activity have been completed mainly by functional analysis of FHOD1 truncation mutants. A hallmark of FHOD1 activation is the formation of thick actin stress fibres (Gasteier et al., 2003; Takeya et al., 2008). FHOD1 contains an N-terminal GBD, FH3 domain, and helical domain (Figure 1.2). Deletion of the C-terminus autoregulatory domain of FHOD1, results in an active FHOD1 variant. Overexpression of this active FHOD1 variant in NIH 3T3 cells induces formation of thick actin stress fibres (Gasteier et al., 2003). Conversely, depletion of FHOD1 resulted in loss of stress fibres, further implying a role for FHOD1 in stress fibre formation (Schulze et al., 2014).

Stress fibre formation has been attributed to FH1 and FH2 domains of FHOD1, since deletion of the FH1 and FH2 in the active FHOD1 variant abolished stress fibre formation (Gasteier et al., 2003). Recently, it was demonstrated that the helical domain of FHOD1 could also mediate stress fibre formation. Expression of FHOD1 lacking the N-terminus GBD-FH3 domain stimulated formation of stress fibres, whereas expression of FHOD1 lacking both the GBD-FH3 and helical domain did not induce stress fibre formation (Schulze et al., 2014). Collectively, these studies suggest that multiple domains within FHOD1 contribute to stress fibre formation.
In vitro studies demonstrated that expression of the full length FHOD1 or the active FHOD1 variant stabilized actin filaments and prevented actin depolymerization (Schonichen et al., 2013). Consistent with these observations, depletion of FHOD1 during cell spreading resulted in unstable protrusions that often collapsed (Iskratsch et al., 2013). This suggests that FHOD1 may act as a capping protein to protect the growing barbed ends of actin filaments. As expected, the activity of active FHOD1 was not affected by inhibition of the Arp2/3 complex, suggesting that FHOD1 induces stress fibres independently of Arp2/3 (Gasteier et al., 2003).

FHOD1 has also been implicated in promoting cell migration since expression of FHOD1 in NIH3T3 cells increased cell migration towards type-1 collagen (Koka et al., 2003). Furthermore, in a wound-healing assay, only 30% of wounds recovered in FHOD1-depleted cells (Iskratsch et al., 2013). The Drosophila FHOD1 homolog, knittrig, also plays a role in cell migration. Generation of knittrig mutants resulted in reduced viability, alluding to the essentiality of knittrig in Drosophila development (Lammel et al., 2014). Wound migration assays in macrophages of knittrig mutants revealed reduced macrophage migration and cell spreading (Lammel et al., 2014). Interestingly, upon challenge with E. coli, knittrig expression was increased by 11 fold in Drosophila hemocytes, suggesting a potential role for knittrig in host-pathogen interactions (Johansson et al., 2005). The mechanism by which knittrig contributes to host-pathogen interactions remains elusive. Furthermore, the contribution of FHOD1 to mammalian cells challenged with bacteria has never been studied and will be a major focus of my thesis.
Figure 1.2 Domain structure of FHOD1. The N-terminus of FHOD1 contains a GBD and FH3 domain. Binding of FH3 with the C-terminus DAD maintains FHOD1 in an inactive state. Phosphorylation of the C-terminus is sufficient to alleviate the intramolecular interaction between DAD and FH3. The helical domain, FH1 and FH2 domain have been previously suggested to promote stress fibre formation. The GBD-FH3 domain appears to mediate actin stress fibre localization.
1.8 Formins and the Arp2/3 complex collaborate to regulate the actin cytoskeleton

The ability of formins to promote the formation of long unbranched actin filaments has generally caused them to be linked with stress fibre formation \textit{in vivo} (Hotulainen and Lappalainen, 2006; Oakes et al., 2012; Takeya et al., 2008; Watanabe et al., 1999). A growing number of studies, however, have described a role for formins in the generation of lamellipodia and filopodia through their interactions with other cytoskeletal remodelling factors (Beli et al., 2008; Block et al., 2012; Bosch et al., 2007; Lee et al., 2010; Okada et al., 2010; Pfender et al., 2011; Quinlan et al., 2007; Sarmiento et al., 2008; Schulze et al., 2014; Suraneni et al., 2012; Tojkander et al., 2011).

In some cases these interactions are antagonistic rather than mutualistic. For example, in HeLa cells and MTLn3 adenocarcinoma cells, EGF-induced stimulation of lamellipodia formation requires activation of the Arp2/3 complex and concomitant inhibition of DRF activity (Beli et al., 2008; Sarmiento et al., 2008). The mechanisms underlying DRF inhibition are cell-type specific and work either through repression of RhoA/mDia1 signalling (Sarmiento et al., 2008) or through a direct inhibitory interaction between the DRF mDia2 and the type I NPF WAVE2 (Beli et al., 2008). Profilin may also serve as another regulatory mechanism to dictate formin or Arp2/3-driven actin elongation. In yeast, addition of profilin inhibited Arp2/3-mediated formation of actin patches, and induced a concomitant increase in formin-mediated formation of contractile rings, suggesting that the presence of profilin promotes formin-mediated actin polymerization (Suarez et al., 2015). The mechanism by which profilin inhibits Arp2/3-mediated actin elongation may be a result of sequestration of G-actin by profilin away from the Arp2/3 complex (Rotty et al., 2015; Suarez et al., 2015). Consistent with this
observation, the profilin to actin ratio appears to dictate whether actin elongation will be carried out by formins or the Arp2/3 complex (Burke et al., 2014).

In other contexts, formin and Arp2/3 complex cooperation, rather than competition, is required for lamellipodia, filopodia or stress fibre assembly (Tojkander et al., 2011). In B16F1 melanoma cells, mDia2 depletion inhibits lamellipodia formation (Yang et al., 2007) while depletion of the DRF FMNL2 decreases the rate of lamellipodial protrusion (Block et al., 2012). In both cases, however, lamellipodial dynamics are dependent on Arp2/3-induced actin polymerization and not formin-induced nucleation. Instead the ability of formins to protect the growing barbed ends of Arp2/3 nucleated filaments is crucial for proper lamellipod formation and cell migration. A similar cooperative Arp2/3- and formin-dependent mechanism has been proposed for the generation of filopodial-like structures in reconstituted lipid bilayers in vitro (Lee et al., 2010). Whether this collaboration translates to an in vivo system, has yet to be explored.

1.9 The actin cytoskeleton is a major target of bacterial pathogens

Microbial pathogens have been shown to modulate the host actin cytoskeleton during infection (Rottner et al., 2005; Stevens et al., 2006; Welch and Way, 2013). This includes both intracellular (e.g. Shigella flexneri, Listeria monocytogenes and Salmonella enterica serovar Typhimurium) as well as extracellular pathogens (enteropathogenic Escherichia Coli) (Stevens et al., 2006; Welch and Way, 2013). Modulation of the host cytoskeleton has been intrinsically linked to the pathogenic lifestyle of these microorganisms and has been the subject of intense study. For example, the actin cytoskeleton is exploited during bacterial invasion, intracellular replication and actin-based motility (de Souza Santos and Orth, 2015; Truong et al., 2014). A great deal has been learned about eukaryotic cell biology from the study of microbial pathogens.
ActA, a virulence factor protein expressed in *Listeria monocytogenes* (*L. monocytogenes*), was the first NPF identified for the Arp2/3 complex (Welch et al., 1997b; Welch et al., 1998). ActA deficient mutants of *L. monocytogenes* are unable to undergo actin-based motility (ABM) in the cytosol of host cells (Kocks et al., 1992). ActA exploits the Arp2/3 complex by acting as a functional mimic of the type I NPF, N-WASP (Welch et al., 1997b). The Arp2/3 complex has garnered much attention in its role in host-pathogen interactions and many subsequent discoveries focused on new findings of other bacterial pathogens that hijack the Arp2/3 complex for actin polymerization (de Souza Santos and Orth, 2015; Stevens et al., 2006; Welch and Way, 2013). However, while most studies of bacterial pathogenesis focused on exploitation of the Arp2/3 complex, recent studies have begun to examine the role of formins.

### 1.10 Rho GTPases

Rho GTPases are part of the Ras superfamily of GTPases and are important regulatory components of signal transduction pathways. Rho GTPases have been demonstrated to be involved in cell cycle regulation, cell motility, as well as phagocytosis (Jaffe and Hall, 2005; Ridley, 2012). There is much to be learned about the specific host targets of each Rho GTPase.

Rho GTPases have intrinsic GTPase activity allowing for GTP hydrolysis. Thus far, 20 Rho GTPases have been identified in mammalian cells (Table 1.2). Four of the Rho GTPase family members do not exhibit intrinsic Rho GTPase activity: Rnd1, Rnd2, RhoE and RhoH, but are predicted, or known to be capable of binding GTP (Table 1.2) (Jaffe and Hall, 2005; Riento et al., 2005; Vega and Ridley, 2007). Rho GTPases undergo post-translation modifications such as, geranylgeranylation or farnesylation at their C-terminal CAAX motif (Jaffe and Hall, 2005). Post-translational modifications have important roles in regulating localization of Rho GTPases.

Since Rho GTPases play important roles in a wide variety of signal transduction pathways, they are tightly regulated. Rho GTPases cycle between an active GTP bound-state
and an inactive GDP-bound state (Figure 1.3). Activation of Rho GTPases is induced by Rho guanine nucleotide exchange factors (GEFs). Rho GEFs induce Rho GTPase activation by catalyzing the exchange of GDP for GTP (Jaffe and Hall, 2005; Ridley, 2012). The intrinsic GTPase activity of Rho GTPases is very slow and requires the activity of Rho GTPase activating proteins (GAPs) to accelerate the process of GTP hydrolysis. Rho guanine nucleotide dissociation inhibitors (GDIs) represent another aspect of Rho GTPase regulation. Rho GDIs bind to the isoprenyl group of Rho GTPases to prevent membrane binding and thus, inhibits spontaneous activation of Rho GTPases (Garcia-Mata et al., 2011). The mammalian genome encodes 85 GEFs, 80 GAPs and 3 GDIs (Burridge and Wennerberg, 2004; Jaffe and Hall, 2005). Given that the mammalian genome encodes more GEFs and GAPs than Rho GTPases, this suggests that GEFs and GAPs may be subjected to spatiotemporal regulation. Vav2 and Tiam1 are both capable of promoting Rac1 activation, suggesting that redundancy between Rho GEFs also exists (Yang et al., 2012). Furthermore, activation of PI3K was previously demonstrated to require Tiam1 (Rac1 GEF), Fgd1 (Cdc42 GEF) and SGEF (RhoG GEF) alluding to the possibility that multiple GEFs may cooperate to elicit Rho GTPase activation (Yang et al., 2012).

Of the 20 mammalian Rho GTPases identified, only Rac1, RhoG and Cdc42 have been extensively studied in the context of S. Typhimurium invasion (Aiastui et al., 2010; Criss et al., 2001; Patel and Galan, 2006). Furthermore, siRNA-mediated knockdown of Rac1, RhoG or Cdc42 does not completely ablate S. Typhimurium invasion (Aiastui et al., 2010; Patel and Galan, 2006). This brings about the question as to whether other host Rho GTPases can play a role during S. Typhimurium invasion.
<table>
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<th><strong>Rho GTPase</strong></th>
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<tr>
<td>RhoA</td>
<td>- stress fibre formation</td>
<td>ROCKI, ROCKII, mDia1, mDia2</td>
<td>Geranylgeranylation (GG)</td>
<td>(Copeland and Treisman, 2002; Sadok and Marshall, 2014)</td>
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<td>RhoB</td>
<td>- regulates endosomal trafficking</td>
<td>PDK1</td>
<td>GG, farnesylation (F), palmitoylation (P)</td>
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<td>RhoC</td>
<td>- cell migration</td>
<td>FMNL2, FMNL3, ROCKI, ROCKII</td>
<td>GG</td>
<td>(Kitzing et al., 2010; Vega et al., 2011)</td>
</tr>
<tr>
<td>Rac1</td>
<td>- lamellipodia formation - NADPH oxidase activation - phagocytosis</td>
<td>PAK1, FMNL1, WAVE</td>
<td>GG</td>
<td>(Yayoshi-Yamamoto et al., 2000)</td>
</tr>
<tr>
<td>Rac2</td>
<td>- lamellipodia formation - NADPH oxidase activation - cell migration</td>
<td>PAK1-3, WAVE</td>
<td>GG</td>
<td>(Steffen et al., 2013)</td>
</tr>
<tr>
<td>Rac3</td>
<td>- lamellipodia formation</td>
<td>PAK1-3, WAVE</td>
<td>GG</td>
<td>(Steffen et al., 2013)</td>
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<tr>
<td>RhoG</td>
<td>- lamellipodia formation - phagocytosis</td>
<td>ELMO, kinectin</td>
<td>GG</td>
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<td>RhoJ</td>
<td>- filopodia formation - recycling of endocytic compartments</td>
<td>N-WASP, PAK1-3</td>
<td>F, GG and P</td>
<td>(de Toledo et al., 2003; Ho et al., 2013; Vignal et al., 2000)</td>
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<tr>
<td>RhoQ</td>
<td>- filopodia formation</td>
<td>N-WASP, Exo70</td>
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<td>(Dupraz et al., 2009; Fujita et al., 2013)</td>
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<td>Cdc42</td>
<td>- filopodia formation</td>
<td>PAK, N-WASP</td>
<td>GG; Cdc42H undergoes palmitoylation</td>
<td>(Kang et al., 2008; Sadok and Marshall, 2014)</td>
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<tr>
<td>Rnd1</td>
<td>- lacks GTPase activity</td>
<td>p190RhoGAP</td>
<td>F</td>
<td>(Oinuma et</td>
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Table 1.2 Rho GTPases and actin dynamics. Adapted from (Vega and Ridley, 2007).
Figure 1.3 Regulation of Rho GTPases. When bound to GDP, Rho GTPases are in an inactive state. GEFs catalyze the exchange of GDP for GTP, thereby inducing Rho GTPase activation. Due to the low intrinsic GTPase activity of Rho GTPases, GAPs are required to catalyze GTP hydrolysis, causing Rho GTPases to cycle back to its GDP bound state. Another arm of regulation is the binding of Rho GTPases to Rho GDIs. Rho GDIs sequester Rho GTPases to the cytosol and prevent their activation.
1.11 *Salmonella enterica* serovar Typhimurium: Impact of infection

*Salmonella enterica* are Gram negative bacterial pathogens that pose a major health hazard to the population. The two major clinical manifestations of *Salmonella enterica* infection are gastroenteritis and typhoid fever (Ohl and Miller, 2001). Typhoid fever is a systemic disease and is characterized by progression of fever, headache, malaise, and bowel discomforts (constipation or diarrhea) (Parry et al., 2002). Gastroenteritis is limited to the small intestine and symptoms typically include diarrhea, nausea and vomiting (Coburn et al., 2007).

*Salmonella enterica* are organized into subspecies, and subsequently divided into serovars. *Salmonella enterica* serovars are classified based on structures of their flagellin, lipopolysaccharides, and carbohydrates (Coburn et al., 2007). Over 2500 serovars have been identified to date. *Salmonella enterica* serovars each have a specific host range and also different clinical outcomes. For example, *S.* Typhi, *S.* Paratyphi, and *S.* Sendai infections are limited to humans and infection results in typhoid fever (Bonnermann and Rosener, 1975). In contrast, *S.* Typhimurium is capable of infecting both humans and animals. *S.* Typhimurium infection in humans induces gastroenteritis, whereas *S.* Typhimurium infection in mice results in a systemic disease resembling typhoid fever (Haraga et al., 2008; Ohl and Miller, 2001).

*S.* Typhimurium is a food borne pathogen that is transmitted via ingestion of contaminated food and water (Haraga et al., 2008). Clinical manifestations of infection in healthy individuals include gastroenteritis – a condition in which individuals suffer from nausea, diarrhea, vomiting, abdominal pain, and muscle pain (Scallan et al., 2011). Severe complications from infection may also arise, such as reactive arthritis, a debilitating inflammatory disorder with the potential to become a chronic manifestation (Hannu et al., 2002; Steckelberg et al., 1988). Generally, those with a healthy immune system recover from *S.* Typhimurium infection without medical intervention. However, infection with *S.* Typhimurium in immunocompromised
individuals can be potentially fatal (Feasey et al., 2012; Gordon, 2008). In addition, an emergence of antibiotic resistant strains of *S. Typhimurium* results in increased morbidity, mortality, as well increased financial burden on our healthcare system (Conly, 2002; Majowicz et al., 2010).

1.12 The *S. Typhimurium* Type 3 secretion systems

An important feature of the virulence strategy used by *S. Typhimurium*, is its ability to infect host cells using a specialized needle-like apparatus, the Type 3 secretion system (T3SS). The needle apparatus of the T3SS creates a translocon pore that inserts into the host plasma membrane to allow for translocation of bacterial proteins (called ‘effectors’) into the host cell cytosol (Kubori et al., 2000; Shea et al., 1996). The base of the needle-like apparatus spans the inner and outer membrane of *S. Typhimurium*.

*S. Typhimurium* expresses two T3SS that are encoded on different regions of the genome and expressed at different stages of infection (Haraga et al., 2008; Kubori et al., 1998). The SPI-1 T3SS is expressed early in infection to promote bacterial adherence to host cells, and subsequent invasion. The SPI-2 T3SS is expressed after *S. Typhimurium* invasion and SPI-2 secreted effectors contribute to bacterial replication in specialized vacuoles, cell-to-cell spread in tissues, and are required for systemic infection in mice (Coombes et al., 2005; LaRock et al., 2015). In this thesis I will focus on the SPI-1 T3SS and its role in the initial stages of *S. Typhimurium* infection.

