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THE EPITHELIAL TRANSMEMBRANE PROTEIN PERP IS REQUIRED FOR
INFLAMMATORY RESPONSES TO S. TYPHIMURIUM INFECTION

A Dissertation Presented

By

KELLY NOELLE HALLSTROM

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

OCTOBER 28, 2015

IMMUNOLOGY AND MICROBIOLOGY PROGRAM

October 28, 2015

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ABSTRACT

Salmonella enterica subtype Typhimurium (*S. Typhimurium*) is one of many non-typhoidal *Salmonella enterica* strains responsible for over one million cases of salmonellosis in the United States each year. These *Salmonella* strains are also a leading cause of diarrheal disease in developing countries. Non-typhoidal salmonellosis induces gastrointestinal distress that is characterized histopathologically by an influx of polymorphonuclear leukocytes (PMNs), the non-specific effects of which lead to tissue damage and contribute to diarrhea.

Prior studies from our lab have demonstrated that the type III secreted bacterial effector SipA is a key regulator of PMN influx during *S. Typhimurium* infection and that its activity requires processing by caspase-3. Although we established caspase-3 activity is required for the activation of inflammatory pathways during *S. Typhimurium* infection, the mechanisms by which caspase-3 is activated remain incompletely understood. Most challenging is the fact that SipA is responsible for activating caspase-3, which begs the question of how SipA can activate an enzyme it requires for its own activity.

In the present study, we describe our findings that the eukaryotic tetraspanning membrane protein PERP is required for the *S. Typhimurium*-induced influx of PMNs. We further show that *S. Typhimurium* infection induces PERP accumulation at the apical surface of polarized colonic epithelial cells, and that this accumulation requires SipA. Strikingly, PERP accumulation occurs in the absence of caspase-3 processing of SipA, which is the first time we have shown

SipA mediates a cellular event without first requiring caspase-3 processing. Previous work demonstrates that PERP mediates the activation of caspase-3, and we find that PERP is required for *Salmonella*-induced caspase-3 activation.

Our combined data support a model in which SipA triggers caspase-3 activation via its cellular modulation of PERP. Since SipA can set this pathway in motion without being cleaved by caspase-3, we propose that PERP-mediated caspase-3 activation is required for the activation of SipA, and thus is a key step in the inflammatory response to *S. Typhimurium* infection. Our findings further our understanding of how SipA induces inflammation during *S. Typhimurium* infection, and also provide additional insight into how type III secreted effectors manipulate host cells.

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Figure 1.1 Key Intercellular Junction Complexes

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PREFACE

Parts of this thesis that have appeared in separate publications include:

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Contributions of all authors can be found within the preface of each chapter.

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CHAPTER I

Introduction

1.1 *Salmonella* Significance in Human Health

Salmonella enterica are Gram negative facultative anaerobic bacteria that cause systemic and localized gastrointestinal illnesses through the contamination of food and water sources with fecal matter from infected hosts. *Salmonella enterica* serovar Typhi and Paratyphi cause systemic typhoid disease in humans, and are responsible for nearly 30 million illnesses each year in underdeveloped countries (Griffin and McSorley, 2011). In comparison, there are about 2000 non-typhoidal *Salmonella enterica* serovars. Serovars from this group cause localized gastrointestinal salmonellosis in humans, as well as in other hosts. Symptoms of gastrointestinal salmonellosis include nausea, vomiting, abdominal cramping, and diarrhea, which may contain mucus or blood. Salmonellosis is typically self-limiting and lasts about 3-5 days in otherwise healthy individuals, although those who are immunocompromised can develop systemic and fatal infections (Graham et al., 2000). The CDC estimates there are over 1 million cases of salmonellosis in the United States alone each year, making it a significant public health concern.

Upon consumption of contaminated food or drinks, *Salmonella* bacteria travel through the gastrointestinal tract and colonize the intestines. Studies have shown that *Salmonellae* induce ileocolitis following colonization of the distal ileum (Altier, 2005). Following colonization, *Salmonellae* invade intestinal epithelial

cells and induce pathogenesis via genes encoded on *Salmonella* pathogenicity islands (SPIs). Pathogenicity islands are areas of a pathogen's genome that are enriched for genes required for pathogenesis. Pathogenicity islands, which can be found in a variety of pathogens (Hallstrom and McCormick, 2014), are thought to be transmitted horizontally from pathogens. Together, *Salmonella* Typhi and non-typhoidal *Salmonellae* possess at least 21 SPIs (Sabbagh et al., 2010). The two most well-characterized are SPI-1 and SPI-2, each of which encodes machinery for type III secretion systems (T3SS), T3SS1 and T3SS2, respectively.

The T3SS is a common virulence mechanism that is specific to Gram negative bacteria and is essentially a molecular syringe that evolved from the flagellar basal body. The T3SS basal body is embedded in the bacterial cell membrane. Extending outward into the extracellular space, the hollow, needle-like channel interacts with the host cell membrane. Proteins at the tip of the needle, called translocases, form a translocon pore in the host cell membrane. Through this pore, bacterial effector proteins are translocated into the host cell. It is these translocated effectors that perturb host cell signaling pathways in ways that lead to pathogenesis. In general, SPI-1 is responsible for promoting *Salmonella* entry into intestinal epithelial cells (Bajaj et al., 1996) and SPI-2 is responsible for mediating intracellular *Salmonella* survival (Ochman et al., 1996), although there is some evidence for overlap between these two systems (Brawn et al., 2007; Lawley et al., 2006).

While *Salmonella* Typhi is a considerable public health concern, the remainder of this introduction will focus on non-typhoidal *Salmonella* (collectively referred to from here as *Salmonellae*) infections. The following sections will describe how *Salmonella* overcomes and, in some cases, subverts host defenses to promote its own program of pathogenesis. Of particular importance will be the way in which *Salmonella* induces and exploits inflammatory responses for its own survival and dissemination.

1.2 General Biology of the Human Intestinal Tract

Gut Microbiome

The intestinal tract is colonized by the resident gut microbiome. This population of microbes consists of over 100 trillion organisms dominated largely by *Lactobacillus*, *Bacteroides*, and *Firmicutes* (Backhed et al., 2005). The gut microbiome provides colonization resistance by competing with *Salmonellae* for nutrients, and thus can help protect the host from invasion. Indeed, in mouse models of colitis, mice are treated with streptomycin prior to being infected with non-Typhi *Salmonellae*. Failure to do so results in a systemic illness that more closely resembles that of human *S. Typhi* infections as opposed to localized gastroenteritis typical of non-Typhi infection (Barthel et al., 2003b). It is inferred that this response is due to the antibiotic-mediated clearance of the microbiome, which then better enables *S. Typhimurium* to colonize and invade murine

intestinal epithelial cells. Other work has shown that the microbiome is key for *Salmonella* clearance as well (Endt et al., 2010).

Epithelial Barrier

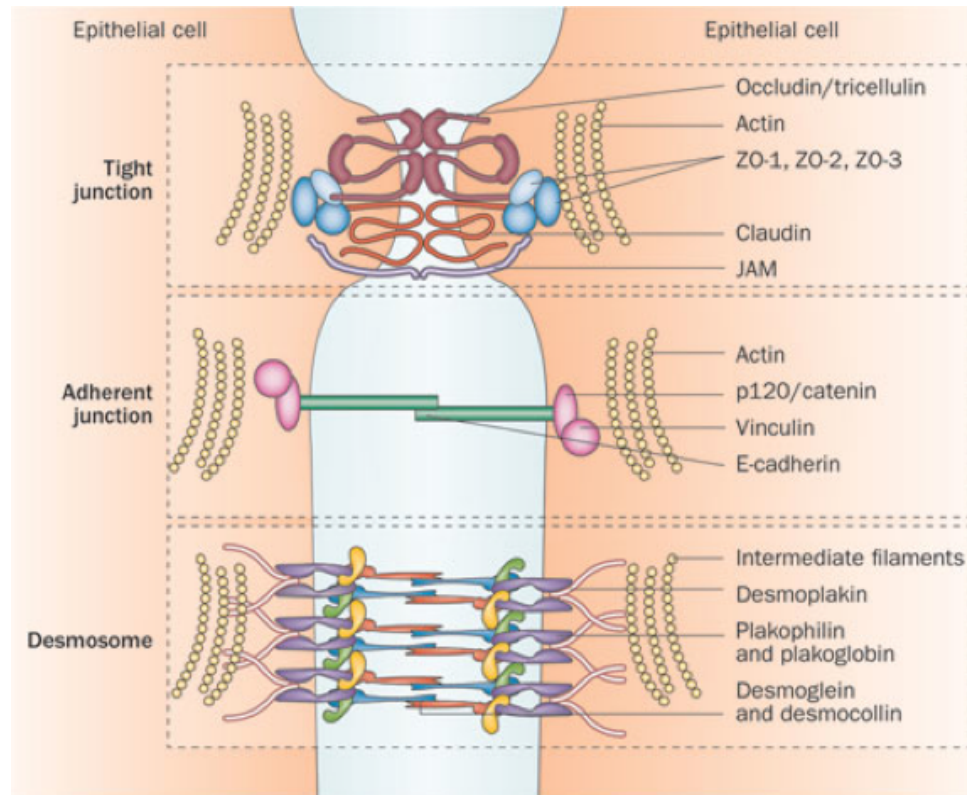
The inner surface of the intestine is lined with a monolayer of polarized columnar epithelial cells. The monolayer is comprised of a variety of cell types that are important for the general health and function of the intestinal epithelium. This includes Paneth cells, which secrete antimicrobial molecules; Goblet cells, which produce mucins that form a protective mucus layer over the epithelium; enterocytes, which are involved with nutrient absorption and chloride secretion; entero-endocrine cells, which secrete hormones and facilitate food digestion; M, or microfold cells, which serve as sentinels in the small intestine; and stem cells, which give rise to the aforementioned cell types.

The monolayer forms a protective barrier between the luminal environment and submucosa, which is regulated by intercellular junction protein complexes (see Figure 1.1). Protein complexes critical to maintaining epithelial barrier integrity include tight junctions, adherens junctions, and desmosomes. Barrier integrity is crucial, as it is the barrier that prevents luminal contents such as food, microflora, and pathogens from gaining access to the underlying tissues where they could potentially cause inflammation and systemic illness. Therefore, transport through the monolayer is carefully regulated via tight junction complexes. Tight junctions seal the environment between cells referred to as the

Figure 1.1 Key Intercellular Junction Complexes

Protein complexes that are critical to maintaining epithelial barrier integrity include tight junctions, which seal off the paracellular space; adherens junctions which promote adhesion; and desmosomes, which absorb mechanical stress. Tight junctions are composed of claudin, occludin, junction associated molecules (JAM), and Coxsackievirus and Adenovirus Receptor (CAR) proteins, and are anchored to the actin cytoskeleton by zona occluden (ZO) proteins (Furuse et al., 1998); (Itoh et al., 1999); (Tsukita and Furuse, 1999). A key component of adherens junctions is E-cadherin, which can homodimerize and thus interact with E-cadherin on neighboring cells to form clusters that associate with actin, thus promoting adhesion between cells. Desmosomes are composed of cadherins, which promote adhesion, and structural proteins such as desmoplakin, desmoglein, and plakoglobin (Hatsell and Cowin, 2001). Desmosomes help to absorb mechanical stress.

Figure 1.1 Key Intercellular Junction Complexes



(Neunlist et al., 2013)

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paracellular space and regulate passage through it in a gate-like manner (Figure 1.1). Tight junctions exhibit size and charge selectivity, and thus are critical regulators of water and ion balance in the intestines as well as overall epithelial barrier integrity (Turner, 2009). Adherens junctions promote adhesion, while desmosomes absorb mechanical stress (Figure 1.1).

1.3 Salmonella Utilizes its T3SS To Manipulate Host Cells

Upon reaching the intestinal environment, *Salmonellae* process environmental signals that activate expression of the T3SS1 via the transcription factor HilA (Bajaj et al., 1996). While pH, osmolarity, and oxygen levels are known to regulate SPI-1 expression (Bajaj et al., 1996), oxygen appears to be the most important regulator of this system (Lee and Falkow, 1990); (Thompson et al., 2006). *Salmonellae* then use the effectors secreted through the T3SS1 to penetrate the epithelial barrier and invade intestinal epithelial cells in order to establish a replicative niche.

Disruption of the Paracellular Pathway

Given that the tight junction is the regulator of paracellular passage, it is perhaps not surprising that *Salmonellae* have evolved a mechanism to disrupt the tight junction in order to facilitate its dissemination in the host. Infection of cultured monolayers with *Salmonella* induces a loss of barrier integrity (Boyle et al., 2006). It was found that this is due to disruption of the tight junction primarily

by the activity of T3SS1 effectors SopE and SopE2. These effectors cause the tight junction proteins ZO-1 and occludin to accumulate in the cytoplasm as opposed to localizing to the lateral cell membrane (Boyle et al., 2006). SopE and SopE2 are both GEFs, and activate Cdc42 and Rac1 GTPases (Hardt et al.); (Stender et al., 2000). Upon activation, these GTPases disrupt the function and structure of tight junctions and promote increased paracellular movement (Braga, 2002). The increased permeability resulting from the loss of tight junctions promotes bacterial translocation via the paracellular pathway to the basolateral surface (Kohler et al., 2007). Thus, uncoupling tight junctions is a critical way by which *Salmonellae* facilitate dissemination in the host. Evidence also shows that *Salmonellae* take advantage of transcytotic trafficking routes in M cells to reach the submucosal environment (Tam et al., 2008); (Jones et al., 1994).

It is important to note that these mechanisms of breaching the epithelial layer are shared across other pathogenic enterobacteria. For example, *Shigella flexneri* induces altered localization of tight junction complex components occludin and claudin-1, which leads to leaky epithelial barriers (Sakaguchi et al., 2002). *Shigella* is also known to invade M cells (Perdomo et al., 1994). Further, several strains of pathogenic *Escherichia coli* are known to disrupt tight junctions and subsequently induce loss of epithelial barrier integrity by inducing loss of or redistribution of occludin, claudins, and ZO proteins (Thanabalasuriar et al., 2010); (Philpott et al., 1998); (Guignot et al., 2007). Accordingly, breaching the epithelial barrier appears to be an important and conserved pathogenic strategy

for enteric bacteria.

Salmonellae Utilize Epithelial Cells as Replicative Niches

Salmonellae contain a wide array of effectors secreted through the T3SS1 that mediate invasion via various mechanisms (Table 1.1). *Salmonellae* encode three proteins that function as T3SS1 translocases: SipB, SipC, and SipD (Collazo and Galan, 1997). Absence of these proteins does not prevent effector secretion nor expression, but does preclude the translocation of effectors into the host cell (Collazo and Galan, 1997). Data indicate that the translocases facilitate bacterial attachment and formation of a pore in the host cell membrane (Scherer et al., 2000); (Lara-Tejero and Galan, 2009). The absence of SipB, SipC, or SipD renders *Salmonella* invasion-deficient, which is likely due at least in part to these mutants being unable to translocate effectors required for invasion (Kaniga et al., 1995a); (Kaniga et al., 1995b); (Wood et al., 1996).

The main mechanism for promoting *Salmonella* entry is via the action of T3SS1 effectors perturbing actin dynamics. These perturbations cause membrane ruffling, in which the host plasma membrane extends outward and around the bacterium, leading to its uptake into the cell in what becomes a *Salmonella* containing vacuole (SCV). That *Salmonellae* possess an array of effectors capable of directly or indirectly manipulating actin and plasma membrane dynamics provides a striking example of how *Salmonellae* have

Table 1.1 *Salmonella* T3SS Effectors and Their Roles in Pathogenesis

A summary of T3SS effectors whose functions in *Salmonella* Typhimurium pathogenesis have been identified.

Table 1.1 *Salmonella* T3SS Effectors and Their Roles in Pathogenesis

Effector	Location	Function	T3SS Apparatus
AvrA	SPI-1	Downregulates <i>Salmonella</i> -induced inflammation (Collier-Hyams et al., 2002); (Ye et al., 2007).	1
SipA	SPI-1	Key regulator of inflammatory responses, including promoting neutrophil migration to the apical surface. Cooperates with SipC to induce actin rearrangements that facilitate <i>Salmonella</i> invasion. Also suspected to promote SCV formation. Undergoes cleavage by caspase-3 (Zhou et al., 1999); (Wall et al., 2007); (Brawn et al., 2007); (Srikanth et al., 2010)	1
SipB	SPI-1	Component of T3SS-1 translocon; facilitates effector translocation (Kaniga et al., 1995b); (Hayward et al., 2000)	1
SipC	SPI-1	SPI-1 translocon component; facilitates effector translocation. Induces actin bundling to promote invasion. Likely regulator of exocyst-mediated trafficking to the cell surface (Kaniga et al., 1995b); (McGhie et al., 2001); (Nichols and Casanova, 2010)	1
SipD	SPI-1	Component of T3SS-1 translocon; facilitates effector translocation (Lara-Tejero and Galan, 2009)	1

Table 1.1 continued

SopA	Downstream of phsA-C	E3 ubiquitin ligase that may promote escape from SCV and promotes neutrophil migration. Also required during invasion (Wood et al., 2000); (Raffatellu et al., 2005); (Zhang et al., 2006)	1
SptP	SPI-1	Functions as a GAP, and is a tyrosine phosphatase. Reverses effects of SopE and SopE2 (Stebbins and Galan, 2000)	1
SopE	Bacteriophage	Promotes membrane ruffling and disrupts tight junctions by acting as a GEF for Rac-1 and Cdc42 (Hardt et al., 1998a); (Boyle et al., 2006)	1
SopE2	Centisomes 40-42	Promotes membrane ruffling and disrupts tight junctions by acting as a GEF for Cdc42 (Stender et al., 2000); (Boyle et al., 2006)	1
SopB	SPI-5	Inositol polyphosphate phosphatase that promotes macropinocytosis, regulates SCV localization, and promotes fluid secretion (Norris et al., 1998); (Hernandez et al., 2004)	1
SopD	Centisome 64	Promotes invasion and fluid secretion (Zhang et al., 2002); (Raffatellu et al., 2005)	1/2
SspH1	Bacteriophage Gifsy-3	E3 ubiquitin ligase (Rytönen and Holden, 2007)	1/2
SpvC	pSLT	A phosphothreonine lyase required for complete virulence in murine models (Mazurkiewicz et al., 2008)	1/2

Table 1.1 continued

SpiC	SPI-2	Helps regulate T3SS-2 secretion (Yu et al., 2002)	2
SseF	SPI-2	SCV regulation (Abrahams et al., 2006)	2
SseG	SPI-2	SCV positioning (Abrahams et al., 2006)	2
SspH2	SPI-12	E3 ubiquitin ligase (Quezada et al., 2009)	2
PipB2	Centisome 60.3	Promotes Sif extension (Knodler and Steele-Mortimer, 2005)	2
SifA	potABCD operon	Sif formation and membrane integrity (Stein et al., 1996); (Beuzon et al., 2000)	2
SopD2	pflAB operon	Sif formation and promotes bacterial replication in mouse macrophages (Jiang et al., 2004)	2
SseJ	Bacteriophage	Negatively regulates Sifs and antagonizes SifA-mediated stability of SCV (Waterman and Holden, 2003); (Ruiz-Albert et al., 2002)	2
SpvB	pSLT	Depolymerizes actin filaments in vitro (Lesnick et al., 2001)	2

evolved to co-opt host signaling pathways.

In addition to its translocation functions, SipC binds to actin and plays an important role in membrane ruffling by promoting actin nucleation and bundling, which leads to increased actin polymerization (Hayward and Koronakis, 1999). Although SipA is not required for invasion (Kaniga et al., 1995a), it has been shown that SipA works cooperatively with SipC to modulate actin (McGhie et al., 2001). However, as SipA deficient strains of *Salmonella* do still induce some membrane ruffling (Higashide et al., 2002a), it is likely that the role of SipA in modulating actin dynamics is not critical, but rather auxiliary in nature.

Other effectors promote invasion by indirectly manipulating actin dynamics. As mentioned earlier, the effectors SopE and SopE2 function as GEFs that activate Cdc42 and Rac1 GTPases, and they are required for membrane ruffling and invasion (Hardt et al., 1998a); (Stender et al., 2000). In addition to regulating the localization of tight junction components as described earlier, Cdc42 and Rac1 are responsible for promoting actin assembly via activities that lead to Arp2/3-mediated actin nucleation and polymerization.

Once *Salmonellae* enter the cell, they reside in the SCV, which functions as a replicative niche that is critical to dissemination (Szeto et al., 2009). Here, functions attributed to the T3SS2 begin to dominate and promote stability of the SCV as well as intracellular growth. However, there are some functions attributed to T3SS1 effectors in early stages. For example, the T3SS1 effector SopB alters the lipid content of the SCV membrane and in turn excludes membrane markers

required for the fusion of phagosomes and lysosomes (Bakowski et al., 2010). In doing so, SopB is responsible for delaying the maturation of the SCV into a phagolysosome; thus SopB subverts normal cell processes that would otherwise lead to *Salmonella* killing by the host. Further, both SipA and SopB as well as T3SS2 effectors SifA, SseG, and SseF facilitate trafficking of the SCV near the Golgi network, which has been shown to promote bacterial replication within the SCV (Brawn et al., 2007); (Rodriguez-Escudero et al., 2011); (Salcedo and Holden, 2003).

To complete its life cycle, *Salmonellae* must escape from the host cell, which is mediated by the normal cell shedding process. Here, infected cells are extruded into the lumen and undergo an inflammatory cell death, releasing the once intracellular bacteria into the extracellular milieu (Knodler et al., 2010). Escape into the lumen enables the bacteria to invade other cells or to exit the host and participate in the transmission cycle.

1.4 *Salmonella* Exploits Inflammatory Responses

The key pathological hallmark of *Salmonella* invasion is an inflammatory response that is initiated upon recognition of pathogen associated molecular patterns (PAMPs) by an array of receptors. Toll-like receptors (TLRs) are key PAMP receptors, and are largely expressed on the basolateral surfaces of intestinal epithelial cells (Abreu, 2010), likely an adaptation to avoid chronic inflammation via recognition of gut microbiota. With regard to Gram-negative

bacteria, TLR4 is generally known to be a key trigger of immune responses via its interactions with LPS. However, TLR4 is not highly expressed in intestinal cells, and as a result these cells have been shown to have a low response to LPS (Abreu et al., 2002). However during *Salmonella* infection, TLR5 expressed at the basolateral surface detects flagellin molecules translocated across monolayers by the bacteria several minutes after infection, which leads to the production of IL-8 (Gewirtz et al., 2000). Internalized bacteria are detected by intracellular receptors including Nod1, which recognizes peptidoglycan from Gram negative bacteria, and Nod2, which recognizes peptidoglycan structures from both Gram negative and Gram positive bacteria. The absence of these receptors has been linked to reduced inflammatory responses and reduced cytokine production during *Salmonella* infection (Geddes et al., 2010).

Upon recognition of PAMMPs, intestinal epithelial cells and infected monocytes release an array of cytokines that trigger a series of events that induce inflammatory responses, including recruiting polymorphonuclear leukocytes (PMNs) to the site of infection. Inflammation appears to be dependent on an IL-18/IL-23 axis, which is stimulated upon the host recognition of *Salmonella*, and leads to the T-cell mediated amplification of cytokine signaling via the activation of IFN γ (activates macrophages), IL-22 (promotes anti-microbial responses at the epithelium), and IL-17 (promotes cytokine signaling leading to PMN recruitment) production (Godinez et al., 2008). Inflammatory signaling is further amplified as a result of the inflammatory death of extruded

infected intestinal cells; this process leads to the release of IL-18 at the apical surface (Knodler et al., 2010). The cumulative result of the signaling is the recruitment and activation of phagocytes and other effector cells of the immune system. Inflammatory signaling also induces the production and release of antimicrobial effector molecules such as lipocalin-2, which blocks iron uptake by enterobacteria; MUC4, which promotes growth of the mucus layer; and defensins and RegIII lectins, which target the bacterial cell membrane (Santos et al., 2009); (Mukherjee et al., 2014).

The inflammatory response is histopathologically characterized by the influx of PMNs, otherwise referred to as neutrophils. PMNs are phagocytic white blood cells among the first line of defense against pathogens in the early immune response. Pro-inflammatory cytokines released from infected cells induce a series of responses that lead to the recruitment of PMNs to the site of infection. These responses include vasodilation and reduced blood flow rate via TNF α , which promotes tethering of PMNs to the endothelium and “rolling” along the endothelium via loose binding with selectins. Upon activation by chemokines, such as IL-8, PMNs begin to express integrins, which bind tightly to ICAM adhesion molecules on the endothelial surface. Chemokines like IL-8 trigger calcium-dependent signaling pathways that cause integrins to be recycled from the rear of the cell to the front such that new attachments are continuously being formed at the leading edge, and permit migration in the direction of the chemokines. The production of IL-8 during *Salmonella* infection is mediated

largely by the detection of flagellin by TLR5. Some evidence indicates T3SS1 effectors or the apparatus itself can also elicit IL-8 production (Hobbie et al., 1997); (Hardt et al., 1998a), although other data indicates T3SS1 is dispensable for activation of this pathway (Gewirtz et al., 2000). TNF α from infected macrophages induces the expression of ICAMs. Via formation of the inflammasome, infected macrophages also produce IL-18, the effects of which are discussed above, and IL-1 β , which elevates body temperature and triggers the production of chemokines (Cromwell et al., 1992).

The tight binding mediated by the integrins allows the PMNs to extravasate through the endothelial wall of the blood vessels. Binding to IL-8 also activates PMNs and causes them to release metalloproteinases that facilitate their movement through the basement membrane and extracellular matrix after they escape the blood vessel. Once in the tissue, PMNs are then directed to the basolateral surface of the intestinal monolayer via *Salmonella*-mediated production of IL-8 gradients. PMNs are then further recruited to the site of infection at the apical surface of the intestinal epithelia via the concerted effort of multiple effectors. While the process of PMN transendothelial migration is generally well-understood, the mechanisms governing transepithelial migration are still under investigation. It is generally accepted that neutrophils pass through the transepithelial tight junctions via mechanical force facilitated by integrin interactions (Nash et al., 1988). However, while it is known that the integrin CD11b/CD18 plays a key role in this process, its epithelial receptor (or receptors)

has yet to be conclusively determined (Zen et al., 2004).

Upon activation, PMNs also undergo degranulation, a process that leads to the release of anti-microbial products stored in intracellular granules. This includes hydrogen peroxide-mediated reactive oxygen and nitrogen species (ROS and RNS, respectively), which oxidize lipids and lead to cell membrane damage. While these serve chiefly as a defense against pathogens, their effects are non-targeted and non-specific and thus result in collateral damage of healthy intestinal cells. Damaged cells subsequently release cytokines, thus contributing to the overall inflammatory response at the site of infection. Tissue damage at the monolayer has other impacts on *Salmonella* infection, as discussed below.

The recruitment of PMNs to the site of infection procures benefits and costs to both the bacteria and the host. Indeed, although inflammatory responses are typically thought of in the context of benefitting the host, mounting evidence indicates that *Salmonella* benefits from the inflammatory response, and that inducing an inflammatory response is key to its pathogenic strategy.

Host Benefits and Costs From PMN Migration to the Site of Infection

An obvious benefit to the host provided by the influx of PMNs is the control of *Salmonella* infection, as neutropenia has been shown to be a risk factor for systemic spread of *Salmonellae* (Noriega et al., 1994). However, the transepithelial migration of PMNs comes with costs to the host. In order to migrate from the basolateral surface to the apical surface, the PMNs must pass

through the tight intercellular spaces of the monolayer. This process is facilitated by the disruption of the tight junction complex as a consequence of the activities of SopE, SopE2, and SopB, and to some extent SipA. As discussed above, these effectors promote the redistribution of the tight junction components ZO-1 and occludin, and consequentially elicit increased permeability of intestinal cell monolayers (Boyle et al., 2006). With the loosening of the barrier, PMNs can more easily traverse the transepithelial space and reach the site of infection. Thus, although the concerted effort of T3SS effectors to loosen tight junctions benefits *Salmonellae* by promoting dissemination as discussed earlier, a consequence (to the bacteria) of barrier loss is that it promotes PMN entry to the site of infection. This serves as an example of how the host and *Salmonellae* are constantly evolving to subvert and adapt to each other.

However, the transepithelial migration of PMNs comes with costs to the host. The passage of PMNs through the intestinal epithelial monolayer damages the monolayer and causes it to become leaky and contributes to inflammation (Nash et al., 1987). Inflammation is also heightened by the release of damaging reactive oxygen species produced by PMNs in response to pathogens. Additionally, PMNs are inferred to contribute to the secretory diarrhea that occurs in response to *Salmonella* infection; studies have shown that 5'-AMP from PMNs is converted to adenosine at the epithelial cell surface, which is then bound by adenosine receptors at the apical membrane, leading to the synthesis of cAMP. Increased levels of cAMP lead to release of chloride ions into the lumen, which is

followed by a compensatory influx of fluid to accommodate the increased ion concentration (Madara et al., 1993). Further, PMNs may facilitate a diarrheal response as a result of the damage their migration causes to the epithelial barrier; studies with ligated ileal calf loops have shown that the visible appearance of fluid accumulation coincides with the first visible signs of barrier damage (Santos et al., 2001); (Zhang et al., 2003). While the diarrheal response can help clear *Salmonellae* from the gut, it can lead to dehydration if it persists.