1.12.1 *S. Typhimurium* SPI-1 T3SS effectors

The SPI-1 T3SS delivers over 20 different bacterial effectors into the host cytosol to manipulate host signalling pathways in the early stages of infection (Figure 1.4.1). SPI-1 T3SS secretes bacterial effectors early in invasion to promote entry into host cells, macrophage apoptosis, and pro-inflammatory cytokine secretion (LaRock et al., 2015). The SPI-1 T3SS
secreted effectors are essential for bacterial invasion because *S. Typhimurium* harbouring mutations in the translocon apparatus, are unable to secrete SPI-1 T3SS into the host cytosol and cannot infect host cells (Galan et al., 1992; Kaniga et al., 1994; Zeng et al., 2003). Although many bacterial effectors have been identified and are known to contribute to virulence, there remains an incomplete understanding of the host targets for some bacterial effectors.

1.13 The role of actin polymerization in *S. Typhimurium* invasion

*S. Typhimurium* entry begins with bacterial-induced actin rearrangements at the host cell surface. The result is the formation of an actin-rich cell surface protrusion (named ‘invasion ruffle’) that extends beyond the cell surface to engulf the bacteria and drive its entry into the host cell (Finlay et al., 1991). Scanning electron microscopy analysis of the invasion ruffle reveals that it is not uniform, and rather, is comprised of multiple actin structures such as finger-like filopodia and sheet-like lamellipodia that extend from the cell surface (Meyerholz and Stabel, 2003; Velge et al., 2012). Actin rearrangements are essential to bacterial entry since pre-treatment of cells with cytochalasin D – an actin depolymerizing agent - prevents bacterial invasion (Finlay et al., 1991). The precise mechanism by which actin rearrangements at the host plasma membrane occur is not completely understood and will be a major focus of my thesis.
Figure 1.4 Early stages of \textit{S. Typhimurium} invasion. 1. \textit{S. Typhimurium} invasion begins with insertion of the SPI-1 T3SS into the host plasma membrane and subsequent secretion of bacterial effectors into the host cytosol. 2. SopE/E2 and SopB activate host Rho GTPases which will then signal to upstream activators of the Arp2/3 complex. The Arp2/3 complex mediates actin rearrangements at the host plasma membrane to form an invasion ruffle. 3. Upon entry into the host cell, the actin invasion ruffle resolves, and the host plasma membrane returns to basal state. This process is mediated by the bacterial effector SptP, which mimics Rho GAP activity to turn off Rac1 and Cdc42 signalling (Fu and Galan, 1999; Stebbins and Galan, 2000). The bacterial effectors SopB and SopD mediate membrane sealing at the plasma membrane, allowing for bacterial entry into the host cell (Bakowski et al., 2007). SopB localization to the SCV induces production of PI(3)P. This is accomplished by SopB dependent recruitment of Rab5 to trigger recruitment of the PI3K, Vps34, to the SCV (Mallo et al., 2008). At this stage, SopB also prevents lysosomal fusion by reducing the negative charge on the SCV, which induces removal of Rab GTPases that promote lysosomal fusion (Bakowski et al., 2010).
1.14 SopE/E2 and SopB are required for *S. Typhimurium* invasion

SopE, SopE2 (referred herein as SopE/E2) and SopB are bacterial effectors secreted by the SPI-1 T3SS and have essential roles in driving actin cytoskeletal rearrangements at the host cell surface to form invasion ruffles (Hardt et al., 1998; Zhou et al., 2001). *S. Typhimurium* mutants lacking SopE, SopE2 and SopB are unable to induce actin rearrangements, and thus, cannot invade host cells (Zhou et al., 2001). Upon invasion, SopE/E2 and SopB induce activation of host Rho GTPases, Rac1 and Cdc42. SopE/E2 are bacterial guanine nucleotide exchange factors (GEF) that directly activate host Rho GTPases, Rac1 and Cdc42 (Patel and Galan, 2006; Stender et al., 2000). In contrast, SopB is a phosphoinositide phosphatase that utilizes a host GEF to indirectly activate Cdc42 (Patel and Galan, 2006). These Rho GTPases bind to WASP and WAVE, respectively, to activate the host actin nucleator, Arp2/3 complex (Rottner et al., 2010; Stender et al., 2000) (Figure 1.4.2).

Recent data has alluded to an Arp2/3-independent mechanism of *S. Typhimurium* invasion. Depletion of upstream activators of the Arp2/3 complex, or subunits of the Arp2/3 complex did not completely abrogate *S. Typhimurium* invasion (Hanisch et al., 2010; Hanisch et al., 2011; Shi et al., 2005). The involvement of other actin nucleators in invasion ruffle formation has never been studied and comprises a main focus of my thesis.

Previous studies demonstrated that in addition to actin rearrangements at the host plasma membrane, membrane delivery is also needed for invasion ruffle formation (Nichols and Casanova, 2010). The bacterial effector, SipC, makes up a part of the T3SS translocon pore. SipC also binds Exo70, a subunit of the exocyst complex, and promotes its recruitment to the bacterial invasion site (Nichols and Casanova, 2010). Proper assembly of the exocyst complex at the plasma membrane requires activation of the small Ral GTPase, RalA. *In vitro* GST pull-down assays demonstrated that SopE/E2 is needed for RalA activation during *S. Typhimurium* invasion.
invasion (Nichols and Casanova, 2010). SopE/E2 is a bacterial GEF that activates Cdc42 and Rac1 (Hardt et al., 1998; Patel and Galan, 2006; Stender et al., 2000). It is possible that the GEF activity of SopE/E2 also allows for RalA activation during S. Typhimurium invasion. Thus, in addition to promoting actin polymerization at invasion sites, SopE/E2 also promotes delivery of membrane via the exocyst complex to contribute to formation of the invasion ruffle (Figure 1.5).

1.15 The many roles of SopB during S. Typhimurium pathogenesis

While SopB plays an important role in formation of the S. Typhimurium invasion ruffle, its contribution to bacterial pathogenesis does not end there. Previous studies identified a role for SopB in inducing gastric inflammation in animal models, long term systemic infection in mice, inhibition of nuclear mRNA export and an increase in chloride secretion (Feng et al., 2001; Galyov et al., 1997; Lawley et al., 2006). The catalytic activity of SopB appears to be required for many of the phenotypes it regulates during infection.

1.15.1 SopB and its phosphoinositide phosphatase activity

SopB is a phosphoinositide phosphatase and its activity is attributed to a region with homology to mammalian polyphosphate-4 phosphatases. In vitro, SopB can dephosphorylate many phosphoinositides and inositol phosphates (Norris et al., 1998). The amino acid sequence of SopB displays amino acid similarity to human inositol phosphatases. The cysteine residue at position 462 of the SopB protein is essential for its phosphatase activity (Norris et al., 1998).
Figure 1.5 Additional roles of SopE/E2 and SopB in S. Typhimurium invasion. SopE/E2 triggers RalA activation to allow for assembly of the exocyst complex at the plasma membrane. Exocyst complex assembly at the plasma membrane allows for delivery of additional membrane to contribute to invasion ruffle formation. SopB is a phosphoinositide phosphatase that depletes PI(4,5)P$_2$ at the base of the invasion ruffle to produce PI(5)P. PI(5)P production will lead to production of PI(3,4,5)P$_3$/PI(3,4)P$_2$, and subsequent activation of Akt at the invasion ruffle.
Previous studies demonstrated that SopB induced depletion of PI(4,5)P$_2$ at the base of the invasion ruffle (Figure 1.5) (Terebiznik et al., 2002). HeLa cells transfected with PLCδ-PH-GFP, a probe for PI(4,5)P$_2$, demonstrated a lack of GFP intensity at the base of the $S$. Typhimurium invasion ruffle, but an enrichment at the tips of the invasion ruffle (Terebiznik et al., 2002). Furthermore, dephosphorylation of PI(4,5)P$_2$ was dependent on the phosphatase activity of SopB, since infection with a phosphatase inactive SopB mutant (C462S) failed to dephosphorylate PI(4,5)P$_2$ (Terebiznik et al., 2002). PI(4,5)P$_2$ depletion at the base of the invasion ruffle appears to be important for membrane sealing after bacterial entry (Terebiznik et al., 2002).

SopB-mediated depletion of PI(4,5)P$_2$ leads to the formation of PI(5)P. However, SopB also promotes an increased accumulation of PI(3,4)P$_2$ and PI(3,4,5)P$_3$ levels at the site of $S$. Typhimurium invasion (Mallo et al., 2008). The mechanism as to how a phosphoinositide phosphatase is capable of dephosphorylating PI(4,5)P$_2$ and concomitantly initiating production of PI(3,4)P$_2$ and PI(3,4,5)P$_3$ remains elusive. There is much to be learned about the $in$ vivo consequences of the phosphatase activity of SopB.

1.15.2 SopB and Akt activation

Akt is a prosurvival kinase that inhibits apoptosis and regulates cell proliferation (Alessi and Cohen, 1998). It is regulated by being recruited from the cytosol to the plasma membrane via binding of its pleckstrin homology (PH) domain to PI(3,4)P$_2$ and PI(3,4,5)P$_3$, both of which are products of class I phosphoinositide-3-kinase (PI3K) (Bellacosa et al., 1998). Recruitment to the plasma membrane however, is not sufficient for activation. Activation is achieved upon phosphorylation at two residues: S473 is phosphorylated by PDK1 and T308 is phosphorylated by mTORC2 (Bellacosa et al., 1998; Sarbassov et al., 2005). $S$. Typhimurium invasion induces
Akt activation to inhibit apoptosis of the host cell, allowing for bacterial replication within the host cell (Knodler et al., 2005).

Previous studies demonstrated a role for SopB in inducing Akt activation during invasion (Knodler et al., 2005; Steele-Mortimer et al., 2000). Infection with a ΔsopB mutant of S. Typhimurium led to ablation of Akt activation during invasion (Steele-Mortimer et al., 2000). This phenotype is specifically attributed to the phosphatase activity of SopB since infection with a catalytically inactive SopB mutant (C462S) was unable to rescue this defect (Knodler et al., 2005).

Consistent with the observation that the PH domain of Akt binds to PI(3,4)P$_2$ and PI(3,4,5)P$_3$, the production of these phosphoinositides are required for Akt activation during S. Typhimurium invasion. The requirement for PI(3,4)P$_2$ and PI(3,4,5)P$_3$ in SopB-mediated Akt activation was demonstrated by depleting both phosphoinositides during invasion. Dephosphorylation of PI(3,4)P$_2$ and PI(3,4,5)P$_3$ can be achieved via expression of PTEN-A4-YFP, a YFP-tagged construct comprising of the PI3 phosphatase, PTEN (Rahdar et al., 2009). The C-terminus of PTEN-A4 contains mutations which enhance its binding to the plasma membrane and also prevents accumulation of PI(3,4)P$_2$ and PI(3,4,5)P$_3$ (Rahdar et al., 2009). During S. Typhimurium invasion, expression of PTEN-A4-YFP into host cells significantly inhibits Akt activation, alluding to the requirement for PI(3,4)P$_2$ and PI(3,4,5)P$_3$ (Rahdar et al., 2009; Roppenser et al., 2013).

The mechanism by which SopB induces Akt activation is not clearly understood. The PH domain of Akt localizes to the invasion ruffle, suggesting a localized increase in PI(3,4,5)P$_3$ at the invasion ruffle upon bacterial entry (Roppenser et al., 2013). Since SopB is essential for Akt activation, this would suggest that SopB plays a role in increasing PI(3,4,5)P$_3$, at the invasion site. This however, is counterintuitive to the fact that SopB is a known phosphoinositide phosphatase.
A possible mechanism underlying SopB-mediated Akt activation may be via recruitment of PI3K to the invasion ruffle to mediate Akt activation (Figure 1.5). Data from our lab suggests that multiple host kinases are involved in SopB-induced Akt activation. Furthermore, depending on the cell type, there is a differential contribution of host kinases toward SopB-mediated Akt activation (Roppenser et al., 2013). While PI(3,4)P₂/PI(3,4,5)P₃ are products of class I PI3K, Akt activation is still observed in HeLa cells treated with the class I PI3K inhibitors, wortmannin and LY294002, suggesting the involvement of other PI3K (Cooper et al., 2011; Roppenser et al., 2013). Our lab demonstrated that class II PI3K C2β and IPMK are the main contributors to Akt activation in HeLa cells. Contrary to HeLa cells, treatment of mouse embryonic fibroblasts (MEFs) with wortmannin or LY294002 abolishes Akt activation during invasion (Roppenser et al., 2013). This variation between cells suggests that SopB utilizes other regulatory mechanisms to induce Akt activation in MEFs. Together, these data suggest that SopB has evolved a mechanism to activate a broad class of host kinases to initiate the formation of PI(3,4)P₂/PI(3,4,5)P₃ and subsequently activate Akt in multiple mammalian cell types.

1.16 Signal Transduction and Palmitoylation

Palmitoylation is a reversible post-translational modification in which a saturated fatty acid (palmitic acid C16:0) is added onto a cysteine residue (Charollais and Van Der Goot, 2009; Levental et al., 2010) (Figure 1.6). Two forms of palmitoylation exist: S-palmitoylation and N-palmitoylation. S-palmitoylation is the addition of palmitic acid to a cysteine residue via a thioester bond (Charollais and Van Der Goot, 2009; Levental et al., 2010). N-palmitoylation is the result of addition of palmitic acid to an N-terminal cysteine residue, forming an intermediate thioester bond, which continues to undergo a chemical rearrangement to create a stable amide linkage (Linder and Deschenes, 2007). The effect of palmitoylation has been characterized for many integral and transmembrane proteins. For example, palmitoylation of the Ras GTPase, H-
Ras, directs its localization to lipid rafts, allowing for activation of PI3K (Charollais and Van Der Goot, 2009; Eisenberg et al., 2013). Furthermore, palmitoylation of the C-terminus of RhoB is required for its localization to endocytic compartments (Perez-Sala et al., 2009).

Lipid modification of proteins has important implications in protein localization and trafficking. Due to its reversible nature, palmitoylation acts as an important regulator of signalling pathways. For example, protein palmitoylation can influence host protein targeting, protein-protein interactions, and protein trafficking (Charollais and Van Der Goot, 2009). Viruses have been previously demonstrated to exploit host palmitoylated proteins during infection (Blanc et al., 2013; Chazal and Gerlier, 2003). Given that palmitoylated proteins play an important role in signal transduction, they may also serve as ideal candidates which are subjected to manipulation by bacterial pathogens. For example, palmitoylation of the host receptor ANTXR1 is required for receptor-mediated endocytosis of the anthrax toxin produced by *Bacillus anthracis* (Abrami et al., 2006; Young and Collier, 2007). The role of host palmitoylated proteins in the context of *S. Typhimurium* pathogenesis has never been studied.
Figure 1.6 Regulation of S-palmitoylation. Addition of the 16 carbon palmitoyl-CoA group is mediated by palmitoyl acyltransferases (PAT). Palmitoyl CoA is added onto a cysteine residue via a thioester linkage. Palmitoylation is reversible, whereby depalmitoylation is regulated by palmitoylthioesterases (PET).
The lack of a palmitoylation consensus sequence has made it difficult to completely identify all host proteins that are palmitoylated. Protein palmitoylation is mainly analyzed by incorporation of $^{3}$[H]-palmitic acid or the non-radiolabelled probe, 17-octadeynoic acid (17-ODYA) into proteins (Martin and Cravatt, 2009; Martin et al., 2012). Large scale studies and data mining has also allowed for the creation of databases to predict palmitoylated sites (Hu et al., 2011; Ren et al., 2008). New chemical probes are also being developed to allow for identification of palmitoylated proteins and palmitoyl acyltransferases (Zheng et al., 2015).

Palmitoylation is mediated by palmitoyl acyltransferases (PAT), and depalmitoylation is mediated by palmitoylthioesterases (PET). While a palmitoylation consensus sequence has yet to be identified, PATs are characterized by a conserved DHHC motif (Korycka et al., 2012). Thus far, 23 DHHC isoforms have been identified in humans (Korycka et al., 2012). Analysis of GFP-tagged human PAT revealed a diverse pattern of localization, suggesting that protein palmitoylation can occur at multiple sites within the host cell (Ohno et al., 2006). Overexpression of GFP-tagged DHHC constructs revealed that DHHC3 and DHHC4 colocalized with the Golgi marker, GM130. Some DHHC isoforms such as DHHC9 and DHHC12 displayed ER and Golgi localization. Interestingly, DHHC5, DHHC20, and DHHC21 displayed plasma membrane localization, suggesting that palmitoylation may not be restricted to the Golgi apparatus (Ohno et al., 2006).

1.17 Lipid Rafts

Lipid rafts are microdomains within the phospholipid bilayer of membranes that serve important functions in signal transduction and are defined by a region containing high concentrations of cholesterol and sphingolipids (Head et al., 2014; Lingwood and Simons, 2010; Simons and Toomre, 2000). Lipid rafts serve as ‘hubs’ for signalling molecules by
compartmentalizing proteins and receptors needed for downstream activation of signalling pathways.

There are a number of proteins that are recruited to lipid rafts. Receptors, kinases, and scaffold proteins are all capable of concentrating at lipid rafts to activate signal transduction pathways (Head et al., 2014; Simons and Toomre, 2000). Localization of proteins within lipid rafts is dependent on lipid modifications. For example, attachment of a GPI-anchor promotes protein affinity for lipid rafts (Lingwood and Simons, 2010; Varma and Mayor, 1998). In addition, palmitoylation increases the hydrophobicity of proteins, allowing for localization within lipid rafts (Smotrys and Linder, 2004).

While lipid rafts are mainly found in the plasma membrane of the cell, synthesis of lipid rafts is completed within the endoplasmic reticulum or Golgi apparatus of the cell (Prinz, 2002; van Meer, 1989). This suggests that regulatory mechanisms are needed to traffic lipid rafts from the compartment in which it is synthesized towards the plasma membrane. Thus far, Arf6 and Myo1c have both been implicated in delivery of lipid rafts to the plasma membrane (Balasubramanian et al., 2007; Brandstaetter et al., 2012).

1.17.1 Lipid Rafts and bacterial pathogens

Lipid rafts contain a high concentration of signalling molecules, potentially making them appealing sites for bacterial invasion. *Shigella flexneri* (*S. flexneri*) infection results in bacillary dysentery, a disease in which epithelial cells in the lower intestine are damaged, leading to stool containing blood and mucus (Zaidi and Estrada-Garcia, 2014). Similar to *S. Typhimurium*, *S. flexneri* also induces formation of an invasion ruffle to gain entry into the host cell, and previous studies alluded to a role for lipid rafts in promoting *S. flexneri* invasion (Lee et al., 2014; Ohya et al., 2005). Cholesterol and the lipid raft marker, GFP-GPI, were previously demonstrated to be enriched at the *S. flexneri* invasion site (Hayward et al., 2005; Lafont et al., 2002). Consistent
with this observation, *S. flexneri* binding and invasion is significantly decreased upon depletion of cholesterol with methyl-β-cyclodextrin treatment (MβCD) (Lafont et al., 2002). This defect was recapitulated upon infection of *S. flexneri* into Chinese hamster ovary (CHO) cells unable to produce sphingolipids, and thus also incapable of forming lipid rafts (Lafont et al., 2002).

Similarly, *S. Typhimurium* induces localization of cholesterol at the invasion ruffle and this is retained on the SCV for up to 6 h p.i. (Brumell et al., 2001b; Garner et al., 2002). *In vitro* experiments allude to a role for the T3SS effector and translocon component, SipB, in mediating cholesterol binding (Hayward et al., 2005). Cholesterol depletion resulted in significant decrease in *S. Typhimurium* invasion and also prevented translocation of bacterial effectors, demonstrating an importance of cholesterol towards *S. Typhimurium* pathogenesis (Garner et al., 2002; Hayward et al., 2005).

Enrichment of cholesterol at the *S. Typhimurium* invasion site may be indicative of localization of lipid rafts, since recent studies demonstrated localization of GFP-GPI to the invasion ruffle (Brandstaetter et al., 2012). However, the mechanism underlying *S. Typhimurium* mediated delivery of GFP-GPI or cholesterol to the invasion ruffle is still not completely understood. It was recently suggested that Myo1c may contribute to lipid raft delivery, since targeted knockdown of Myo1c significantly prevented localization of GFP-GPI to the plasma membrane, and also inhibited *S. Typhimurium* invasion (Brandstaetter et al., 2012). Collectively, this suggests that delivery of lipid rafts may contribute to *S. Typhimurium* invasion.

While previous studies identified enrichment of cholesterol and lipid rafts to the *S. Typhimurium* invasion site, it is still unclear how bacterial pathogens utilize lipid rafts for pathogenesis.
1.18 THESIS SUMMARY

To promote their pathogenesis, bacteria such as S. Typhimurium utilize multiple mechanisms to hijack host-signalling pathways. While prior studies have focused on this area, we still do not have a complete understanding of how bacteria manipulate host proteins during pathogenesis. The purpose of my thesis was to identify new host factors that are manipulated during the early stages of S. Typhimurium invasion.

Chapter Two characterized a novel pathway involved in S. Typhimurium invasion. I examined the role for the formin, FHOD1 in inducing invasion ruffle formation. Interestingly, FHOD1 is recruited to the invasion ruffle prior to Arp2/3, and this prompted the examination of the contribution of each actin nucleator to invasion ruffle formation. I demonstrate that S. Typhimurium invasion indirectly induces FHOD1 phosphorylation via secretion of its SPI-1 T3SS effectors, SopE/E2 and SopB. Furthermore, while phosphorylation of FHOD1 is not necessary for its localization to the invasion ruffle, FHOD1 phosphorylation is required for S. Typhimurium invasion.

In Chapter Three, I further characterize the role of host Rho GTPases during S. Typhimurium invasion. Although Rac1, Cdc42 and RhoG contribute to invasion, the remaining members of the Rho GTPase family have not been extensively studied. I examined the potential involvement of other Rho GTPases in S. Typhimurium invasion and their manipulation by bacterial effectors. In this thesis, four novel Rho GTPases were identified to localize to the invasion ruffle in a SopB-dependent manner. Further characterization of these Rho GTPases reveals new players in regulating SopB-dependent functions. RhoJ contributes to S. Typhimurium invasion, and RhoB and RhoH contribute to SopB-mediated Akt activation. Furthermore, the lipid raft marker, GFP-GPI localized to the invasion ruffle in a SopB-dependent manner, suggesting that SopB could be manipulating lipid rafts to induce localization of Rho GTPases.
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In Chapter Two, cloning of FHOD1-SR-HA and FHOD1-3A-SR-HA was completed in collaboration with Leo C.K. Wan. Images in Figure 2.3B were acquired with the help of Dr. Mikhail Bashkurov. Experimental design, imaging and analysis of Figure 2.2A-B was completed by Dr. John Copeland.

In Chapter Three, data from Figure 3.1A-C was acquired by Dr. Danielle Brabant and Veronica Canadien. Dr. Danielle Brabant also contributed to Figure 3.4A, Figure 3.5A and Figure 3.5C.


2 Chapter Two: Formin-mediated actin polymerization promotes *Salmonella* invasion

The work presented in this chapter is published in *Cellular Microbiology* (December 2013, 15: 2051-2063). I am extremely grateful to Leo C.K. Wan who helped with generation of the FHOD1-SR-HA and FHOD1-3A-SR-HA constructs. I also wish to acknowledge Dr. Mikhail Bashkurov for help with acquiring the structure illumination microscopy images. Experimental design, imaging and analysis of Figure 2.2A-B was completed by Dr. John Copeland. Figure legends also specify collaborations and data generated by individuals other than myself.

2.1 SUMMARY

*Salmonella* invade host cells using Type 3 secreted effectors, which modulate host cellular targets to promote actin rearrangements at the cell surface that drive bacterial uptake. The Arp2/3 complex contributes to *Salmonella* invasion but is not essential, indicating other actin regulatory factors are involved. Here, I show a novel role for FHOD1, a formin family member, in *Salmonella* invasion. FHOD1 and Arp2/3 occupy distinct microdomains at the invasion site and control distinct aspects of membrane protrusion formation. FHOD1 is phosphorylated during infection and this modification is required for promoting bacterial uptake by host cells. ROCK II, but not ROCK I, is recruited to the invasion site and is required for FHOD1 phosphorylation and for *Salmonella* invasion. Together, my studies reveal an important phospho-dependent FHOD1 actin polymerization pathway in *Salmonella* invasion.
2.2 RESULTS

2.2.1 FHOD1 is required for S. Typhimurium invasion

Recent data has alluded to Arp2/3-independent pathways of S. Typhimurium invasion (Hanisch et al., 2010). Formins also promote actin polymerization within eukaryotic cells, and thus, I sought to analyze their role in S. Typhimurium invasion. I utilized the F1F2Δ1-myc construct, an FH1-FH2 containing derivative of mDia1 in which codons 750-770 are replaced with three alanine codons, as a pan-formin dominant negative construct (Copeland and Treisman, 2002). Cells transfected with the indicated construct were infected with S. Typhimurium for 30 min and immunostained for bacteria. Immunostaining before permeabilization was used to differentiate between intracellular and extracellular bacteria, as previously described (Smith et al., 2007). Bacterial internalization was analyzed in 200 transfected cells in at least three independent experiments. Expression of F1F2Δ1-myc significantly decreased S. Typhimurium invasion relative to eGFP control (Figure 2.1A). Furthermore, expression of F1F2Δ1 inhibited S. Typhimurium invasion to a similar extent as expression of a dominant negative construct of Rac1, Rac1 T17N-CFP, a known inhibitor of S. Typhimurium invasion (Patel and Galan, 2006) (Figure 2.1A). These results implicate formins in S. Typhimurium invasion.

The translocation of SopE/E2 and SopB into the host cytosol results in the activation of Rac1. Of note, Rac1 has been implicated in inducing actin rearrangements at the plasma membrane to create an invasion ruffle (Haraga et al., 2008). Previous studies demonstrated that active Rac1 is sufficient to induce relocalization of FHOD1 from the cytosol to the plasma membrane (Schulte et al., 2008). Thus, I tested the role of FHOD1 in S. Typhimurium invasion. HeLa cells were infected with S. Typhimurium and fixed 10 min p.i.. Using antibodies to FHOD1, I identified strong recruitment of endogenous FHOD1 to actin-rich invasion sites (Figure 2.1B). The ability of F1F2Δ1 to inhibit the function of FHOD1 was also validated.
Co-expression of F1F2Δ1 and an active variant of FHOD1 (FHOD1∆DAD) resulted in decreased formation of actin stress fibres (Figure 2.2A-B).

**Figure 2.1 FHOD1 is required for S. Typhimurium invasion.** Bacterial invasion was assessed in HeLa cells transfected with F1F2Δ1-myc, dominant negative Rac1 T17N-CFP, or eGFP as a control. 200 cells were analyzed for internalized bacteria. Data is normalized to cells transfected with eGFP. * denotes p-value < 0.05, ** denotes p-value < 0.01. (B) HeLa cells were infected with S. Typhimurium and fixed 10 min p.i.. Cells were immunostained for phalloidin and endogenous FHOD1. Scale bar, 5 μm. (C) HeLa cells were transfected with the indicated siRNA, and infected with S. Typhimurium for 30 min. Differential antibody staining was used to identify intracellular and extracellular bacteria. 200 cells were analyzed for internalized bacteria. Data is normalized to cells treated with control siRNA. * denotes p-value < 0.05 and ** denotes p-value < 0.01.
To test the role of FHOD1 in S. Typhimurium invasion, cells were transfected with siRNA targeting FHOD1, Arp3 or both proteins concomitantly. Knockdown was confirmed by immunofluorescence and Western blotting (Figure 2.2C-E). Knockdown of FHOD1 significantly decreased efficiency of S. Typhimurium invasion (Figure 2.1C). Knockdown of Arp3 also significantly decreased S. Typhimurium invasion (Figure 2.1C), in agreement with past findings (Criss and Casanova, 2003; Hanisch et al., 2010).
**Figure 2.2 Dominant negative Dia1 expression inhibits FHOD1-induced stress fibre formation.** (A) Expression of a constitutively active derivative of FHOD1 (FHOD1ΔDAD, green) induces actin stress fibre (red) formation in HeLa cells (upper panels). Co-expression of a dominant negative derivative of Dia1 (F1F2Δ1, white) inhibits FHOD1 induced stress fibre formation (bottom panels). (B) The percent of transfected cells with increased stress fibre formation was determined by immunofluorescence. N=3, error bars represent SEM. (C) HeLa cells were transfected with control or FHOD1 siRNA. 24 h post siRNA transfection, cells were transfected with FHOD1-GFP, where indicated. Lysates were probed with anti-FHOD1 antibody to detect the presence of FHOD1-GFP and endogenous FHOD1. (D) Cells were transfected with either control or FHOD1 siRNA. 24 h post transfection, cells were infected with *S.* Typhimurium for 10 min. Cells were then fixed and immunostained for endogenous FHOD1, *S.* Typhimurium and phalloidin. The same exposure settings were used when imaging endogenous FHOD1 in control and FHOD1 siRNA treated cells. (E) Western blot analysis was used to confirm knockdown of FHOD1 or Arp3. Cells were transfected with indicated siRNA 48h prior to lysis. Cell lysates were prepared as described in Experimental Procedures, and separated on an 8% SDS-PAGE gel. Lysates were probed with antibody recognizing endogenous FHOD1 or Arp3. Antibody against GAPDH was used to confirm equal loading. Figures 2.2A-B were completed by Dr. John Copeland.
2.2.2 FHOD1 and Arp2/3 occupy distinct microdomains at S. Typhimurium invasion site

I examined localization of FHOD1 and Arp3 to the bacterial invasion site with live cell imaging (Figure 2.3A). Cells were co-transfected with FHOD1-GFP and Arp3-mCherry and subsequently infected with NHS-647 labelled S. Typhimurium. Invasion was monitored in real time with spinning-disk confocal microscopy. FHOD1-GFP and Arp3-mCherry both localized to the same invasion ruffle, indicating that both actin nucleators contribute to this form of bacterial uptake.

Using antibodies against endogenous FHOD1 and Arp3, I analyzed the spatial localization of these two actin polymerization factors at the bacterial invasion site. Cells were infected with S. Typhimurium and the invasion site was visualized with LifeAct-GFP (Figure 2.3B). Closer analysis of the invasion site using structured illumination microscopy (SIM) revealed spatial differences between FHOD1 and Arp3, since they localized to different areas. FHOD1 was often observed in puncta associated with the invasion site, and in filamentous structures that extended from the cell surface. In contrast, Arp2/3 was localized to distinct ‘patches’ associated with invasion sites. I conclude that FHOD1 and Arp2/3 occupy distinct microdomains at invasion sites, consistent with previous studies indicating they polymerize actin via distinct mechanisms.
Figure 2.3 FHOD1 and Arp2/3 control distinct aspects of membrane protrusion formation during *S. Typhimurium* invasion. (A) Cells were transfected with FHOD1-GFP and Arp3-mCherry, then subsequently infected with NHS-647 labelled *S. Typhimurium*, 24 hr post-transfection. Invasion was monitored in live cells using confocal spinning disk microscopy. Images are enlarged insets of boxed region in left-hand panels. Times shown (in minutes) are relative to initiation of invasion process. Scale bar, 5 µm. (B) HeLa cells were transfected with LifeAct-GFP and fixed with methanol 10 min p.i.. Cells were immunostained with antibody against endogenous FHOD1 or Arp3. Images were taken with Delta Vision microscope. Scale bar, 5 µm. Images in Figure 2.3B were acquired with the help of Dr. Mikhail Bashkurov.
2.2.3 FHOD1 and Arp2/3 control distinct aspects of membrane protrusion formation

Given that FHOD1 and Arp2/3 occupy distinct microdomains at S. Typhimurium invasion sites, I determined whether this impacted their contributions to invasion ruffle morphology. Using scanning electron microscopy, I examined the morphology of S. Typhimurium invasion sites. Control siRNA treated cells displayed large membrane protrusions at invasion sites, often called “ruffles” or “splashes” (Hanisch et al., 2010) (Figure 2.4). Knockdown of FHOD1 resulted in invasion sites that were smaller in size, while knockdown of Arp3 produced invasion sites with many filopodia-like structures (Figure 2.4).

Next, I examined invasion site morphology using live cell imaging. Cells were transfected with siRNA targeting FHOD1 or Arp3, and LifeAct-GFP was used to visualize F-actin at invasion sites. Upon Arp3 knockdown, S. Typhimurium induced filamentous, filopodia-like protrusions at the host plasma membrane. Of note, the filopodia-like structures visualized by live cell imaging are consistent with results seen with scanning electron microscopy (Figure 2.5A). These protrusions did not coalesce into typical membrane protrusions as seen in control knockdown cells (Figure 2.5A), but rather migrated outwards from the bacterial contact site. Also consistent with the scanning electron microscopy data, I observed formation of smaller invasion ruffles with FHOD1 knockdown cells (Figure 2.5A). Together, these findings demonstrate that FHOD1 and Arp2/3 control distinct aspects of membrane protrusion formation during S. Typhimurium invasion.
Figure 2.4 FHOD1 and Arp3 knockdown results in distinct ruffle morphology phenotypes. Cells were transfected with the indicated siRNA and subsequently infected with S. Typhimurium for 10 min. Scanning electron microscopy images were taken at 24,000x. Scale bar, 2 µm.
Figure 2.5 FHOD1 and Arp3 control distinct aspects of the *S. Typhimurium* invasion ruffle. (A) Cells were transfected with the indicated siRNA and infected with RFP-expressing *S. Typhimurium*. Invasion was monitored in live cells using confocal spinning disk microscopy. Arrows indicate site of invasion. Images are enlarged insets of boxed region in left-hand panels. Times shown (in minutes) are relative to initiation of invasion process. Scale bar, 5µm. (B) Representative image of an invasion ruffle from Control (upper panel) or FHOD1 (bottom panel) siRNA treated cells. Cells were transfected with the indicated siRNA and infected with *S. Typhimurium* for 10 min. Phalloidin was utilized to visualize actin-rich invasion ruffles. Image of the XZ plane was taken at the dashed line on left panel. Arrow indicates invasion ruffle. (C) Volumes of 50 invasion ruffles in three independent experiments were measured using Volocity software and graphed.
2.2.4 FHOD1 promotes actin polymerization at S. Typhimurium invasion ruffles

Using confocal microscopy, I quantified the impact of FHOD1 knockdown on actin polymerization during S. Typhimurium invasion. Cells were transfected with FHOD1 or control siRNA, then infected with bacteria for 10 min. Immunostaining of fixed cells was performed with phalloidin to visualize the F-actin-rich invasion ruffles. Using Volocity software, the volume of 50 invasion sites was analyzed in three independent experiments. FHOD1 knockdown decreased the volume of the invasion ruffle compared to control siRNA treated cells (Figure 2.5B-C). Therefore, FHOD1 promotes actin polymerization at invasion ruffles.