Salmonellae Benefit From PMN Migration to the Site of Infection

While PMNs can phagocytose and kill *Salmonellae*, evidence suggests the bacteria may benefit from PMN influx. As discussed previously, the leaky epithelial barrier that results in part from PMN transmigration can promote *Salmonella* invasion into the subepithelial space, and promote its dissemination into the host (Kohler et al., 2007). Further, while the diarrheal response facilitated by PMNs may serve the host by helping to clear *Salmonellae* from the intestines, it also helps return *Salmonellae* to the environment, from which it can gain access to new hosts. Indeed, SopA, SopB, SopD, SopE2, and SipA have been shown to act together to induce diarrhea (Zhang et al., 2003), indicating that the induction of diarrhea is a key part of *Salmonella*'s pathogenic strategy.

Salmonella Benefits From Intestinal Inflammation

As indicated earlier, *Salmonellae* must outcompete the resident gut microbes in order to colonize the intestines. Mounting evidence in recent years indicates that *Salmonellae* are able to do so as a direct result of inducing inflammation.

This hypothesis was first ventured as a result of a study showing that inflammation promotes *Salmonella* colonization. In this study (Stecher et al., 2007), an avirulent *Salmonella* strain (lacking both T3SS1 and T3SS2) was able to overcome its colonization-deficiency in the presence of inflammatory conditions in mouse intestinal tracts. Ileocolitis was induced by co-infection with wild-type *Salmonella*, or by using infection-independent mouse models of colitis. In each of the three models, the avirulent *Salmonella* was able to overcome colonization resistance caused by the microbiota. Two follow up studies indicate that inflammatory responses help provide nutrients to *Salmonellae* and also reduce resident gut microbe populations.

Winter et al (2010) showed that the NADPH-dependent oxygen radicals released from phagocytes (i.e., respiratory burst) during the inflammatory response to infection is required to oxidize thiosulfate, which is normally present in the gut, to tetrathionate, which can be used by *Salmonellae* as an alternative terminal electron acceptor. This study also showed that a *Salmonella* strain unable to utilize tetrathionate has a markedly reduced ability to colonize intestines compared to wild-type *Salmonella*. Therefore, the ability of *Salmonella*

to use tetrathionate during active states of intestinal inflammation provides it with access to a unique resource that then enables *Salmonellae* to outcompete the resident microbes and colonize the host (Winter et al., 2010).

An additional study (Stelzer et al., 2011) shows that RegIII B, one of the antibacterial, membrane-damaging, lectins released in the intestinal mucosa as part of the inflammatory response to *Salmonella* infection, reduces resident microbe populations and enhances *Salmonella* colonization. This study confirmed that *Salmonella* is resistant to RegIII B killing. Together these observations support the striking hypothesis that *Salmonellae* possess mechanisms to purposely induce inflammatory responses. In doing so, *Salmonella* increases its overall fitness and thereby overcomes colonization resistance, which enables it to induce mechanisms that lead to its dissemination.

1.5 SipA As An Inflammatory Mediator

SipA Induces PMN Transmigration

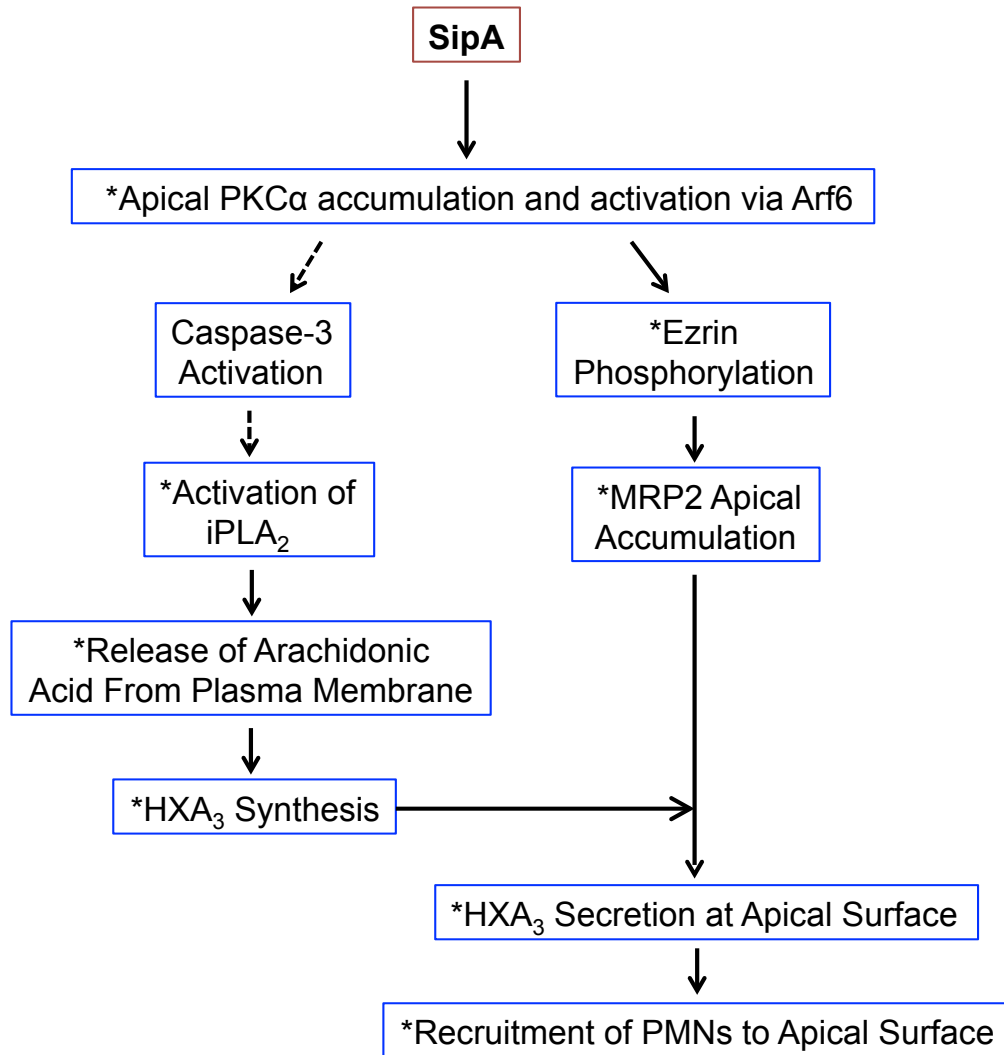
Collective data from several studies indicate that SipA is a key mediator of *Salmonella*-induced inflammation. Infection of monolayers with SipA-deficient *Salmonella* induces a marked reduction in PMN transmigration (Criss et al., 2001a). This observation is corroborated by *in vivo* studies showing the loss of SipA results in a dramatic reduction of inflammation and tissue damage (Zhang et al., 2002); (Wall et al., 2007). The mechanism by which SipA induces the movement of PMNs from the basolateral surface to the apical surface involves

the activation of a signaling pathway that leads to the production and secretion of the chemoattractant hepxilin A3 (HXA₃). HXA₃ is a metabolite of arachidonic acid from the 12-lipoxygenase (12-LOX) pathway, and is a neutrophil chemoattractant that is secreted vectorally from the apical surface of epithelial cells (Mrsny et al., 2004a).

SipA first triggers the apical membrane accumulation of protein kinase C- (PKC)- α (Silva et al., 2004b) via a pathway dependent on the GTPase Arf6 (Criss et al., 2001a). Movement of PKC α to the apical membrane leads to its activation, which is modeled to have two consequences. First, studies by other labs show that PKC α activation leads to activation of caspase-3 (Reyland et al., 2000), which can activate a phospholipase (PLA) isoform, iPLA₂ (Atsumi et al., 2000). Studies from our lab show that iPLA₂ is activated during *Salmonella* infection (Mumy et al., 2008b). The activation of iPLA₂ leads to the release of arachidonic acid from the cell membrane (Atsumi et al., 2000), which then enables it to be processed via the 12-LOX pathway into HXA₃. Further, the activation of PKC α also leads to the phosphorylation of the scaffolding protein ezrin, which we have shown facilitates the apical accumulation of the multidrug resistance-associated transporter-2 (MRP2) (Pazos et al., 2008); (Agbor et al., 2011b). MRP2 is an actin-binding cassette (ABC) transporter associated with resistance to anti-cancer drugs via its role as an efflux pump (Leslie et al., 2005). In the context of *Salmonella* infection, MRP2 facilitates the apical secretion of HXA₃ (Pazos et al., 2008). This pathway is summarized in Figure 1.2.

Figure 1.2 How SipA Triggers PMN Migration

The pathway by which SipA triggers PMN transepithelial migration to the apical surface. Asterisks indicate publications from the McCormick lab group (see main text). Solid lines indicate data collected as a result from *Salmonella* infection assays. Dashed lines indicate inferences supported by publications not involving work with *Salmonella*.

Figure 1.2 How SipA Triggers PMN Migration

Our studies indicated that SipA is a driving force of this pathway, as the absence of SipA blocks several key steps. SipA-deficient strains of *Salmonella* do not induce the movement of PKC α to the apical membrane (Silva et al., 2004b), and they also do not induce ezrin phosphorylation (Agbor et al., 2011b). Further, SipA-deficient strains do not induce the movement of MRP2 to the cell surface (Pazos et al., 2008), and they also induce less HXA₃ secretion (Pazos et al., 2008). Taken together, these data demonstrate that SipA is the key *Salmonella* regulator in this complex pathway that leads to PMN transepithelial migration to the apical surface of infected cells.

SipA Does Not Require Translocation via the T3SS1

While it is known SipA is an important inflammatory mediator, several key questions remain about how it functions. For example, studies describing how T3SSs work indicate that T3SS effectors require delivery into the host cell via the T3SS apparatus in order to impart their functions (Collazo and Galan, 1997); (Galan, 1996); (Hueck et al., 1995). However, while SipA is a T3SS effector, it is able to induce transepithelial migration without being translocated into host cells via the T3SS apparatus (Lee et al., 2000). Indeed, exogenous addition of SipA to the apical surface of intestinal monolayers in the absence of bacteria induces PMN migration in a dose-response manner (Lee et al., 2000), and also induces the pathway described above leading to the accumulation of MRP2 at the apical surface (Silva et al., 2004b). The ability of a T3SS effector to function in the

absence of T3SS-mediated translocation has only been reported one other time; the *Yersinia pseudotuberculosis* effector YopB induces inflammatory responses in the absence of a T3SS translocation channel into the host cell (Viboud et al., 2003).

That our understanding of how T3SSs work remains incomplete is further exemplified by the finding that T3SS effectors can be secreted through the flagellar secretion system. In order for flagella to be assembled, the flagellar basal body must form first in the bacterial cell membrane, from where it protrudes outward into the extracellular space. Flagella subunits are secreted from the basal body and then assemble into the flagellar apparatus. A study in 1999 showed that in the absence of the flagellar basal body in *Yersinia enterocolitica*, the virulence protein YplA, which functions as a phospholipase, was not secreted and no phospholipase activity was detected (Young et al., 1999). A later study confirmed that YplA can be secreted through *Y. enterocolitica* T3SSs and the flagellar secretion system (Young and Young, 2002).

Thus, while the paradigm indicates T3SS effectors require secretion and translocation via T3SS machinery, it is clear that there are exceptions to these rules that highlight how much remains unknown about how these systems function.

Caspase-3 Is Critical to SipA's Functions

As shown in Figure 1.2, there is a critical role for PKC α -mediated activation of caspase-3 in the SipA-driven inflammatory response during *Salmonella* infection. However, recent evidence from our lab indicates a requirement for activated caspase-3 prior to the activation of PKC α .

SipA is a bifunctional protein that has been shown to facilitate actin rearrangements during invasion and also inflammatory responses, as discussed previously. The C-terminus is necessary and sufficient for actin bundling (Myeni and Zhou, 2010). The inflammatory function of SipA has been mapped to its N-terminus, as truncated SipA lacking its C-terminus not only induces inflammation in murine models of *Salmonella* infection, but induces more inflammation than does the full-length SipA protein (Wall et al., 2007). The significance of this observation was made apparent by the later finding that SipA harbors a caspase-3 cleavage site, which separates the N-terminus from the C-terminus, and that cleavage by caspase-3 is required for SipA's inflammatory functions (Srikanth et al., 2010). It was also found that SipA promotes the activation of caspase-3 as well as caspase-3 secretion at the apical surface, suggesting SipA is proteolytically processed prior to entering the host cell (Srikanth et al., 2010). This hypothesis was substantiated by the recovery of cleaved SipA products from the apical media, but not from the infected host cell cytosol (Srikanth et al., 2010). Although caspase-3 activity is typically associated with pro-apoptotic signaling that leads to cell death, the increased activation of caspase-3 during

Salmonella infection occurred independently of apoptosis. This observation is in line with other reports indicating caspase-3 has roles in cell functions not related to apoptosis (Rosado et al., 2006), and that apoptosis of *Salmonella* infected cells does not occur for several hours post infection (Knodler et al., 2005).

Data from (Srikanth et al., 2010) further demonstrate that caspase-3 cleavage sites exist in other bacterial effectors, indicating this may be a common motif amongst these proteins. However, the mechanism by which SipA activates caspase-3 remains unknown. Indeed, it is perplexing that SipA is responsible for inducing caspase-3 activation while also requiring cleavage by caspase-3 to become fully functional.

The exact reason why *Salmonellae* would rely on caspase-3 activity to activate SipA remains unclear, but doing so likely benefits *Salmonellae* in at least two ways. One is that encoding one protein with two functions that can later be separated saves the bacteria the energy of having to encode and regulate two separate proteins. Indeed, the “terminal reassortment” theory proposes that bifunctional effectors were once distinct proteins that over time evolved into one protein (Stavrinides et al.). An additional benefit to relying on caspase-3 regulation is that SipA will only be active under the proper conditions, i.e., once *Salmonella* is present in the intestines. By relying on the host for this regulation, not only is *Salmonella* spared the energy of regulating individual SipA functions itself, but SipA activation is guaranteed to be timed with host invasion.

1.6 Thesis Summary: What Are the Host Factors that Promote the SipA-Caspase-3 Activation Process?

The combined observations from Wall et al, 2007 and Srikanth et al, 2010 indicate that caspase-3 activity is required prior to the PKC α -mediated activation of caspase-3, and thus indicate that SipA activates caspase-3 via an additional, but unknown, pathway. Given the important role of SipA in promoting inflammation during *Salmonella* infection, we sought to identify a host factor(s) that promotes SipA-mediated responses via caspase-3. The goals of this endeavor were to address how SipA can elicit inflammation from the apical surface, and to improve our understanding of the SipA-caspase-3 pathway. Identification of an additional host member key to the inflammatory responses to *Salmonella* infection has implications for novel therapeutic strategies, as well as broadens our general understanding of how *Salmonella* infection occurs and indeed how T3SS effectors impart their functions.

Initial experiments into this inquiry, as discussed in following chapters, indicated SipA binds to the host tetraspanning membrane protein PERP, and that infection with *Salmonella* alters PERP's cellular location. PERP (p53 effector related to PMP-22) is part of a class of membrane proteins that are involved in such functions as apoptosis regulation (Brancolini et al., 2000), myelin regulation (Naef and Suter, 1999), intercellular junctions (Lee et al., 2005), and protein-protein interactions (Wilson et al, 2002). PERP was first identified as a downstream effector of p53-induced apoptosis (Attardi et al., 2000), and has

since been found to promote epithelial barrier integrity due to its localization to desmosomes (Ihrie et al, 2005), regulate caspase activation (Davies et al., 2009), and to regulate tumorigenesis (Beaudry et al., 2010). PERP is also expressed in intestinal epithelial tissue (Franke et al., 2013b). To date, studies on PERP have been relegated largely to its functions in cancer and in epithelial barrier maintenance; thus exploration into functions for PERP during *Salmonella* infection has the prospect of uncovering a novel role for this protein, in addition to improving our understanding of how *Salmonella* co-opts pathways leading to caspase-3 activation to promote pathogenesis.

Specific aims of this study are outlined are as follows:

- 1) Determine the nature of the SipA and PERP Interaction
 - a. Does SipA bind to PERP during *Salmonella* pathogenesis?
 - b. Is PERP present on the apical surface of intestinal epithelial cells?
 - c. Does PERP bind to other T3SS effectors?

- 2) Does PERP regulate SipA-mediated host responses to *Salmonella* infection?
 - a. Is PERP required for PMN transepithelial migration?
 - b. Does PERP promote caspase-3 activation during *Salmonella* infection?

3) Determine How PERP Localization is Regulated During *Salmonella* Infection

- a. What strategies does *Salmonella* employ to modulate PERP localization?
- b. What *Salmonella* effectors regulate PERP localization?

Preface to Chapter II

The data within this chapter were published in the following paper
**PERP, a Host Tetraspanning Membrane Protein, is Required for
Salmonella-Induced Inflammation**

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Kristen Peters, Luminita Paraoan, James E. Casanova, Erik J. Boll and Beth A.
McCormick

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Description of Author Contributions

Kelly Hallstrom: Performed experiments culminating in results displayed in Figures 2.1B-C, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, S2.1, S2.2, S2.3, S2.4, and S2.5, and compilation of the manuscript.

C.V. Srikanth: Performed experiments culminating in results displayed in Figure 2.1A, initial yeast-2-hybrid screen, design of stable PERP knock down cells, and edited the manuscript.

Terence A. Agbor: Provided technical assistance with experiments represented by Figures 2.5A and 2.5B, assisted with design of stable and transient PERP knock down cells, and edited of the manuscript.

Christopher M. Dumont: Assisted C.V. Srikanth with yeast-2-hybrid experiments.

Kristen Peters: Collected microscopy data in Figures 2.6-7 and Figure S2.5, and edited the manuscript.

Luminita Paraoan: Provided technical expertise regarding PERP and edited the manuscript.

James E. Casanova: Provided reagents and technical expertise for experiment represented in Figure 2.1C, and general cell trafficking expertise.

Erik J. Boll: Completed reverse yeast-2-hybrid screen and edited the manuscript.

Beth A. McCormick: Provided technical expertise and edited the manuscript.

CHAPTER II

PERP, a Host Tetraspanning Membrane Protein, is Required for *Salmonella*-Induced Inflammation

2.1 Introduction

Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is one of several *Salmonella enterica* strains responsible for over one million cases of gastrointestinal salmonellosis in the United States each year. The pathological hallmark of *Salmonella*-elicited enteritis is extensive intestinal inflammation, characterized by a substantial polymorphonuclear leukocyte (PMN) infiltrate to the site of infection. While PMNs are integral to innate immunity, poorly controlled intestinal inflammation results in extensive tissue destruction, and in some instances, the formation of crypt abscesses. Such PMN recruitment is coordinated by the epithelial release of an array of proinflammatory cytokines, among which are two potent PMN chemoattractants, interleukin-8 (IL-8) and hepxilin A₃ (HXA₃). IL-8 is secreted basolaterally by epithelial cells in response to not only the bacterial product flagellin but also to a host of *Salmonella* type III secretion system (T3SS) effectors (e.g., SopE, SopB) that increase IL-8 gene expression via nuclear factor kappa B (NF-κB) ((Hobbie et al., 1997) (Hardt et al., 1998a)). The basolateral secretion of IL-8 establishes a stable haptotactic gradient across the lamina propria. This gradient serves to guide PMNs from the lamina propria to the subepithelium, but does not induce movement across the epithelium, as observed in both model epithelia (McCormick et al., 1993)

(McCormick et al., 1995) and a double transgenic mouse model with the ability to induce the expression of human IL-8 (Kucharzik et al., 2005).

Using an *in vitro* model of *S. Typhimurium* infection of human intestinal epithelial cells to study such inflammatory events occurring at the intestinal mucosa, we determined that subsequent PMN transit through the epithelial monolayer to the luminal surface (defined as PMN transepithelial migration) is directed by the eicosanoid HXA₃ (McCormick et al., 1998; Mrsny et al., 2004b). HXA₃ is a potent PMN chemoattractant that is secreted apically in response to the *Salmonella* T3SS effector protein, SipA (Lee et al., 2000; McCormick et al., 1998; Silva et al., 2004a). The key role that SipA plays in inducing epithelial responses resulting in the transepithelial migration of PMNs has also been substantiated using two distinct *in vivo* models of *Salmonella*-induced enteritis (Barthel et al., 2003a; Wall et al., 2007; Zhang et al., 2002). To date, the molecular mechanism underlying these cellular events has revealed that SipA activates a lipid signaling cascade that in turn activates protein kinase c (PKC)- α and 12-lipoxygenase (Lee et al, 2000) (Mumy et al, 2008) in a pathway dependent on ADP-ribosylation factor (ARF)-6 and phospholipase D (PLD) (Criss et al, 2001). These events ultimately lead to apical efflux of HXA₃ (Pazos et al., 2008); (Mrsny et al., 2004b); (Mumy et al., 2008a). HXA₃ is an arachidonic acid-derived hydroxy epoxide that forms a chemotactic gradient across the epithelial tight junction complex, which directs PMNs across the intestinal epithelium to the luminal surface (Mrsny et al., 2004b), the final step in PMN recruitment to the

mucosal lumen. Recently, we found that SipA also triggers the activation of caspase-3, and that caspase-3 activity is required for the SipA-mediated influx of PMNs during *Salmonella* infection (Srikanth et al, 2010).

While such studies have informed us of the nature of the signal transduction pathways induced by SipA that prompt PMN transepithelial migration, the way in which SipA initiates this complex cellular network remains undefined. Through both biochemical and genetic assessment, we have previously determined that T3SS-mediated translocation is not necessary for SipA to elicit inflammation (Lee et al., 2000), but that interaction of SipA at the apical surface of intestinal epithelial cells is sufficient to initiate the cellular events that lead to PMN transepithelial migration. Based on these observations, we hypothesize that SipA need not enter the epithelial cell cytosol to stimulate proinflammatory signal transduction pathways but rather may function extracellularly at the epithelial cell surface (Srikanth et al., 2010; Wall et al., 2007). This hypothesis is also consistent with the bi-functional properties of SipA, which promotes gastroenteritis via two distinct functional domains that activate inflammation and co-regulate mechanisms of bacterial entry by exploiting discreet extracellular and intracellular locations, respectively (Higashide et al., 2002b; Lilic et al., 2003; Wall et al., 2007; Zhou et al., 1999).

To test the hypothesis that SipA modulates a host protein at the apical surface that triggers the induction of PMN transepithelial migration, we used a yeast-two-hybrid (Y2H) strategy to screen a human colonic cDNA library, and

identified the tetraspanning membrane protein p53 Effector Related to PMP-22 (PERP) as a SipA binding partner. PERP was first identified as a p53 effector (Attardi et al., 2000), but has since been shown to play a role in development (Ihrie et al., 2005), caspase activation (Davies et al., 2009; Singaravelu et al., 2009), inflammation, and cancer (Beaudry et al., 2010; Paraoan et al., 2006). Herein, we describe the role of PERP in promoting the SipA-dependent inflammatory response to *S. Typhimurium* infection.

2.2 Materials and Methods

Tissue Culture

T84 or HCT8 polarized monolayers were grown on polycarbonate filters and used 6–8 days after plating. Inverted monolayers (Costar 3421) were used for PMN transmigration assays. Non-inverted monolayers (Costar 3421) were used for microscopy. For biotinylation, cells were seeded on transwells in 100 mm tissue culture dishes (Costar 3419). For co-immunoprecipitations and infection assays, cells were seeded on transwells in six-well plates (Costar 3412).

Use of bacterial strains

S. Typhimurium strains (SL1344, wild-type; EE663, SipA-deficient) were grown as previously described (Lee et al., 2000). One colony was grown in a shaking culture of LB for 6 hours at 37 C. The culture was diluted 1:1000 and grown overnight as a standing culture at 37 C. Morning cultures were then pelleted and washed once with HBSS+, then re-suspended in HBSS+ at 10x the

concentration of the overnight culture prior to use. SipB-deficient ($\Delta SipB$) *S.* Typhimurium was grown in the same manner as the SipA-deficient strain (Wall et al, 2007). pSipA (SipA complemented, expresses HA-tagged SipA), AJK63 (SipA complemented, expresses high level of HA-tagged SipA), and CSM (SipA complement expressing SipA caspase-3 site mutant on pBH plasmid) were grown in the presence of 50ug/mL ampicillin. Unless otherwise indicated, cells were infected at an MOI of 100:1 for one hour. The pET3a-GST plasmid containing the GST-tagged C-terminus of SipC (Nichols and Casanova, 2010) was transformed into BL21 cells and maintained in the presence of 50ug/mL ampicillin.

In vitro Infection

HCT8 cell monolayers were infected apically with SL1344 or left non-infected in HBSS+ for 1 hour. The cells were then washed with HBSS+ to remove bacteria and lysed. Lysates were prepared in whole cell lysis buffer (150mM NaCl; 25mM Tris, pH 8; 1mM EDTA; 1% NP-40; 5mM Na₃VO₄, 20mM NaF, 0.8mM PMSF, and protease inhibitor cocktail). Homogenized lysate supernatants were normalized via Bradford Assay, boiled in loading dye supplemented with β -mercaptoethanol, separated by SDS-PAGE, and immunoblotted for the desired proteins. GAPDH (Millipore) was used as a loading control where indicated.

PERP siRNA Construct Design

The pSUPER vector (Oligoengine) was used to generate a PERP siRNA construct following methods in (Brummelkamp et al., 2002). Oligonucleotides contained a specific human PERP sequence (GI: 222080101: 184-765), its reverse complement (in italics) separated by a short spacer region, and *BglIII* or *HindIII* restriction sites. PERPKO_F1GATCCCC AAGATGACCTTCTGGGCAA TTCAAGAGA TTGCCCAGAAGGTCATCTT TTTTGGAAA and PERPKO_R1 AGCTTTTCCAAAAA AAGATGACCTTCTGGGCAA TCTCTTGAA TTGCCCAGAAGGTCATCTT GGG and for a random control sequence, 5'-GATCCCCCGACAAGCTTGAATTTATTTCAAGAGAATAAATTCAAGCTTGTC GGTTTTTGGAAA-3' and 5' AGCTTTTCCAAAAACCGACAAGCTTGAATTTA TTCTCTTGAAATAAATTCAAGCTTGTCTGGGGG-3'.

Transfection of HCT8 intestinal epithelial cells

For stable PERP knockdowns, HCT8 cells were transfected with the modified pSUPER using Lipofectamine 2000 (Invitrogen) per manufacturer's instructions in RPMI 1640 without serum (Invitrogen), incubated in RPMI with 8% v/v FBS then passaged into fresh media with neomycin-G418 (Sigma-Aldrich). Cells underwent two additional cycles of growth/passage in G418 prior to use. For transient PERP knockdowns, siRNA against human PERP and a non-targeting pool were obtained from Dharmacon. HCT8's were transfected with 20nM siRNA using Lipofectamine 2000 in OptiMem serum-free media for 24 hours.

Split Ubiquitin Yeast-Two-Hybrid Screen

The Dual-hunter split ubiquitin yeast-2-hybrid kit was used per manufacturer's instructions (Dualsystems Biotech AG). Coding DNA for SipA was cloned into the BAIT plasmid (pDHB1) and transformed into yeast reporter strain NMY51. A human colonic cDNA library (Dualsystems Biotech AG) was transformed into the bait-expressing yeast per manufacturer's protocols. For the reverse yeast-2 hybrid assay, the coding DNA of PERP was cloned into pDHB1 while SipA was cloned into the PREY plasmid (pPR3-N).

HA-Tagged SipA Isolation

An overnight culture of AJK63 (*Salmonella* Typhimurium SL1344 expressing HA-tagged SipA) was back-diluted then centrifuged at 6000 rpm. The supernatant was passed through an Amicon Centrifugal Filter Unit (Millipore UFC900324). We kept the concentrated volume left in the top chamber and added one tablet of Protease Inhibitor Complete Mini (Roche). We then prepped the HA column by adding 0.5mL of HA-Affinity matrix (Roche), and equilibrated per manufacturer instructions. The sample was then run through the column, followed by washing. Finally, the HA-tagged protein was eluted with 1mg/mL of HA-peptide. Samples were analyzed for concentration and stored at -80 C.

Immunoprecipitations

Normalized lysates from T84 cells infected with AJK63 or Δ SifA/SifA-pBH (*Salmonella* Typhimurium SL1344 expressing HA-tagged SifA) were immunoprecipitated for HA-SipA or HA-SifA, respectfully, using Protein A/G Agarose Plus beads (Santa Cruz) and anti-HA or IgG isotype control antibodies (Abcam). The presence of PERP was determined via western blot following cell lysis procedures as described above.