2.2.5 S. Typhimurium invasion induces FHOD1 phosphorylation

Under basal conditions, FHOD1 is maintained in an inactive state via an intramolecular interaction between its N and C terminus. Phosphorylation of S1131, S1137 and Thr1141 by Rho kinase (ROCK) mitigates the intramolecular interaction and leads to FHOD1 activation (Hannemann et al., 2008; Takeya et al., 2008). I first determined whether FHOD1 phosphorylation contributes to S. Typhimurium invasion. Using an antibody against phosphorylated Thr1141 on FHOD1, I examined localization of phosphorylated FHOD1 during S. Typhimurium invasion. Cells were transiently transfected with LifeAct-GFP and subsequently infected with S. Typhimurium for 10 mins. Analysis of bacterial invasion sites suggested that FHOD1 is active at the S. Typhimurium invasion site (Figure 2.6A). A time-course experiment revealed that FHOD1 phosphorylation was maximal at 20 min p.i. and decreased thereafter (Figure 2.6B-C). Calyculin A, a protein phosphatase inhibitor, was used as a positive control. Thus, S. Typhimurium infection is sufficient to induce FHOD1 phosphorylation.
Figure 2.6 FHOD1 is phosphorylated during *S. Typhimurium* invasion. (A) HeLa cells were transiently co-transfected with FHOD1-HA and Life Act-GFP, to visualize actin-rich invasion ruffles. Cells infected with *S. Typhimurium* for 10 min were subsequently immunostained with phosphoFHOD1 (pThr1141) antibody. Scale bar, 5µm. (B) Cells transfected with FHOD1-HA, were infected with *S. Typhimurium* and lysed at indicated time points. Whole cell lysates were probed with a phosphoFHOD1 (pThr1141) antibody. Treatment of cells with 50 nM Calyculin A (Serine/Threonine phosphatase inhibitor) was used as a positive control. HA blotting validated equal loading. (C) Densitometry was performed for three independent experiments. Levels of FHOD1 phosphorylation was normalized to total FHOD1. Statistical analysis was performed with one-way ANOVA and post-hoc Bonferroni’s test. * denotes p < 0.05 and ** denotes p < 0.01.
2.2.6 FHOD1 phosphorylation is required for S. Typhimurium invasion

We generated an siRNA-resistant plasmid (FHOD1-SR-HA) encoding wild type FHOD1-HA carrying silent mutations to eliminate the siRNA target sequence. Western blot analysis confirmed that expression levels of FHOD1-SR-HA were unaltered upon siRNA treatment (Figure 2.7A). Next, we modified FHOD1-SR-HA using site-directed mutagenesis to replace the serine/threonine residues (S1131, S1137 and Thr1141) in FHOD1 with alanine residues, thereby generating a kinase-insensitive FHOD1 that is also siRNA-resistant (FHOD1-3A-SR-HA). Western blot analysis confirmed that FHOD1-3A-SR-HA could no longer be phosphorylated (Figure 2.7B).

To elucidate a functional role for FHOD1 phosphorylation during S. Typhimurium invasion, I quantified localization of FHOD1-SR-HA and FHOD1-3A-SR-HA to invasion ruffles. Cells were transfected with FHOD1-SR-HA or FHOD1-3A-SR-HA and subsequently infected with S. Typhimurium for 10 mins. 100 invasion ruffles in three independent experiments were scored for the presence or absence of either FHOD1-SR-HA or FHOD1-3A-SR-HA. There was no difference in recruitment of either FHOD1-SR-HA or FHOD1-3A-SR-HA to the invasion ruffles (Figure 2.8A-B). This suggests that FHOD1 phosphorylation is not essential for localization to invasion ruffles during S. Typhimurium invasion.

I next analyzed the role of FHOD1 phosphorylation in invasion of S. Typhimurium. Cells transfected with FHOD1-3A-SR-HA were infected with S. Typhimurium and bacterial internalization was analyzed in 200 cells in three independent experiments. Expression of FHOD1-3A-SR-HA was sufficient to inhibit bacterial internalization to levels comparable to dominant negative Rac1, Rac1 T17N-CFP (Figure 2.9A). There was no difference in bacterial invasion between FHOD1-HA and FHOD1-SR-HA. Thus, it appears that FHOD1-3A-SR-HA can act as a dominant negative towards S. Typhimurium invasion.
Figure 2.7 FHOD1-3A-SR-HA is not phosphorylated during S. Typhimurium invasion. (A) Cells were transfected with control or FHOD1 siRNA. 24 h post siRNA transfection, cells were transfected with the indicated expression plasmid (FHOD1-HA or FHOD1-SR-HA). Lysates were probed with anti-HA and equal loading was validated with antibody against β-tubulin. (B) Cells were first transfected with the indicated expression plasmid and subsequently infected with S. Typhimurium for 20 min. Uninfected cells were used as a control. Lysates were probed with phosphoFHOD1 (Thr1141) antibody and equal loading was validated with antibody against HA. FHOD1-3A-SR-HA and FHOD1-SR-HA were generated in collaboration with Leo C.K. Wan.
Figure 2.8 FHOD1 phosphorylation is not required for localization to S. Typhimurium invasion ruffle. (A) HeLa cells transfected with FHOD1-SR-HA or FHOD1-3A-SR-HA were infected with S. Typhimurium and fixed 10 min p.i. Cells were immunostained with antibody against S. Typhimurium, phalloidin, and anti-HA antibody was used to visualize expression of the construct. (B) The number of invasion ruffles with recruitment of FHOD1-SR-HA or FHOD1-3A-SR-HA was determined in three independent experiments.
Figure 2.9 FHOD1 phosphorylation is necessary for S. Typhimurium invasion. (A) Cells were transiently transfected with indicated variants of FHOD1-HA constructs and infected with S. Typhimurium for 30 min. Three independent experiments were conducted with 200 cells analyzed per experiment. eGFP and Rac1 T17N-CFP were used as controls. Bacterial internalization was normalized against eGFP. ** denotes p < 0.01. (B) Cells were co-transfected with FHOD1 siRNA and FHOD1-SR-HA, FHOD1-3A-SR-HA, or no vector. Control siRNA was used as a control, and bacterial internalization for each condition was normalized against its respective control. ** denotes p < 0.01. FHOD1-SR-HA and FHOD1-3A-SR-HA were generated in collaboration with Leo CK Wan.
I next sought to determine whether FHOD1-3A-SR-HA could rescue the defect in *S. Typhimurium* invasion upon FHOD1 knockdown. Cells were co-transfected with control or FHOD1 siRNA, together with FHOD1-SR-HA or FHOD1-3A-SR-HA. Bacterial internalization was analyzed in 200 cells in three independent experiments. Co-transfection of FHOD1 siRNA and FHOD1-SR-HA rescued *S. Typhimurium* invasion, relative to no vector treated cells (Figure 2.9B). Consistent with our earlier finding that FHOD1-3A-SR-HA acts as a dominant negative allele, co-transfection of FHOD1 siRNA and FHOD1-3A-SR-HA had no significant impact on invasion relative to no vector treated cells. This suggests that FHOD1-3A-SR-HA is incapable of rescuing the impairment of *S. Typhimurium* invasion in FHOD1 knockdown cells. Together, these data indicate that phosphorylation of FHOD1 is necessary for *S. Typhimurium* invasion but not its localization to invasion ruffles.

### 2.2.7 ROCK II mediates FHOD1 phosphorylation during *S. Typhimurium* invasion

There are two human Rho kinase isoforms: ROCK I and ROCK II. Recent studies have suggested isoform-specific functions for Rho kinases (Coleman et al., 2001; Sebbagh et al., 2001; Yoneda et al., 2005). Therefore, I analyzed the localization of both Rho kinases during *S. Typhimurium* invasion using antibodies recognizing endogenous proteins. I observed specific recruitment of ROCK II, but not ROCK I, to the invasion site (Figure 2.10A, upper panel and bottom panel, respectively).

The role of each Rho kinase in FHOD1 regulation was analyzed with siRNA targeted knockdown of ROCK I, ROCK II, or both kinases concomitantly, which was confirmed with Western blot analysis (Figure 2.11). Knockdown of ROCK II significantly decreased FHOD1 phosphorylation during *Salmonella* invasion, whereas knockdown of ROCK I had only a modest effect (Figure 2.10B-C). The Rho kinase inhibitor Y27632, which is not isoform selective, also inhibited FHOD1 phosphorylation, as expected.
Figure 2.10 ROCK II is required for FHOD1 phosphorylation during S. Typhimurium invasion. (A) HeLa cells were infected with S. Typhimurium and immunostained for endogenous ROCK II (upper panels) or ROCK I (bottom panels). Life Act-GFP was transfected to allow for visualization of actin-rich invasion ruffles. Scale bar, 5µm. (B) Cells were treated with the indicated siRNA or 20 µM Y27632 and transfected with FHOD1-HA. Cells were subsequently infected for 20 min with S. Typhimurium and lysates were probed with a phospho-FHOD1 (pThr1141) antibody. For treatment with Y27632, cells were pre-treated with 20 µM Y27632 for 30 min at 37°C, and infected for 20 min with S. Typhimurium in the presence of 20 µM Y27632. Blotting with HA confirmed equal loading. (C) Densitometry from three independent experiments was analyzed. Levels of FHOD1 phosphorylation was normalized to total FHOD1. Statistical analysis was performed with one-way ANOVA and post-hoc Dunnett’s test. * denotes p < 0.05 and ** denotes p < 0.01. (D) Cells were treated with indicated siRNA or 20 µM Y27632 prior to infection with S. Typhimurium. Data is normalized to cells treated with Control siRNA. * denotes p-value < 0.05 and ** denotes p < 0.01.
**Figure 2.11 Knockdown of ROCK isoforms.** Cells were transfected with indicated siRNA 48h prior to lysis and lysates were prepared as described in Experimental Procedures. Lysates were probed with antibody against endogenous ROCK I or ROCK II. Equal loading was confirmed with antibody against β-tubulin.

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I next assessed the role of each Rho kinase in bacterial invasion. Similar to previous findings (Hanisch et al., 2011), Y27632 significantly decreased *Salmonella* invasion (Figure 2.10D). However, specific knockdown of ROCK I had no effect, whereas knockdown of ROCK II significantly decreased invasion (Figure 2.10D). Thus, ROCK II plays a specific role in *Salmonella* invasion and is the main mediator of FHOD1 phosphorylation.

### 2.2.8 The Type 3 secreted bacterial effectors SopB and SopE/E2 mediate FHOD1 phosphorylation

Since RhoA is a known activator of ROCK I and ROCK II, (Vega et al., 2011; Vega and Ridley, 2007) I analyzed the role of RhoA in FHOD1 phosphorylation during *S. Typhimurium* invasion. Cells were transfected with siRNA to target expression of RhoA, then infected with *S. Typhimurium*. Levels of FHOD1 phosphorylation were then analyzed by Western blot (Figure 2.12A, Figure 2.13A). Knockdown of RhoA resulted in significant decrease in FHOD1 phosphorylation during *S. Typhimurium* invasion. Consistent with this observation, overexpression of constitutively active mutant of RhoA was sufficient to induce FHOD1 phosphorylation (Figure 2.13B). These findings indicate that RhoA contributes to FHOD1 phosphorylation during infection.

Bacterial T3SS effectors are known to activate host Rho family GTPases, and thus, I aimed to identify which T3SS effectors induce FHOD1 phosphorylation. Cells were transfected with FHOD1-HA and infected with wild-type *S. Typhimurium* and the indicated mutants. Relative to wild type (WT) bacteria, Δ*sopB* and Δ*sopE/E2* mutants were less efficient in inducing FHOD1 phosphorylation (Figure 2.12B). The MOI of Δ*sopB* and Δ*sopE/E2* mutants were adjusted to obtain invasion efficiencies comparable to WT. I next determined whether SopB and SopE/E2 are required for FHOD1 recruitment to invasion sites. Cells were transfected with FHOD1-GFP and infected for 10 min with the indicated *S. Typhimurium* mutant. Cells were immunostained for FHOD1-GFP, *S. Typhimurium*, and phalloidin was used to visualize...
the F-actin-rich invasion ruffles. Invasion ruffles were scored for the presence or absence of FHOD1-GFP. In comparison to WT S. Typhimurium, both ΔsopB and ΔsopE/E2 were significantly impaired in FHOD1 recruitment to the invasion ruffle (Figure 2.13C).

I also addressed whether FHOD1 contributes to a specific T3SS effector invasion pathway. Cells were transfected with siRNA specific to FHOD1 or control siRNA and subsequently infected with ΔsopB or ΔsopE/E2 mutant bacteria. Knockdown of FHOD1 resulted in decreased S. Typhimurium invasion of both ΔsopB and ΔsopE/E2 mutants (Figure 2.12C). These data are consistent with the demonstrated ability of SopE/E2 and SopB to activate RhoA during infection (Hanisch et al., 2011; Hardt et al., 1998; Patel and Galan, 2006). In summary, these results indicate that the Type 3 secreted effectors SopE/E2 and SopB both contribute to FHOD1 activation at invasion sites through their ability to activate RhoA in the host cell.
Figure 2.12 The Type 3 Secreted bacterial effectors SopB and SopE/E2 mediate FHOD1 phosphorylation. (A) Cells were transfected with FHOD1-HA and the indicated siRNA. 48hr after siRNA transfection, cells were infected with S. Typhimurium and lysates were probed with phosphoFHOD1 (pThr1141) antibody. HA blotting validated equal loading. Three independent experiments were performed and graph of densitometry results is shown. FHOD1 phosphorylation levels were normalized to total FHOD1 and subsequently to Control siRNA group. Statistical analysis was performed with one-way ANOVA and post-hoc Bonferroni test. *** denotes p < 0.001. (B) HeLa cells were transiently transfected with FHOD1-HA and subsequently infected with WT, ΔsopB, or ΔsopE/E2 S. Typhimurium for 20 min. Lysates were probed with a phosphoFHOD1 (pThr1141) antibody. HA blotting validated equal loading. (C) HeLa cells were transfected with Control or FHOD1 siRNA and subsequently infected with the indicated S. Typhimurium mutant for 30 min. Data is normalized to its respective control.
Figure 2.13 RhoA is necessary for FHOD1 phosphorylation during S. Typhimurium invasion. (A) Cells were co-transfected with FHOD1-HA and RhoA siRNA. Invasion with S. Typhimurium was allowed to proceed for 20 min and cell lysates were prepared as described in Experimental procedures. Lysates were probed with anti-phosphoFHOD1 (Thr1141) and HA blotting validated loading. (B) HeLa cells were co-transfected with FHOD1-HA and active RhoA. Cell lysates were prepared as described in Experimental Procedures, and separated on an 8% gel. Lysates were probed with an antibody against phospho-FHOD1. Expression of the CFP-tagged active Rho GTPase was confirmed by probing with an antibody against GFP. Total FHOD1 protein was validated with an antibody against HA. Calyculin A treatment was used as a positive control. Cells were co-transfected with FHOD1-HA and eGFP. Prior to lysis, cells were treated with 50mM of Calyculin A. (C) Cells were transfected with FHOD1-GFP and infected with the indicated S. Typhimurium mutant for 10 min. Phalloidin was used to visualize actin-rich invasion ruffles. Three independent experiments were performed and 100 invasion ruffles were scored for the presence or absence of FHOD1-GFP. Statistical analysis was performed with one-way ANOVA and post-hoc Dunnett’s test. ** denotes p < 0.01 and *** denotes p < 0.001
2.3 DISCUSSION

While the Arp2/3 complex has garnered much attention for its role in mediating cytoskeletal rearrangements during bacterial pathogenesis, Arp2/3-independent mechanisms are beginning to be explored. For example, intercellular spread of *Shigella flexneri* utilizes a mDia1-dependent mechanism of actin polymerization (Heindl et al., 2010). *Rickettsia rickettsii* utilizes a formin mimic, Sca2, to promote intracellular motility and intercellular spreading (Haglund et al., 2010). *Vibrio cholera* utilizes a T3SS effector with formin-like activity, VopF, to promote intestinal colonization (Tam et al., 2007). Here, I demonstrate a role for formins early in the bacterial invasion process. I show that upon infection, *S. Typhimurium* induces FHOD1 localization and activation at actin-rich invasion sites to promote its entry into host cells.

My findings indicate that FHOD1 and Arp2/3 control distinct aspects of invasion. I observed filopodia-like structures in Arp3 knockdown cells, whereas FHOD1 knockdown cells displayed invasion ruffles that were smaller in volume. Recent data has demonstrated that FHOD1 can act simultaneously to cap actin filaments and to bundle F-actin, thereby stabilizing actin filaments (Schonichen et al., 2013). This is consistent with my finding of decreased volume of invasion ruffles in FHOD1 knockdown cells. It is possible that the actin filaments making up a *S. Typhimurium* invasion ruffle are less stable upon knockdown of FHOD1, thus resulting in a decreased volume of the invasion ruffle as well as decreased *S. Typhimurium* invasion. Furthermore, consistent with my findings, ArpC3<sup>−/−</sup> fibroblasts display filopodia structures and are unable to undergo directed movement towards a chemoattractant (Suraneni et al., 2012). In the context of *S. Typhimurium* invasion, Arp2/3 is similarly needed to ensure the invasion ruffle is directed towards and forms around the bacteria. FHOD1 may be driving the formation of filopodia-like structures that I observed in Arp3 knockdown cells. My data provides insight into the previous observation that structures resembling both filopodia and
lamellipodia can be observed during *S. Typhimurium* invasion (Meyerholz and Stabel, 2003). Importantly, both FHOD1 and Arp2/3 are required for optimal invasion of host cells by *S. Typhimurium*. I conclude that the two actin polymerization factors play different, but complementary roles in bacterial invasion.

*S. Typhimurium* induced FHOD1 phosphorylation via a process mainly mediated by ROCK II. Furthermore, my experiments reveal a role for ROCK II, but not ROCK I, in *S. Typhimurium* invasion. This is consistent with previous data demonstrating phosphorylation of ROCK II, but not ROCK I, during *S. Typhimurium* invasion (Rogers et al., 2011). I speculate that the difference in the role of the two Rho kinase isoforms in *S. Typhimurium* invasion is due to the unique ability of ROCK II to bind to phosphatidylinositol 3,4,5 phosphate (PIP3) (Yoneda et al., 2005). During *S. Typhimurium* invasion, SopB triggers production of PIP3 in host cells (Mallo et al., 2008). Thus, it is likely that PIP3 production allows for specific recruitment of ROCK II to actin-rich invasion ruffles, whereby it aids in mediating actin rearrangements to promote *S. Typhimurium* invasion.