The SipC-PERP pulldowns were performed in accordance with Nichols and Casanova, 2010, with minor modifications. An over night culture of BL21 *E. coli* expressing the pET3a-GST plasmid containing the GST-tagged C-terminus of SipC was centrifuged at 6,000 RPM. The pellet was resuspended in lysis buffer (25mM Tris, 3mM DTT, 1mM PMSF), sonicated, and centrifuged at 14,000 RPM at 4 C for 1 hour. The cleared supernatants were then incubated with Glutathione sepharose 4B affinity matrix beads (GE Healthcare) prepared according to manufacturer instructions for 2 hours at room temperature. Whole cell lysates from HCT8 cells were then incubated with the SipC-GST-bound beads over night at 4C with end-over-end rotation. After washing steps with 1x PBS, the GST-SipC protein complexes were eluted with reduced glutathione. The eluates were then diluted in 4X tricine loading dye, boiled, and examined via western blot for the presence of SipC-GST (not shown) and PERP.

Biotinylation

Apical cell surface biotinylation was performed using the protocol described by (Agbor et al., 2011a). Following 1 hour infection, the apical surface of HCT8 monolayers was labeled with biotin (Thermo Scientific) at 4C. Labeling of the basolateral surface was blocked with acetate (Thermo Scientific). The cells were then lysed, homogenized, normalized for protein concentration via the Bradford assay, and incubated with streptavidin beads overnight at 4C in order to pull down apically-labeled proteins. After washing, the beads were then boiled to release bound biotinylated apical proteins. The apical protein-enriched samples were then immunoblotted for PERP (Santa Cruz SC-67184). The level of PERP expression was quantified via densitometric analyses using FIJI. Data are displayed as a ratio of PERP expression from cells following infection with a mutant OR left non-infected compared to the level of PERP from cells following infection with SL1344 (WT *Salmonella* Typhimurium). PERP expression following SL1344 infection was set to 1 for these analyses. Densitometric graphs displayed are of three representative experiments showing reproducible trends. The raw data from these analyses are shown in section C.1.

For brefeldin A experiments, cells were exposed to 150uM brefeldin A in HBSS+ for 1 hour prior to infection (Lippincott-Schwartz et al., 1990); (Caumont et al., 2000). The brefeldin A was then removed, and the cells were washed once prior to infection. For cycloheximide experiments, cells were exposed to 2mg/mL of

cycloheximide in HBSS+ prior to infection, a concentration previously shown to block protein synthesis in HCT8 cells (Thorpe et al., 1999). The cycloheximide was then removed, and the cells were washed once prior to infection. Blots displayed are of one representative experiment showing reproducible trends.

PMN Transepithelial Migration Assays

Stable PERP Knockdowns:

PMN migration assays were carried out as described (McCormick et al., 1993) using p11 (PERP knockdown), and p24 (vector control) monolayers. Briefly, inverted monolayers were infected with SL1344 for 1 hour at the apical surface, or left non-infected in HBSS+ buffer. Freshly isolated human polymorphonuclear leukocytes (PMNs) were then added to the basolateral surface and allowed to migrate to the apical surface over two hours. Where indicated, fMLP served as a transmigration stimulus in the absence of infection. The number of migrated PMNs was quantified via a colorimetric assay using myeloperoxidase as an indicator of the presence of PMNs. Assays were performed in triplicate and are presented as one replicate showing reproducible trends across at least three experiments.

Antibody Blocking:

PERP blocking was performed according to (Zen et al., 2004) with some modifications. HCT8 cells were infected at the apical surface with SL1344 for 40

minutes. After washing, 25ug/mL of anti-PERP (Santa Cruz), IgG control (Abcam), or the mitochondrial marker MTCO-1 (Abcam) were added to the basolateral surface for 30 minutes prior to addition of PMNs and maintained during migration. Where indicated, fMLP, IL-8, or LTB4 was used to induce migration in the absence of infection. The number of migrated PMNs was quantified via a colorimetric assay using myeloperoxidase as an indicator of the presence of PMNs. Assays were performed in triplicate and are presented as one replicate showing reproducible trends across three experiments.

Activated caspase-3 Assay

Following transient PERP knockdown, cells were infected for two hours then lysed and analyzed for caspase-3 activity via the BioVision colorimetric caspase-3 activity assay per manufacturer's instructions (BioVision; Srikanth et al, 2010). Data is displayed as a percent increase in activated caspase-3 levels following infection compared to levels at basal state, and shows values averaged across three experiments.

Fluorescent Wide Field Microscopy

For examination of PERP apical accumulation, T84 monolayers were grown on permeable filters and infected with SL1344, EE633, CSM, pSipA, treated exogenously with HA-tagged SipA, or left in HBSS+ buffer (non-infected) for one hour. The filters were washed in 1% PBS, fixed with 1% paraformaldehyde in

PBS for 15 minutes, quenched with NH_4Cl in PBS for 15 minutes, then permeabilized in 0.1% Triton in PBS for 5 minutes, with washing steps in between. The filters were then blocked with 5% NGS in PBS for 1 hour, followed by staining with primary antibody against PERP overnight at 4C. The filters were stained the next day with secondary Alexa Fluor 488 (Life Technologies), Alexa Fluor 568 (Life Technologies) and phalloidin Alexa Fluor 647 (Life Technologies) for 1 hour at room temperature in the dark. Filters were then mounted using SlowFade Gold with DAPI and maintained in the dark at 4C.

Immunofluorescence samples were imaged using a Nikon Ti-E wide field fluorescent microscope (Nikon Instruments, Melville, NY) with a 60X objective using a Photometrics QuantEM wide field camera at room temperature. Widefield Z-stacks were taken with 0.4 μm Z slices using the filter pores to differentiate the basolateral from the apical surface of the monolayer. The entire monolayer was imaged in this manner, with at least 5 random distinct areas imaged for each sample. Images were acquired with the Nikon Elements SW version 4.13 software. Quantification of the PERP staining pattern was done with Z volume projections processed using the Nikon Elements SW version 4.13 software, encompassing the whole monolayer. Phalloidin staining was used to determine cell number. The level of PERP staining was quantified using FIJI to count punctae in each image as in (Ruck et al., 2011); (Chen et al., 2006), using four images per condition. Data displayed indicate average number of punctae per imaged field for each condition in a representative experiment. To better

determine apical or basolateral location of the punctae, a line was added to the Z projections during processing to bisect the Z volume of the monolayer.

Designation of PERP staining in an apical compartment is in accordance with other reports showing staining of apical compartments (Ivanov et al., 2004); (Cario et al., 2002).

Confocal Microscopy

Mouse Colon Tissue:

Sections of the proximal colons from 6 week old C57BL/6 mice were removed and snap frozen in OCT media, then cut into 5mm sections on glass slides.

Sections were fixed in 4% PFA, quenched with 50mM ammonium chloride, then permeabilized with 0.5% triton X in PBS. Sections were then blocked with blocking buffer (5% normal goat serum in PBS) for one hour at room temperature, followed by overnight incubation with anti-PERP antibody (Abcam 5986) in blocking buffer at 4C. The next day, the sections were washed with blocking buffer, then incubated with secondary Alexa Fluor 488 (Life Technologies) at room temperature for one hour. Sections were then washed in blocking buffer, mounted with SlowFade Gold with DAPI, and viewed under a Leica TCS SP-5 Confocal microscope (Leica Microsystems, Buffalo Grove, IL) using a 40x oil objective with 1x digital zoom (Leica LASAF Software, Leica Microsystems, Buffalo Grove, IL). All samples were imaged as 0.2um Z stacks. Images were processed using FIJI (NIH, Bethesda, MD). Animals were treated in

accordance with institutional IACUC protocols. The image shown is of one 0.2um Z stack taken from one of 4 proximal colon samples.

PERP and Rab25 Colocalization:

Polarized T84 monolayers were infected with SL1344 for 1 hour and stained as described above for PERP with the exception that 0.2% saponin in NGS was used to permeabilize in place of Triton-x to help preserve endosome structures. Determination of PERP colocalization with Rab25 (AB32004) was performed using a Leica TCS SP-5 Confocal microscope (Leica Microsystems, Buffalo Grove, IL) using a 63x oil objective with 6x digital zoom (Leica LASAF Software, Leica Microsystems, Buffalo Grove, IL). For increased resolution of PERP localization, the pinhole was decreased to 0.5 airy units (AU) for all imaging and all samples were imaged as 0.2mm Z stacks. Images shown are representative of 3 images taken from random fields per sample. Post-imaging, images were processed using FIJI (NIH, Bethesda, MD) with single 0.2 mm slices selected from the quarter most apical sections to show colocalization of Rab25 (AlexaFluor 568, red pseudocolor) and PERP (AlexaFluor 488, green pseudocolor), along with F-actin (phalloidin AlexaFluor 647, blue pseudocolor) to show cellular structure. The level of colocalization was determined with Manders' coefficient analyses in FIJI. Images and Manders' coefficients displayed are a representative set from one of three experiments.

Data Analysis

Data presentation is described for each experiment above. Where indicated, p values were calculated using the Student's t-test, and values of <0.05 were considered statistically significant. In cases where datasets contained more than two groups, one-way ANOVA analyses were performed first, followed by individual Student's t-test analyses to determine which treatment groups differed from the control. Error bars represent standard error.

2.3 Results

PERP is a Binding Partner of SipA

Previous studies have identified the *S. Typhimurium* effector SipA as an important mediator of the immune inflammatory response that results in PMN influx. The fact that our prior studies found purified SipA to directly activate this response has prompted us to consider whether SipA might engage a receptor at the host cell surface (Lee et al., 2000). Since we infer that this putative receptor represents the initiation site of the transcellular PMN signaling cascade, identification of a functional receptor will be crucial for understanding SipA's involvement in controlling intestinal inflammation. We used a split ubiquitin based Y2H (protein-protein interaction) analysis system (Dualsystems Biotech) (Dirnberger et al., 2008) (Stagljar et al., 1998), with full length SipA as bait and a human colonic cDNA-based library as prey. Approximately 4×10^6 transformants were screened and selected based on two growth reporters. Candidate interacting partners were then selected using a Lac-Z based colorimetric reporter

assay. The screen yielded seven positive clones out of which PERP was represented three times (Table 2.1). Using the Lac-Z reporter assay, we confirmed the PERP-SipA interaction in a reverse Y2H assay in which SipA was sub-cloned into the prey vector, and PERP of the initial screen was used as bait. Furthermore, we used a biochemical approach to demonstrate PERP-SipA interactions. Model human colonic epithelial cells were infected with a wild type *S. Typhimurium* strain expressing an HA-tagged SipA protein (AJK63). Immunoprecipitation of infected cell lysates with an anti-HA antibody specifically resulted in the pull-down of PERP, as immunoprecipitation with an isotype control IgG antibody yielded neither HA-SipA nor PERP (Figure 2.1A). We also performed the pull down with another *S. Typhimurium* T3SS effector, SifA, also tagged with HA to ensure our observation was not due to non-specific recognition of the HA-tag by the PERP antibody (Figure 2.1B).

Since this data supports our contention that PERP is a SipA binding partner, we next examined the specificity of the PERP-SipA interaction by testing whether PERP binds to the *Salmonella* protein SipC, a component of the T3SS1 translocon. SipC is not only required for the translocation of *Salmonella* effectors into the host cell (Collazo and Galan, 1997) and for *Salmonella* invasion (Myeni and Zhou, 2010), but also SipC and SipA are known to have cooperative roles during invasion (McGhie et al., 2001). As shown in Figure 2.1C, passage of HCT8 lysates across beads bound to the GST-labeled C-terminus of SipC (Nichols and Casanova, 2010) resulted in the specific pull down of PERP,

Table 2.1: SipA Interacting Partner Candidates

Five potential SipA-binding candidates were identified from our yeast-two-hybrid screen. Most candidates have been identified as membrane proteins with various functions pertaining to cell stress and death regulation. Out of these candidates, PERP was the only one to be pulled out multiple times from our screen.

Table 2.1: SipA Interacting Partner Candidates

SipA Interacting Partner Candidates Identified Via Y2H Screen			
Clone	Gene Name	Function	Reference
1.	SERP1	ER Stress response	Yamaguchi et al, 1999
2.	DERP2	cell death regulation	Oka et al, 2008
3.	TMEM87	Unknown	
4.	TMEM147	Interacts with nicalin-NOMO complex	Dettmer et al, 2010
5.	PERP*	p53 effector, regulates Caspase-3 activation	Attardi et al, 2000; Davies et al, 2009

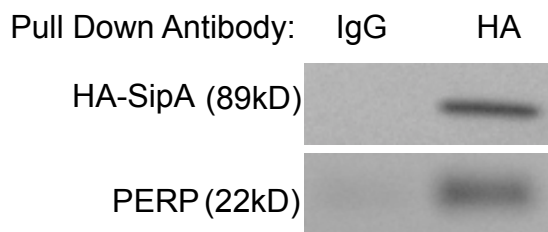
*Multiple hits

Figure 2.1: SipA and PERP are Binding Partners

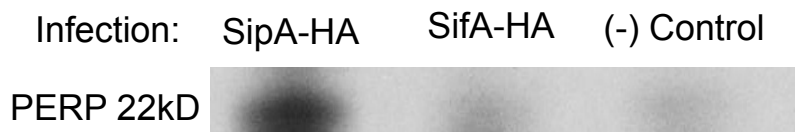
A. T84 lysates infected with an HA-tagged SipA containing strain of *S. Typhimurium* were pulled down with IgG or anti-HA antibody, and then probed for PERP. Only the HA-SipA pull down samples resulted in a PERP band. B. The specificity of the PERP-SipA interaction was confirmed by a pull down of HA-tagged SipA and HA-tagged SifA. Cells were infected with *S. Typhimurium* expressing HA-tagged SipA or HA-tagged SifA, or left non-infected as a non-specific control ((-) control) and lysed. Lysates were pulled down with anti-HA antibody and probed for PERP. The SipA-HA lysates resulted in a PERP band, while the SifA-HA lysates resulted only in a faint band of background intensity. C. Passage of HCT8 lysates through glutathione beads conjugated to the GST-tagged c-terminus of SipC (“conjugated”) resulted in the specific pull down of PERP (“eluted”), as a passage of the lysates through non-conjugated beads fails to result in the pull down of PERP.

Figure 2.1: SipA and PERP Are Binding Partners

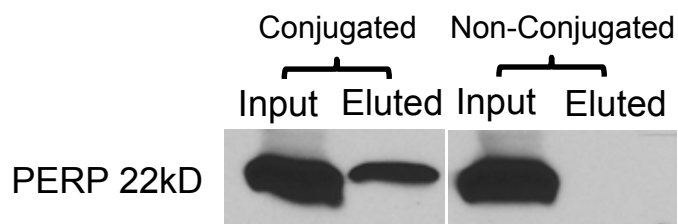
A



B



C



suggesting that PERP is able to interact with two *Salmonella* proteins that function during early stages of *Salmonella* pathogenesis and that PERP may have a role mediating these events. However, the precise mechanism(s) remain unknown.

Functional Roles of PERP in the Promotion of the Inflammatory Response to Salmonella Infection

PERP is a tetraspanning membrane protein that belongs to the PMP-22(Gas3)/EMP family (Attardi et al., 2000), which includes PMP-22 and the epithelial membrane proteins (EMP) 1, 2, and 3. Detection of PERP as an interacting partner with SipA piqued our interest given that PERP has been documented to regulate inflammatory signaling pathways (Beaudry et al., 2010), as well as to regulate the activation of caspase-3 (Singaravelu et al., 2009) (Davies et al., 2009). Since we have previously shown the *Salmonella* effector, SipA, induces inflammatory pathways that lead to the recruitment of PMNs to the site of infection, we sought to determine the extent to which PERP might also be involved in governing these processes during infection with *S. Typhimurium* using our *in vitro* PMN migration assay (see Materials and Methods). Following infection, polarized colonic cell monolayers were exposed to 25ug/mL of anti-PERP antibody, anti-MTCO-1 antibody (mitochondrial marker – used as an irrelevant isotype control) or IgG isotype control antibody prior to adding freshly isolated human peripheral blood PMNs.

As shown in Figure 2.2A, the presence of anti-PERP antibody decreased the ability of *S. Typhimurium* to induce PMN transepithelial migration by 90%. This result was specific to exposure with the PERP antibody, as treatment with control antibodies MTCO-1 (mitochondrial marker) and IgG (isotype control) did not similarly inhibit *Salmonella*-induced PMN transmigration. Addition of the PERP antibody in the absence of a chemoattractant signal has no impact on PMN transmigration (Supplemental Figure 2.1).

As a complementary approach, we performed PMN transepithelial migration assays using PERP siRNA knockdown cells (p11) paired with an siRNA vector-control (p24). PMN transepithelial migration across the PERP knockdown monolayers in response to *S. Typhimurium* infection was reduced by 40% as compared to the vector control cells (Figure 2.2B). Although these studies suggest that PERP is involved in facilitating PMN transmigration in response to *Salmonella* infection, PERP might also play a role in other intestinal inflammatory conditions beyond that of *Salmonella* infection where PMN migration is a key pathological feature.

We modeled such conditions *in vitro* via addition of formyl-Methionyl-Leucyl-Phenylalanine (fMLP), a PMN chemoattractant, to our polarized monolayers in the absence of infection. fMLP is regularly used as a positive control for PMN migration, and mimics formylated peptides from bacteria. As shown in Figure 2.3, PMN transepithelial migration in response to an imposed gradient of fMLP across cells treated with the PERP antibody (Figure 2.3A),

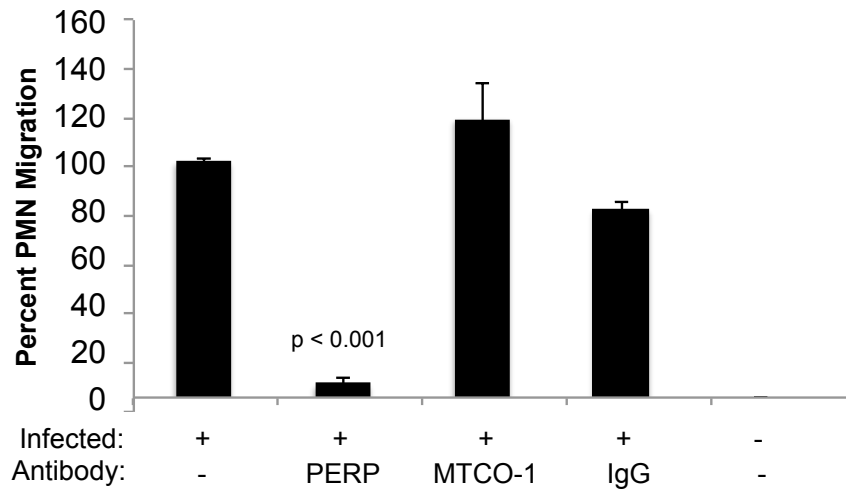
Figure 2.2: PERP Promotes the Inflammatory Response to *Salmonella***Infection**

A. Polarized HCT8 cells were infected with wild-type *S. Typhimurium* and then exposed to 25ug/mL of PERP, MTCO-1 (mitochondrial marker, control), or IgG (control) antibodies at the basolateral surface or left in HBSS+ buffer prior to addition of PMNs at the basolateral surface. Values are expressed as the percentage of PMN transmigration compared to PMN transmigration across infected cells not treated with antibodies (set to 100%) from one of three representative experiments performed in triplicate. B. Lines of stable PERP knockdown cells and control cells were generated via transfection of PERP siRNA (inset). Values are expressed as the percentage of PMN transmigration compared to PMN transmigration across infected vector control cells (set to 100%) from one of three representative experiments performed in triplicate. The PERP knockdown cells showed no defect in barrier function compared to the vector control cells (data not shown). Error bars represent \pm standard errors and p values less than 0.05 according to Student's T-test were considered statistically significant.

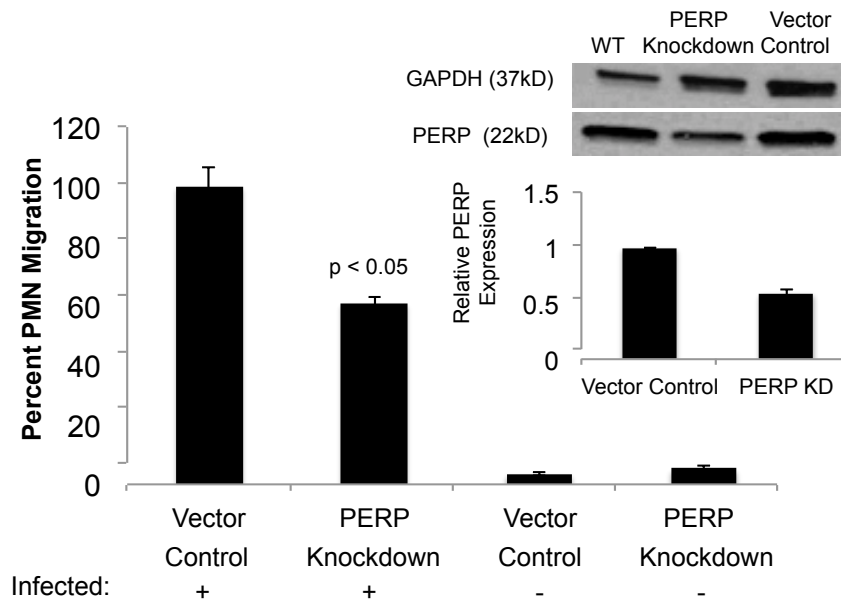
Figure 2.2: PERP Promotes the Inflammatory Response to *Salmonella*

Infection

A



B



or across the PERP knockdown cells (Figure 2.3B) was reduced approximately 90% and 35%, respectively.

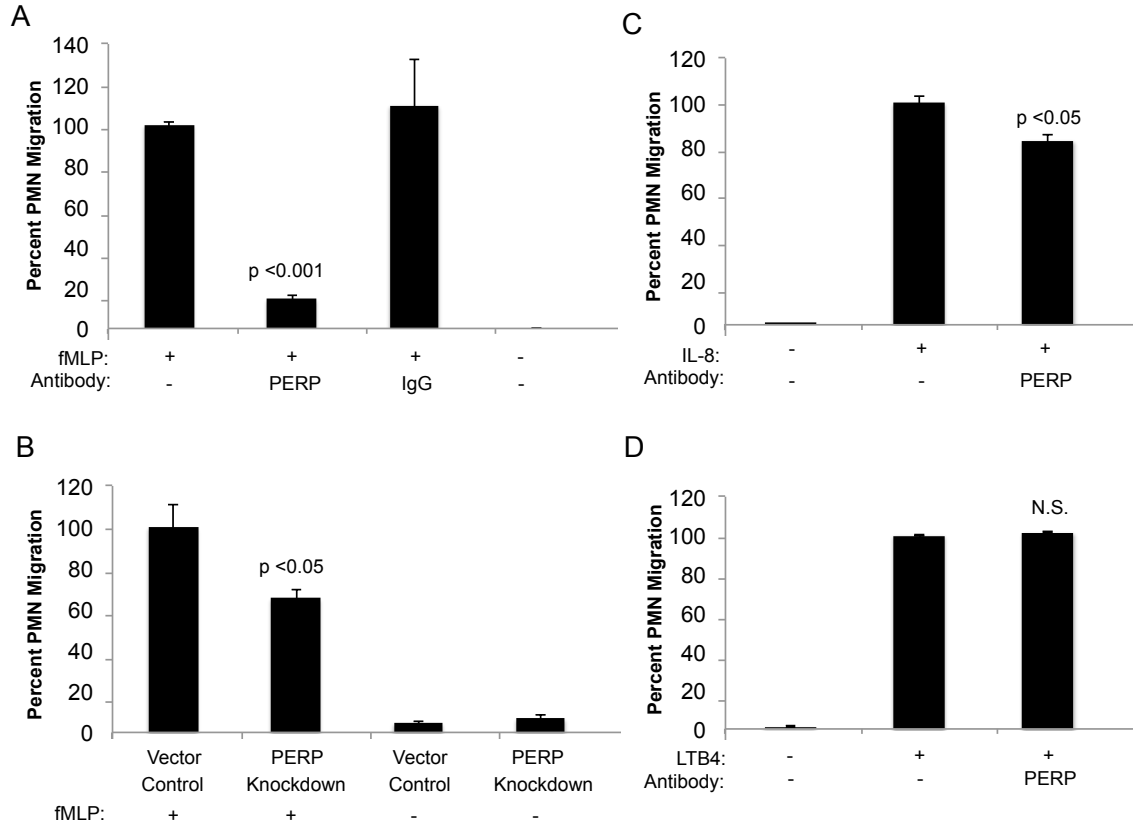
We also probed the function of PERP during PMN transmigration in response to other chemoattractants known to be secreted by intestinal epithelial cells, such as IL-8 and leukotriene B₄ (LTB₄). We found that PERP-antibody treatment of HCT8 monolayers prior to inducing PMN transmigration through imposed gradients of IL-8 or LTB₄ at the apical surface (see Experimental Procedures for details) resulted in a modest, though statistically significant inhibitory impact on IL-8-induced migration (Figure 2.3C), but not on LTB₄-induced migration (Figure 2.3D). Together, these results indicate PERP has a broad, though not universal, role in regulating PMN migration.

The *Salmonella* effector protein, SipA, promotes gastroenteritis via two distinct functional motifs that trigger not only inflammation but also mechanisms of bacterial entry (Wall et al., 2007). Moreover, we also recently found that during infection of colonic epithelial cells, SipA is responsible for the activation of caspase-3 (Srikanth et al., 2010). This enzyme is essential for SipA cleavage at a specific recognition motif, dividing the protein into its two functional domains (Srikanth et al., 2010). Such studies further revealed that cleavage of the SipA caspase-3 motif is central for promoting proinflammatory responses, and that caspase-3 is required during pathogenesis given that *Salmonella* is less virulent in caspase-3 knockout (caspase-3^{-/-}) mice (Srikanth et al., 2010). Since prior studies have indicated that increased levels of PERP lead to caspase-3

Figure 2.3: PERP Promotes PMN Migration

Migration was stimulated by the addition of formyl-methionyl-leucyl-phenylalanine (fMLP), IL-8, or LTB₄. Presence of the PERP antibody (A) specifically reduced fMLP –induced PMN migration by about 90%. Similarly, migration across PERP-knockdown monolayers (B) was reduced by about 35%. Presence of the PERP antibody also reduced IL-8-induced migration by about 20% (C), though had no impact on migration induced by LTB₄ (D). Values are expressed as the percentage of PMN transmigration compared to PMN transmigration across cells treated with chemoattractant but not with PERP antibody (set to 100%) from one of three representative experiments performed in triplicate. Error bars show standard error. p values less than 0.05 according to Student's T-test were considered statistically significant.

Figure 2.3: PERP Promotes PMN Migration



activation (Davies et al., 2009), we next sought to determine the extent to which PERP plays a role in *Salmonella*-induced activation of caspase-3.

Using a colorimetric caspase-3 bioactivity assay kit, we evaluated the extent to which PERP regulates caspase-3 activation in an HCT8 line of transient PERP-knockdown cells (Figure 2.4A) in the presence of *S. Typhimurium* infection. We observed an increase of 86.7% +/- 5.3 (standard error, $p < 0.05$) in the level of *S. Typhimurium* -induced activated caspase-3 in the vector control cells compared to only a 57.8% +/- 4.5 (standard error) increase in *S. Typhimurium* -induced activated caspase-3 in the PERP knockdown cells (Figure 2.4B). Since the partial knockdown of PERP resulted in about a 30% decrease in the ability of *S. Typhimurium* to induce caspase-3, these results indicate that PERP is necessary but not sufficient for caspase-3 activation during *Salmonella* infection.