*S. Typhimurium* invasion provides an excellent model to study actin dynamics during host-pathogen interactions. I demonstrate that efficient *S. Typhimurium* invasion requires the concerted efforts of FHOD1 and Arp2/3, revealing an intricate interplay between these two actin nucleators. Since FHOD1 is ubiquitously expressed, it may also be required for host cell invasion by other bacterial pathogens.
2.4 EXPERIMENTAL PROCEDURES

2.4.1 Cell Culture

HeLa cells were obtained from ATCC and maintained in growth medium [DMEM (HyClone) supplemented with 10% FBS (Wisent)] at 37°C in 5% CO2. HeLa cells were seeded at 2.5 x 10^4 cells per well in 24-well tissue culture plates containing coverslips or at 7.5 x 10^4 cells per 6 cm dish, 16–24 h before use. Late-log bacterial cultures were used for infecting HeLa cells as outlined previously (Szeto et al., 2009). Briefly, bacteria were pelleted at 10,000x g for 2 min and resuspended in PBS. The inoculum was diluted and added to HeLa cells at 37°C for 10 min. The cells were then washed extensively and fixed as per indicated time points.

For experiments related to quantification of cells with actin stress fibres, HeLa cells were plated at a density of 150,000 cells/well on acid-washed coverslips in 6-well plates. Cells were transfected the next day using PEI as previously described (Vaillant et al., 2008). Briefly, 1.5 µg total plasmid DNA (0.3 µg GFP-FHOD1ΔDAD + 1.2 µg myc-F1F2Δ1) was diluted in 50 µL Optimem, 5 µL of 1 mg/mL PEI was added and the mixture was incubated for 25–30 minutes at room temperature. The DNA/PEI mix was added to cells in 1 mL of Optimem and left for 5 hours under normal culture conditions. At the end of 5 hours the media was replaced with 2 mL of DMEM supplemented with 0.5% FCS. Cells were prepared for immunofluorescence as previously described (Young et al., 2008). Briefly, cells cultured on acid-washed glass coverslips were fixed for 10 minutes directly in 2 mL of 4% para-formaldehyde freshly prepared in 1xPBS. Following fixation, the cells were permeabilized for 20 minutes in 0.3% Triton-X-100, 5% Donor Bovine Serum (DBS) in 1xPBS. The coverslips were washed in 1xPBS and incubated with the appropriate primary antibody in 0.03% Triton-X-100, 5% DBS in 1xPBS for 1 hour at room temperature. The coverslips were washed 3 times in 1xPBS and then incubated with secondary antibody in the same solution for 1 hour at room temperature. After washing in 1xPBS the coverslips were mounted in Vectashield with DAPI and sealed with nail polish.
Primary antibody: mouse anti-myc, 1:500 dilution (Santa Cruz Biotech); secondary antibody: Cy5 Donkey anti-mouse, 1:200 (Jackson Labs). F-actin was detected with TRITC phalloidin, 1:200 (Invitrogen).

2.4.2 Bacterial Strains and Plasmids

Bacteria used in this study are as follows: S. Typhimurium 1344 (Hoiseth and Stocker, 1981), RFP expressing S. Typhimurium 1344 (Birmingham and Brumell, 2006), M202 (ΔsopE ΔsopE2 mutant) (Stender et al., 2000), and ΔsopB (Steele-Mortimer et al., 2000). For invasion experiments, WT bacteria was diluted to 1:100, ΔsopB was diluted to 1:100 and ΔsopE/E2 was diluted to 1:50. For transfection of HeLa cells, Genejuice (Novagen), Xtreme Gene 9 (Roche), or Jet Prime (Polyplus Transfection) transfection reagents were used as per manufacturer’s protocols. Constructs used were FHOD1-HA (Westendorf et al., 1999) (gift from Dr. Jennifer Westendorf, Mayo Clinic, USA) and Arp3-mCherry (gift from Dr. Kenneth Campellone, University of California, USA). Arp3-mCherry was generated by replacing the EGFP in Arp3-GFP (Welch et al., 1997a) with mCherry (Shaner et al., 2004). FHOD1-GFP was cloned into pcDNA-dest53 vector backbone and was a generous gift from Jonathan Lee (University of Ottawa). F1F2Δ1-myc was described previously (Copeland and Treisman, 2002). All Rho GTPase constructs used were previously described (Heo et al., 2006; Heo and Meyer, 2003).

FHOD1-HA was generated using standard recombinant DNA protocols as previously described (Westendorf et al., 1999). Cloning of FHOD1-SR-HA and FHOD1-3A-SR-HA was completed in collaboration with Leo C.K. Wan. The Quikchange II site-directed mutagenesis kit (Agilent) was used to generate siRNA-resistant FHOD1-SR-HA and FHOD1-3A-SR-HA mutant constructs. Primers 5’ CAT GAT GCC CAC GGA AGA GGA AAG ACA AAA AAT CGA AGA GGC TCA GCT GGC CAA C 3’ and 5’ GTT GGC CAG CTG AGC CTC TTC GAT TTT TTG TCT TTC CTC TTC CGT GGG CAT CAT G 3’ were used to generate the
FHOD1-SR-HA clone. Primers 5'CGC AAG CTGCC CGC AAG CC CGC AAG GCT TTG AGA AGG GCG TTG AAG AG3' and 5'CTT CAA CGT CCT TCT CAA AGA CTT GCG GTT GCC GCG GGA ACG CTT GCG TTC3' were used to generate FHOD1-3A-SR-HA clone.

2.4.3 RNA interference
HeLa cells were seeded into 24-well culture plates at $5 \times 10^4$ cells per well and transfected 4 h later using Oligofectamine (Invitrogen). The control siRNA was siCONTROL Non-Targeting siRNA #2 (Dharmacon). FHOD1-directed siRNA (5'-GAA GAG CGG CAG AAG AUU GAG GAA-3) used in this work was obtained from Sigma Aldrich (Takeya et al., 2008). Arp3-directed siRNA was obtained from Applied Biosystems (siRNA ID #130829). ROCK I (5'-GAG GCT CAA GAC ATG CTT A-3') and ROCK II (5'-GGC ATC GCA GAA GGT TTA T-3') siRNA were both obtained from Dharmacon. A concentration of 100 nM of total siRNA was used in each knockdown. Medium was changed 24 h after transfection, and HeLa cells were infected with S. Typhimurium 48 h after transfection.

2.4.4 Immunofluorescence Microscopy and Antibodies
Cells were fixed with 2.5% paraformaldehyde in PBS for 10 min at 37°C or with methanol for 5 min at -20°C, where indicated. Fixed cells were immunostained as previously described (Brumell et al., 2001a). Immunostaining before permeabilization was used to differentiate between intracellular and extracellular bacteria (Smith et al., 2007). Coverslips were mounted onto glass slides using DakoCytomation fluorescent mounting medium and imaged using a Quorum WaveFX-X1 spinning disc confocal system (Quorum Technologies Inc., Guelph, Canada) and Volocity software (Improvision). Images were imported into Adobe Photoshop and assembled in Adobe Illustrator.
For deconvolution microscopy, coverslips were mounted onto glass slides using Prolong Gold mounting medium (Invitrogen). Three-dimensional image data sets were acquired on an imaging system (DeltaVision Elite, Applied Precision) equipped with an IX71 microscope (Olympus), a CCD camera (CoolSNAP 1024x1024; Roper Scientific), and 60x/1.42 NA plan-Apochromat oil immersion objectives (Olympus) using 1x1 binning. Z stacks (0.2 μm apart for each optical section) were collected, computationally deconvolved using the softWoRx software package (v5.0, Applied Precision). Deconvolution microscopy images were acquired with the help of Dr. Mikhail Bashkurov.

Immunofluorescence staining of endogenous FHOD1 was completed with anti-FHOD1 (ECM Biosciences, Cat No. FM3521) and anti-phosphoFHOD1 (pThr1141) (ECM Biosciences, Cat No. FP3481). Salmonella O antisera (BD Difco) was used for immunostaining of S. Typhimurium (Cat No. 225341). Anti-Arp3 was a generous gift from Dr. Kenneth Campellone (University of California, USA). Immunofluorescence staining of Rho kinases was completed with anti-ROCK I (BD Biosciences, Cat No. 611136), and anti-ROCK II (Upstate, 05-841). Anti-HA (Covance, Cat No. MMS-101R), anti-β tubulin (Sigma Cat. No. T4026), and anti-GAPDH (Millipore, Cat No. MAB374) were used to validate loading for Western blot analysis. All fluorescent secondary antibodies were AlexaFluor conjugates from Molecular Probes (Invitrogen). Y27632 (Calbiochem, Cat No. 688000) and Calyculin A (LC Laboratories, Cat No. C-3987) were used to pre-treat cells, where indicated.

2.4.5 Scanning Electron Microscopy

Cells were seeded at a density of 5 x 10^4 and transfected with the indicated siRNA 4 hours later. Invasion was carried out 48 h after transfection. Cells were transfected with S. Typhimurium for 10 minutes. Samples were fixed in 2% glutaraldehyde in cacodylate buffer, rinsed in buffer and dehydrated in a graded ethanol series. The samples were critical point dried
in a Bal-tec CPD030 critical point dryer, mounted on aluminum stubs, gold coated in a Denton Desk II sputter coater and examined in an FEI XL30 SEM.

2.4.6 Western Blots

Cells were lysed in 1% Triton-X 100, 50mM Tris pH 7.4, 150mM NaCl, and 1mM EDTA. Lysis buffer was supplemented with protease inhibitors (10µg/ml aprotinin, 10µg/ml leupeptin, 1µM pepstatin A, 1mM PMSF) and 1mM DTT. Sample buffer (60mM Tris pH6.8, 5% glycerol, 1% SDS, 2% β-mercaptoethanol, 0.02% bromophenol blue) was added to the suspension, and samples boiled for 10 min. Samples were separated on 8% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked in 5% skim milk overnight. Primary antibodies were incubated for 1 h at room temperature. Secondary antibodies used were conjugated to horseradish peroxidase (HRP) and were purchased from Sigma.

2.4.7 Live cell imaging

Cells were grown on 2.5 cm coverslips, co-transfected 12–16 h before invasion with FHOD1-GFP and Arp3-mCherry or LifeAct-GFP constructs and preincubated with RPMI-1640 media (supplemented with L-glutamine, HEPES, no bicarbonate; Wisent) with 10% FBS at 37°C for 20min. Cells were infected with RFP-expressing SL1344 bacteria. In brief, 1mL of late log bacterial suspension was extensively washed with PBS. The entire bacterial suspension was used for infection. Time-lapse confocal z-stacks of the cells were imaged using a Quorum WaveFX-X1 spinning disc confocal system (Quorum Technologies Inc., Guelph, Canada). Images were processed using Volocity software (Improvision).

2.4.8 Statistical Analysis

Statistical analyses were conducted using GraphPad Prism v5.0. The mean ± standard error of the mean (SEM) is shown in figures, and $P$ values were calculated using one sample t-test or one-way ANOVA, where indicated. A $p$-value of less than 0.05 was considered
statistically significant and is denoted by *. p < 0.01 is denoted by ** and p < 0.001 is denoted by ***.
Chapter Three: *Salmonella* exploits host palmitoylation-dependent signalling pathways

The data in this chapter consists of unpublished data. I am extremely grateful to Dr. Danielle Brabant and Veronica Canadien who conducted the initial Rho GTPase screen to generate Figures 3.1A-C. Dr. Danielle Brabant also contributed to Figures 3.4A, 3.5A and 3.5C. Nancy Zhu contributed to Figure 3.9. Figure legends also specifically indicate collaborations and data generated by individuals other than myself.

3.1 SUMMARY

*Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) utilizes type 3 secretion systems (T3SS) to deliver its virulence factors, called effectors, into host cells during infection (Coburn et al., 2007; Haraga et al., 2008). The T3SS effectors promote bacterial invasion into host cells and the generation of a replicative niche. The T3SS effector SopB plays an important role in *Salmonella* pathogenesis through its lipid phosphatase activity (Knodler et al., 2005; Mallo et al., 2008; Steele-Mortimer et al., 2000; Terebiznik et al., 2002; Zhou et al., 2001). SopB has been shown to activate a wide variety of host signalling pathways during infection, including factors that promote bacterial invasion and Akt activation (Cooper et al., 2011; Feng et al., 2001; Patel and Galan, 2006; Roppenser et al., 2013; Steele-Mortimer et al., 2000).

However, the mechanism by which SopB regulates diverse host signalling pathways during infection is unclear. Here, I demonstrate that SopB can exploit host palmitoylation-dependent signalling pathways during infection. RhoJ is required for *S.* Typhimurium invasion, and RhoB and RhoH contribute to Akt activation. Furthermore, I demonstrate that palmitoylation plays an important role in recruitment of Rho GTPases during *S.* Typhimurium invasion. Many palmitoylated proteins are targeted to lipid rafts and these lipid microdomains are required for *S.* Typhimurium invasion (Brandstaetter et al., 2012; Brumell et al., 2001b; Garner et al., 2002;
Hayward et al., 2005; Lingwood and Simons, 2010; Simons and Toomre, 2000). Here, I show that recruitment of lipid rafts to *S. Typhimurium* invasion sites requires SopB, providing a mechanism for delivery of palmitoylated Rho GTPases. These studies identify a novel mechanism by which SopB exploits palmitoylated proteins in lipid rafts to engage in diverse signalling pathways in host cells.
3.2 RESULTS

3.2.1 SopB-dependent recruitment of Rho GTPases to the *Salmonella* invasion site

Of the 20 members of the Rho GTPase family, only Rac1, Cdc42 and RhoG have been extensively studied in the context of *S. Typhimurium* infection (Aiastui et al., 2010; Criss et al., 2001; Patel and Galan, 2006). Thus, a screen was conducted to identify Rho GTPases recruited to the *S. Typhimurium* invasion site. Henle intestinal epithelial cells were transfected with CFP-tagged Rho GTPases and infected with *S. Typhimurium*. Consistent with previous studies demonstrating a role for Rac1 in *S. Typhimurium* invasion, we observed an enrichment of Rac1-CFP to the invasion ruffle (Aiastui et al., 2010; Patel and Galan, 2006). Our screen identified four Rho GTPases that are recruited to the invasion site: RhoB, RhoD, RhoH and RhoJ (Figure 3.1A-B). To verify that recruitment of these Rho GTPases was not an artefact of overexpression, I visualized recruitment of RhoB/D/H/J with antibodies to the endogenous proteins (Figure 3.2). Similar to overexpressed constructs, endogenous RhoB/D/H/J were also recruited to the *S. Typhimurium* invasion site (Figure 3.2).
Figure 3.1 SopB mediates recruitment of RhoB, RhoD, RhoH and RhoJ to invasion sites. (A) Henle cells were transfected with the indicated Rho GTPase construct and infected with WT S. Typhimurium. Cells were immunostained for GFP, Salmonella, and phalloidin was used to visualize actin-rich invasion ruffles. Arrows indicate site of invasion. (B) Quantification of Rho GTPase screen. 100 actin rich invasion ruffles were scored for the presence or absence of the indicated Rho GTPase. Statistical analysis was performed with one-way ANOVA and post-hoc Dunnett’s test. (C) Henle cells were transfected with the indicated Rho GTPase construct and infected with WT, ΔsopB, ΔsopB + pSopB, or ΔsopB + pSopB C462S. The number of invasion ruffles with recruitment of the indicated Rho GTPase was determined in three independent experiments. Statistical analysis was performed with two-way ANOVA. * denotes p< 0.05 and *** denotes p < 0.001. Data presented in Figure 3.1A-C was generated in collaboration with Dr. Danielle Brabant and Veronica Canadien.
Figure 3.2 Localization of endogenous Rho GTPases to S. Typhimurium invasion site.
 Henle cells were infected with S. Typhimurium and fixed with 10% trichloroacetic acid (TCA). Cells were immunostained with antibodies against endogenous Rho GTPase. Wheat germ agglutinin (WGA) was utilized to stain the membrane and visualize S. Typhimurium invasion sites.
The T3SS effector SopB plays a major role in modulating host signal transduction cascades during *S. Typhimurium* infection (Feng et al., 2001; Knodler et al., 2005; Steele-Mortimer et al., 2000). We observed that mutants lacking SopB (ΔsopB, deleted for the *sopB* gene) were significantly impaired in their ability to induce the recruitment of RhoB/D/H/J to invasion sites (Figure 3.1C). In contrast, recruitment of Rac1 was not affected, consistent with prior studies showing that this GTPase is activated by the T3SS effectors SopE and SopE2 (Criss et al., 2001; Patel and Galan, 2006). Complementation of the ΔsopB mutant by expression of the wild type *sopB* gene from a low copy plasmid restored recruitment of RhoB/D/H/J to invasion sites, confirming that SopB is contributes to this phenotype. In contrast, expression of a catalytically inactive mutant of SopB (C462S) lacking phosphatase activity was not sufficient to complement recruitment of RhoB, RhoH and RhoJ to invasion sites during infection by the ΔsopB mutant. Thus, SopB facilitates the recruitment of a subset of Rho family GTPases to invasion sites in a manner that requires its catalytic activity.