PERP Accumulates at the Apical Surface in a SipA-Dependent Manner

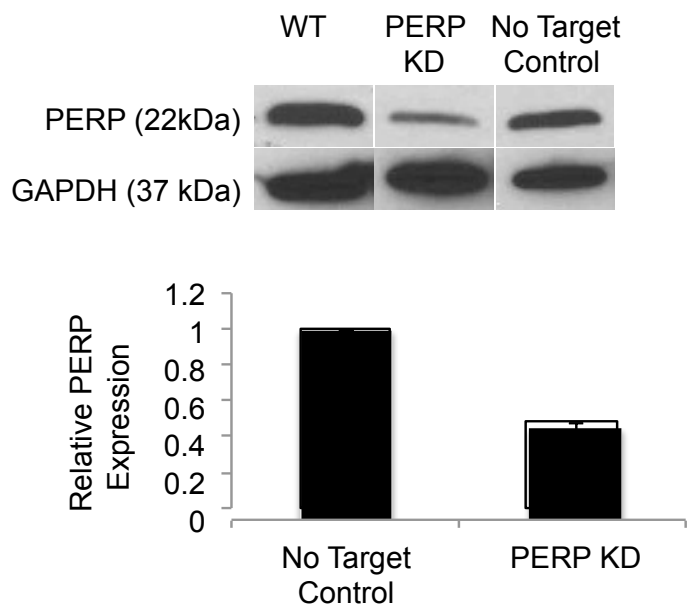
Thus far, our observations show that during infection with *S. Typhimurium*, PERP not only plays a crucial role in governing PMN recruitment but is also involved with the activation of caspase-3. PERP, as a tetraspanning membrane protein, has also been shown to localize to desmosomes and to promote epithelial barrier integrity (Ihrle et al., 2005). More recently, PERP was shown to localize to peri- and interdesmosomal regions termed “tessellate junctions” in

Figure 2.4: PERP Promotes Caspase-3 Activity During *Salmonella* Infection

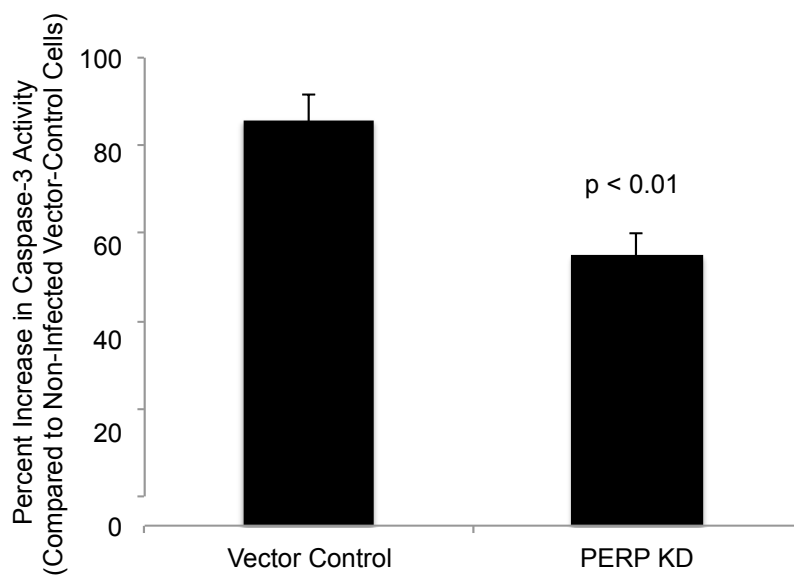
A. PERP was transiently knocked down in HCT8 cells. A no-targeting control was used to confirm specificity. By this method, PERP was reproducibly knocked down by about 50%. B. Levels of activated caspase-3 in response to *S.* Typhimurium infection were reduced by about 30% in the PERP-knockdown cells. Numbers are expressed as percent of activated caspase-3 relative to activated caspase-3 levels in non-infected, vector control cells across three experiments performed in triplicate. Error bars show \pm standard error. *p* values less than 0.05 according to Student's T-test were considered statistically significant.

Figure 2.4: PERP Promotes Caspase-3 Activity During *Salmonella* Infection

A



B



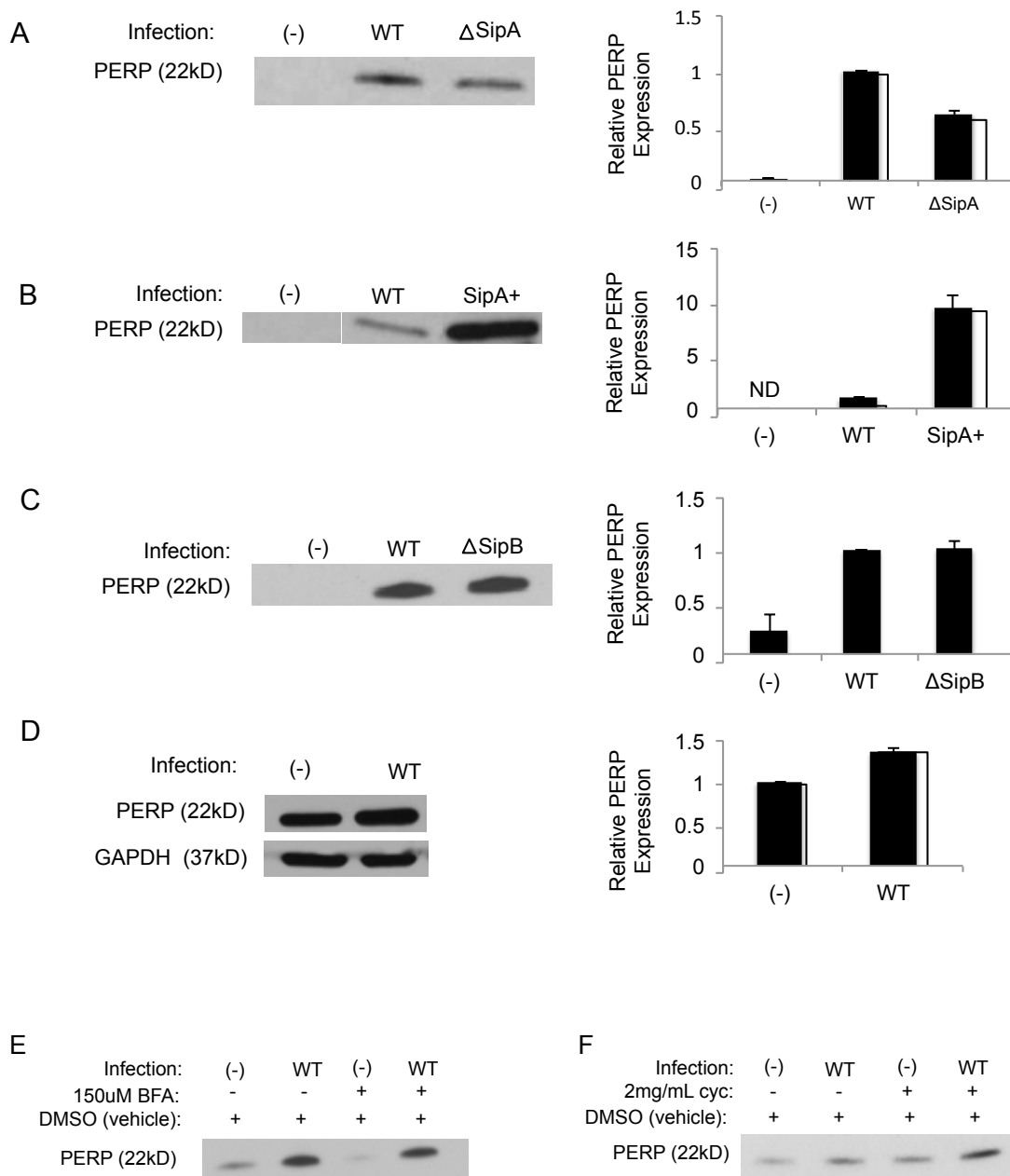
stratified epithelia, as well as to desmosomes in bovine intestinal epithelium (Franke et al., 2013a). Consistent with this, we also observed PERP expression on the mucosal surface of mouse proximal colon tissue (Supplemental Figure 2.2).

Since aberrant localization of transmembrane proteins is linked to numerous human diseases, we examined whether PERP is redistributed during infection with *S. Typhimurium*. The apical surface of polarized cell monolayers was selectively biotinylated following infection with wild type *S. Typhimurium* or mock infection with buffer. This method permits us to identify changes in protein expression specifically at the apical surface in response to *S. Typhimurium* infection. As shown in Figure 2.5, PERP accumulates at the apical surface of polarized colonic epithelial cells in response to wild-type infection. Moreover, the involvement of SipA in the accumulation of PERP at the apical surface is evidenced by infection with the isogenic SipA-deficient strain, which results in less PERP accumulation at this location compared to wild type infection (Figure 2.5A). By contrast, infection with a SipA-complemented strain correlated with greater PERP accumulation at the apical surface as compared to the wild type strain (Figure 2.5B). These results indicate SipA is necessary, though likely not sufficient under normal infection conditions, to induce PERP accumulation at the apical surface during *S. Typhimurium* infection. The accumulation of PERP at the apical surface also appears to be a directed cellular event since a similar

Figure 2.5: PERP Accumulates at the Apical Cell Surface In a SipA-dependent Manner

Polarized HCT8 monolayers were infected with wild-type (WT), SipA-deficient (Δ SipA), SipA-complemented (SipA+), or SipB-deficient (Δ SipB) *S. Typhimurium*, or left uninfected (-) in HBSS+ for one hour, and the apical cell surfaces were biotinylated, pulled down with streptavidin, and Western blotted for PERP (A-C). D. Whole cell lysates from non-infected and WT-infected HCT8s were probed for overall PERP expression. GAPDH serves as a loading control. Densitometry confirms a minor increase in PERP expression in response to wild-type infection. E. Cells were treated with 150uM of brefeldin A (BFA) for one hour or left untreated in HBSS+ plus DMSO (vehicle control) for one hour prior to infection. PERP expression at the apical surface was examined as explained for panels A-C. F. Cells were treated with 2mg/mL of cycloheximide (cyc) for one hour or left untreated in HBSS+ plus DMSO (vehicle control) for one hour prior to infection. PERP expression at the apical surface was examined as explained for Figures A-C. While it is noted that the basal level of PERP in Figure 5E is comparatively higher than the basal level of PERP in Figure 5A-C, we interpret this difference as normal variation seen when using different stocks of cultured HCT8 cell lines. Regardless of this observed difference, we are able to consistently reproduce results showing PERP accumulates at the apical surface in response to *S. Typhimurium* infection. Densitometry analyses show the change in PERP expression induced by infection with mutant or complemented strains compared to the change in PERP expression induced in WT-infected cells. The level of PERP in WT infected cells was set to 1 for biotinylations; for Figure D, the level of PERP in non-infected cells was set to 1. Densitometry graphs are of three representative experiments showing reproducible trends. Statistics for densitometry across experiments can be found in Table C.1. "ND" means none detected. Error bars show +/- standard error.

Figure 2.5: PERP Accumulates at the Apical Cell Surface In a SipA-dependent Manner



assessment of PERP distribution to the basolateral surface showed only a minute increase (Supplemental Figure 2.3A).

Our previous results demonstrated that purified *S. Typhimurium* SipA protein could trigger the PMN migration response in the absence of the type III secretion and translocation factors, such as SipB and SipC, suggesting that this effector does not need to be translocated via the T3SS to initiate the events that lead to PMN transepithelial migration (Lee et al., 2000). To examine whether PERP also accumulates to the apical surface in the absence of bacterially-translocated SipA, we took a genetic approach using an isogenic $\Delta sipB$ non-polar deletion mutant. This strain expresses native SipA from the chromosomal *sipA* locus and is capable of secreting effector proteins, but cannot translocate them into the host cell cytosol (Wall et al., 2007). Moreover, we have previously reported on the secretion profile of SipA from the $\Delta sipB$ non-polar deletion strain, confirming that this strain secretes identical amounts of SipA compared to the parent wild type *S. Typhimurium* strain (SL1344) (Wall et al., 2007). As shown in Figure 2.5C and consistent with our prior studies (Lee et al., 2000) (Wall et al., 2007); (Srikanth et al., 2010) infection with the $\Delta sipB$ non-polar deletion mutant failed to disrupt *S. Typhimurium*-mediated PERP localization to the apical surface. Thus, these observations provide important genetic-based evidence to further substantiate our contention that SipA does not need to be bacterially translocated into the epithelial cell cytosol but rather acts extracellularly to induce host cell responses, including PERP accumulation at the apical surface.

Because *Salmonella* enters host cells by a mechanism characterized by membrane ruffling and actin cytoskeleton rearrangements at sites of invasion, we further confirmed that our observation of PERP localizing to the apical surface was not simply due to leakage of biotin through to intercellular junctions. To control for this possibility, we evaluated the adherens junction protein, E-cadherin, during infection with *S. Typhimurium*. As shown in Supplemental Figure 2.3B, we failed to detect biotin labeling of E-cadherin in response to wild type *S. Typhimurium* infection, again demonstrating the specific detection of apically-located PERP.

Lastly, to determine whether our observations were due to redistribution of PERP or the result of an overall increase in protein expression in response to *S. Typhimurium* infection, we examined the total level of PERP expression in non-infected cells compared to wild-type infected cells. As shown in Figure 2.5D we detected a modest increase in PERP protein expression in response to *S. Typhimurium* infection. Such an increase is minute compared to the prominent increase in PERP protein expression found at the apical epithelial surface in response to *S. Typhimurium* infection, indicating that the apical accumulation of PERP is due to its redistribution to the apical surface rather than to an increase in overall PERP expression. To confirm accumulation of PERP at the apical surface of *S. Typhimurium* -infected cells is not due to new protein synthesis, we performed the cell surface biotinylation experiments with the addition of brefeldin A, a drug known to block the anterograde transport of proteins from the

endoplasmic reticulum to the Golgi apparatus. If the apical increase of PERP during *Salmonella* infection is due to the delivery of newly synthesized PERP to this location, we would expect treatment with brefeldin A to block this response. As shown in Figure 2.5E, treatment with brefeldin A reduces the baseline amount of PERP at the apical surface compared to cells not treated with the drug, as would be expected; however, despite brefeldin A treatment, we still observed a considerable increase in PERP expression at the apical surface in response to infection. This observation was further confirmed by the failure of treatment with cycloheximide, which prevents new protein synthesis, to block apical accumulation of PERP in response to infection (Figure 2.5F). Taken together, these results indicate that new protein synthesis does not explain the apical accumulation of PERP. Rather, we propose that *S. Typhimurium* triggers apical PERP accumulation by redistributing it to the apical surface.

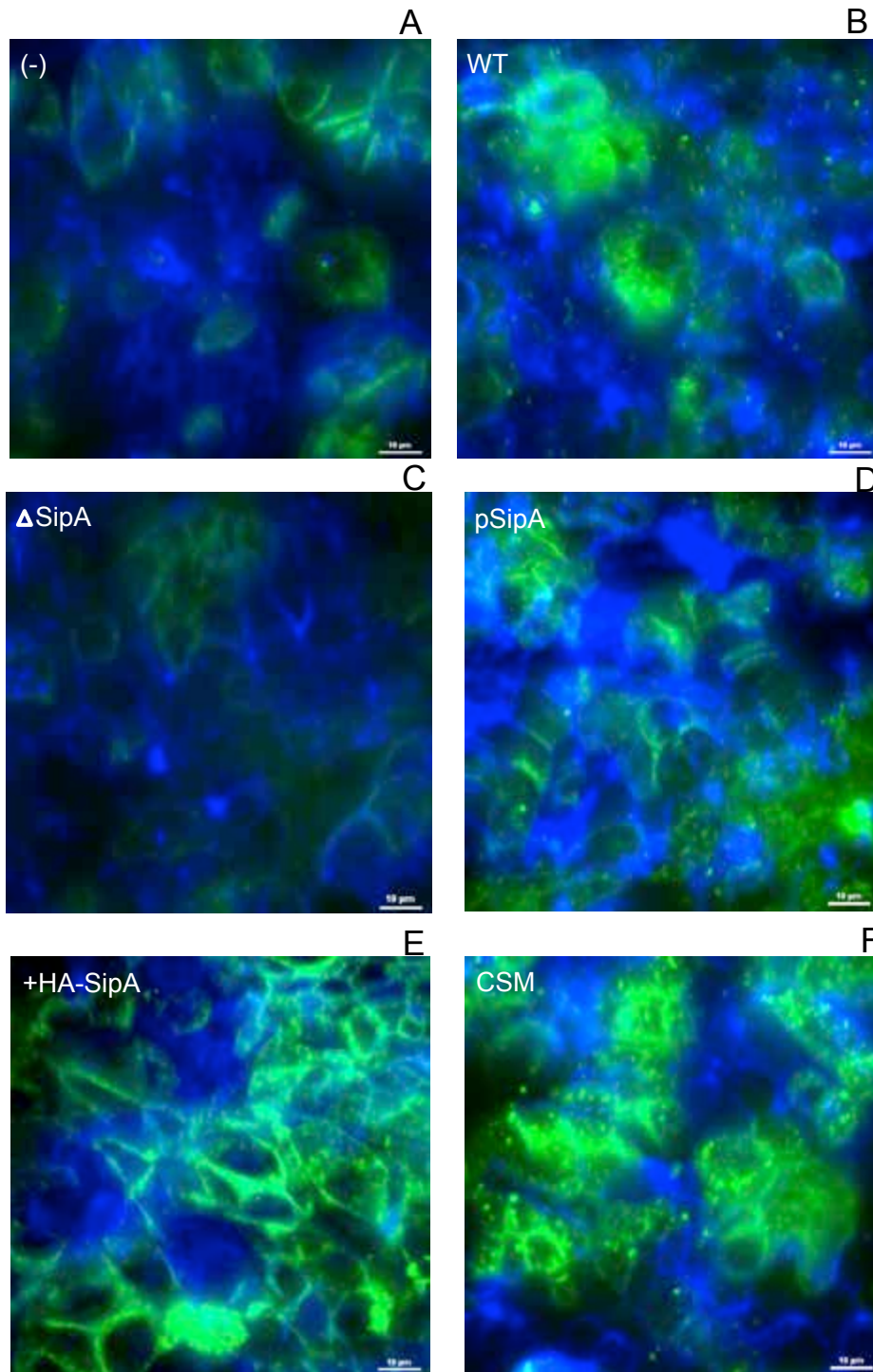
Mechanism Governing PERP Localization

We have begun to examine the molecular mechanism governing the apical accumulation of PERP in response to *S. Typhimurium* infection. Using wide-field fluorescent microscopy, we observed that PERP exhibits a mostly punctate staining pattern in response to *Salmonella* infection (Figure 2.6A-B). This pattern is consistent with staining patterns of proteins found localized to membrane-bound vesicles (Wang et al, 2000). Given that PERP is a membrane protein and that it has been previously found to localize to the secretory pathway,

Figure 2.6: PERP Accumulates in the Apical Compartment In Response to *Salmonella* Infection

T84 monolayers were treated with (A) buffer only (-), or infected with (B) wild type *S. Typhimurium* (WT), (C) SipA-deficient *S. Typhimurium* (Δ SipA), (D) SipA-complemented *S. Typhimurium* (pSipA), (E) treated apically with 20ug/mL of HA-tagged SipA (Lee et al, 2000) (+HA-SipA), or (F) *S. Typhimurium* expressing a caspase-3 site mutant SipA in the Δ SipA background (CSM). Cells were stained with an antibody against PERP followed by secondary conjugated to Alexa Fluor 488 (green), and with phalloidin conjugated to Alexa Fluor 647 (projected blue). The volume plots imaged at 60x magnification show PERP located at the apical compartment, and that PERP exhibits a punctate staining pattern. The staining was quantified via FIJI (Supplemental Figure 2.4). There is more PERP in the apical compartment in response to WT infection and CSM infection compared to buffer-only non-infected cells. The PERP level in response to infection with the SipA-deficient strain is comparable to what we see in the non-infected cells, which is rescued by infection with the SipA-complemented strain. Exogenous treatment with HA-tagged SipA also induces PERP accumulation into the apical compartment as seen with WT *S. Typhimurium* infection. Bar represents 10um. Images are taken from one of three representative experiments showing reproducible trends.

Figure 2.6: PERP Accumulates in the Apical Compartment in Response to *Salmonella* Infection

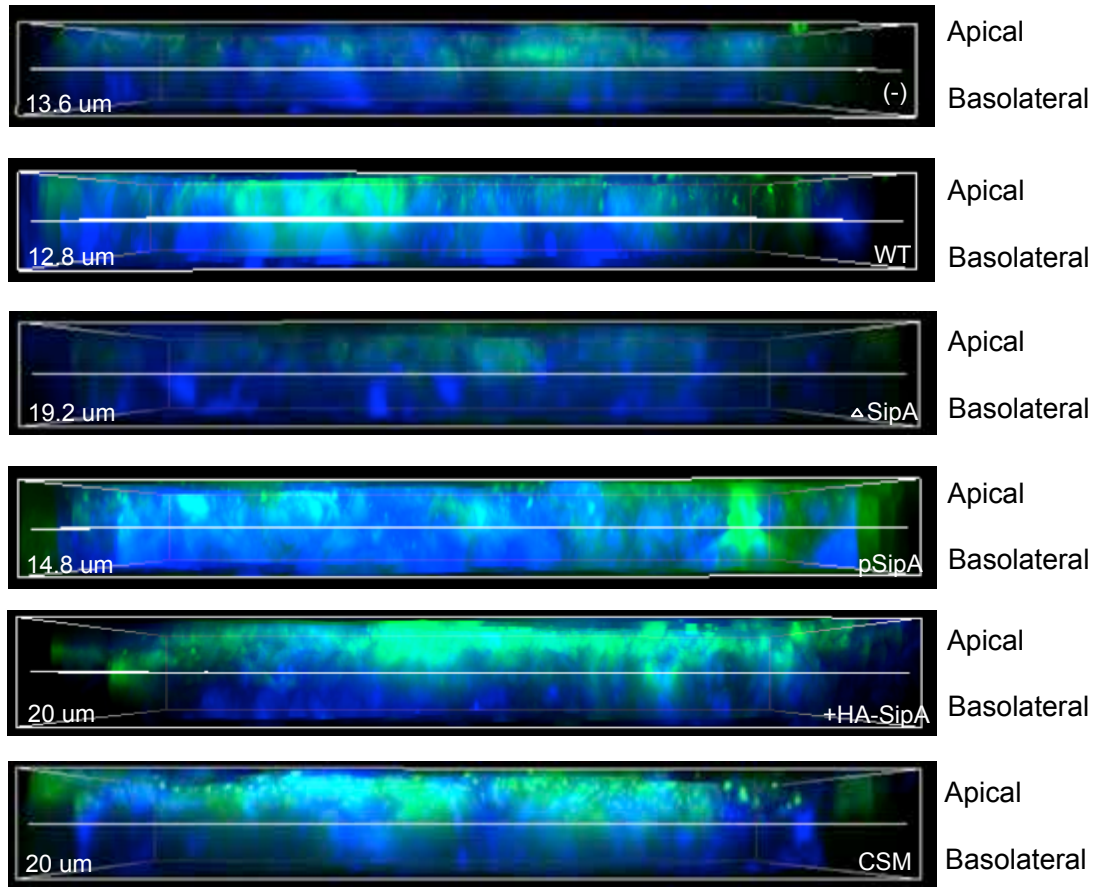


we would expect it to be found in membrane-bound vesicles. The amount of PERP punctae was quantified with the FIJI software (Supplemental Figure 2.4), and found to be significantly increased in response to *S. Typhimurium* infection compared to non-infected cells. The PERP punctae were consistently found to be located in the apical compartment of the cells (Figure 2.7), providing further evidence that *S. Typhimurium* -induced PERP redistribution occurs at the apical surface (Figure 2.5). Additionally, the increase in PERP appeared to be at least in part dependent on SipA, as cells infected with the isogenic SipA-mutant strain showed less PERP staining, a level similar to that seen in the non-infected cells (Figure 2.6C). Further, infection with the SipA complemented strain rescued the PERP staining pattern (Figure 2.6D). These data corroborate our previous observations in Figure 2.5 that show a role for SipA in *S. Typhimurium* -induced PERP accumulation at the apical surface. Exogenous addition of purified HA-tagged SipA at concentrations previously shown to trigger PMN migration to the same degree as wild-type *S. Typhimurium* infection (Lee et al, 2000) also induced apical PERP accumulation (Figure 2.6E and Supplemental Figure 2.4), further indicating that extracellular SipA is capable of triggering PERP redistribution.

It has been documented that increased levels of PERP lead to caspase-3 activation (Davies et al., 2009). Because of the finding that SipA plays a role in the redistribution of PERP during *S. Typhimurium* infection, and since we previously showed the proinflammatory function of SipA requires the activation of

Figure 2.7: PERP Punctae are Apically Located

T84 cells were treated as indicated in Figure 2.6. The side view of the monolayer volume plots show the punctate staining is mostly apical (above the bisecting Z-plane line). The μm values at the top left of the images indicate the thickness of the respective monolayer. The location of punctae were found to be apical across all samples. Green color indicates PERP staining, and blue color indicates phalloidin staining.

Figure 2.7: PERP Punctae are Apically Located

caspase-3, we next investigated the extent to which SipA processing by caspase-3 is necessary to induce the redistribution and accumulation of PERP to the apical surface. We therefore infected polarized colonic epithelial cell monolayers with an isogenic *S. Typhimurium* Δ SipA strain complemented with SipA in which the caspase-3 recognition motif was changed in the key aspartic acid at position four to alanine (DEVD \rightarrow DEVA; termed caspase site mutant: csm-SipA), rendering SipA insensitive to caspase-3 cleavage (Srikanth et al, 2010). As shown in Figure 2.6F, we found that the CSM-SipA strain induced a PERP punctate staining pattern comparable to that of wild-type *Salmonella* infection. This result suggests that SipA does not depend on caspase-3 cleavage to alter PERP localization, and indicates SipA is able to promote PERP redistribution prior to being cleaved by caspase-3. These observations build upon our initial report of the role of caspase-3 activity during *Salmonella* infection providing new insight into the point at which specific events in *Salmonella* infection are required to promote pathogenesis. Further, they provide the first mechanistic insight into how SipA promotes caspase-3 activation; we propose that SipA-mediated PERP redistribution to the apical surface causes PERP to accumulate at this location, which subsequently leads to PERP-mediated activation of caspase-3.

2.4 Discussion

PERP is a tetraspanning membrane protein that belongs to the PMP-22(Gas3)/EMP family (Attardi et al., 2000). Although PERP was first reported to be a downstream effector of p53 (Attardi et al., 2000) more recent studies have found PERP to play a critical role not only in maintaining epithelial barrier integrity (Ihrie et al., 2005), but also in regulating genes involved in inflammation (Beaudry et al., 2010). In the current study, we now identify a new role for PERP in the pathogenesis of the enteric pathogen *S. Typhimurium*. Of particular interest, we show that PERP associates with the *S. Typhimurium* T3SE SipA and regulates PMN transmigration during infection.

Precisely how SipA initially interacts with PERP remains to be determined and our current efforts are focused on understanding the biochemistry of the SipA-PERP interactions, including the domains responsible. Nevertheless, some inferences can be made based on our findings. One possibility is that PERP becomes part of a protein complex at the plasma membrane. We reason this to be the case since in addition to PERP, we also identified four other potential SipA binding partners that were less represented in the yeast-two hybrid screen (Table 2.1). Tetraspanning proteins are well documented to complex with other tetraspanins, integrins, immunoglobulin proteins, signaling enzymes, or co-receptors to impart a variety of functions (reviewed in (Hemler, 2001; Maecker et al., 1997). That we also see SipC binding to PERP suggests PERP may facilitate the initial interactions of T3SS1 effectors with the apical surface. However,

further work validating the physiological relevance of SipA and SipC binding to PERP is required before we can begin to understand why the binding occurs.

That our observed functions of PERP during *S. Typhimurium* infection appear to be consistent with the reported activities of SipA in triggering intestinal inflammation characterized by PMN transepithelial migration raises the question of whether SipA subverts PERP functional activities. The fact that PERP is involved in facilitating PMN transmigration in response to *S. Typhimurium* infection is moreover consistent with previous studies showing that PERP regulates the expression of various inflammation-associated gene products (Beaudry et al., 2010). Among these is Chi3L1, which is expressed in inflamed mucosa, particularly in Crohn's disease and ulcerative colitis patients, and appears to promote bacterial adhesion to colonic epithelial cells (Mizoguchi, 2006). PERP was also found to regulate Ccl20 (or MIP-3-alpha), which is expressed in intestinal epithelia associated with Peyer's Patches and aids in the attraction of natural killer cells, memory T cells, and immature dendritic cells to the site of inflammation (Hoover et al., 2002). Moreover, we have also found PERP to be increased in both a murine model of *Salmonella* colitis as well as in a dextran sodium sulfate chemically-induced colitis; in the former, infection with the SipA mutant strain resulted in PERP expression levels that were similar to background control levels (unpublished observations; Hallstrom and McCormick).

The molecular mechanism by which PERP supports PMN transmigration is still under investigation. We are exploring the possibilities that PERP interacts

with a ligand or receptor on the surface of PMNs in order to enable their transmigration to the apical surface, or activates (or de-activates) signaling pathways that promote PMN transmigration (Chin et al., 2008). Unpublished observations from our lab have also shown that PERP is able to bind to itself and may be expressed on PMNs. Since PERP is known to localize to desmosomes, this raises the interesting possibility that PERP could facilitate PMN migration by promoting PMN interactions with junctional proteins expressed by intestinal epithelial cells. Such activity, if confirmed, would indicate PERP could have a significant role in other intestinal inflammatory conditions beyond that of *Salmonella* infection where PMN migration is a key pathological feature. Given the different requirements for PERP in PMN migration (Figures 2.2 and 2.3), we propose PERP's roles in mediating PMN migration may depend on the specific trigger. Our proposal for how PERP mediates PMN migration in the context of *S. Typhimurium* infection is presented in the model at the end of this section.

Our data also indicate that PERP regulates caspase-3 activation during *S. Typhimurium* infection (Figure 2.4). This observation is consistent with our previous studies where we identified that caspase-3-dependent processing of type III secreted effectors plays an important role in *Salmonella* pathogenesis (Srikanth et al, 2010). Of note, the SipA effector itself was found to be necessary and sufficient to promote activation of caspase-3 (Srikanth et al., 2010) in a process independent of the apoptotic cascades. Given that prior studies have shown that PERP is linked to the activation of caspase-3 (Davies et al., 2009),

we postulate that SipA-induced caspase-3 activity occurs through a PERP-dependent pathway. Indeed, in addition to showing PERP is required for *Salmonella*-induced PERP accumulation at the apical surface, our data also shows that the SipA caspase-3 cleavage site is dispensable for PERP accumulation at the apical surface (Figure 2.6F), indicating that caspase-3 cleavage of SipA and the subsequent inflammatory events mediated by cleaved SipA (Srikanth et al., 2010) occur after PERP redistributes to the apical surface.

It is evident that infection with *S. Typhimurium* prompts the accumulation of PERP to the apical surface and one mechanism that may account for the redistribution of PERP is subversion of its trafficking pathway to the apical surface. This is supported by our data herein that show blocking new protein synthesis or blocking the delivery of newly synthesized proteins to the Golgi apparatus is not sufficient to block *S. Typhimurium*-mediated PERP accumulation at the apical surface. In polarized cells, the apical recycling pathway is a key mode of regulating trafficking to the cell surface. The endosome recycling pathway has long been known to facilitate the shuttling of proteins, including junctional proteins (Lock and Stow, 2005), back and forth from intracellular to membrane locations, and plays a fundamental role in maintaining cellular polarity (reviewed in (Golachowska et al., 2010b; Perret et al., 2005)). Further, endosomal pathways are well-known to be involved and perturbed in the response to *Salmonella* infection (Dukes et al., 2006a); (Brawn et al., 2007); (Bakowski et al., 2007). In addition to finding that the PERP staining pattern

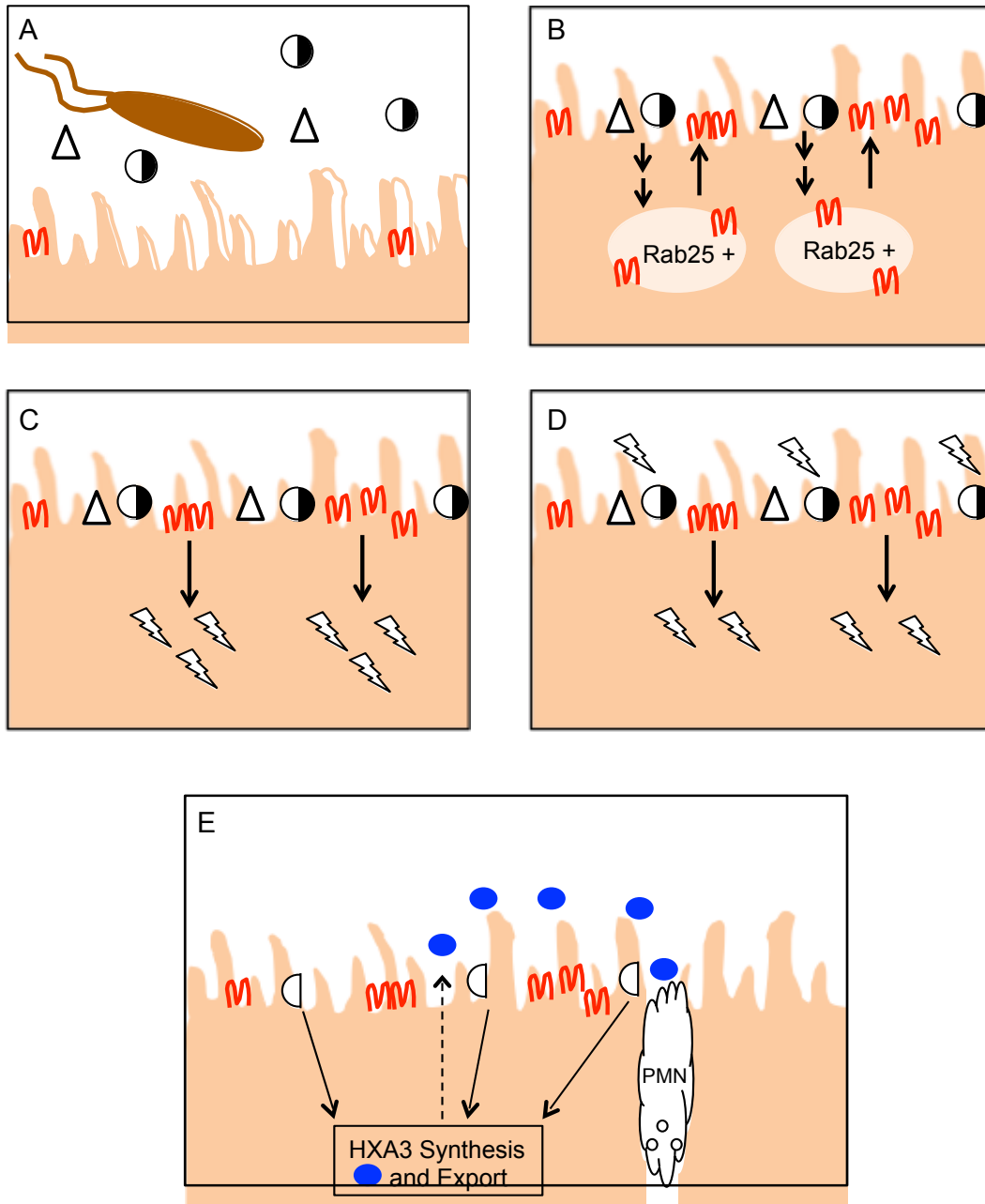
indicates it is in membrane-bound vesicles, we also observed PERP to co-localize with the apical recycling endosome marker Rab25 (Supplemental Figure 2.5), inviting speculation that *Salmonella* perturbs the cellular trafficking of PERP through a pathway involving the endosome recycling system. This hypothesis is supported by our previous studies, which demonstrate a requirement for ARF6 in *S. Typhimurium*-induced PMN transepithelial migration and localization of this small GTPase to the apical site of bacterial entry (Criss et al., 2001b). The nexus between these observations is that ARF6 is involved in the endocytosis and membrane recycling of a subset of membrane proteins, as well as in remodeling of the cortical actin cytoskeleton (D'Souza-Schorey et al., 1995); (Frank et al., 1998); (Radhakrishna and Donaldson, 1997); (Radhakrishna et al., 1999); (Boshans et al., 2000). ARF6 is also highly expressed in polarized epithelial cells, where it localizes primarily to the apical brush border and apical early endosomes (Altschuler et al., 1999); (Londono et al., 1999).

Our studies reveal a critical role for PERP in the pathogenesis of *S. Typhimurium*, and for the first time demonstrate that SipA, a type III secreted protein, can bind to a host protein that is present at the apical surface during infection. More detailed investigations are required to further the understanding of the regulation underlying the SipA-mediated accumulation of PERP at the apical surface, including the possible role of the endosomal pathway. Nevertheless, we propose a model (Figure 2.8) that describes our observations for how PERP functions during *Salmonella* infection. SipA and other *Salmonella*

Figure 2.8: Model for PERP's Role in the Inflammatory Response to *Salmonella* Infection

- A. *Salmonella* at the apical surface releases SipA (black and white circles) and other effectors (triangles). Some PERP (red M's) is already present at the apical surface.
- B. T3SS effectors trigger the apical accumulation of PERP, likely through a pathway involving apical recycling endosomes (beige circles marked Rab25+).
- C. PERP accumulation at the apical surface causes an overall increase in PERP expression, which induces the activation of caspase-3 (lightning bolts).
- D. In accordance with previously published results (Srikanth et al, 2010), activated caspase-3 is released at the apical surface where it cleaves SipA and liberates its proinflammatory N-terminus.
- E. As previously described (Wall et al, 2007), the SipA N-terminus (white half circle) triggers pathways leading to the production and formation of HXA₃ (blue circle), which forms a chemotactic gradient that triggers PMN transepithelial migration to the apical surface.

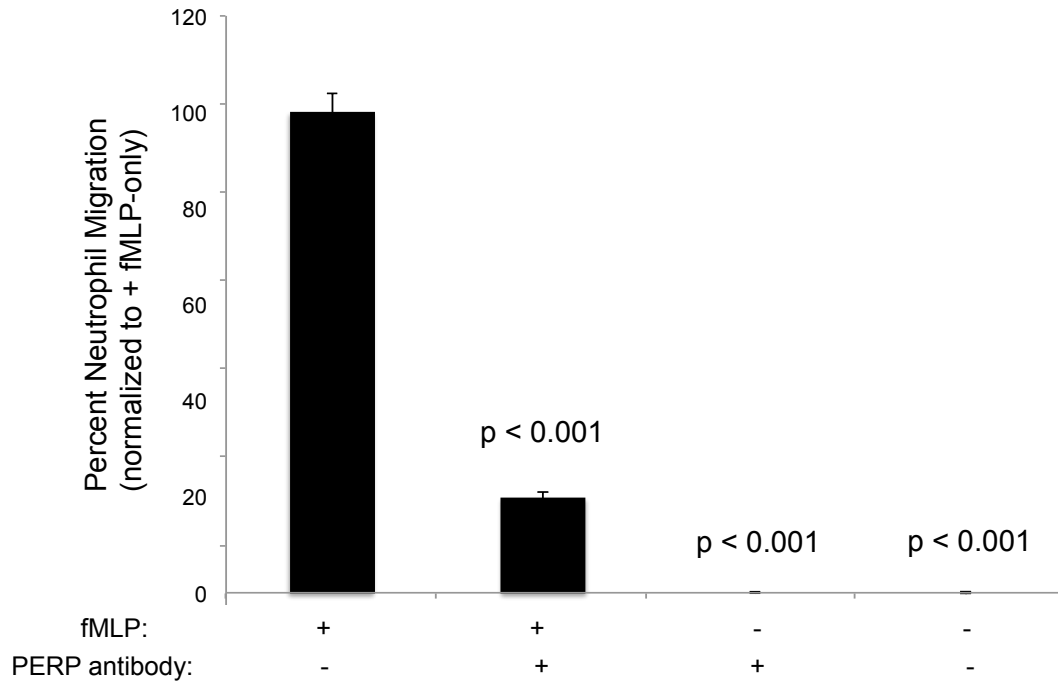
Figure 2.8: Model for PERP's Role in the Inflammatory Response to *Salmonella* Infection



effectors direct PERP to accumulate at the apical surface, likely due to a pathway involving apical recycling endosomes (Figure 2.8, panels A and B). The increased delivery of PERP to the apical surface interferes with its normal trafficking route and subsequently keeps the protein out of degradation pathways, thereby leading to an overall increase in the amount of PERP in the cell. The increased PERP level triggers the activation of caspase-3, as shown in Figure 2.8C, and subsequent release of activated caspase-3 at the apical surface (Figure 2.8D). In accordance with our previously published work (Srikanth et al, 2010), SipA is cleaved by caspase-3 at the apical surface (Figure 2.8E), thus liberating the pro-inflammatory N-terminal domain, which we previously show is responsible for the SipA-mediated inflammatory response to *Salmonella* infection, including PMN transepithelial migration (Wall et al, 2007). Therefore, our data support our contention that PERP, via its role in activating caspase-3, is the link between SipA-mediated activation of caspase-3 and SipA-mediated inflammatory responses. While there is still much to be learned about the role PERP plays in inflammatory conditions, we have now taken the first steps to show that this tetraspanning membrane protein plays a pivotal role in the pathogenesis of *Salmonella* infection.

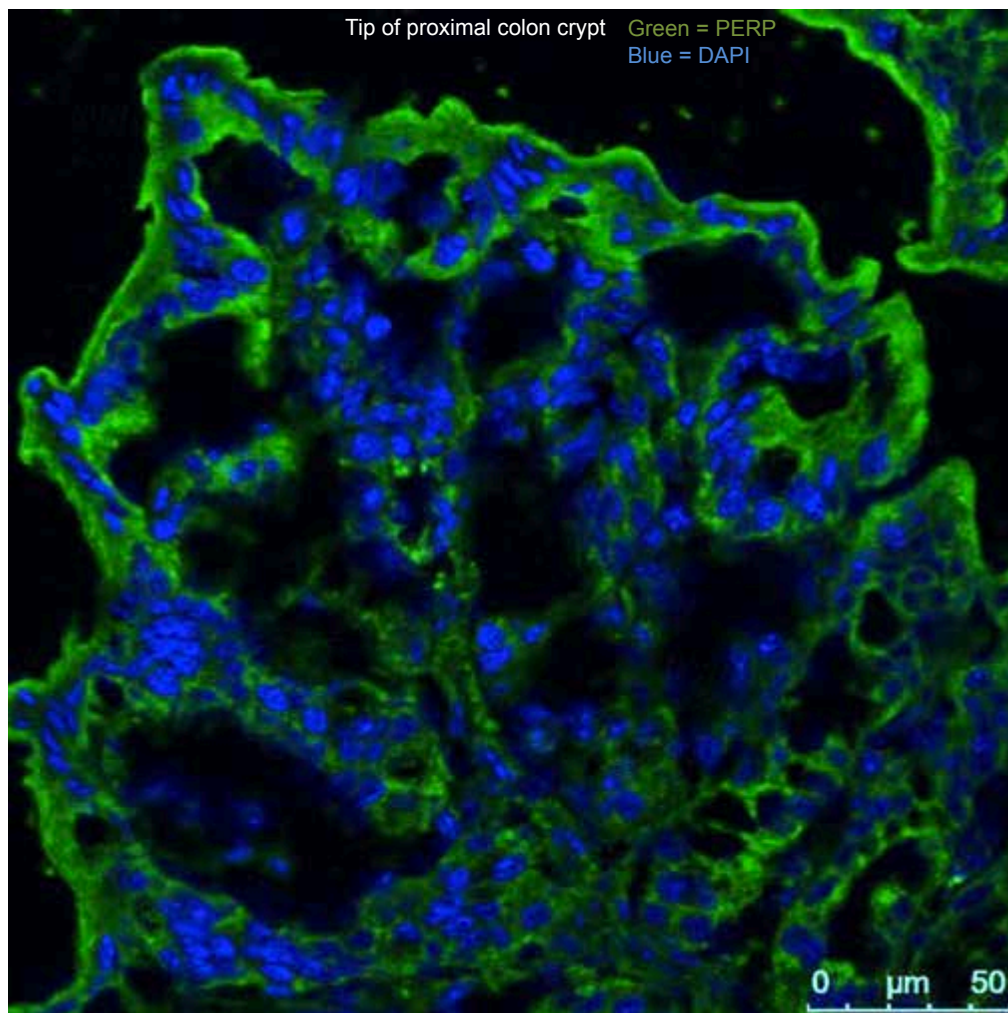
Supplemental Data Figure S2.1: PERP Antibody Migration Control

Polarized HCT8 monolayers were treated with the PMN chemoattractant fMLP (set to 100%) or left untreated, and exposed to 25ug/mL of anti-PERP antibody or incubated in HBSS+. Cells incubated with anti-PERP antibody only (no fMLP or infection) did not induce PMN transmigration, indicating treatment with anti-PERP antibody alone does not impact PMN transmigration. Data shown are of one of three experiments performed in triplicate.

Supplemental Data Figure S2.1: PERP Antibody Migration Control

Supplemental Figure S2.2: Expression of PERP in Mouse Colon

5uM cryosections of mouse proximal colon were stained for PERP (green) or DAPI (blue). We detect the presence of PERP throughout the tissue, including at the mucosal surface where epithelial cells would be exposed to *Salmonella* infection.

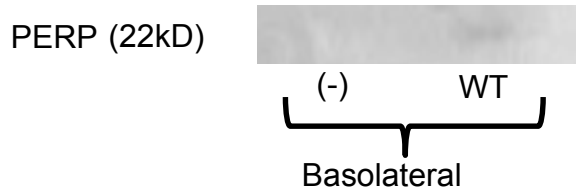
Supplemental Figure S2.2: Expression of PERP in Mouse Colon

Supplemental Figure S2.3: Controls for Apical Surface Biotinylation

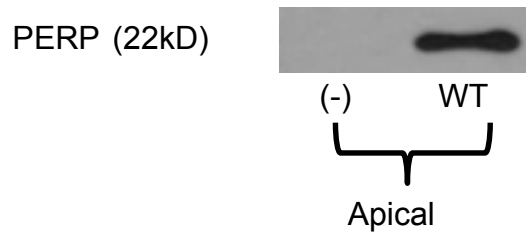
A. Polarized HCT8 monolayers were infected (WT) or left uninfected (-) in HBSS+ for one hour, and the basolateral surface was biotinylated, pulled down via streptavidin, and analyzed for PERP via Western blot. B. Polarized HCT8 monolayers were infected (WT) or left uninfected (-) in HBSS+ for one hour, and the apical surface was biotinylated, pulled down via streptavidin, and analyzed for PERP or E-cadherin. "WCL" refers to a whole cell lysate sample showing the E-cadherin band.

Supplemental Figure S2.3: Controls for Apical Surface Biotinylation

A



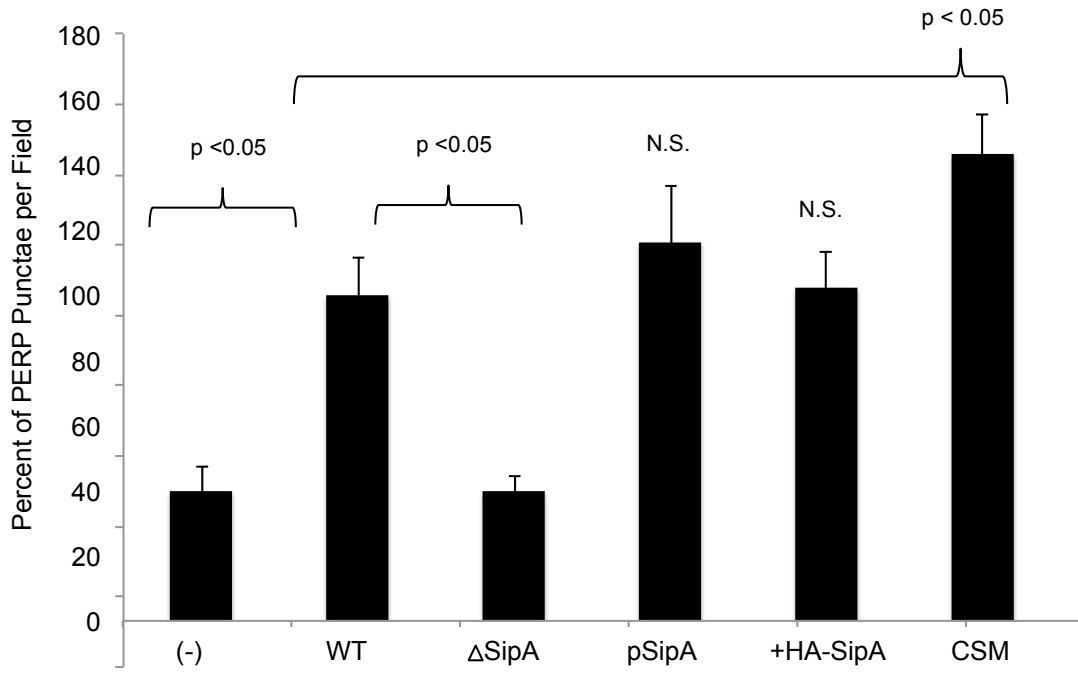
B



Supplemental Figure S2.4: Quantification of Punctate PERP Staining in Response to *Salmonella* Infection

The level of PERP punctate staining was quantified with FIJI using the same size filter for the punctae across all samples. Data from each treatment group were compared to data from WT-infected cells, which was set to 100%. Student's T test analyses were used to statistically evaluate data from WT samples to the remaining groups. WT infection induced more PERP punctae than cells left untreated or cells infected with the SipA-deficient strain (Δ SipA). Error bars indicate standard error. p values less than 0.05 were considered statistically significant. N.S. indicates no statistical difference between the indicated group and WT. Values shown are from a set of images from one of three experiments showing reproducible trends.

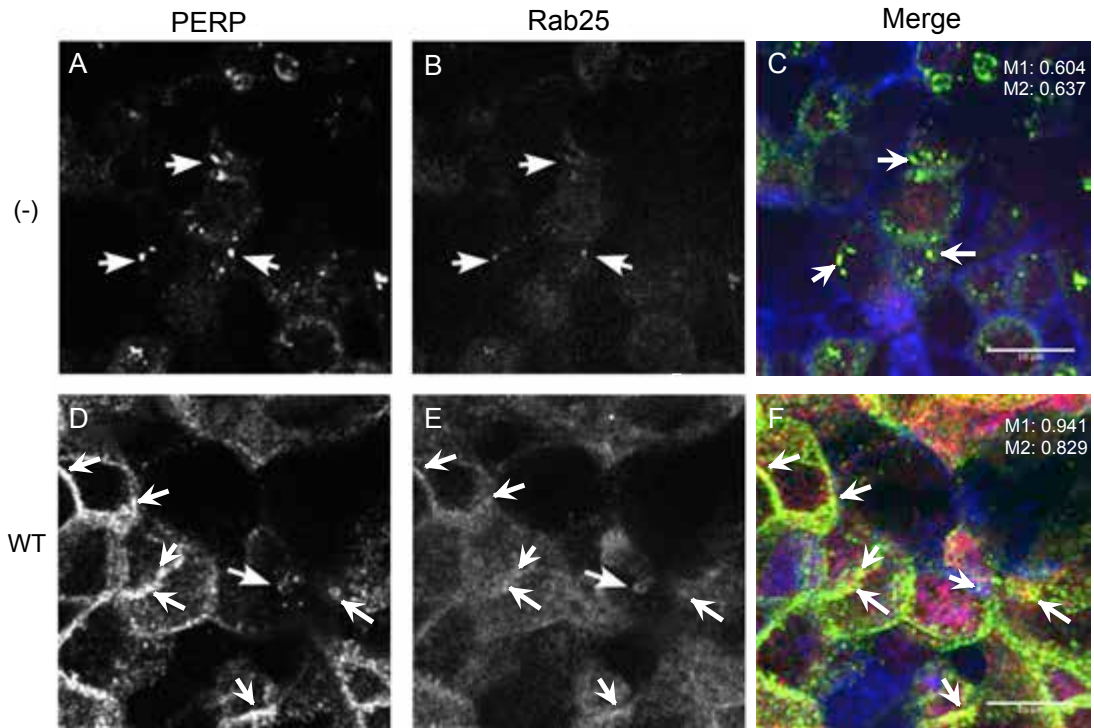
Supplemental Figure S2.4: Quantification of Punctate PERP Staining in Response to *Salmonella* Infection



Supplemental Figure S2.5: PERP Co-Localizes with Apical Recycling Endosomes

T84 monolayers were treated with HBSS+ only (-) or infected with wild type *Salmonella* (WT). The cells were stained with an antibody against PERP followed by secondary conjugated to Alexa Fluor 488 (green pseudocolor), and with an antibody against Rab25 followed by secondary conjugated to Alexa Fluor 568 (red pseudocolor). PERP and Rab25 co-localize in non-infected (C) and infected (F) cells (white arrows), indicating the apical recycling endosome pathway is at least partly responsible for PERP localization. The level of colocalization was determined via Manders' coefficient analyses, where M1 refers to PERP comparison to Rab25 staining, and M2 refers to Rab25 comparison to PERP staining. Images are of one set from one of three experiments. Blue pseudocolor indicates phalloidin staining to show location of cells

Supplemental Figure S2.5: PERP Co-Localizes with Apical Recycling Endosomes



Preface to Chapter III

The data within this chapter have been submitted for publication in Gut Microbes

The Type Three Secreted Effector SipC Regulates the Trafficking of PERP During *Salmonella* Infection.

Kelly N. Hallstrom and Beth A. McCormick

Description of Author Contributions

Kelly Hallstrom: Experiments culminating in results displayed in Figures 3.1-3.3,
and compilation of the manuscript.

Beth A. McCormick: Provided technical expertise and edited the manuscript.

Chapter III

The Type Three Secreted Effector SipC Regulates the Trafficking of PERP During *Salmonella* Infection.

3.1 Introduction

Salmonella enterica subtype Typhimurium (*S. Typhimurium*) is one of several *Salmonella enterica* strains responsible for over a million cases of salmonellosis in the United States each year, and is also a leading cause of diarrheal disease in developing countries. Salmonellosis is typified by gastrointestinal distress, including abdominal cramping, nausea, fever, vomiting, and most prominently, diarrhea that may contain mucus or blood. Intestinal inflammation is further characterized histopathologically by a prevailing influx of polymorphonuclear leukocytes, the non-specific effects of which lead to tissue damage and also contribute to diarrhea (Madara et al., 1993).

Salmonella species, like other Gram-negative pathogens, have evolved a sophisticated virulence mechanism called a type III secretion system (T3SS), which is responsible for the delivery of a series of bacterial effectors into host cells with the capability of hijacking eukaryotic cell functions. While the T3SS apparatus is highly conserved, the translocated effectors are unique proteins with very specialized functions central to virulence. In particular, *Salmonella* effector proteins impart a variety of effects on the host cell including triggering *Salmonella* invasion by interfering with actin dynamics (McGhie et al., 2001), and inducing inflammatory responses ((Lee et al., 2000); (Zhang et al., 2002); (Wall et al.,

2007)).

Our prior studies have revealed that the type III secreted effector SipA is a key regulator of intestinal inflammation during *S. Typhimurium* infection and that it is able to impart this function in the host without being translocated into the host cell via the T3SS ((Lee et al., 2000); (Wall et al., 2007); (Hallstrom et al., 2015)). That SipA is able to carry out its proinflammatory functions without being translocated via the T3SS indicated to us that SipA might be modulating a host cell component at the apical cell surface. In line with this hypothesis, we recently reported that SipA binds to the mammalian tetraspanning membrane protein PERP, which we show accumulates at the apical surface of colonic epithelial cells during *S. Typhimurium* infection in a SipA-dependent manner (Hallstrom et al., 2015). PERP (p53-effector related to PMP-22) was first identified as a p53 effector (Attardi et al., 2000), and has since been shown to have roles in development (Ihrie et al., 2005), caspase activation (Davies et al., 2009), and cancer (Paraoan et al., 2006). Functionally, we identified PERP to be involved in proinflammatory pathways required for *S. Typhimurium*-induced PMN migration, uncovering a previously unknown role for PERP in *Salmonella* pathogenesis.

While SipA is involved in eliciting PERP accumulation at the apical surface of polarized colonic epithelial cells, we noted that it is not the only *S. Typhimurium* factor to be involved. This is because infection with Δ SipA *S. Typhimurium* shows an incomplete, reduction in the level of PERP accumulation as compared to wild type infection (Hallstrom et al., 2015), indicating involvement

of another virulence factor. Since it has been previously documented that SipA and SipC cooperate to promote *Salmonella* invasion (McGhie et al., 2001), we examined the extent to which SipC influences PERP accumulation at the apical surface of colonic epithelial cells. Herein, we describe our observations that SipC also contributes to the accumulation of PERP at the cell surface. To date, studies of SipC have largely focused on its role in perturbing actin dynamics and its role in effector translocation during *Salmonella* invasion of host cells. Our study is the first to reveal SipC also plays a role in regulating the accumulation of a host membrane protein at the cell surface during *S. Typhimurium* infection.

3.2 Materials and Methods

Tissue Culture

HCT8 polarized monolayers were seeded on transwells in 100 mm tissue culture dishes (Costar 3419) and used 6-8 days later.

Use of Bacterial Strains:

Wild type *S. Typhimurium* (SL1344) was grown as previously described (Lee et al, 2000). HilA-deficient (Δ HilA), SipC-deficient (Δ SipC), and the double SipA/SipC-deficient (Δ SipA Δ SipC) strains are isogenic to SL1344, and were grown in the same manner. The pSipC and pHilA isogenic complementing strains were grown in the same manner in the presence of 50ug/mL ampicillin. Cells were infected for 1 hour at an MOI of 100:1.

Construction of Mutants and Complemented Strains

The Δ SipC has been previously described (Wall et al., 2007). Despite its inability to translocate effectors into host cells, the Δ SipC secretes wild-type levels of effectors. The pSipC complemented strain was constructed by placing the whole SipC gene into the pBH expression vector (described in Lee et al, 2000) via the EcoRI and HindIII restriction digest sites. The gene with the appropriate digest sites was constructed via IDT. The Δ SipA Δ SipC double mutant was constructed by replacing SipC with a chloramphenicol resistance cassette in the SipA-mutant strain as described in (Datsenko and Wanner, 2000). Briefly, primers were designed to amplify the chloramphenicol cassette from pKD3 with end regions of homology to the N and C termini of SipC. This method leads to replacement of SipC with the chloramphenicol cassette following recombination. The resulting PCR product was gel-purified using the QiaQuick Gel Extraction Kit (Qiagen, 28704). The SipA deletion strain was then transformed with the pKD46 plasmid, which expresses the lambda Red system for recombination in the presence of arabinose, and then transformed with the purified PCR product via electroporation. Transformants were selected on chloramphenicol-containing media, and sequenced for verification of replacement of SipC with the chloramphenicol cassette. The HilA-deficient strain has previously been described (vv341; (Hueck et al., 1995)). The pHilA complemented strain was constructed as described above for the pSipC complement, with the exception

that the EcoRI and KpnI sites were used.