### 3.2.2 RhoJ promotes *S. Typhimurium* invasion

Given that RhoB, RhoD, RhoH and RhoJ were recruited to the *S. Typhimurium* invasion ruffle in a SopB-dependent manner, we examined their roles in known SopB-dependent phenotypes. SopB-mediated Rho GTPase activation contributes to formation of the *S. Typhimurium* invasion ruffle, thereby promoting bacterial invasion (Patel and Galan, 2006; Zhou et al., 2001). The role of each Rho GTPase in *S. Typhimurium* invasion was analyzed in Henle cells. Henle cells were transfected with siRNA to target expression of each Rho GTPase, and then infected with WT *S. Typhimurium* (Figure 3.4A). Consistent with prior studies, Rac1 knockdown significantly inhibited bacterial invasion (Criss et al., 2001; Patel and Galan, 2006). Interestingly, there was also a significant decrease in *S. Typhimurium* invasion upon RhoJ knockdown (Figure 3.4A), suggesting a role for RhoJ in promoting *S. Typhimurium* invasion.
Figure 3.3 SiRNA-targeted knockdown of Rho GTPases. (A) Cells were transfected with indicated siRNA 48h prior to lysis and lysates were prepared as described in Experimental Procedures. Lysates were probed with antibody against endogenous Rho GTPases. Equal loading was confirmed with antibody against β-tubulin or GAPDH. (B) Cells were transfected with RhoJ siRNA and RNA was extracted 48h post-transfection. RT-PCR was performed to measure relative gene expression of RhoJ in control or RhoJ siRNA treated cells. (C) Cells were transfected with RhoH siRNA and RNA was extracted 48h post-transfection. RT-PCR was performed to measure relative gene expression of RhoH in control or RhoH siRNA treated cells. Data generated in Figure 3.3A was generated in collaboration with Dr. Danielle Braissant.
**Figure 3.4** *S. Typhimurium invasion induces RhoJ activation.* (A) Henle cells were transfected with siRNA against the indicated Rho GTPase and infected with *S. Typhimurium* for 30 min. Differential antibody staining was utilized to distinguish between intracellular and extracellular bacteria. 200 cells were scored for bacterial internalization. (B) Henle cells were transfected with the indicated RhoJ construct and infected with *S. Typhimurium* for the indicated time points. Binding of activated RhoJ to PAK-PBD-GST beads was determined by immunoblotting. (C) Cells were transfected with RhoJ-CFP or RhoJ CA-CFP and infected with the indicated *S. Typhimurium* strain. Activated RhoJ was determined by binding to PAK-PBD-GST beads. Numbers are densitometry analysis of RhoJ activation relative to no invasion control. (D) Cells were infected with the indicated *S. Typhimurium* mutant and fixed 10 mins p.i. Cells were immunostained for *S. Typhimurium* and with antibody against endogenous Arp3. Phalloidin was used to visualize actin-rich invasion ruffles. 100 invasion ruffles were analyzed for Arp3 localization. * denotes p < 0.05. Data generated in Figure 3.4A, B and D was completed in collaboration with Dr. Danielle Brabant.
I next asked the question whether *S. Typhimurium* invasion induces RhoJ activation. Activated RhoJ binds to p21-binding domain (PBD) of p21-activated kinase (PAK). RhoJ activation was therefore assessed via binding to PAK-PBD-GST bound beads. Cells were transfected with CFP-RhoJ, the active variant of RhoJ (CFP-RhoJ CA) or the dominant negative variant of RhoJ (CFP-RhoJ DN), and subsequently infected with *S. Typhimurium* for the indicated time points. Cell lysates were incubated with PAK-PBD-GST, and probed for CFP to analyze RhoJ activation. As expected, CFP-RhoJ CA bound to PAK-PBD-GST, whereas CFP-RhoJ DN did not. In the context of invasion, RhoJ activation was maximal at 15 min p.i. and decreased thereafter, suggesting that *S. Typhimurium* induces RhoJ activation early in the invasion process (Figure 3.4B). RhoJ was found to be activated in response to *S. Typhimurium* infection in a manner that required the T3SS effectors SopB and SopE/E2 (Figure 3.4C). Activated RhoJ is known to bind N-WASP, which activates the Arp2/3 complex (Vignal et al., 2000). Consistent with this model, it was observed that SopB is required for recruitment of the Arp2/3 complex to invasion sites (Figure 3.4D). Thus, this data suggests that RhoJ plays an important role in promoting *S. Typhimurium* invasion.

### 3.2.3 RhoB and RhoH contribute to Akt activation during *S. Typhimurium* invasion

Akt is a central regulator of cell proliferation and differentiation (Follo et al., 2015). Growth factor-induced activation of Akt involves its recruitment to the plasma membrane by binding of its pleckstrin homology (PH) domain to the products of PI3-kinases, including phosphatidylinositol 3,4-bisphosphate (PI(3,4)P$_2$) and PI(3,4,5)P$_3$ (Alessi and Cohen, 1998; Bellacosa et al., 1998; Follo et al., 2015). Akt activation also requires its phosphorylation by PDK1 (threonine 308) and mTORC2 (serine 473) (Alessi and Cohen, 1998; Bellacosa et al., 1998; Sarbassov et al., 2005). *S. Typhimurium* induces rapid activation of Akt during infection,
to promote epithelial cell survival, allowing bacteria to establish an intracellular replicative niche within the host cell (Knodler et al., 2005; Kuijl et al., 2007).

Infection with ΔsopB does not induce Akt activation, suggesting an essential role for SopB during S. Typhimurium invasion (Cooper et al., 2011; Knodler et al., 2005; Roppenser et al., 2013). However, the mechanism by which SopB mediates Akt activation is poorly understood. SiRNA targeted expression of RhoD in fibroblasts, decreases PDGF-induced Akt activation, and inhibition of RhoB results in a significant decrease of Akt activation (Adini et al., 2003; Nehru et al., 2013). This provides precedence for a role of Rho GTPases in Akt activation. However, the contribution of Rho GTPases to Akt activation during S. Typhimurium invasion has never been assessed.

To determine whether Rho GTPases contribute to Akt activation, I analyzed the role of Rho GTPases in SopB-mediated Akt activation by targeting their expression with siRNA prior to infection. Cell lysates were probed with phospho-specific antibodies that recognize the phosphorylated S473 residue of Akt as an indicator of Akt activation. Knockdown of RhoB and RhoH resulted in a significant decrease in Akt activation (Figure 3.5A-B). Together, these findings indicate that SopB exploits host Rho GTPases to promote Akt activation.
Figure 3.5 Rho GTPases contribute to SopB-dependent phenotypes during infection. (A) Cells were transfected with the indicated siRNA and infected with WT or ΔsopB S. Typhimurium and lysed at 30 mins. Cell lysates were probed with a phospho-Akt (S473) antibody. Blotting with pan-Akt antibody validated equal loading. (B) Densitometry of three independent experiments was performed. Data is normalized to Control siRNA treated cells. * denotes p < 0.05, ** denotes p < 0.01, and *** denotes p < 0.001.
3.2.4 Palmitoylated proteins play a role in SopB-dependent phenotypes

Palmitoylation is a post-translational modification in which palmitic acid is added to a cysteine residue of a protein (Charollais and Van Der Goot, 2009; Smotrys and Linder, 2004). The reversible nature of palmitoylation allows it to have important contributions towards protein trafficking and localization. Previous studies have demonstrated that RhoB and RhoJ are both amenable to palmitoylation (Heo et al., 2006; Perez-Sala et al., 2009). Furthermore, the use of a palmitoylation prediction algorithm, CSS-Palm (http://csspalm.biocuckoo.org/) (Ren et al., 2008), reveals that RhoH contains a predicted palmitoylation site in its C-terminal domain.

Since these Rho GTPases play an important role in SopB-mediated phenotypes, I hypothesized that SopB might take advantage of the post-translational modification that they share in common.

To test this hypothesis, I first asked whether palmitoylated proteins play a role in S. Typhimurium invasion. 2-bromopalmitate has been extensively used to study the consequences of inhibiting palmitoylation of proteins such as Ras GTPase and Rho GTPases (Chenette et al., 2005; Jennings et al., 2009; Trenchi et al., 2009; Webb et al., 2000). Henle cells were pretreated with 2-bromopalmitate and subsequently infected with S. Typhimurium. Immunofluorescence staining prior to permeabilization was utilized to differentiate between intracellular and extracellular bacteria. Treatment with 2-bromopalmitate significantly inhibited S. Typhimurium invasion relative to untreated control cells, suggesting the involvement of palmitoylated proteins in bacterial uptake by host cells (Figure 3.6A).

SopB contains a phosphatase domain that can efficiently dephosphorylate phosphoinositides and inositol phosphates in vitro (Mallo et al., 2008; Mason et al., 2007; Norris et al., 1998). During infection of host cells, SopB mediates dephosphorylation of PI(4,5)P$_2$, its main substrate (Mallo et al., 2008; Mason et al., 2007).
Figure 3.6 Palmitoylated proteins play a role in SopB-dependent phenotypes. (A) Henle cells were infected with S. Typhimurium in the presence or absence of 2-bromopalmitate. 200 cells were scored for bacterial internalization. Averages ± SEM are shown. * denotes p < 0.05. (B) Cells were transfected with PH-Akt-GFP, treated with 2-bromopalmitate and infected with S. Typhimurium for 10 mins. Cells were immunostained for GFP, Salmonella, and phalloidin was used to visualize actin-rich invasion ruffles (arrows). Untreated cells served as controls. Scale bar, 11µm. (C) At least 50 invasion ruffles were scored for the presence or absence of PH-Akt-GFP. Arrows indicate site of invasion. ** denotes p < 0.01. (D) Intensity of PH-Akt-GFP at the invasion ruffle was analyzed using Volocity Software. PH-Akt-GFP intensity was normalized to the volume of the invasion ruffle, and to background intensity of the cell. *** denotes p < 0.001.
Somewhat paradoxically, SopB also induces the formation of PI(3,4,5)P$_3$ and PI(3,4)P$_2$ at the invasion site by a mechanism that is not understood (Mallo et al., 2008). SopB-mediated production of PI(3,4,5)P$_3$ and PI(3,4)P$_2$ is required for Akt activation during *S.* Typhimurium invasion, since depletion of these phosphoinositides with the PTEN-A4 construct inhibits SopB-mediated Akt activation (Roppenser et al., 2013).

I examined the role of protein palmitoylation in SopB-mediated PI(3,4,5)P$_3$/PI(3,4)P$_2$ production during *S.* Typhimurium invasion. The production of PI(3,4,5)P$_3$/PI(3,4)P$_2$ was monitored by expression of a GFP fusion to the PH domain of Akt (PH-Akt-GFP) (Weernink et al., 2000). I observed that 2-bromopalmitate significantly reduced the number of invasion ruffles with PH-Akt-GFP localization, suggesting that protein palmitoylation is required for PI(3,4,5)P$_3$/PI(3,4)P$_2$ production at the *S.* Typhimurium invasion site (Figure 3.6B-C). To control for possible volume changes in the *S.* Typhimurium invasion ruffle upon 2-bromopalmitate treatment, the GFP intensity of PH-Akt-GFP at the invasion ruffle was analyzed and normalized to the volume of the invasion ruffle (Figure 3.6D). After normalizing PH-Akt-GFP against the volume of the invasion ruffle, a significant decrease in PH-Akt-GFP intensity at the invasion site was observed. Thus, 2-bromopalmitate inhibits PI(3,4,5)P$_3$ production at the invasion site and palmitoylated proteins contribute to PI(3,4,5)P$_3$ production during *S.* Typhimurium invasion.

### 3.2.5 Palmitoylation of RhoB is required for localization to the invasion ruffle

The mechanism underlying 2-bromopalmitate inhibition of palmitoylation remains elusive. Thus, to control for possible off-target or non-specific effects of 2-bromopalmitate, I analyzed recruitment of a palmitoylation-defective RhoB. RhoB is dually palmitoylated at C189 and C192 (Perez-Sala et al., 2009). RhoB also undergoes isoprenylation on C193, which is a
prerequisite for palmitoylation (Aicart-Ramos et al., 2011; Perez-Sala et al., 2009). I analyzed localization of GFP-tagged RhoB mutants in which these cysteine residues were mutated to serine residues, thereby preventing lipid modification. I observed significantly less recruitment of the isoprenyl-deficient RhoB mutant (RhoB C193S) to S. Typhimurium invasion sites. The palmitoyl-deficient RhoB mutant (RhoB C189S C192S) contains an isoprenyl motif, allowing it to bind to membranes, but it was recruited significantly less to S. Typhimurium invasion sites (Figure 3.7A-B). This suggests that isoprenylation of RhoB is not sufficient for localization to the invasion ruffle, and that palmitoylation of RhoB is the critical post-translational modification required for its localization to the invasion site.

3.2.6 Lipid rafts are recruited to the S. Typhimurium invasion ruffle in a SopB-dependent manner

Palmitoylation of proteins increases their hydrophobicity, allowing them to localize to lipid rafts (Blaskovic et al., 2014; Smotrys and Linder, 2004). Previous studies demonstrated localization of cholesterol and the lipid raft marker, GFP-GPI to the S. Typhimurium invasion site, and lipid rafts are required for efficient bacterial uptake (Brandstaetter et al., 2012; Brumell et al., 2001b; Hayward et al., 2005). Given that RhoB is palmitoylated and localizes to the S. Typhimurium invasion site in a SopB-dependent manner, I sought to determine whether lipid raft recruitment to the invasion sites is dependent on SopB.

I observed recruitment of the lipid raft marker GFP-GPI to invasion sites following infection with WT bacteria (Figure 3.8A-B; Figure 3.9), consistent with previous observations (Brandstaetter et al., 2012). Recruitment of GFP-GPI was significantly impaired in cells infected with ΔsopB mutant bacteria. Complementation of the ΔsopB mutant by expression of the wild type sopB gene from a low copy plasmid restored recruitment of GFP-GPI to invasion sites, confirming that SopB is needed for this phenotype. In contrast, expression of a catalytically inactive mutant of SopB (C462S) lacking phosphatase activity was not sufficient to complement
recruitment of GFP-GPI during infection by the ΔsopB mutant. Thus, SopB mediates the recruitment of a subset of lipid rafts to invasion sites in a manner that requires its catalytic activity.

A high concentration of signalling molecules within lipid rafts allow them to serve as important signalling hubs (Head et al., 2014; Simons and Toomre, 2000). The localization of the lipid raft marker to the S. Typhimurium invasion site, prompted me to determine whether other signalling molecules also localize to the invasion site. Ras GTPases are regulated in a similar manner to Rho GTPases and are also amenable to palmitoylation modification, allowing them to associate with lipid rafts (Colicelli, 2004; Eisenberg et al., 2013). Furthermore, Ras GTPases have been previously implicated in Akt activation (Colicelli, 2004; Wurtzel et al., 2012). This prompted me to determine whether Ras GTPases are also recruited to the S. Typhimurium invasion ruffle. I observed recruitment of the Ras GTPases, R-ras1, R-ras2 and N-Ras to the S. Typhimurium invasion ruffle (Figure 3.10), suggesting that lipid rafts may be used by S. Typhimurium to recruit the necessary signalling molecules to the invasion site for its pathogenesis.
Figure 3.7 RhoB palmitoylation is needed for enrichment at *S. Typhimurium* invasion site

(A) Cells were transfected with the indicated RhoB-GFP construct and infected with *S. Typhimurium* for 10 mins. Cells were immunostained for GFP, *Salmonella* and phalloidin. Arrows indicate *S. Typhimurium* invasion site. (B) 100 invasion ruffles were scored for the presence or absence of the indicated RhoB-GFP construct. *** denotes p < 0.001.
Figure 3.8 SopB mediates recruitment of lipid rafts to S. Typhimurium invasion sites (A) Henle cells were transfected with GFP-GPI and infected with the indicated *Salmonella* mutant for 10 mins. Cells were immunostained for GFP, *Salmonella*, and phalloidin was used to visualize actin-rich invasion ruffles. Arrow indicates S. Typhimurium invasion sites. (B) In three independent experiments, localization of GFP-GPI was analyzed in 100 invasion ruffles. *** denotes p < 0.001.
Figure 3.9 Line scan analysis of GFP-GPI localization to S. Typhimurium invasion site.

Cells were transfected with GFP-GPI and infected with WT or ΔsopB S. Typhimurium. F-actin was visualized with phalloidin staining. Line scan analysis was performed for (A) WT and (B-C) ΔsopB. Graphs on right panel indicate intensity of GFP-GPI (green) relative to actin (red) at S. Typhimurium invasion sites.
Figure 3.10 Ras GTPases are recruited to the *S. Typhimurium* invasion site. Henle cells were transfected with the indicated Ras GTPase and infected with *S. Typhimurium*. Cells were immunostained with antibody against GFP, *Salmonella* and phalloidin. Invasion ruffles were analyzed for the localization of the indicated Ras GTPase. *** denotes p < 0.001. Data in Figure 3.9 was generated in collaboration with Nancy Zhu.
3.3 DISCUSSION

Rho GTPases are important molecular switches within the host cell and play important roles in signal transduction. This is the first study to analyze localization of members of the Rho GTPase family that have never been studied in *S. Typhimurium* invasion. 15 Rho GTPase family members were analyzed for their localization to the *S. Typhimurium* invasion site. Results from the screen identified four novel Rho GTPases enriched at the invasion site. RhoB, RhoD, RhoH and RhoJ localize to the invasion site in a SopB-dependent manner. My data suggests that *S. Typhimurium* promotes RhoB recruitment to induce Akt activation during invasion. This is consistent with previous findings demonstrating that RhoB contributes to trafficking of PDK1-bound endosomes and Akt activation (Adini et al., 2003; Flynn et al., 2000).