Biotinylation

Apical cell surface biotinylation was performed using the protocol described in Chapter 2 (Hallstrom et al., 2015). Briefly, following infection, the apical surface of HCT8 monolayers was labeled with biotin (Thermo Scientific, 21335) at 4C. Labeling of the basolateral surface was blocked with acetate (Thermo Scientific, 26777). The cells were then lysed, homogenized, normalized for protein concentration via Bradford assay, and incubated with streptavidin beads (Thermo Scientific, 20347) overnight at 4C in order to pull down apically-labeled proteins. The apically-enriched lysates were then immunoblotted for PERP (Santa Cruz, SC-67184). For drug treatments, cells were exposed to 1ug/mL of cytochalasin D (Sigma Aldrich, C8273-1MG) (Finlay et al., 1991) OR 80uM dynasore (Tocris, 2897) ((Pietila et al., 2010); (Martinez-Argudo and Jepson, 2008)) in HBSS+, or DMSO in HBSS+ (vehicle control) for 1 hour prior to infection and during infection.

Invasion Assay

To confirm the invasion-blocking effects of cytochalasin D in the HCT8 cell line, we performed a gentamicin protection assay generally following the guidelines in (Finlay et al., 1991). Cells were pretreated with 1ug/mL of cytochalasin D or the vehicle (DMSO) for 1 hour prior to infection and maintained during the 1 hour

infection with SL1344. After washing away adherent bacteria, the cells were incubated with 0.1mg/mL of gentamicin for 90 minutes, then washed and lysed with 1% Triton X in PBS. 10-fold serial dilutions were then plated in triplicate. Serial dilutions of the bacterial suspension used to infect the cells were also plated to determine percent invasion as a result of each condition.

Data Presentation

Densitometric analyses for Western blots are presented as the results from three replicates showing reproducible results. Densitometric analyses for these experiments can be found in Table C.2. Densitometry was performed using FIJI. The p-value for the invasion assay was obtained via Student's T-test.

3.3 Results

Type Three Secreted Effectors Drive PERP Accumulation

We observed that in addition to the type III secreted effector SipA other *S. Typhimurium* virulence determinants might be required to induce the full level of PERP accumulation at the apical surface of colonic epithelial cells observed in response to wild type infection (Hallstrom et al, 2015). We first considered the T3SS located on *Salmonella* pathogenicity island (SPI)-1, given that the SPI-1 T3SS (T3SS1) is largely associated with early invasion and pathogenicity events (Galan, 1996). The key regulator of this system is the transcription factor HilA,

without which the T3SS1 needle apparatus is not formed and the transcription of several T3SS1 effectors is blocked, thus rendering *Salmonella* invasion-deficient (Eichelberg and Galan, 1999). To examine the importance of the T3SS1 in PERP accumulation at the apical surface, we employed an apical surface biotinylation assay ((Strohmeier et al., 1997); (Hallstrom et al., 2015)), which allows us to selectively label the apical surface of polarized cells and examine changes in protein expression at this cellular location in response to infection. As shown in Figure 3.1A, infection of HCT8 colonic epithelial cells with our HilA-deficient strain nearly abolishes apical PERP accumulation in response to infection. Complementation of the HilA mutant (Figure 3.1B) also rescued the ability of *S. Typhimurium* to promote the accumulation of PERP. This result shows that additional key factors governing PERP accumulation at the apical surface of colonic epithelial cells infected with *S. Typhimurium* are associated with T3SS1.

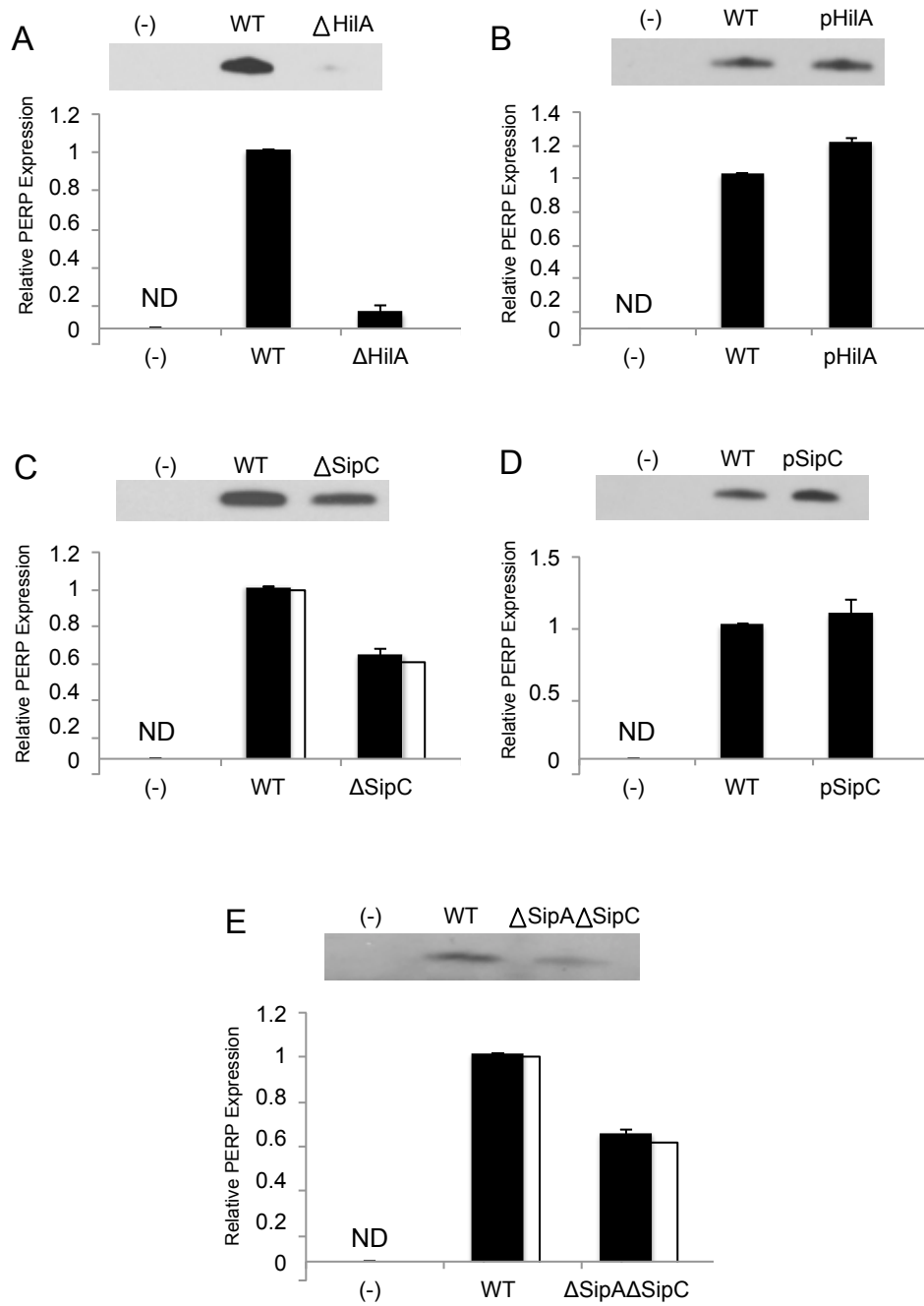
SipC Promotes PERP Accumulation at the Apical Surface of Colonic Epithelial Cells

Since there is precedence for the T3SS1 effectors SipA and SipC cooperating to promote *Salmonella* invasion due to interference with actin dynamics (McGhie et al., 2001), we next examined the extent to which SipC functions in PERP apical accumulation during infection. To observe changes in PERP protein levels at the apical surface in response to infection, we compared

Figure 3.1: PERP Accumulation at the Apical Membrane Surface is Mediated by the Type Three Secretion System

A-E: Polarized HCT8 monolayers were infected with wild-type (WT), HilA-deficient (Δ HilA), HilA complemented (pHilA), SipC-deficient (Δ SipC), SipC complemented (pSipC), or double SipA and SipC-deficient (Δ SipA Δ SipC) strains of *Salmonella*, or left uninfected (-) in HBSS+ for 1 hour. The apical cell surfaces were biotinylated, and whole cell lysates were pulled down with streptavidin beads, and Western blotted for PERP. The level of PERP intensity in cells following infection with mutant strains or complemented strains was compared to the level of PERP intensity following WT infection to obtain a ratio of PERP expression. WT PERP expression level was set to 1. Densitometric measurements are of three representative experiments showing reproducible results. Error bars show +/- standard error. "ND" indicates none detected. Densitometric data for replicates can be found in Table C.2

Figure 3.1: PERP Accumulation at the Apical Membrane Surface is Mediated by the Type Three Secretion System



wild type *S. Typhimurium* versus an isogenic *Salmonella* Δ SipC strain (Wall et al., 2007). As shown in Figure 3.1C, the absence of SipC causes less PERP to accumulate at the apical surface compared to levels observed in response to wild type infection; complementation with a plasmid expressing SipC rescued the effect, confirming the result is SipC-specific (Figure 3.1D). To our knowledge, this represents the first time SipC has been shown to have a direct role in regulating the accumulation of a host membrane protein at the surface of polarized colonic epithelial cells during *Salmonella* infection.

We next sought to determine whether SipC and SipA act via the same or independent pathways to induce PERP accumulation in response to *S. Typhimurium* infection. To accomplish this, we generated a Δ SipA Δ SipC isogenic double mutant strain via replacement of SipC with a chloramphenicol resistance cassette (Datsenko and Wanner, 2000) in our SipA mutant strain, and then examined PERP accumulation employing the biotinylation assay. We reasoned that if SipA and SipC act via independent pathways to induce PERP accumulation, one would expect to see an additive effect from the double mutant compared to our observations with either individual mutant. However, if SipA and SipC were to act via the same pathway, we would envision the Δ SipA Δ SipC double mutant not to cause any additional loss of PERP accumulation as compared to either individual mutant. As shown in Figure 3.1E, we observed a comparable reduction in PERP accumulation from the double Δ SipA Δ SipC mutant as compared to either single mutant (Figure 3.1C; (Hallstrom et al.,

2015)). This result suggests that SipA and SipC act along the same pathway to induce PERP accumulation to the apical surface of colonic epithelial cells during *S. Typhimurium* infection.

PERP Accumulation is Due to an Exocytic Trafficking Event

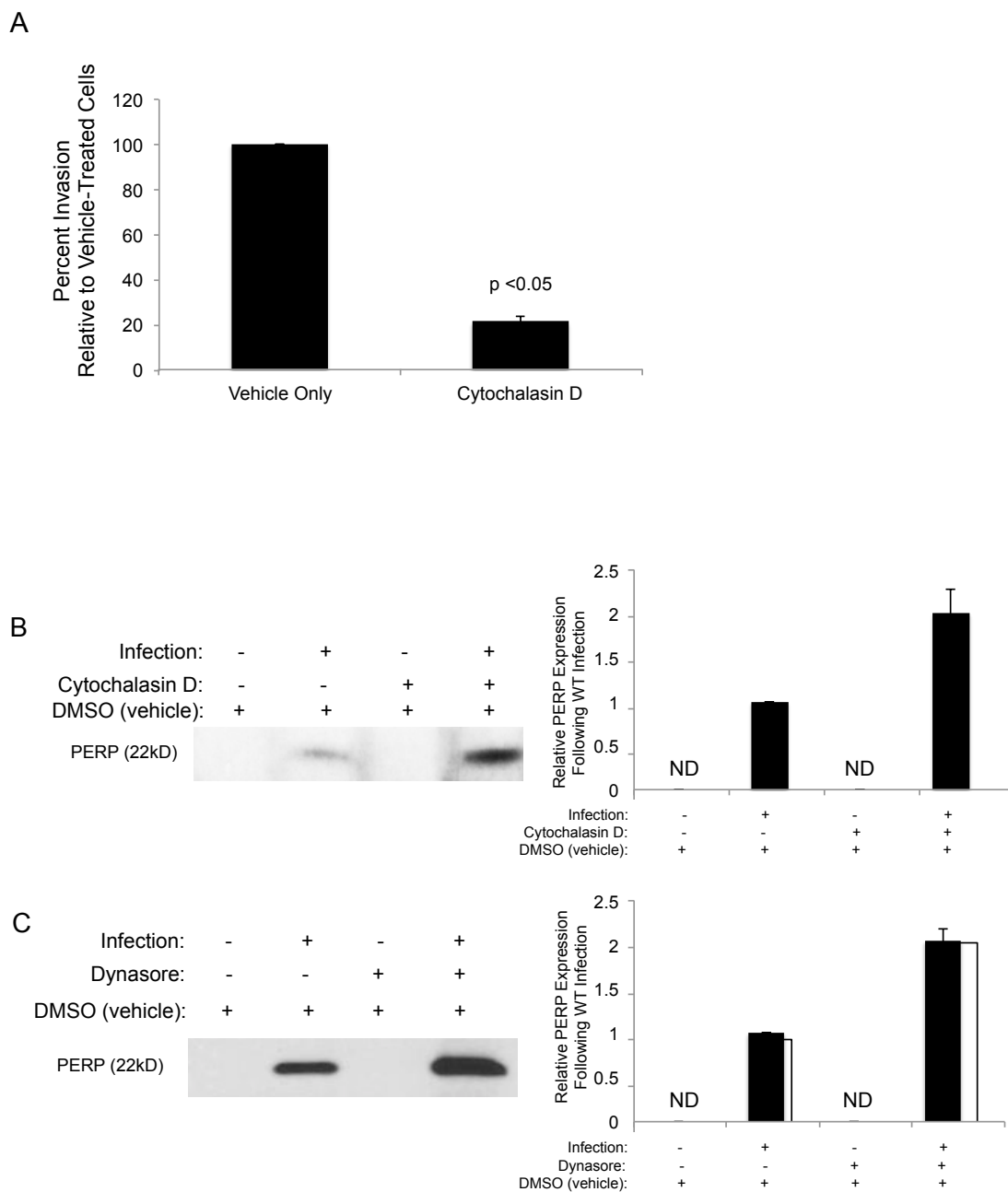
We next investigated whether PERP accumulation is dependent on actin-mediated mechanisms associated with *Salmonella* invasion by treating cells with the actin-disrupting drug cytochalasin D. Treatment of host cells with cytochalasin D is well documented to reduce *Salmonella* invasion by blocking the ability of actin to polymerize and form membrane ruffles, an event that is mediated during *Salmonella* invasion via the activities of SipA and SipC among other effectors (Finlay et al., 1991). We confirmed this observation in the HCT8 cell line (Figure 3.2A). Further, we inferred that if the actin-modulating activities of SipA and SipC were to be critical for PERP accumulation, then interfering with actin dynamics via cytochalasin D treatment should cause a reduction in PERP accumulation. Rather, we found that treatment with cytochalasin D caused an additional increase in PERP accumulation at the apical surface in response to *S. Typhimurium* infection (Figure 3.2B). This observation suggests that invasion of *S. Typhimurium* into host cells is not required for PERP accumulation, and also implies that the actin-modulating functions of *Salmonella* effectors, such as SipA and SipC, may also be dispensable for PERP accumulation. Although further investigation is required to achieve full understanding of the precise roles each

Figure 3.2: PERP Accumulation Occurs Independently of *Salmonella* Invasion, and Dynamin-mediated Endocytosis

A. Polarized HCT8 monolayers were treated with 1ug/mL of cytochalasin D or DMSO only (vehicle only) prior to infection and during infection. The amount of invading bacteria in each condition was quantified via a gentamicin protection assay (see Methods). Data displayed are from one representative experiment performed in triplicate.

B-C. Polarized HCT8 monolayers were treated with cytochalasin D (B) or dynasore (C) or with DMSO only (vehicle) prior to and during a 1 hour *Salmonella* infection. The apical cell surfaces were then biotinylated, and whole cell lysates were pulled down with streptavidin beads, and Western blotted for PERP. The level of PERP intensity in infected cells following treatment with either drug was compared to the level of the PERP intensity in infected cells treated with the vehicle to obtain a ratio of PERP expression. PERP expression level from infected, vehicle-treated cells was set to 1. Densitometric measurements are of three representative experiments showing reproducible results. Error bars show +/- standard error. "ND" indicates none detected. Densitometric data for all replicates can be found in Table C.2.

Figure 3.2: PERP Accumulation Occurs Independently of *Salmonella* Invasion, and Dynamin-mediated Endocytosis



effector plays in inducing PERP accumulation, our studies start to shed light on the molecular mechanisms by which PERP accumulates at the apical surface.

We previously reported that PERP accumulation at the apical surface of colonic epithelial cells infected with *S. Typhimurium* was likely due to a trafficking event involving existing cellular PERP stores, as inhibiting new protein synthesis did not block PERP accumulation (Hallstrom et al., 2015). Building upon this observation, we next sought to determine whether *S. Typhimurium* was preventing endocytosis, consequently causing PERP to accumulate at the apical surface, or inducing exocytosis, and thus targeting PERP-containing vesicles to the apical surface.

To distinguish between these two pathways, we treated HCT8 cells with 80uM of the dynamin-blocking drug dynasore. Dynamin is an integral part of clathrin-mediated endocytosis, and potentially caveolae-mediated endocytosis, as it functions to essentially “pinch off” newly forming vesicles (Ferguson and De Camilli, 2012). Dynasore is routinely used to block endocytosis at the cell surface (Macia et al., 2006). If *Salmonella* prevents endocytosis, we would expect that treatment with dynasore would have no additional effect on the level of PERP accumulation. However, we found that blocking endocytosis alone with dynasore was not sufficient to induce PERP accumulation at the apical surface, and, moreover, that *S. Typhimurium* infection has an additional effect in the presence of this drug (Figure 3.2C). This observation supports the hypothesis that *S. Typhimurium* induces PERP accumulation via an endocytosis-independent

pathway. Indeed, this is supported by our cytochalasin D observations, as this drug has also been shown to block clathrin-mediated endocytosis at the apical surface of polarized epithelial cells (Gottlieb et al., 1993). As shown in Figure 3.2B, cytochalasin D treatment alone is not sufficient to induce PERP accumulation. Given our previous observations that PERP co-localizes with apical recycling endosomes (Hallstrom et al., 2015), which transport new intracellular material to the surface of polarized cells (Golachowska et al., 2010a) we propose that *S. Typhimurium* causes PERP to accumulate at the apical surface by triggering PERP-containing vesicles to traffic to the apical surface.

3.4 Discussion

Previous studies of SipA and SipC have mainly focused on their roles in regulating *Salmonella* invasion and inflammatory responses to infection and, in the case of SipC, effector translocation. Such studies have shown that both effectors are capable of perturbing actin dynamics in ways that promote growth of the host cell plasma membrane outward and around *Salmonella*, thus permitting the bacteria to invade the cell (McGhie et al., 2001). Furthermore, SipA, and to some extent SipC, has been shown to be a key regulator of the inflammatory response to *Salmonella* infection ((Lee et al., 2000); (Wall et al., 2007)). However, our work investigating the role of SipC in PERP apical accumulation is the first to show how this effector induces the apical accumulation of a host transmembrane protein.

Our observation that PERP accumulation at the apical surface is almost entirely dependent on the type III secretion system is congruent with what is known about these systems. The type III secreted effectors promote disease by facilitating cell attachment and entry, suppressing the host immune/defense response, and modulating other aspects of host cell biology. Consequently, type III secreted effectors play a prominent role in bacterial pathogenesis and host-association. In the absence of HilA, and thus in the absence of a key regulator of the T3SS1 and its effectors, *Salmonella* invasion is significantly reduced (Eichelberg and Galan, 1999). It is notable that treatment with cytochalasin D in the presence of *Salmonella* does not block PERP accumulation at the cell surface, as cytochalasin D is known to block invasion ((Finlay et al., 1991); this report). Multiple conclusions may be drawn from this observation. First, although cytochalasin D treatment inhibits invasion, most likely by interfering with the actin polymerization required for *Salmonella* engulfment (Finlay and Falkow, 1988), it does not prevent either the secretion nor translocation of type three secreted effectors (Akopyan et al., 2011). This observation suggests that invasion is dispensable for PERP accumulation as long as the effectors are still able to access the cell, and further supports the notion that type III secreted effectors, including SipA and SipC, play an active role in PERP accumulation at the apical surface.

Cytochalasin D treatment is also well known to block endocytosis as discussed above. Therefore, our observation that cytochalasin D treatment alone

does not induce PERP accumulation is in agreement with our finding that blocking dynamin-mediated endocytosis with dynasore (Figure 3.2C) is also insufficient to induce PERP accumulation. From these experiments, we can infer that blocking endocytosis does not lead to PERP accumulation at the cell surface within the time frame studied. Since infecting with *S. Typhimurium* induces an additive effect on PERP accumulation in the presence of either drug, we postulate that *S. Typhimurium* does not facilitate PERP accumulation at the apical surface through a mechanism that disrupts its endocytic uptake. This assertion is consistent with previous reports indicating that *Salmonella* requires endocytic processes to invade host cells ((Pietila et al., 2010); (Martinez-Argudo and Jepson, 2008)). Further, we previously showed that blocking new protein synthesis does not prevent *S. Typhimurium*-induced PERP accumulation (Hallstrom et al., 2015). Based on these observations, we propose that *S. Typhimurium* prompts PERP redistribution to the apical surface by inducing the delivery of PERP-containing exocytic vesicles to the apical surface.

In considering how exocytosis is regulated at the cell surface, involvement of the exocyst complex becomes a key event. The exocyst is an octomeric protein complex that regulates tethering of vesicles to the new membrane location, thus promoting their eventual fusion (Heider and Munson, 2012). This process becomes important at the cell surface during *Salmonella* infection as incorporation of new vesicles at the cell surface provides an extra store of membrane at this cellular location. This is thought to help promote the membrane

ruffling events that promote *Salmonella* engulfment into the intestinal epithelial cell (Nichols and Casanova, 2010). Indeed, involvement of the exocyst in *Salmonella* invasion has been demonstrated, and it is known that exocyst components accumulate around areas of *Salmonella* invasion (Nichols and Casanova, 2010). Furthermore, SipC likely mediates such directed accumulation given that this effector binds to at least three exocyst components (Nichols and Casanova, 2010). These observations favor the hypothesis that SipC provides a “docking site” for the exocyst to form and thus direct the trafficking of vesicles to the cell surface (Nichols and Casanova, 2010).

Our data indicate that SipC and SipA act via the same pathway to induce PERP accumulation (Figure 3.1). In keeping with this, SipA can be coupled to exocyst trafficking as well. This is supported by reports indicating SipA induces the apical accumulation of the GTPase Arf6 (Criss et al., 2001a), and also GTP-bound Arf6 directs the exocyst complex to the cell surface via its interaction with Sec10 (Prigent et al., 2003). Moreover, Arf6-mediated recruitment of the exocyst appears to be directed to areas of plasma membrane growth and remodeling (Prigent et al., 2003). These observations are consistent with a model for SipA directing the apical recruitment of Arf6 (Criss et al., 2001a), and thereafter Arf6 eliciting exocyst recruitment to sites of apical membrane ruffling during *Salmonella* invasion.

While we identified SipA (Hallstrom et al., 2015) and SipC to play key roles in PERP accumulation at the apical surface, our data indicate that at least

one other type III secreted effector is involved with this process. In this respect, the effector SopE is an interesting consideration. This effector is important for *Salmonella* invasion by acting as a guanine exchange factor (GEF) for the Rho-GTPase Cdc42 (Hardt et al., 1998b). SopE's activation of Cdc42 is thought to explain this effector's role in promoting the cytoskeletal rearrangements that lead to membrane ruffling (Hardt et al., 1998b). Of note, the exocyst member Sec3 is a downstream effector of Cdc42, and Exo70 likely is as well (He and Guo, 2009), thus connecting SopE to exocyst activity. Furthermore, Nichols and Casanova (2010) showed that SopE is required for the activation of RalA, a GTPase known to regulate exocyst activity. Nevertheless, additional experimentation is necessary to identify the other T3SS effector(s) responsible for PERP accumulation, as well as their mechanisms of action.

In light of the above conclusions, Figure 3.3 depicts a working model for how we envision *S. Typhimurium* effectors trigger PERP accumulation at the cell surface. First, *S. Typhimurium* invades at the apical surface of colonic epithelial cells where SipA (red) and SipC (blue) act to trigger the formation of the exocyst complex (yellow) (Figure 3.3A). The exocyst complex permits the tethering (and thus eventual fusion) of vesicles from apical recycling endosomes containing Rab25 and PERP (purple M's) to the apical surface (Figure 3.3B; (Hallstrom et al., 2015)). The newly recruited vesicles then fuse to the apical plasma membrane, as indicated by the darker shading at the apical surface in Figure 3.3C. This fusion leads to the incorporation of PERP at the apical surface.

Figure 3.3: *Salmonella* Induces PERP Accumulation by Triggering Increased Exocytosis: A Model

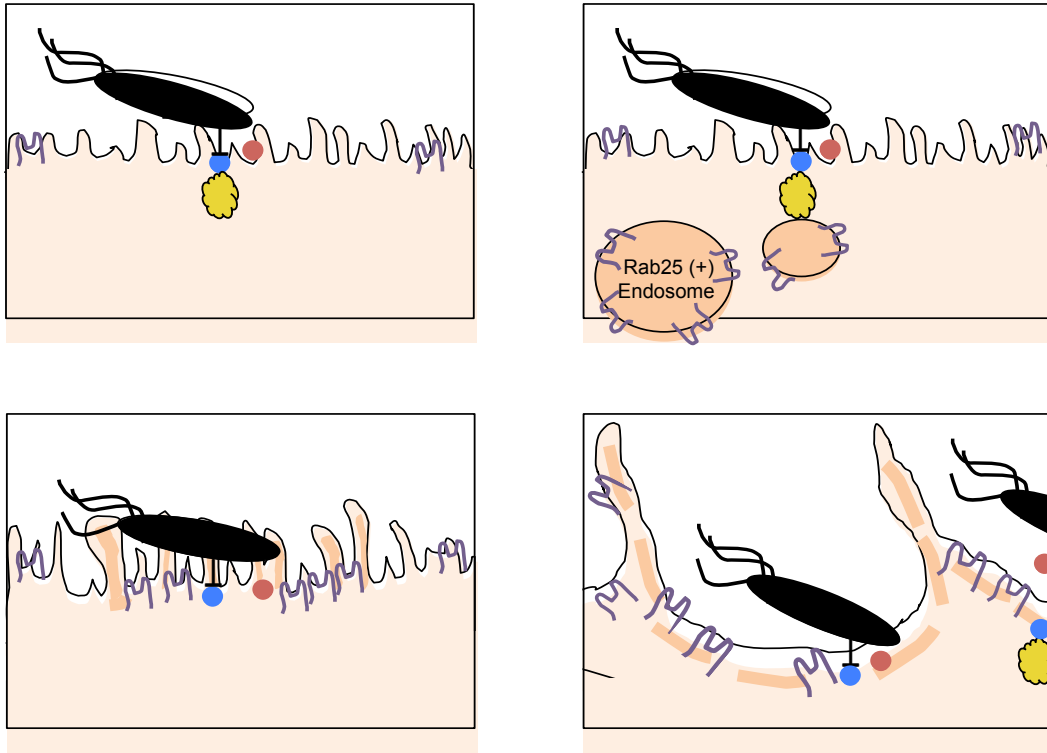
Panel A: *Salmonella* invades at the apical surface of colonic epithelial cells, where it secretes and translocates type III secreted effectors including SipC (blue dot) and SipA (red dot). These effectors trigger the formation of the exocyst complex (yellow dot) at the apical surface.

Panel B. The fully formed exocyst mediates increased trafficking of vesicles from apical recycling endosomes containing Rab25 ((Hallstrom et al., 2015)) and PERP (purple M) to the cell surface, and their eventual fusion to the plasma membrane.

Panel C. The fused vesicles (shaded regions), as well as PERP, become incorporated into the plasma membrane at the apical surface.

Panel D: The extra membrane provided by vesicle fusion permits membrane ruffles to form, thus allowing for *Salmonella* invasion. Repeated delivery of vesicles causes PERP to accumulate at the apical surface.

Figure 3.3: *Salmonella* Induces PERP Accumulation by Triggering Increased Exocytosis: A Model



Additionally, with the extra store of membrane provided by the fusion of vesicles at the cell surface, membrane ruffling occurs thus permitting *Salmonella* to enter the cell (Figure 3.3D). As multiple bacteria infect a cell, more vesicles will traffic to the apical surface, thus causing an accumulation of PERP at this cellular location.

Several questions remain to be asked of this model, including the identity of the remaining T3SS effectors responsible for PERP accumulation at the apical surface, as well as the timing of each event with respect to *S. Typhimurium*'s invasion strategy and overall induction of pathogenesis. Nevertheless, the data presented in this report suggest that *S. Typhimurium* exploits type three secreted effectors, namely SipA and now SipC, to induce exocytic trafficking events that lead to the accumulation of PERP at the cell surface.

CHAPTER IV

Discussion

4.1 Introduction

The findings described herein address the question of how SipA promotes the caspase-3 activity that is required for inflammatory responses to *S. Typhimurium* infection. Our results from this work have led to an increased understanding of *Salmonella* pathogenesis mechanisms, and have broadened our understanding of the roles of PERP in the host cell. In this section, I discuss key findings from this project that led to a new model describing how we envision PERP to promote SipA-mediated inflammatory responses to *Salmonella* infection.

4.2 PERP Accumulation Upon *Salmonella* Infection

PERP Accumulates at the Apical Surface of Colonic Epithelial Cells

One of our first observations was that PERP accumulates at the apical surface of colonic epithelial cells in response to *S. Typhimurium* infection (Chapter 2). As discussed previously, PERP is known to localize to and help organize desmosomes, which are one of the key basolaterally-located intercellular junction complexes that help maintain epithelial barrier integrity (Figure 1.1). Loss of PERP from desmosomes causes displacement of other desmosome components and, subsequently, poor epithelial barrier integrity (Ihrie et al., 2005); (Nguyen et al., 2009). That *S. Typhimurium* infection causes PERP

to accumulate at the apical surface is in keeping with observations of *Salmonella* disrupting the localization of other epithelial junction complex proteins. For example, the T3SS effectors SopE and SopE2, and to some extent SopB and SipA, disrupt the epithelial barrier by inducing mis-localization of ZO-1, occludin and E-cadherin, which are key tight junction components (Boyle et al., 2006). As disruption of the tight junction is known to facilitate bacterial translocation (Kohler et al., 2007), it is clear that disrupting the organization of intercellular junction complexes is a key pathogenic strategy of *Salmonella*, and indeed other enteropathogens as discussed in Chapter 1, that promotes bacterial dissemination in the host.

It is important to note that knocking down PERP expression in the HCT8 cell line does not appear to impact barrier function; if it did, then we would expect an increase in PMN migration across the PERP knockdown monolayers, when in fact we see a decrease. This may reflect the fact that we knock down expression as opposed to completely knocking out PERP as was done in the above-mentioned desmosome study (Ihrie et al., 2005), and the level of PERP remaining in our cells is sufficient to keep desmosomes intact. From this observation, we can infer that *Salmonella*-induced distribution of PERP at the apical surface, as opposed to it being delivered to desmosomes, may not necessarily impact barrier function. Further, given the fact that PERP loss reduces PMN migration, it is likely that PERP regulates *Salmonella*-induced PMN migration in ways that do not involve its functions in maintaining epithelial

barrier integrity; the ways by which this may occur are discussed in later sections.

PERP Accumulation at the Apical Surface is Due to Exocytosis

We found PERP is present in apical recycling endosomes (Chapter 2), which indicated to us that *S. Typhimurium* induces PERP accumulation at the apical surface by causing it to redistribute to this cellular location. In support of this hypothesis is our data showing that *Salmonella*-induced PERP accumulation at the apical surface occurs even when new protein synthesis is blocked (Chapter 2), and that blocking endocytosis (Chapter 3) alone is insufficient to induce detectable PERP accumulation at the apical surface. These data further show that *S. Typhimurium* induces additional PERP accumulation when endocytosis is already biochemically blocked compared to when endocytosis is not blocked; this observation supports the contention that *S. Typhimurium* induces PERP accumulation via a route that does not involve blocking endocytosis. Rather, we propose *S. Typhimurium* triggers PERP accumulation at the apical surface by driving its redistribution there via exocytic trafficking events.

In further support of this argument, it is known that *Salmonella*'s invasion strategy requires active endocytosis. As discussed previously, *Salmonellae* enter non-phagocytic intestinal epithelial cells by triggering membrane ruffling, which leads to *Salmonella* uptake via an endocytic event called macropinocytosis. Thus if *Salmonellae* blocked endocytosis, they would not be able to enter intestinal

epithelial cells. Indeed, biochemically blocking endocytosis reduces *Salmonella* invasion (Pietila et al., 2010); (Finlay et al., 1991), thus highlighting the important role of active endocytosis in *Salmonella* uptake.

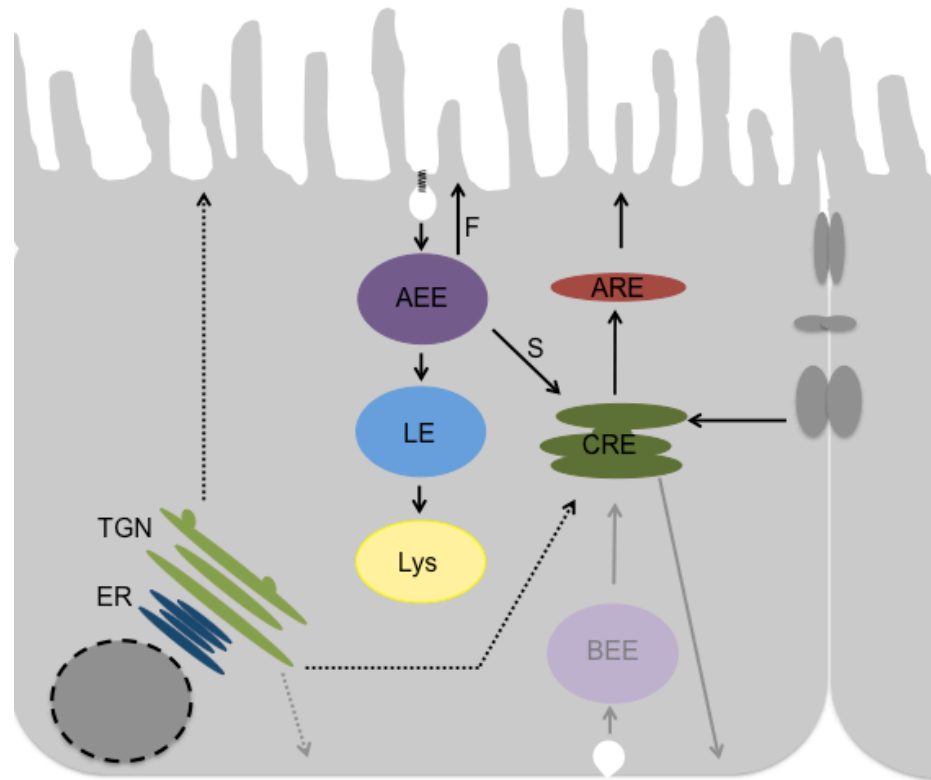
However, delivery of plasma membrane to the surface of cells via exocytic vesicles is well-known to compensate for the loss of plasma membrane from endocytic events. Moreover, evidence indicates that delivery of exocytic vesicles to sites of *Salmonella* invasion supports the endocytic uptake of these bacteria into host cells by providing additional plasma membrane (Nichols and Casanova, 2010). Thus, the manner by which *Salmonellae* hijack and utilize the endocytic and exocytic pathways for invasion bolsters our proposal that PERP accumulation at the apical surface is not due to *Salmonella* blocking its endocytosis, but rather due to *Salmonella* mediating its delivery to the apical surface via exocytic vesicles.

Endocytosis and exocytosis are tightly regulated events, as these processes are responsible for the proper localization of proteins and other cellular components based on the needs of the cell at any given point. While general mechanisms for endocytic and exocytic trafficking are comparable between cells, these networks take on a distinct organization in polarized cells (see Figure 4.1). Indeed, endocytic and exocytic events are key for maintaining polarity in epithelial cells; distinct apical and basolateral compartments are responsible for delivering and receiving cargo to and from these cellular locations (Golachowska et al., 2010a).

Figure 4.1: Endocytic and Exocytic Trafficking in Polarized Cells

This model displays an overview of the endosomal/exocytic pathway in polarized cells with an emphasis on the apical routes. Newly synthesized proteins can travel directly (dashed lines) to the apical surface from the trans-Golgi network (TGN), or can be delivered directly to the common recycling endosome (CRE), which is a tubular compartment that sorts cargo to be delivered to the apical surface basolateral cargo to be delivered to the basolateral surface. Cargo from the plasma membrane also enters the CRE following uptake from the cell surface. With regard to the apical surface, cargo in vesicles first enter into apical early endosomes (AEE), which form as the result of the fusion of endocytic vesicles. Some cargo can be immediately returned to the cell surface (fast route, F), or enter the recycling pathway for more regulated return to the cell surface (slow route, S). Cargo that are not returned to the surface by either route remain in the early endosomes which mature into late endosomes (LE) and eventually lysosomes (Lys) where the cargo is degraded. Cargo that is delivered to the CRE, which arises from early endosomes, is then believed to be sorted laterally for return to the apical surface via apical recycling endosomes (ARE), which are also believed to be tubular. Because the recycling endosome is an additional step in the return of cargo to the plasma membrane surface, it is thought that this compartment serves as a holding pattern for cargo that requires a specific signal prior to its return to the surface. Whether or not recycling endosomes are distinct organelles, or are segregated tubule extensions of early endosomes remains up for debate, although the existence of recycling compartments has been demonstrated by the presence of specific RabGTPases, such as Rab11 and Rab25 as markers. Endocytosis and exocytosis at the basolateral surface (faded lines) are believed to occur via the same general mechanisms, although basolateral recycling endosomes have not yet been identified. Gray ovals joining the two cells indicate tight junction, adherens junction, and desmosome complexes.

Figure 4.1: Endocytic and Exocytic Trafficking in Polarized Cells



Recycling endosomes are inferred to arise during the maturation process of early endosomes (also known as sorting endosomes) (Grant and Donaldson, 2009). These compartments play a role in regulating the rate at which cargo is returned to the cell surface. For example, studies show that while a portion of endocytosed transferrin is returned almost immediately to the cell surface via vesicles from early endosomes, another portion is retained in recycling endosomes, and return from this compartment takes several minutes depending on the cell type (Mayle et al., 2012). Thus recycling endosomes are thought to serve as a holding pattern for certain cargo that require a cellular signal to be returned to the cell surface, and therefore play an important role in regulating trafficking pathways.

Disruptions to the endocytosis and exocytosis pathways cause mis-localization of cargo and can be harmful to the cell. Thus, it is no small feat that *Salmonellae* have evolved ways of co-opting these networks in a way that does not immediately lead to death of the infected cell. In addition to its ability to utilize a cell's endocytic and exocytic pathways to invade, *Salmonellae* perturb the endosomal-lysosomal pathway to promote its intracellular survival. After cellular invasion, it is known that *Salmonella* effectors delay maturation of the resulting *Salmonella* containing vacuole (SCV) from a phagosome into a phagolysosome (Brawn et al., 2007); (Bakowski et al., 2010). Doing so is crucial for the intracellular replication of *Salmonella*. Yet, despite these early impacts on these

pathways, apoptosis induced by *Salmonella* is delayed 16-24 hours post infection (Kim et al., 1998); (Knodler et al., 2005).

Therefore, our inference that PERP arrives at the cell surface as a result of *Salmonella*-induced exocytosis is in agreement with *Salmonella*'s known strategies of manipulating endocytic and exocytic pathways to promote pathogenesis. Furthermore, our findings indicate that in addition to impacting vesicle formation and maturation in ways that, respectively, promote *Salmonella* uptake and intracellular survival, manipulating these pathways can conceivably lead to aberrant localization of proteins, such as PERP, which has the potential to provide additional benefits to *Salmonella* as discussed later.

Our observation that PERP is found in apical recycling endosomes in polarized colonic epithelial cells was determined by its co-localization with Rab25, a marker for apical recycling endosomes in polarized cells (Wang et al., 2000); (Casanova et al., 1999). A previous study in keratinocytes showed PERP is taken up into early endosomes, as demonstrated by its co-localization with the early endosome marker EEA1 (Nguyen et al., 2009); however, the authors of that study did not investigate whether PERP could be recycled back to the plasma membrane following its uptake. Therefore, that we find PERP localized to an endosomal compartment that regulates the delivery of cargo back to the cell surface not only supports our contention that *S. Typhimurium* drives PERP accumulation by triggering its delivery to the apical surface, but also furthers our understanding of how PERP localization is regulated in the cell. Such information

is likely to be useful in ongoing studies governing PERP's regulation and function in the context of epithelial barrier integrity and during disease states such as tumorigenesis (Ihrie et al., 2005); (Nguyen et al., 2009); (Beaudry et al., 2010).

4.3. PERP Trafficking is Mediated by the SPI-1 T3SS

We show that PERP apical accumulation is dependent on the SPI-1 type three secretion system (T3SS1), as the loss of the T3SS1 regulator, HilA, nearly completely abolished *Salmonella*-mediated PERP accumulation (Chapter 3). Furthermore, we found that the T3SS1 effectors SipA and SipC promote PERP accumulation at the apical surface (Chapter 2 and Chapter 3). This is the first time SipC has been shown to modulate the localization of a host transmembrane protein to the apical surface.

Interestingly, although SipA and SipC have previously been shown to act cooperatively in promoting actin rearrangements that facilitate *Salmonella* entry (McGhie et al., 2001), our studies here suggest they do not cooperate to promote PERP accumulation at the apical surface and, furthermore, that they act via the same pathway. Based on our findings and reports from other labs, we propose SipA and SipC promote PERP accumulation at the apical surface by inducing formation of the exocyst.

As discussed above, our data support the contention that PERP is trafficked to the apical surface via an exocytic route. In order for an exocytic vesicle to become incorporated into the new membrane compartment, it must

first be tethered to the membrane. The exocyst protein complex is the key regulator of vesicle tethering at the plasma membrane. The exocyst has two plasma membrane-localized components that function as docking sites, Exo70 and Sec3, and the remaining five components are inferred to be carried on vesicles arriving at the plasma membrane (Heider and Munson, 2012). Previous reports show that the exocyst is key for mediating delivery of cargo from recycling endosomes to the plasma membrane. For example, the loss of exocyst components *sec5*, *sec6*, and *sec15* from *Drosophila* epithelial cells causes E-cadherin to build up in recycling endosomes, and blocks it from being delivered to the plasma membrane (Langevin et al., 2005).

A previously published report showed that SipC binds directly to one of the membrane components of the exocyst, Exo70. The authors also showed that Exo70 and other exocyst components accumulate at sites of *Salmonella* invasion. Since SipC facilitates *Salmonella* invasion by acting at the cell surface, the authors propose that SipC directs the location of Exo70, and subsequently the formation of the exocyst, at sites of *Salmonella* invasion (Nichols and Casanova, 2010). The authors contend that by inducing the formation of the exocyst at sites of invasion, *Salmonella* induces the increased trafficking of exocytic vesicles to the apical surface where they provide extra stores of membrane to support *Salmonella* uptake via macropinocytosis. Based on these findings, we propose SipC causes PERP to accumulate at the apical surface by driving the delivery of PERP-containing exocytic vesicles to sites of *Salmonella*

invasion.

Our data presented in Chapter 3 indicate SipC and SipA act along the same pathway to induce PERP accumulation at the apical surface. As with SipC, we can connect SipA to the exocyst pathway, and we infer this is due to SipA's role in mediating Arf6 localization. As discussed in Chapter 1, reports from our lab demonstrate that SipA directs a pathway that requires the accumulation of Arf6 at the apical surface (Criss et al., 2001a); (Silva et al., 2004a). Separately, it was shown that Arf6 binds to exocyst components Sec10 and Sec15 at sites of phagocytosis (Niedergang et al., 2003). Furthermore, (Prigent et al., 2003) showed that Arf6 recruits the exocyst to the plasma membrane, likely due to its interactions with Sec10. Thus the collective data from these studies support our proposal that SipA promotes the accumulation of Arf6 at the apical membrane, and this accumulation leads to Arf6-mediated exocyst formation and subsequent delivery of PERP-containing vesicles to sites of *Salmonella* invasion.

These published observations support the argument that SipA and SipC can mediate vesicle delivery to the apical surface via modulation of the exocyst. Further, our data indicating that SipA and SipC induce PERP accumulation via the same pathway is in agreement with the fact that both SipA and SipC can be connected to modulating exocyst activity. Moreover, that SipA and SipC can both be connected to factors regulating exocytosis lends additional support for our contention that PERP accumulates at the apical surface due to its delivery in exocytic vesicles. It is interesting to note that if PERP is delivered to the apical

surface via exocytic vesicles, then we may expect to see an increase in the expression levels of other transmembrane proteins shuttled via exocytic vesicles to the apical surface in response to *Salmonella* infection.

Is SipC's Translocation Function Important for PERP Trafficking?

The fact that infection with the Δ SipA Δ SipC strain did not completely abolish PERP accumulation suggests that other T3SS1 members are required for this process. This argument is substantiated by our observation that infection with the Δ HilA strain caused an additional loss of PERP accumulation at the apical surface. HilA is a transcription factor that regulates T3SS1 formation and the activation of T3SS1 effectors (Saini et al., 2010); therefore the Δ HilA strain lacks the function of T3SS1 members beyond just SipA and SipC. That we see a requirement for multiple effectors in mediating PERP trafficking to the apical surface is expected, given that T3SS effectors often have overlapping and redundant roles. Indeed, SopB (Zhou et al., 2001), SopE (Hardt et al., 1998a) and SopE2 (Stender et al., 2000) have been shown to be important for actin-rearrangements required for invasion. Redundancy ensures that important functions are carried out, even if one effector is missing or ineffective in a given host. Given that SopE has already been shown to promote the activity of the exocyst-associated GTPase RaIA (Nichols and Casanova, 2010), we propose that the additional absence of SopE from the Δ HilA strain may explain why infection with the strain lacking HilA induces even less PERP accumulation

compared to infection with strains lacking SipA and/or SipC.

While we postulate that exocyst activity is regulated specifically by T3SS1 effectors, our data leave room for at least one additional consideration. As discussed, SipC has multiple roles during *Salmonella* pathogenesis: promoting *Salmonella* invasion via regulating actin dynamics, modulating exocyst formation, and translocating effectors into the host cell. If the translocation of effectors, such as SopE, is important for PERP accumulation, then one might expect a Δ SipC strain to behave functionally as a Δ HilA strain, as the absence of SipC would thwart delivery of the T3SS1 effectors to the interior of the host cell. However, this is not what we observe to take place, as the absence of SipC does not reduce PERP accumulation at the apical surface in response to *Salmonella* infection to the same degree as does the absence of HilA.

One possible explanation for this is that the translocation function of SipC is in fact not required for PERP accumulation at the apical surface. Indeed, this is supported by our observations that exogenous SipA added to HCT8 cells in the absence of bacteria is sufficient to induce apical PERP accumulation (Chapter 2), indicating SipA does not require T3SS1-mediated translocation into the host cell to induce PERP accumulation at the apical surface. Further, if effectors have to be translocated into the cell to induce PERP accumulation, then we would expect SipB, another T3SS1 effector translocase, to be required; yet, we see SipB is dispensable for PERP accumulation at the apical surface (Chapter 2). Thus in the context of PERP accumulation, SipC's role in modulating vesicle

trafficking via the exocyst may be more important than its role as a T3SS1 translocase.

However, that explanation does not fully address why the absence of HilA has a worse effect on *Salmonella*-induced PERP accumulation compared to the effect caused by the absence of SipC. If SipC's translocase functions are not required for PERP accumulation, then one explanation for the difference in PERP accumulation seen following infection with the Δ SipC versus the Δ HilA strains is that the T3SS1 apparatus itself, which can form in the absence of SipC but not in the absence of HilA, triggers a response in the host cell that leads to PERP accumulation. Indeed, the T3SS1 has been shown to mediate the delivery of flagellin across epithelial monolayers, indicating that this machinery has roles in *Salmonella* pathogenesis separate from its functions in translocating T3SS1 effectors into host cells (Sun et al., 2007); (Gewirtz et al., 2001).

Nevertheless, the discrepancy seen between the Δ SipC and the Δ HilA strains is likely to be better understood once the remaining *Salmonella* T3SS1-associated factors that promote PERP accumulation are identified. Furthermore, knowing which T3SS1 proteins are required to induce PERP accumulation will help establish the relative importance of SipC's functions in effector translocation and exocyst modulation in this process. Until then, we cannot rule out the additional possibility that both functions are important. It is well established that T3SS effectors can have multiple roles in pathogenesis that are distinct from each other (Dean, 2011). SipC's roles in both effector translocation and

Salmonella invasion have already been discussed. Further, SipA has been shown to stabilize actin filaments and prevent their depolymerization via its C-terminal domain acting as a “molecular staple” (Lilic et al., 2003); its N-terminal end, however, has been shown to be sufficient to cause SipA-induced inflammatory responses (Wall et al., 2007). SopB facilitates *Salmonella* invasion by activating Cdc42 (Zhou et al., 2001), and delays SCV maturation into a phagolysosome by excluding maturation markers (Dukes et al., 2006b); (Bakowski et al., 2010). Moreover, the translocation factor, YopB, of *Yersinia pseudotuberculosis* has been shown to induce pro-inflammatory signaling pathways in a manner that is independent of its established pore-forming functions (Viboud et al., 2003). These examples highlight the fact that one effector can play more than one role in a host cell either by the activity of the same or different functional domains.

4.4 PERP is Required for Caspase-3 Activation During *Salmonella* Infection, and Implications of PERP Accumulation.

Our study first started out with the goal of determining how SipA induces the activation of caspase-3 while also requiring caspase-3 cleavage to induce PMN transmigration. That SipA promotes activation of caspase-3 and simultaneously requires cleavage by this protease to be fully functional highlights the complexity of the signaling that occurs between SipA and the host cell. Indeed, a mechanism for how this may occur remained elusive, until we

discovered PERP is required for caspase-3 activation during *S. Typhimurium* infection (Chapter 2).

Previous reports have shown that increased PERP expression causes increased levels of activated caspases, including caspase-3 (Davies et al, 2009; Singaravelu et al, 2009). Since the mechanism by which PERP regulates caspase-3 activity remains unknown, it is impossible to know which PERP-mediated caspase-3 activation pathways are affected during *Salmonella* infection. Nevertheless, based on what is known about PERP and caspase-3 activation, we contend that the apical accumulation of PERP is processed by the cell as an over-abundance of PERP; this then leads to increased activation of caspase-3. Indeed, examination of PERP levels in whole cell lysates following infection showed a modest increase in PERP levels compared to non-infected cells (Chapter 2). Since our data indicate that *S. Typhimurium* does not cause apical accumulation of PERP by inducing new protein synthesis, we propose that the increase in PERP expression in whole cell lysates is due to the redistribution of PERP to the apical surface, and consequential removal from its normal trafficking route that would carry it into degradative pathways. As a result, *S. Typhimurium* causes PERP to accumulate in the host cell.

It is critical to note that apical PERP accumulation is dependent on SipA, but not dependent on the caspase-3 cleavage of SipA, as shown in Chapter 2. Further, we detect PERP accumulation at the apical surface after 1 hour of infection, yet we do not detect *Salmonella*-induced activation of caspase-3 until

after two or more hours of infection (Srikanth et al, 2010; Chapter 2). This tells us 1) that PERP accumulation at the apical surface occurs independently of caspase-3 mediated SipA processing, and 2) that PERP apical accumulation can thus occur prior to the point at which SipA triggers activation of caspase-3. These combined observations support our argument that SipA-mediated modulation of PERP leads to caspase-3 activation. Thus, our cumulative findings provide the first mechanistic insight into how SipA can induce caspase-3 activation prior to requiring cleavage by it.

4.5 PERP Promotes an Inflammatory Response to *Salmonella* Infection

One of our key findings is that PERP plays a fundamental role in facilitating transepithelial PMN migration in response to *S. Typhimurium* infection (Chapter 2). This observation is particularly exciting because not only does it identify a previously unknown player in the inflammatory response to *Salmonella* infection, but it also ascribes a previously unknown function to PERP. Strikingly, an earlier study indicated PERP might downregulate some inflammatory signaling pathways in a skin carcinoma model (Beaudry et al., 2010). However, our work is the first to demonstrate a distinct proinflammatory function for PERP, and is the first to show a role for PERP in the response to a bacterial infection. While our data may appear to conflict with the previous report, studies have shown PERP's functions to vary depending on cell type. Although PERP was first identified as a p53-effector of apoptosis (Attardi et al., 2000), it was later found to

be dispensable for p53-dependent apoptosis in certain fibroblasts, but required for p53-dependent apoptosis in thymocytes (Ihrie et al., 2003). Thus the specific roles of PERP may differ depending on the cell type being investigated.

PERP is required for PMN transepithelial migration in response to bacterial signals (*Salmonella* and fMLP) indicating that it could be important for mediating PMN migration in response to bacterial infections in general. Since PERP is known to be expressed in a variety of epithelial tissues (epidermal keratinocytes and oral epithelia in mice: (Ihrie et al., 2005); bovine hepatocytes, human lung epithelium, bovine urogenital epithelium, bovine intestinal epithelium: (Franke et al., 2013b)), it would be worth exploring whether PERP is required for PMN transepithelial migration in responses to non-enteric infections of mucosal surfaces as well, such as those caused by *P. aeruginosa*, and *S. pneumoniae*. Such experiments would show whether PERP's proinflammatory functions are restricted to *Salmonella* infection or to the intestinal tract, or exist throughout the body in response to a variety of pathogenic onslaughts.

Intriguingly, PERP was important for PMN transepithelial migration in response to some signals, but not all of the ones we tested. Indeed, LTB₄ and IL-8 are both potent signaling molecules that act on PMNs to trigger their migration, yet PERP was dispensable for migration that occurred in response to LTB₄ and only marginally required for migration that occurred in response to IL-8. In comparison, the lack of PERP caused a significant reduction in PMN transepithelial migration in response to fMLP. Why PERP is required for

migration in response to certain chemotactic signals but not others is difficult to ascertain at this point. The nuances of PERP activity during PMN migration resulting from different triggers will be better understood as we learn more about how PERP functions in the cell.

With regard to how PERP promotes PMN transepithelial migration in response to *Salmonella* infection, our data support the argument that PERP does so by mediating caspase-3 activation. As discussed above, we show that PERP is required for caspase-3 activation during *S. Typhimurium* infection, and we have previously shown that caspase-3 activation is required for SipA-mediated inflammatory responses, including PMN transepithelial migration. Thus, we contend that by promoting the activation of caspase-3, PERP promotes the proteolytic processing of SipA that enables it to induce PMN transepithelial migration and other inflammatory responses.

An additional possibility, and one that is not exclusive of a caspase-3 mediated role, is that PERP functions as a binding partner for PMNs as they pass through the transepithelial space. Indeed, another desmosome component, JAM-C, has been proposed to function as the elusive epithelial receptor for the PMN integrin CD11b/CD18, which is known to be required for transepithelial migration (Zen et al., 2004). If PERP does function as a PMN binding partner, that offers one explanation of why the addition of anti-PERP antibodies causes such a striking reduction in *Salmonella*-induced transepithelial PMN migration (Chapter 2); they may interfere with PERP's ability to bind to PMNs. However,

this possibility does not negate our findings that PERP is critical to the activation of caspase-3 during *S. Typhimurium* infection, nor does it exclude our contention that PERP promotes PMN transepithelial migration via its activation of caspase-3, which then leads to activation of SipA. Indeed, it is possible that PERP could have multiple ways of regulating PMN transepithelial migration depending on the specific signal and PERP's cellular location. In support of this hypothesis, a study in skin cell carcinoma indicates PERP has at least two roles in suppressing cancer progression: 1) inducing apoptosis and 2) mediating cell-cell adhesion (Beaudry et al., 2010). In that study, the loss of PERP promoted the development of UV-induced skin tumors and also promoted tumor progression, likely due to loss of desmosome stability. Thus, it would not be surprising to find that PERP has multiple mechanisms for modulating inflammatory responses to *Salmonella* infection as well.

Our finding that PERP has a role in mediating PMN transepithelial migration broadens our understanding of PERP's functions in the host cell, which to date have been limited largely to apoptosis, and epithelial barrier integrity and development. Given that PERP appears to be an inflammatory regulator in intestinal tissue, it may be worth investigating whether PERP functions in mediating inflammation in chronic intestinal inflammation conditions as well, such as Crohn's disease and ulcerative colitis.

4.6 *Salmonella* Activates Caspase-3 via the Modulation of PERP Trafficking:

A Model

On the basis of the discussion of our results and current literature above, we present a model of how we envision *Salmonella* to induce caspase-3-dependent inflammation outlined below and summarized in Figure 4.2.