RhoJ was recruited to the *S. Typhimurium* invasion site in a SopB-dependent manner and was also needed for invasion. RhoJ belongs to the Cdc42 family of Rho GTPases and also binds to N-WASP and PAK, both of which are upstream of the Arp2/3 complex (Sadok and Marshall, 2014; Vignal et al., 2000). It is therefore possible that RhoJ promotes *S. Typhimurium* invasion via activation of the Arp2/3 complex. Depletion of RhoJ in melanoma cells prevented phosphorylation of p41 ARC, a subunit of the Arp2/3 complex required for complex formation (Ho et al., 2013; Vadlamudi et al., 2004). This process was dependent on RhoJ dependent activation of PAK (Ho et al., 2013). Furthermore, it was previously demonstrated that decreased phosphorylation of p41 ARC perturbs Arp2/3 complex activity, and results in decreased F-actin formation (Vadlamudi et al., 2004). Given that the Arp2/3 complex plays an important role in *S. Typhimurium* invasion, future studies should interrogate the potential role of RhoJ in promoting Arp2/3 complex formation during *S. Typhimurium* invasion. This can be achieved by analyzing the levels of phosphorylated p41 ARC in RhoJ siRNA treated cells during *S. Typhimurium* invasion.
Endocytosis of the anthrax toxin requires palmitoylation of the host receptor, ANTXR1 (Abrami et al., 2006). This alludes to an important role for palmitoylated proteins in mediating host-pathogen interactions. Consistent with this, our studies demonstrate that S. Typhimurium invasion and PI(3,4,5)P₃ production is dependent on host palmitoylated proteins. Furthermore, I demonstrate that palmitoylation of RhoB is required for localization to the invasion ruffle.

In the context of S. Typhimurium invasion, 2-bromopalmitate inhibited S. Typhimurium invasion and PI(3,4,5)P₃ production at the invasion site. Future studies should complement this data by utilizing alternative methods to disrupt palmitoylation cycling. Signal transduction by palmitoylated proteins require cycling between its palmitoylated and depalmitoylated states. For example, inhibition of palmitoylation turnover prevented G-protein coupled receptor function and signalling (Jia et al., 2014). Thus, it will be of importance to also analyze the effect of inhibiting palmitoylthioesterases to prevent depalmitoylation during S. Typhimurium invasion.

Similar to Rho GTPases, Ras GTPases are also amenable to palmitoylation (Eisenberg et al., 2013). Ras GTPase palmitoylation is required for transduction of downstream signalling pathways. For example, a palmitoylation-deficient R-Ras is incapable of promoting serum-mediated Akt activation (Wurtzel et al., 2012). Preliminary analysis of Ras GTPase localization reveals an enrichment of R-Ras1, R-Ras2 and N-Ras at the S. Typhimurium invasion site. Future studies should interrogate the functional relevance of Ras GTPase recruitment to the invasion site. SopB-mediated Akt activation should be analyzed upon knockdown of candidate Ras GTPases. Redundancy amongst Ras GTPase family members may exist, since R-Ras1, R-Ras2 and N-Ras have been previously demonstrated to activate Akt (Haigis et al., 2008; Wurtzel et al., 2012). Thus, multiple Ras GTPases may need to be simultaneously targeted to analyze their contributions to SopB-mediated Akt activation.

Ras GTPase palmitoylation has important implications towards Ras GTPase signalling and localization. Previous studies demonstrated that Ras GTPase palmitoylation is required to
induce serum-mediated Akt activation (Wurtzel et al., 2012). It will be important to analyze whether palmitoylation-deficient Ras GTPases can induce Akt activation during S. Typhimurium invasion. Based on previous observations, I hypothesize that R-Ras and N-Ras may also contribute to SopB-mediated Akt activation. This will have important implications and may suggest that SopB hijacks multiple host-signalling pathways to mediate Akt activation.

This study identifies new Rho GTPase players in S. Typhimurium pathogenesis. Rho GTPase manipulation has been previously demonstrated for Enteropathogenic *Escherichia coli* (EPEC) and *Shigella flexneri* (Bulgin et al., 2009; Ohya et al., 2005). It is possible that RhoB, RhoD, RhoH and RhoJ may also play a role in pathogenesis of other bacterial pathogens.
3.4 EXPERIMENTAL PROCEDURES

3.4.1 Cell culture

Henle cells were obtained from ATCC and maintained in growth medium [DMEM (HyClone) supplemented with 10% FBS (Wisent)] at 37°C in 5% CO₂. Henle cells were seeded at 2.5 x 10⁴ cells per well in 24-well tissue culture plates containing coverslips. Late-log bacterial cultures were used for infecting Henle cells as outlined previously (Szeto et al., 2009). Briefly, bacteria were pelleted at 10,000x g for 2 min and resuspended in PBS. The inoculum was diluted and added to Henle cells at 37°C for 10 min. The cells were then washed extensively and fixed as per indicated time points.

3.4.2 Bacterial Strains and Plasmids

Bacteria used in this study are as follows: S. Typhimurium 1344 (Hoiseth and Stocker, 1981), M202 (ΔsopE ΔsopE2 mutant) (Stender et al., 2000), ΔsopB (Steele-Mortimer et al., 2000), ΔsopB + pSopB (Knodler et al., 2002), and ΔsopB + pSopB C462S (Steele-Mortimer et al., 2000). For invasion experiments, WT bacteria was diluted to 1:100, ΔsopEΔsopE2 was diluted to 1:50 and all ΔsopB mutants were diluted to 1:100. For transfection of Henle cells, Genejuice (Novagen) or Jet Prime (Polyplus Transfection) transfection reagents were used as per manufacturer’s protocols. GFP-GPI and PAK-PBD-GST was a gift from Dr. Sergio Grinstein. All Rho GTPase and Ras GTPase constructs used were previously described (Heo et al., 2006; Heo and Meyer, 2003). All RhoB constructs used were previously described (Perez-Sala et al., 2009).

3.4.3 RNA interference

Henle cells were seeded into 24-well culture plates at 5 x 10⁴ cells per well and transfected the next day using Oligofectamine (Invitrogen). A concentration of 100 nM of total siRNA was used in each knockdown. Medium was changed 24 h after transfection, and Henle
cells were infected with S. Typhimurium 48 h after transfection. siRNA used in this study are as follows: control siRNA (Sigma Life Science, #SIC001), Rac1 siRNA (ThermoScientific, L-003560-00-0005), RhoB siRNA (Sigma Life Science, # SASI_Hs01_00130025), RhoD siRNA (Sigma Life Science, SASI_Hs01_00186023), RhoH siRNA (Sigma Life Science, # SASI_Hs02_00337332), and RhoJ siRNA (Sigma Life Science, SASI_Hs01_00074414).

3.4.4 Immunofluorescence microscopy and Antibodies

Cells were fixed with 2.5% paraformaldehyde in PBS for 10 min at 37°C. TCA fixation was utilized for immunostaining of endogenous Rho GTPases, as previously described (Hayashi et al., 1999). Fixed cells were immunostained as previously described (Brumell et al., 2001a). Immunostaining before permeabilization was used to differentiate between intracellular and extracellular bacteria (Smith et al., 2007). Coverslips were mounted onto glass slides using DakoCytomation fluorescent mounting medium and imaged using a Quorum WaveFX-X1 spinning disc confocal system (Quorum Technologies Inc., Guelph, Canada) and Volocity software (Improvision). Images were imported into Adobe Photoshop and assembled in Adobe Illustrator.

Salmonella O antisera (BD Difco) was used for immunostaining of S. Typhimurium (Cat No. 225341). All fluorescent secondary antibodies were AlexaFluor conjugates from Molecular Probes (Invitrogen). Anti-Arp3 was a generous gift from Dr. Kenneth Campellone (University of California, USA. Antibodies used for endogenous Rho GTPases are as follows: anti-RhoB (Santa Cruz, Cat. No sc-180), anti-RhoD (Santa Cruz, Cat. No. sc-365241), anti RhoH (Sigma, Cat. No. SAB4501761), anti-RhoJ (abcam, Cat. No. ab57584), anti-RhoG (Millipore, Cat. No. 04486), anti-Rac1 (BD Biosciences, Cat. No. 610651), and anti-Cdc42 (Cell Signalling, Cat. No. 2466). Immunostaining of GFP or CFP-tagged constructs was visualized with anti-GFP.
(Molecular Probes, Cat. No. A11122). 2-bromopalmitate (Sigma Life Science, Cat. No. 238422) was used to pre-treat cells, where indicated.

3.4.5 Western Blot Analysis

Cells were lysed in 1% Triton-X 100, 50mM Tris pH 7.4, 150mM NaCl, and 1mM EDTA. Lysis buffer was supplemented with protease inhibitors (10µg/ml aprotinin, 10µg/ml leupeptin, 1µM pepstatin A, 1mM PMSF) and 1mM DTT. Sample buffer (60mM Tris pH6.8, 5% glycerol, 1% SDS, 2% b-mercaptoethanol, 0.02% bromophenol blue) was added to the suspension, and samples boiled for 10 min. Samples were separated on 12% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked in 5% skim milk overnight. Primary antibodies were incubated for 1 h at room temperature. Akt activation was assessed with anti-phospho Akt (S473) (Cell Signalling, Cat. No. 9271), and total Akt was visualized with anti-pan Akt (Cell Signalling, Cat. No. 4691). Anti-β tubulin (Sigma Cat. No. T4026) and anti-GAPDH (Millipore #MAB374) were used to validate loading for Western blot analysis. Secondary antibodies used were conjugated to horseradish peroxidase (HRP) and were purchased from Sigma.

3.4.6 Statistical Analysis

Statistical analyses were conducted using GraphPad Prism v5.0. The mean ± standard error mean (SEM) is shown in figures, and P values were calculated using one sample t-test, one-way ANOVA or two-way ANOVA, where indicated. A p-value of less than 0.05 was considered statistically significant and is denoted by *. p < 0.01 is denoted by ** and p < 0.001 is denoted by ***.
4 Chapter Four: DISCUSSION

Parts of this chapter contains material that was reprinted with permission from Bioessays, 7: 687-96, Truong et al., Bacterial subversion of host cytoskeletal machinery: hijacking formins and the Arp2/3 complex, 2014.

The purpose of this thesis was to further characterize the early stages of S. Typhimurium invasion. Chapter Two of this thesis identified a novel host molecular machinery involved in S. Typhimurium invasion. In Chapter Three, I further elucidated the signalling pathways that occur during the early stages of S. Typhimurium invasion. In this chapter I will discuss the findings of my research and put them into the context of the current state of knowledge in the field.

4.1 Dynamic model of invasion ruffle formation by S. Typhimurium

This study provides a new model for invasion ruffle formation. Live imaging analysis revealed recruitment of FHOD1 prior to Arp2/3 during S. Typhimurium invasion. Furthermore, recruitment of FHOD1 led to formation of filopodia at the site of invasion. My data suggests that actin nucleators undergo spatiotemporal regulation by S. Typhimurium. It begins with FHOD1 recruitment to form filopodia first, then recruitment of Arp2/3 to form lamellipodia (Figure 4.1).

4.1.1 How do formins and the Arp2/3 complex collaborate during Salmonella invasion?

I demonstrated that the Arp2/3 complex is not the sole mediator of actin rearrangements during S. Typhimurium invasion. Rather, there appears to be an intricate collaboration between the Arp2/3 complex and the formin, FHOD1, during formation of the actin-rich invasion ruffle (Truong et al., 2013). Similar to the Arp2/3 complex, FHOD1 is also recruited to the invasion
ruffle and knockdown of FHOD1 impairs S. Typhimurium invasion (Truong et al., 2013). While FHOD1 activation during S. Typhimurium invasion is also dependent on SopB and SopE/E2, the downstream signalling events leading to FHOD1 activation and phosphorylation appear to be different from the pathways activating the Arp2/3 complex (Truong et al., 2013).

Interestingly, I demonstrated that Arp2/3 and FHOD1 contribute to different aspects of invasion ruffle morphology (Truong et al., 2013). Live imaging microscopy and scanning electron microscopy revealed that siRNA-mediated knockdown of the Arp2/3 complex resulted in invasion ruffles with expansive filopodia-like structures that are characteristic of formin-mediated actin elongation (Figure 4.2). In contrast, siRNA-mediated knockdown of FHOD1 resulted in lamellipodia-like invasion ruffles that were smaller in volume. I also demonstrated that recruitment of FHOD1 to the invasion ruffle occurs prior to that of the Arp2/3 complex (Truong et al., 2013). These results demonstrate the intricate cross-talk between FHOD1 and the Arp2/3 complex during formation of the invasion ruffle. Since formin-mediated actin elongation prevents capping of actin filaments (Higgs, 2005; Pring et al., 2003; Romero et al., 2004; Schonichen and Geyer, 2010), this allows for quick formation of actin filaments and may be beneficial for S. Typhimurium to first recruit FHOD1 to the invasion ruffle. Arp2/3 complex mediated actin elongation requires a pre-existing actin filament. Thus, it is possible that upon invasion, S. Typhimurium recruits FHOD1 first to create actin filaments for the Arp2/3 complex to bind to and mediate branched actin elongation. Future studies should further explore the regulation of FHOD1 and the Arp2/3 complex and identify whether FHOD1 is needed for recruitment of the Arp2/3 complex to the S. Typhimurium invasion ruffle.
**Figure 4.1 Dynamic model of S. Typhimurium invasion ruffle formation**

1. S. Typhimurium invasion begins with translocation of bacterial effectors into the host cytosol.
2. Invasion ruffle formation begins with FHOD1-mediated filopodia formation. SopB and SopE/E2 activate FHOD1 via a RhoA-ROCKII pathway.
3. Following FHOD1-mediated filopodia formation, Arp2/3 is recruited to the S. Typhimurium invasion site to induce lamellipodia formation.
Figure 4.2 FHOD1 and Arp2/3 have different roles at the *S. Typhimurium* invasion site. FHOD1 and Arp2/3 both localize throughout the *S. Typhimurium* invasion ruffle (left panel) and contribute to its formation. Knockdown of FHOD1 (middle panel) resulted in stunted invasion ruffles that are smaller in volume relative to the control. Upon Arp2/3 knockdown (right panel), *S. Typhimurium* invasion ruffles take on a filopodia-like structure.
4.2 A potential role for formins in later stages of *S. Typhimurium* pathogenesis?

After entry into the host cell, *S. Typhimurium* enters a protective niche, termed the *Salmonella* Containing Vacuole (SCV) (Haraga et al., 2008). The early SCV (30 min p.i.) has characteristics that resemble early endosomes. For example, both early endosomes and the SCV are marked by an enrichment of the GTPase, Rab5 (Mallo et al., 2008; Nielsen et al., 1999).

At 4h p.i. a meshwork of F-actin associates with the SCV (Meresse et al., 2001; Unsworth et al., 2004). The association of the SCV with F-actin is required for intracellular bacterial replication and maintenance of membrane integrity around the bacteria (Unsworth et al., 2004). It was further demonstrated that the SPI-2 secreted effector, SteC contributes to formation of actin around the SCV via activation of the MAP kinase, MEK (Odendall et al., 2012; Poh et al., 2008). Whether actin nucleators contribute to *de novo* actin polymerization around the SCV remains elusive. The role for Arp2/3 in formation of F-actin around the SCV was ruled out because inhibition of Arp2/3 with dominant negative constructs of N-WASP and WAVE, activators of Arp2/3, did not affect the number of F-actin$^+$ SCV (Unsworth et al., 2004).

Transmission electron microscopy (TEM) analysis of the F-actin around the SCV revealed formation of unbranched actin filaments, reminiscent of formin-mediated actin polymerization (Meresse et al., 2001). Upon knockdown of mDia1, no difference in the number of SCVs associated with F-actin was found (Unsworth et al., 2004). However, given that 15 mammalian formins have been identified to date, a more extensive and comprehensive study may reveal a role for other formins in F-actin formation around the SCV. The diaphanous-related formin, hDia2C, is a splice variant of Dia2 and localizes to endosomes in a RhoD-dependent manner (Gasman et al., 2003). Furthermore, previous studies identified localization of FRLα (FMNL1) to the phagocytic cup in macrophages undergoing phagocytosis of IgG-opsonized red blood cells (RBCs) (Seth et al., 2006). Localization of hDia2C and FRLα/FMNL1
to endosomes and the phagocytic cup, respectively, makes them potential candidates for F-actin formation around the SCV.

4.3 Involvement of formins in other forms of bacterial invasion

*Borrelia burgdorferi* (*B. burgdorferi*) is a bacterial pathogen transmitted to humans by tick bites and infection results in Lyme disease, a multi-systemic disease affecting the peripheral and central nervous systems, the skin, and joints (Radolf et al., 2012). At the site of invasion, *B. burgdorferi* is taken up by macrophages through a coiling-phagocytosis mechanism. F-actin rich filopodia-like structures, called pseudopods, extend from the surface of macrophages and coil around the bacteria to drive its entry into the macrophage (Linder et al., 2001). Given that the pseudopod is rich in F-actin, the role of the Arp2/3 complex in formation of the pseudopod was analyzed. Immunofluorescence staining of human macrophages revealed localization of the Arp2/3 complex and WASP along the pseudopod surrounding *B. burgdorferi* (Linder et al., 2001). Expression of dominant negative variants of Cdc42Hs or Rac1 (activators of WASP) significantly decreased the number of *B. burgdorferi* induced-pseudopods on infected macrophages, suggesting that coiling phagocytosis of *B. burgdorferi* proceeds via a signalling pathway mediated by Cdc42Hs or Rac1 induced Arp2/3 activation (Figure 4.3).