1. *Salmonella* effectors induce exocyst formation at the apical surface of intestinal epithelial cells (Nichols and Casanova, 2010).
2. Vesicles containing PERP from apical endosomes traffic to the apical surface (Chapter 2 and Chapter 3).
3. PERP accumulates at the apical surface by the action of *Salmonella* T3SS1 effectors on the exocytic pathway (Chapter 2 and Chapter 3).
4. Apical PERP accumulation leads to increased levels of total cellular PERP, which leads to activation of caspase-3 (Chapter 2; Davies et al, 2009; Singaravelu et al, 2009).
5. Activation of caspase-3 by PERP leads to proteolytic processing of SipA, and promotes SipA's pro-inflammatory functions leading to transepithelial PMN migration (Chapter 2; Srikanth et al, 2010).

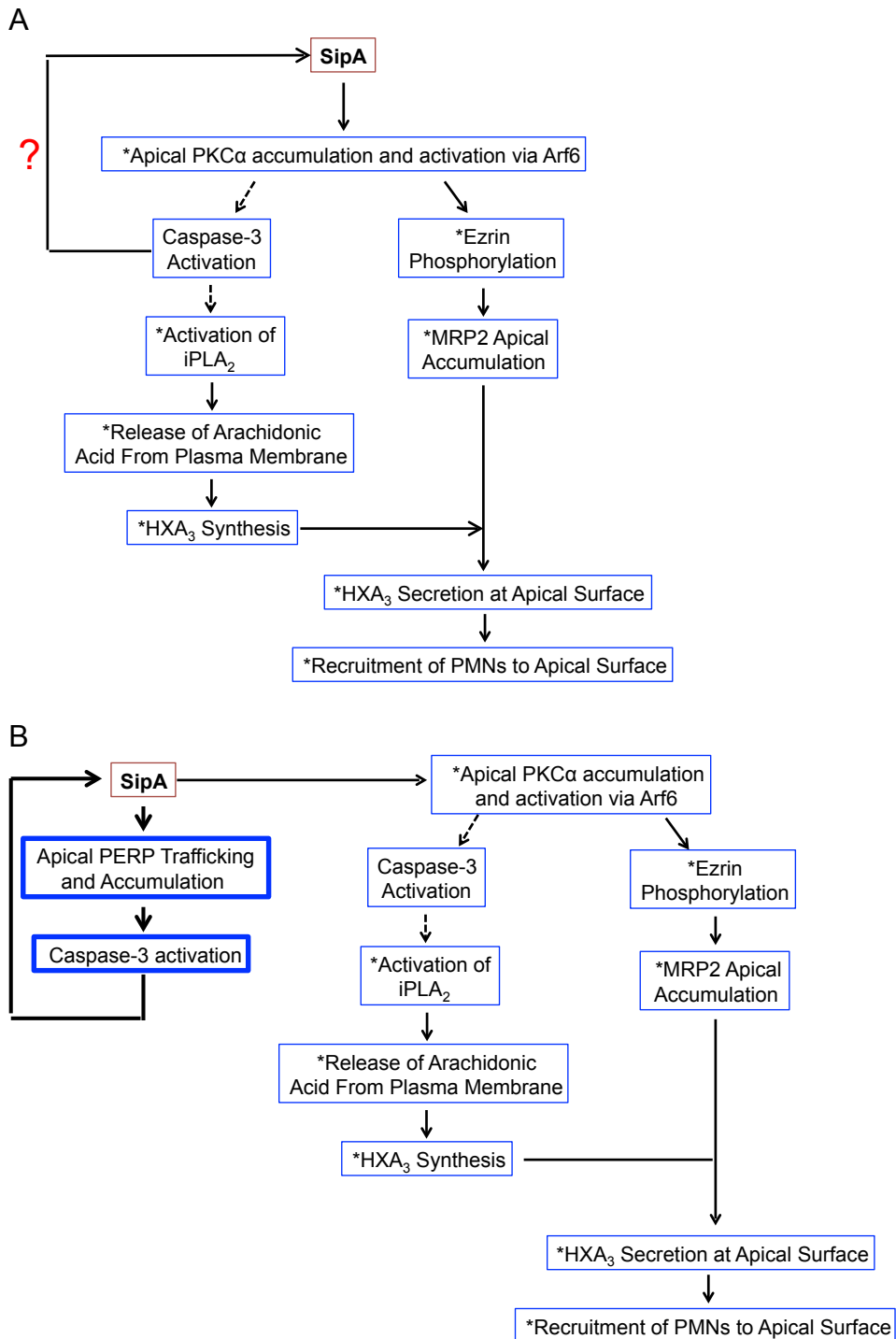
As described in Chapter 1, our earlier model indicated that caspase-3 activity is required for the SipA-mediated activation of iPLA₂, which leads to the

Figure 4.2: Models of PERP and Caspase-3 Activation During *Salmonella* Infection

A. This model illustrates our previous understanding of how caspase-3 is activated and required during *Salmonella* infection. Previous reports indicated that SipA both activates caspase-3 and requires caspase-3 activity in order to induce inflammatory responses, including recruitment of PMNs to the apical surface. A more detailed discussion of this pathway can be found in Chapter 1. The manner by which SipA activates caspase-3 to promote its own activation has remained unknown.

B. The model presented here shows how the data presented herein, highlighted in bold boxes and arrows, addresses the question of how SipA triggers caspase-3 activation. We showed that SipA induces PERP accumulation at the apical surface, which we propose leads to its accumulation within the cell. Previous papers show that increased PERP expression leads to increased activity of caspase-3, and indeed we see PERP is required for caspase-3 activation during *S. Typhimurium* infection. Strikingly, caspase-3 cleavage of SipA is NOT required for it to induce PERP apical accumulation, thus indicating that PERP-mediated caspase-3 activation occurs prior to SipA processing. This finding supports our model here that argues SipA-mediated redistribution and subsequent accumulation of PERP is the manner by which SipA induces caspase-3 activation.

Figure 4.2: Models of PERP and Caspase-3 Activation During *Salmonella* Infection



synthesis of HXA₃ (Figure 4.2a). However, our previous finding that the N-terminus of SipA alone is capable of activating pathways that lead to HXA₃ synthesis indicated that the SipA N-terminus is liberated via caspase-3 processing *prior* to the initiation of the PKC α -mediated caspase-3 activation cascade. We show that accumulation of PERP at the apical surface occurs without SipA needing to be cleaved by caspase-3. This observation supports our proposal that the accumulation of PERP at the apical surface provides the means by which activation of caspase-3 can occur prior to SipA cleavage. Thus, we propose that vesicle trafficking to the apical surface to support *Salmonella* uptake serves the additional purpose of facilitating SipA processing by causing PERP accumulation at this location, which then leads to caspase-3 activation.

Another inference that may be made from our new model is that there are at least two time points at which caspase-3 activity is required (Figure 4.2b). The first point is for cleavage of SipA, and the second is for the PKC α -mediated pathway of HXA₃ production. Whether or not the pathways leading to activation of caspase-3 at these time points overlap or are independent of each other remains to be determined. However, the requirement for two caspase-3 activation pathways may help explain why we don't see a more drastic decrease in PERP-mediated caspase-3 activity during *Salmonella* infection in our PERP knockdown cells. Further, the existence of distinct early (PERP-mediated) and late (PKC α -mediated) caspase-3 activation pathways explains how SipA can be cleaved by caspase-3 prior to activating PKC α .

4.7 Future Directions

As alluded to above, many questions remain with regard to how different parts of our new model function and intersect. Perhaps the biggest question is whether SipC plays an active role in mediating caspase-3 activation during *Salmonella* infection. Indeed we infer this is the case since, like SipA, SipC mediates PERP accumulation at the apical surface, which we propose plays a role in PERP-mediated caspase-3 activation. While we have demonstrated a role for SipA in caspase-3 activation (Srikanth et al., 2010), a role for SipC in this process has yet to be examined. However, previous work from our lab shows that SipC is required for PMN transepithelial migration (Wall et al., 2007). This was surprising, given that the absence of SipA nearly completely abrogates PMN transepithelial migration (Lee et al., 2000). Nevertheless, the effector SopA has also been shown to be an important mediator of the PMN response to *Salmonella* infection (Srikanth et al., 2010) indicating that the pathways by which PMNs are recruited to the apical surface during *Salmonella* infection are complex and remain to be completely understood. Therefore, a role for SipC in mediating caspase-3 activation is plausible, and would help explain why it is important for PMN responses.

An additional question worth pursuing is the pathway by which PERP triggers caspase-3 activation. Our previous study (Srikanth et al., 2010) indicated that increased caspase-8 activity is not detected in response to *Salmonella* infection. Thus, while a previous study indicated increased caspase-8 activation

was correlated with increased PERP expression (Davies et al., 2009), activation of caspase-8 does not appear to be the pathway by which PERP leads to caspase-3 activation in our system. However, caspase-3 can also be activated by activated caspase-9 and caspase-10. A previous study has shown a correlation between PERP expression and caspase-9 activation (Singaravelu et al., 2009), and so it is worth examining whether caspase-9 increases with *Salmonella* infection. Caspase-10 activation during *Salmonella* infection would also be worth exploring. Such experiments would not only yield a better understanding of how caspase-3 is activated during *Salmonella* infection, but would provide further understanding as to how PERP regulates caspase-3 activation in general. Indeed, caspase-8 and caspase-10 are associated with the extrinsic apoptotic pathway whereas caspase-9 is associated with the intrinsic apoptotic pathway. Knowing which apoptotic pathway PERP participates in would help improve our general understanding how of PERP triggers apoptosis.

4.8 Summary

Our reported findings enlighten our understanding of how SipA mediates inflammatory responses to *Salmonella* infection, and provide the first mechanistic insight addressing how SipA can simultaneously trigger the activation of caspase-3 while also requiring cleavage by this enzyme to induce inflammation. These results build upon our current understanding of how T3SS effectors interact with host cells and perturb host cell signaling pathways to induce

pathogenesis. Moreover, our data provide insight into the functions of PERP. Our findings build upon previous reports indicating PERP is important for normal cell homeostasis and apoptosis, as we now show here that PERP is key for mediating inflammatory responses to *S. Typhimurium* infection. Indeed our results highlight not only what bacteria can teach us about mechanisms of pathogenesis, but also how they can be used as tools to provide insight into eukaryotic cell pathways.

APPENDIX A

Is PERP the SipA Apical Surface Receptor?

While we found PERP can bind to SipA (Chapter 2), the physiological relevance of this interaction remains to be determined, and thus it is not included in our model in Chapter 4. Nevertheless, the finding does merit discussion here, as it paves the way for further investigation into whether PERP is the apical surface SipA receptor.

Finding that PERP and SipA interact was eye-opening, as this is the first demonstration of SipA binding to a eukaryotic membrane protein that localizes to the apical surface during *Salmonella* infection, and furthers our understanding of how SipA interacts with host cells during pathogenesis. Interestingly, we did not detect binding of PERP to SifA, a *Salmonella* effector protein translocated via T3SS2, which is the T3SS generally used to promote intracellular survival. However, we did detect binding between PERP and SipC (Chapter 2), which is a component of the T3SS1, although this was not done under infection conditions so the physiological relevance remains to be verified. Nevertheless, it would be interesting to examine whether other T3SS1 effectors are also capable of binding to PERP to help us better understand whether PERP's interaction with SipA has a role specific to this effector's functions, or whether there is a more universal role for PERP binding to T3SS1 effectors.

While it is tempting to speculate that PERP is an important SipA binding partner that promotes SipA-mediated inflammatory responses, the pathological

importance of PERP binding to SipA remains to be determined. Ideally, it would be best to mutate sites on SipA and PERP suspected to be important for the interaction, examine whether the interaction fails as a result, and then examine the physiological consequences of the failed interaction. However, while the structures of some regions of SipA have been determined (Lilic et al., 2003), its entire structure has yet to be resolved. Further, while PERP's membrane domains have been mapped, its final conformation within the cell also remains to be determined.

Further, our data do not exclude the hypothesis that the PERP-SipA interaction occurs within the host cell. Our attempts to verify the cellular location of this interaction via modifications to the biotinylation assay or via microscopy yielded inconclusive results. We suspect this may be due in part to the level of SipA present at the apical surface of a cell at any given point being below the detection level required for our methods.

Perhaps the evidence that argues most strongly against PERP acting as the apical SipA receptor is the finding that SipA is required for PERP accumulation at the apical surface (Chapter 2). This observation is particularly confounding as it is difficult to imagine how SipA can simultaneously require binding to PERP at the apical surface *and* induce its apical accumulation. Nevertheless, *Salmonella Typhi* has been shown to utilize CFTR as its host cell receptor *and* to trigger accumulation of this protein to the apical surface of infected cells (Lyczak and Pier, 2002). Thus it is not impossible that PERP is a

SipA receptor, and that interaction between these two proteins induces a signaling cascade that leads to PERP trafficking events. Indeed, it was initially difficult to imagine how SipA could simultaneously require *and* trigger caspase-3 activation to induce inflammatory responses, but we now have a model that explains how that might occur. While key questions remain regarding the relevance of the PERP and SipA interaction, it is important to not lose sight of the fact that finding such an interaction occurs serves to broaden our understanding of how SipA, and possibly other T3SS effectors, interact with host cells.

APPENDIX B

Baseline PERP Behavior

Introduction

Since the range of PERP's functions within the cell remain unknown, we thought it prudent to confirm that knocking down PERP expression did not alter the ability of *Salmonella* to invade colonic epithelial cells. If it did, this would raise additional questions as to how PERP regulates epithelial responses to *Salmonella* infection. The specificity of the PERP antibody was also confirmed.

Materials and Methods

Invasion assay

Description of stable PERP knockdown design, and of invasion assay methods are detailed in Chapter 2.

Western blotting

Whole cell lysates from wild type HCT8 cells and HCT8 cells transfected with PERP siRNA, the no targeting control, or the PERP-GFP construct were prepared and Western blotted for PERP as described in Chapter 2.

Transfection

HCT8 cells were transfected with 3ug of the PERP-GFP construct (kind gift from Dr. Luminita Paraoan) using Lipofectamine 2000 per the manufacturer's instructions.

Confocal Microscopy

Following transfection with the PERP-GFP construct (green), cells were washed with PBS, then fixed in 4% PFA, quenched with 50mM ammonium chloride, then permeabilized with 1% triton in PBS. The cells were then blocked with blocking buffer (3% BSA in PBS) for one hour followed by staining with phalloidin AlexaFluor 647 (red), and mounting with SlowFade Gold containing DAPI (blue). Images of the transfected cells were collected from a Leica TCS SP-5 Confocal microscope as described for the "PERP and Rab25 Colocalization" image collection in Chapter 2. Post imaging, 0.2 mm image slices were processed using FIJI.

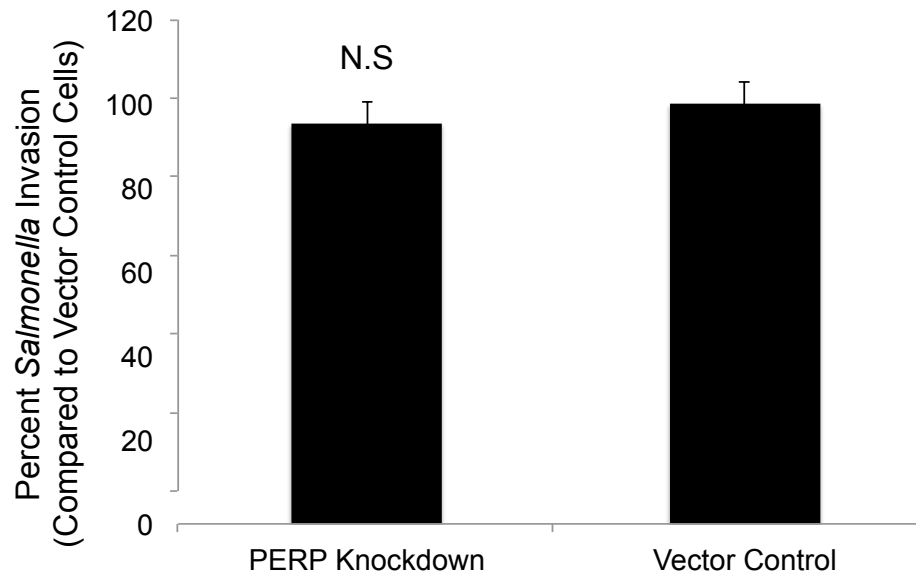
Results

PERP Knockdown Cells Are Not Invasion-Defective

As shown in Figure B.1, knocking down PERP expression (Figure 2.2) does not affect the ability of *Salmonella* to invade the HCT8 colonic epithelial cell line.

Figure B.1: PERP Knock Down Cells Are Not Invasion Deficient

An invasion assay was performed on stable PERP knockdown cells and vector control cells. The level of *Salmonella* invasion is reflected as percent invasion compared to invasion levels in vector control cells (set to 100%). Data shown are of one representative of three experiments performed in triplicate. Error bars show +/- standard error.

Figure B.1: PERP Knock Down Cells Are Not Invasion Deficient

PERP Antibody Specificity

As shown in Figure B.2A, we typically observed three bands upon staining for PERP via western blot. One of these corresponds to the expected molecular weight of PERP, 22kD. The remaining two bands are visible at about 33kD and about 52kD. The identity of these two higher bands remains undetermined. However, as shown in Figure B.2B, the appearance of the 22kD band is reduced in the PERP knockdown cells, as is expected. Further, upon transfecting HCT8 cells with a PERP-GFP construct, we observe the appearance of a band that runs at about the size of a band expected from the addition of a 27kD GFP tag to the 22kD PERP protein (Figure B.2C).

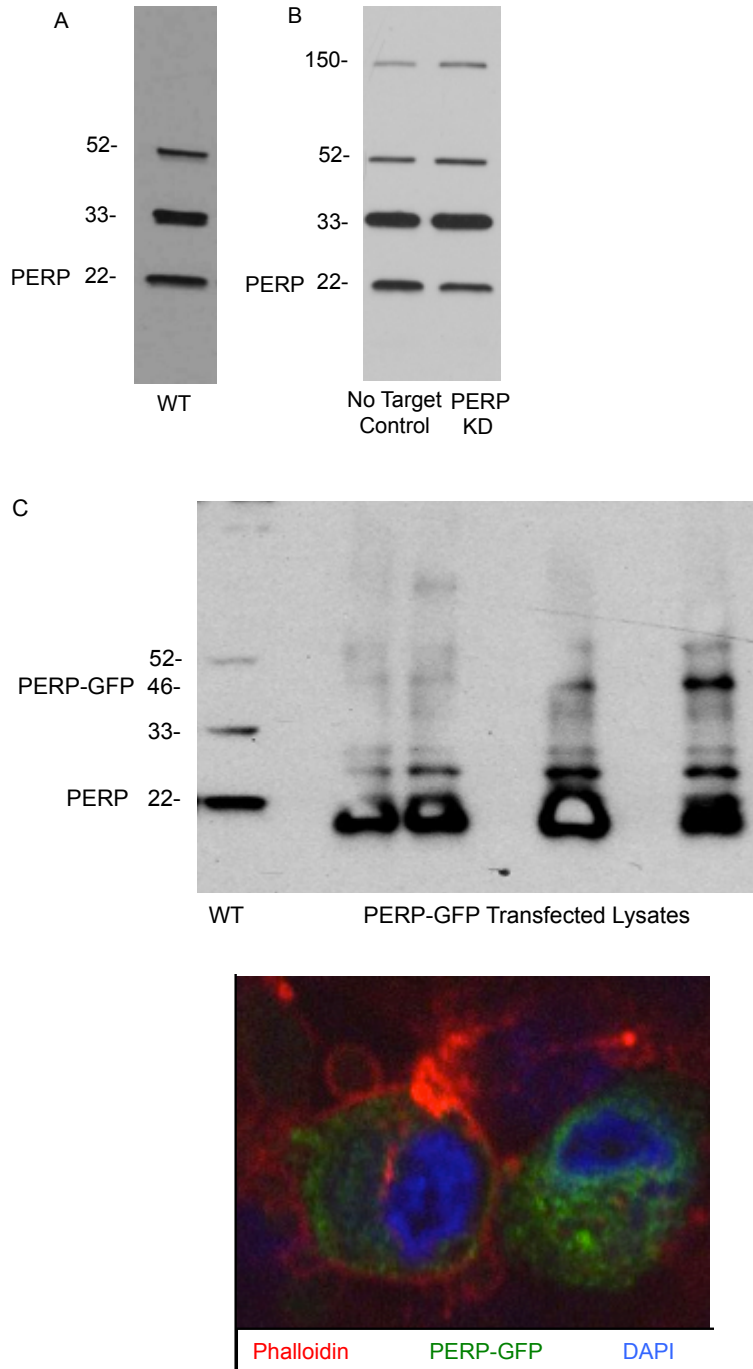
Conclusions

That knocking down PERP expression does not impact the ability of *Salmonella* to invade colonic epithelial cells indicates that PERP is not required for *Salmonella* invasion. This is in line with our data showing that PERP is required for inflammatory responses (caspase-3 activation and PMN transmigration), which are often controlled by separate pathways from those controlling invasion.

As shown in Figure B.2, the PERP antibody detects PERP. It should be noted that transfecting the PERP-GFP construct into HCT8 cells induced morphological changes in the cells that were characteristic of early signs of

Figure B.2: PERP Antibody Specificity

- A. Western blot of PERP from wild type whole cell HCT8 lysates showing three bands at 22kD, 33kD, and 52kD.
- B. Western blot of PERP from whole cell lysates of HCT8 cells transfected with a no targeting siRNA control, or with PERP siRNA.
- C. Western blot of PERP from lysates of wild type HCT8 cells, or HCT8 cells transfected with the PERP-GFP construct. Fluorescent microscopy panel shows cells transfected with the PERP-GFP construct (green) and stained with phalloidin (red) and DAPI (blue).

Figure B.2: PERP Antibody Specificity

apoptosis, including membrane blebbing (Figure B.2C, confocal image). This observation was previously reported (Davies et al., 2009) and is likely due to increased levels of PERP protein due to the presence of the PERP-GFP construct. Due to these changes, no experiments with this construct in the context of *Salmonella* infection were run.

APPENDIX C

Raw Data for Densitometry

Introduction

Due to the inherent variability between western blots, densitometric values in Chapter 2 and Chapter 3 were presented as ratios of protein expression from one representative experiment. Presenting the values as ratios rather than in the raw densitometric values from each experiment allowed us to make comparisons across multiple experiments.

The following tables show the raw densitometric values from three replicates of these experiments. These tables also show the ratios of PERP expression in response to infection with wild-type *Salmonella* compared to PERP expression in response to infection with mutant or complemented strains, or PERP expression in response to treated cells compared to non-treated cells. In cases where PERP was detected at the apical surface without infection, the ratio of PERP expression following infection compared to baseline PERP expression was first determined before comparing PERP expression levels following wild-type infection versus infection with mutants or complemented strains. The tables include the average, standard deviation, and standard error for the ratios across these experiments calculated via Prism software. Densitometric values expressed are values obtained after background subtraction. ND indicates no detectable expression. n/a indicates not applicable. Table headings indicate the corresponding figure in the main text.

C.1 Densitometric Data for Chapter 2

Figure 2.2B: PERP Expression in Stable PERP Knockdown Cells

Replicate	Vector Control	PERP Knockdown	Ratio of PERP Expression (Knockdown to Vector Control)
1	27.136	14.587	0.537
2	96.5	63.5	0.658
3	15.178	8.962	0.591
Mean			0.595
Standard Deviation			+/- 0.061
Standard Error			+/- 0.035

Figure 2.4A: PERP Expression in Transient PERP Knockdown Cells

Replicate	No Target Control	PERP Knockdown	Ratio of PERP Expression (Knockdown to No Target Control)
1	64.004	34.455	0.538
2	41.047	18.993	0.463
3	47.087	21.151	0.449
Mean			0.483
Standard Deviation			+/- 0.048
Standard Error			+/- 0.028

Figure 2.5A: Apical Surface PERP Expression Following Infection with WT and Δ SipA *Salmonella*

Replicate	(-)	WT	Ratio of PERP Expression (WT to (-))	Δ SipA	Ratio of PERP Expression (Δ SipA to (-))	Ratio of PERP Expression (Δ SipA to WT)
1	ND	47.13	n/a	30.705	n/a	0.651
2	6.78	74.46	10.98	42.473	6.27	0.57
3	ND	38.39	n/a	22.694	n/a	0.591
Mean						0.604
Standard Deviation						+/- 0.042
Standard Error						+/- 0.024

Figure 2.5B: Apical Surface PERP Expression Following Infection with WT and Δ SipA *Salmonella* Complemented with High-Expressing SipA pBH Plasmid

Replicate	(-)	WT	SipA+	Ratio of PERP Expression (SipA+ to WT)
1	ND	7.303	67.907	9.3
2	ND	10.29	77.35	7.52
3	ND	6.847	79.228	11.57
Mean				9.462
Standard Deviation				+/- 2.032
Standard Error				+/- 1.173

Figure 2.5C: Apical Surface PERP Expression Following Infection with WT and Δ SipB *Salmonella*

Replicate	(-)	WT	Ratio of PERP Expression (WT to (-))	Δ SipB	Ratio of PERP Expression (Δ SipB to (-))	Ratio of PERP Expression (Δ SipB to WT)
1	ND	55.811	n/a	63.93	n/a	1.14
2	7.648	23.655	3.09	21.088	2.76	0.89
3	21.213	44.223	2.08	44.32	2.09	1.004
Mean						1.011
Standard Deviation						+/- 0.125
Standard Error						+/- 0.072

Figure 2.5D: PERP Expression from Non-Infected and WT Infected Whole Cell Lysates

Replicate	(-)	WT	Ratio of PERP Expression (WT to (-))
1	44.92	65.057	1.44
2	55.613	71.552	1.28
3	35.186	48.061	1.36
Mean			1.363
Standard Deviation			+/- 0.08
Standard Error			+/- 0.046

C.2 Densitometric Data For Chapter 3

Figure 3.1A: Apical Surface PERP Expression Following Infection with WT and Δ HilA *Salmonella*

Replicate	(-)	WT	Δ HilA	Ratio of PERP Expression (Δ HilA to WT)
1	ND	51.691	1.709	0.033
2	ND	41.993	5.406	0.129
3	ND	24.758	3.018	0.121
Mean				0.094
Standard Deviation				+/- 0.053
Standard Error				+/- 0.031

Figure 3.1B: Apical Surface PERP Expression Following Infection with WT and Δ HilA *Salmonella* Complemented with HilA on the pBH Plasmid

Replicate	(-)	WT	pHilA	Ratio of PERP Expression (pHilA to WT)
1	ND	16.033	19.742	1.231
2	ND	20.406	24.987	1.224
3	ND	35.702	41.463	1.161
Mean				1.206
Standard Deviation				+/- 0.0385
Standard Error				+/- 0.0223

Figure 3.1C: Apical Surface PERP Expression Following Infection with WT and Δ SipC *Salmonella*

Replicate	(-)	WT	Δ SipC	Ratio of PERP Expression (Δ SipC to WT)
1	ND	55.924	36.119	0.646
2	ND	42.455	23.09	0.544
3	ND	73.721	46.331	0.628
Mean				0.606
Standard Deviation				+/- 0.054
Standard Error				+/- 0.031

Figure 3.1D: Apical Surface PERP Expression Following Infection with WT and Δ SipC *Salmonella* Complemented with SipC on the pBH Plasmid

Replicate	(-)	WT	pSipC	Ratio of PERP Expression (pSipC to WT)
1	ND	21.53	22.221	1.032
2	ND	63	59.56	0.9454
3	ND	33.288	42.489	1.27
Mean				1.082
Standard Deviation				+/- 0.168
Standard Error				+/- 0.097

Figure 3.1E: Apical Surface PERP Expression Following Infection with WT and Δ SipA Δ SipC *Salmonella*

Replicate	X	WT	Δ SipA Δ SipC	Ratio of PERP Expression (Δ SipA Δ SipC to WT)
1	ND	71.096	45.079	0.634
2	ND	8.56	5.446	0.636
3	ND	60.1	35.196	0.585
Mean				0.618
Standard Deviation				+/- 0.028
Standard Error				+/- 0.017

Figure 3.2B: Apical Surface PERP Expression Of Non-Infected (-) and WT-Infected (WT) Cells With or Without Cytochalasin D (Cyto D) Treatment

Replicate	(-) DMSO	WT DMSO	(-) Cyto D	WT Cyto D	Ratio of PERP Expression (WT Cyto D to WT DMSO)
1	ND	24.576	ND	58.626	2.385
2	ND	11.842	ND	25.239	2.131
3	ND	68.32	ND	102.746	1.50
Mean					2.01
Standard Deviation					+/- 0.455
Standard Error					+/- 0.263

Figure 3.2C: Apical Surface PERP Expression Of Non-Infected (-) and WT-Infected (WT) Cells With or Without Dynasore Treatment

Replicate	(-) DMSO	WT DMSO	(-) Dynasore	WT Dynasore	Ratio of PERP Expression (WT Dynasore to WT DMSO)
1	ND	42.093	ND	74.937	1.78
2	ND	42.345	ND	93.91	2.22
3	ND	53.099	ND	112.777	2.12
Mean					2.041
Standard Deviation					+/- 0.230
Standard Error					+/- 0.133

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