Recent research has identified a role for formins, FMNL1, mDia1 and DAAM1, in coiling phagocytosis of *B. burgdorferi* (Hoffmann et al., 2014; Naj et al., 2013). Immunofluorescence staining with antibodies against endogenous FMNL1 and mDia1, revealed an enrichment of FMNL1 and mDia1 on *B. burgdorferi* induced pseudopods (Naj et al., 2013). Interestingly, while FMNL1 localized to both the tip and along the pseudopod, mDia1 mainly localized to the tip of the pseudopod (Naj et al., 2013). Live imaging analysis revealed that during *B. burgdorferi* invasion, DAAM1 is first observed in filopodia associated with initial capture of *B. burgdorferi* (Figure 4.3). Following formation of the coiling pseudopod, DAAM1
displays similar localization patterns as FMNL1 and is found enriched along the length of the pseudopod. This may suggest that DAAM1 is involved in capturing *B. burgdorferi* at the surface of the macrophage prior to pseudopod formation and in formation of the coiling pseudopod.

Knockdown of FMNL1, mDia1 or DAAM1 with siRNA led to a significant decrease in the number of *B. burgdorferi* induced pseudopods and a corresponding decrease in invasion (Hoffmann et al., 2014; Naj et al., 2013). Interestingly, simultaneous knockdown of FMNL1, mDia1, and DAAM1 did not result in an additive decrease in *B. burgdorferi* invasion relative to single knockdown of each formin. This suggests that FMNL1, mDia1 and DAAM1 may act in the same pathway during pseudopod formation (Fig. 4.3).
Figure 4.3 Exploitation of formins and the Arp2/3 complex by bacterial pathogens. *B. burgdorferi* invasion induces coiling phagocytosis in the infected macrophage. DAAM1 appears to play a role in formation of filopodia to initiate capture of *B. burgdorferi*. Following capture, a coiling pseudopod extends from the macrophage to initiate *B. burgdorferi* invasion. mDia1 has a predominant localization to the tip of the pseudopod. In contrast, FMNL1, DAAM1 and the Arp2/3 complex are found along the length and the tip of the pseudopod. Adapted from Bioessays, 7: 687-96, Truong et al., Bacterial subversion of host cytoskeletal machinery: hijacking formins and the Arp2/3 complex, 2014.
Given that the Arp2/3 complex also localizes to the coiling pseudopod, it will be interesting to determine localization of the Arp2/3 complex, FMNL1, mDia1, and DAAM1 relative to each other on the *B. burgdorferi* pseudopod. This may provide further insight into the specific role each actin nucleator has on pseudopod formation. Similar to *S. Typhimurium*, it is possible that there exists a spatial and temporal collaboration between the Arp2/3 complex and formins in coiling phagocytosis of *B. burgdorferi*. Deducing the role of each actin nucleator will require analysis of the pseudopod morphology when either the expression of the Arp2/3 complex, FMNL1, mDia1 or DAAM1 is knocked down.

The uptake of bacteria into macrophages by coiling phagocytosis was first described for *Legionella pneumophilia* (Horwitz, 1984). It is possible that *L. pneumophila* also employs formins and the Arp2/3 to mediate coiling phagocytosis into host macrophages. Furthermore, it will be important to elucidate the host signalling pathway leading to coiling phagocytosis. Recent research has demonstrated that dendritic cells also utilize coiling phagocytosis for uptake of yeast cells (Neumann and Jacobson, 2010), suggesting that coiling phagocytosis also plays a role in the innate immune system. As such, it will be of great benefit to attain a greater understanding of the host regulatory pathway leading to coiling phagocytosis.

### 4.4 Signalling during early stages of *S. Typhimurium* invasion

The purpose of Chapter Three was to further characterize the signalling events that occur during early stages of *S. Typhimurium* invasion via localization analysis of overexpressed Rho GTPases to the *S. Typhimurium* invasion ruffle. This analysis revealed that in addition to Rac1 and Cdc42, RhoB, RhoD, RhoH and RhoJ are also recruited to the invasion site. I further demonstrate that localization of lipid rafts to the invasion site could explain the mechanism behind the recruitment of Rho GTPases to the invasion ruffle (Figure 4.4). This model demonstrates the unique ability of one bacterial effector to modulate membranes to promote
pathogenesis. Early in invasion, SopB manipulates membrane through recruitment of lipid rafts, allowing for a concentration of signalling molecules at the plasma membrane that are needed during S. Typhimurium pathogenesis. Later in invasion, SopB once again manipulates membrane by decreasing the negative charge on the SCV, to induce removal of Rab GTPases that promote lysosomal fusion (Figure 4.4).
Figure 4.4 Model of RhoB, RhoD, RhoH and RhoJ recruitment to the S. Typhimurium invasion site. Recruitment of RhoB/D/H/J to the S. Typhimurium invasion site is SopB-dependent. Palmitoylation of Rho GTPases promotes their localization to the invasion site. SopB also mediates lipid raft recruitment to the S. Typhimurium invasion ruffle. Palmitoylation of Rho GTPases may facilitate their ability to bind to lipid rafts allowing them to be recruited to the S. Typhimurium invasion site in a SopB-dependent manner.
4.5 RhoB and Akt activation during *S. Typhimurium* invasion

Akt activation occurs via phosphorylation at two residues: T308 by PDK1 and S473 by mTORC2 (Bellacosa et al., 1998; Sarbassov et al., 2005). In the context of *S. Typhimurium* invasion, PDK1 is needed for Akt activation, although the mechanism underlying PDK1 activation during invasion remains elusive (Roppenser et al., 2013). Previous studies identified a role for RhoB in PDK1 regulation and activation. RhoB interacts indirectly with PDK1 and sequesters it from the cytosol to endosomes in fibroblasts (Flynn et al., 2000). Formation of the RhoB-PDK1 complex is dependent on the GTP-bound state of RhoB and its ability to bind membranes since treatment of cells with the isoprenyl inhibitor, mevastatin, prevented complex formation between RhoB and PDK1 (Flynn et al., 2000).

The relevance of this RhoB-PDK1 complex has not been tested in the context of *S. Typhimurium* invasion. My data suggests that RhoB contributes to Akt activation during *S. Typhimurium* invasion via a mechanism that is as yet, uncharacterized (Figure 3.5). It is possible that during *S. Typhimurium* invasion, recruitment of RhoB allows for the localization of PDK1 to the invasion site, and thus Akt activation. Future studies should analyze whether knockdown of RhoB affects PDK1 localization during *S. Typhimurium* invasion and whether *S. Typhimurium* invasion is sufficient to induce complex formation between RhoB and PDK1.

4.6 Role of RhoB in Akt activation during *S. flexneri* invasion?

Depletion of PI(4,5)P$_2$ by *S. flexneri* results in accumulation of PI(5)P, and thereby activation of PI3K (Pendaries et al., 2006). The result is an increased production of PI(3,4,5)P$_3$ allowing for Akt activation. This process of Akt activation by IpgD parallels those of SopB, although the PI3K involved in PI(3,4,5)P$_3$ production may vary between the two pathogens (Pendaries et al., 2006; Roppenser et al., 2013). My data suggests a role for RhoB in Akt activation during *S. Typhimurium* invasion. It may be possible that IpgD may also target RhoB
to induce Akt activation during *S. flexneri* invasion. Future studies should interrogate how siRNA-targeted knockdown of RhoB affects Akt activation during *S. flexneri* invasion.

### 4.7 Akt activation during *S. Typhimurium* invasion: remaining questions

SiRNA-targeted knockdown of RhoB resulted in a significant but modest decrease in Akt activation, alluding to the involvement of other signalling molecules. Furthermore, I demonstrate a role for SopB-dependent localization of lipid rafts to the invasion ruffle (Figure 3.8). A high concentration of signalling molecules are found within lipid rafts, allowing lipid rafts to act as signalling hubs to induce activation of downstream signalling pathways (Head et al., 2014; Levental et al., 2010; Lingwood and Simons, 2010). Could it be possible that SopB induces localization of lipid rafts to the invasion ruffle to bring all necessary signalling molecules to the site of invasion?

Ras GTPases are another family of GTPases made up of proteins H-, N-, K- and R-ras. Ras GTPases are regulated in similar manner to Rho GTPases, and also contain a CAAX motif at their C-terminus that is amenable to lipid modifications (Colicelli, 2004). H-, N- and R-ras are palmitoylated and can be found within lipid rafts (Colicelli, 2004; Eisenberg et al., 2013; Parton and Hancock, 2004). Furthermore, Ras GTPases have been described to activate Akt. For example, expression of dominant negative K-ras or N-ras results in attenuated Akt activation (Haigis et al., 2008).

R-ras contributes to cell spreading and membrane ruffling, both of which are dependent on its ability to undergo lipid modifications (Wurtzel et al., 2012). R-ras colocalizes with the PI(3,4,5)P₃ probe, PH-Akt, at EGF induced membrane ruffles. Furthermore, palmitoylation deficient, but not isoprenylation-deficient R-ras displayed defects in Akt activation. Collectively, this alludes to an important role for palmitoylation of R-ras in Akt activation (Wurtzel et al., 2012).
Preliminary data presented in Chapter Three suggests that palmitoylation plays an important role in PI(3,4,5)P₃ production at the invasion ruffle (Figure 3.6). Given that Ras GTPases contribute to Akt activation, and also undergo palmitoylation, recruitment of Ras GTPases to the S. Typhimurium invasion ruffle was analyzed. Preliminary data demonstrates recruitment of N-ras and the isoforms R-ras1, R-ras2 to the invasion ruffle (Figure 3.10). The functional relevance of Ras GTPases to Akt activation during S. Typhimurium invasion should be determined by siRNA-targeted knockdown of Ras GTPases. It will also be of relevance to determine whether lipid modifications of Ras GTPases are needed for Akt activation during S. Typhimurium invasion, since preliminary data suggests that palmitoylation has an important role in PI(3,4,5)P₃ production (Figure 3.6).

4.8 Mechanism of lipid raft recruitment by SopB?

Lipid rafts are domains within the plasma membrane that contain a high concentration of cholesterol and sphingolipids (Head et al., 2014). Lipid rafts are very dynamic and undergo constant endocytosis and exocytosis of membrane to allow for recycling of proteins required during cellular processes such as cell spreading and pathogen invasion (Brandstaetter et al., 2012; Nichols et al., 2001). Myo1c has been demonstrated to play a role in recycling of lipid rafts, since siRNA-targeted knockdown of Myo1c resulted in endocytosis of the lipid raft marker, GFP-GPI, towards a perinuclear region of the cell (Brandstaetter et al., 2012).

Previous studies demonstrated localization of cholesterol and the lipid raft marker, GFP-GPI to the S. Typhimurium invasion site (Brandstaetter et al., 2012; Brumell et al., 2001b; Garner et al., 2002; Hayward et al., 2005). Furthermore, depletion of cholesterol with MβCD or targeted knockdown of Myo1c resulted in a significant decrease in S. Typhimurium invasion (Brandstaetter et al., 2012; Garner et al., 2002). Collectively, this suggests that S. Typhimurium
invasion relies on lipid raft recycling. Whether lipid raft recycling is manipulated by *S. Typhimurium* effectors, has never been studied.

I identified a role for SopB in inducing localization of lipid rafts to the invasion ruffle (Figure 3.8-3.9). However, the mechanism as to how this is achieved still remains unanswered. It is possible that SopB may target Myo1c to allow for delivery of lipid rafts to the plasma membrane. RalA is a GTPase from the Ral family of GTPases and is a known activator of Myo1c (Brandstaetter et al., 2012). RalA not only localizes to the invasion ruffle, but siRNA-targeted knockdown of RalA significantly inhibits *S. Typhimurium* invasion (Brandstaetter et al., 2012; Nichols and Casanova, 2010). While previous studies revealed a role for SopE in inducing RalA activation (Nichols and Casanova, 2010), the bacterial effector that mediates localization of RalA to the invasion ruffle still remains to be elucidated. Future studies should address whether RalA recruitment to the invasion ruffle is SopB-dependent, and whether this recruitment results in activation of Myo1c.

### 4.9 Lipid rafts and Akt activation?

*S. Typhimurium* is capable of infecting many different cell types, but it is unclear how SopB is able to selectively engage specific pathways in different cell types to activate Akt. Previous data from our lab demonstrated that in different cell types, different PI3K contribute to Akt activation during *S. Typhimurium* invasion (Roppenser et al., 2013). Specifically, HeLa cells appear to utilize multiple host PI3K to induce Akt activation (Roppenser et al., 2013). A possible explanation for this may be that *S. Typhimurium* utilizes lipid raft recruitment as a general mechanism to target multiple host kinases or their upstream activators to the invasion site to induce Akt activation. By removing the dependency on a single kinase, *S. Typhimurium* can recruit lipid rafts to the invasion site, and with it localize PI3K that are specifically expressed in that cell type. This general mechanism of targeting lipid rafts, may also explain
why *S.* Typhimurium is capable of inducing Akt activation in a variety of cell types. Analysis of how disruption of lipid rafts in different cell types affect Akt activation may help determine if lipid raft recruitment is a general mechanism utilized by *S.* Typhimurium to activate Akt.

### 4.10 Differential involvement of Rho GTPases in polarized cells?

*S.* Typhimurium is a food-borne pathogen that upon infection, transits through the stomach to invade the intestinal epithelial layer, which comprises mainly of polarized enterocyte cells (Haraga et al., 2008; LaRock et al., 2015). Microfold cells (M cells) are found in the vicinity of enterocytes and contribute to the maintenance of the mucosal immune system (Mabbott et al., 2013).

*S.* Typhimurium infection begins with invasion at the apical surface of M cells. As infection progresses, *S.* Typhimurium invasion imparts cytotoxic effects on M cells and induces their destruction (Jones et al., 1994). The destruction of M cells introduces gaps in the intestinal lining, allowing for *S.* Typhimurium to infect both the apical and basolateral surfaces of the neighbouring enterocytes (Jones et al., 1994). *S.* Typhimurium can infect the apical and basolateral plasma membrane at equal efficiencies (Criss et al., 2001).

Interestingly, different Rho GTPases contribute to apical and basolateral infection by *S.* Typhimurium. *S.* Typhimurium invasion of MDCK cells revealed a requirement for Rac1, but not Cdc42, for invasion of the apical plasma membrane (Criss et al., 2001). In contrast to infection with non-polarized cells, in which Rac1 and Cdc42 both contribute to invasion, neither Rac1 nor Cdc42 were required for basolateral invasion by *S.* Typhimurium (Aiastui et al., 2010; Criss et al., 2001; Patel and Galan, 2006). Analysis of the bacterial effector involved, revealed a role for SopE in triggering Rac1 activation at the apical plasma membrane during infection (Criss et al., 2001). SopE was also demonstrated to contribute to basolateral infection of *S.*
Typhimurium, since Δ sopE displayed a reduction in basolateral invasion relative to their WT counterparts (Criss et al., 2001).

Overall, one may speculate that infection of polarized cells may require activation of a different subset of Rho GTPases, compared to non-polarized cells. The contribution of each member within the Rho GTPase family to apical and basolateral S. Typhimurium infection should also be examined. A localization analysis such as the one presented in Chapter Three, may reveal new Rho GTPase players in mediating S. Typhimurium infection in polarized cells.

4.11 Role of Rho GTPases in other bacterial pathogens?

*S. flexneri* is a foodborne bacterial pathogen that induces damage to the small intestine upon infection. Similarly to S. Typhimurium, *S. flexneri* invasion is mediated by translocation of bacterial effectors through a T3SS and formation of an invasion ruffle (De Geyter et al., 2000; Ohya et al., 2005). Of note, *S. flexneri* encodes an effector, IpgD, which shares homology with SopB (Pendaries et al., 2006). IpgD also contains phosphoinositide phosphatase activity, induces Akt activation, and contributes to actin reorganization during invasion ruffle formation (Garza-Mayers et al., 2015; Pendaries et al., 2006).

Recent studies identified that IpgD is capable of recruiting Arf6, a GTPase from the Arf family of GTPases (Garza-Mayers et al., 2015). Recruitment of Arf6 was dependent on the phosphatase activity of IpgD. Whether IpgD is capable of recruiting GTPases from the Rho family of GTPases has never been studied. Given that SopB and IpgD share homology and have similar functional activities, future studies should determine whether RhoB, RhoD, RhoH, and RhoJ are also recruited to the *S. flexneri* invasion site. Alternatively, a comprehensive analysis of Rho GTPases that localize to the *S. flexneri* invasion site may reveal novel Rho GTPases involved in *S. flexneri* pathogenesis.
Similarly to *S. Typhimurium*, *S. flexneri* also forms an invasion ruffle to mediate bacterial entry (De Geyter et al., 2000; Lee et al., 2014). Previous studies demonstrated an enrichment of GFP-GPI and cholesterol at the *S. flexneri* invasion site (Hayward et al., 2005; Lafont et al., 2002). Furthermore, MβCD treatment inhibited *S. flexneri* binding and invasion (Lafont et al., 2002). The mechanism underlying recruitment of lipid rafts to the *S. flexneri* invasion site remains elusive. Given that IpgD is a homologue of SopB, future studies should analyze the role of IpgD in promoting lipid raft recruitment to the *S. flexneri* invasion sites. Similar to SopB, IpgD may target lipid rafts to enrich Rho GTPases at the invasion site.
5 CONCLUSION

*S. Typhimurium* invasion involves manipulation of multiple host proteins. While many host targets of *S. Typhimurium* have been identified, there still remains an underrepresentation of the host proteins that are targeted during invasion. Through my studies, I add to the list of host proteins involved in the early stages of *S. Typhimurium* invasion. Characterization of the role of FHOD1 in *S. Typhimurium* invasion provides us with a new dynamic model of invasion ruffle formation. Furthermore, identification of novel Rho GTPases at the invasion ruffle, demonstrates that multiple host signalling pathways are utilized by *S. Typhimurium* to gain entry into host cells. These studies provide insight on how fundamental host molecular machineries and signalling molecules are manipulated by *S. Typhimurium* during invasion. Many bacterial pathogens also manipulate the host actin cytoskeleton and Rho GTPases during invasion, and thus, these findings may aid in further understanding mechanisms of invasion by other bacterial pathogens.
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