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CHARACTERIZATION OF THE CASPASE-3 CLEAVAGE MOTIF OF THE *SALMONELLA* TYPHIMURIUM EFFECTOR PROTEIN SIFA AND ITS ROLE IN PATHOGENESIS

A Dissertation Presented
By
SAMIR PATEL

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

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CHARACTERIZATION OF THE CASPASE-3 CLEAVAGE MOTIF OF THE *SALMONELLA* TYPHIMURIUM EFFECTOR PROTEIN SIFA AND ITS ROLE IN PATHOGENESIS

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SAMIR PATEL

The signatures of the Dissertation Defense Committee signify completion and approval as to the style and content of the Dissertation

_____
Beth McCormick, Ph.D, Thesis Advisor

_____
Sanjay Ram, MD, Member of Committee

_____
Egil Lien, Ph.D, Member of Committee

_____
Javier Irazoqui, Ph.D, Member of Committee

_____
Christina Faherty, Ph.D, Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

_____
Christopher Sassetti, Ph.D, Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requires of the school.

_____
Mary Ellen Lane, Ph.D,
Dean of the Graduate School of Biomedical Sciences

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ABSTRACT

Salmonella enterica serovar Typhimurium (*S*. Typhimurium) is a Gram-negative facultative anaerobe that induces severe inflammation resulting in gastroenteritis. In the case of *S.* Typhimurium infection, induction of an inflammatory response has been linked to its primary virulence mechanism, the type III secretion system (T3SS). The T3SS secretes protein effectors that exploit the host's cell biology to facilitate bacterial entry and intracellular survival, and to modulate the host immune response.

One such effector, SifA, is a bi-functional T3SS effector protein that plays an important role in *Salmonella* virulence. The N-terminal domain of SifA binds SifA-Kinesin-Interacting-Protein (SKIP), and via an interaction with kinesin, forms tubular membrane extensions called Sif filaments (Sifs) that emanate from the *Salmonella* Containing Vacuole (SCV). The C-terminal domain of SifA harbors a WxxxE motif that functions to mimic active host cell GTPases. Taken together, SifA functions in inducing endosomal tubulation in order to maintain the integrity of the SCV and promote bacterial dissemination. Since SifA performs multiple, unrelated functions, the objective of this study was to determine how each functional domain of SifA becomes processed.

In the present study, we demonstrate that a linker region containing a caspase-3 cleavage motif separates the two functional domains of SifA. To test the hypothesis that processing of SifA by caspase-3 at this particular site is required for function and proper localization of the effector protein domains, we developed two tracking methods to analyze the intracellular localization of SifA.

We first adapted a fluorescent tag called phiLOV that allowed for T3SS mediated delivery of SifA and observation of its intracellular colocalization with caspase-3. Additionally, we created a dual-tagging strategy that permitted tracking of each of the SifA functional domains following caspase-3 cleavage to different subcellular locations. The results of this study reveal that caspase-3 cleavage of SifA is required for the proper localization of functional domains and bacterial dissemination. Considering the importance of these events in *Salmonella* pathogenesis, we conclude that caspase-3 cleavage of effector proteins is a more broadly applicable effector processing mechanism utilized by *Salmonella* to invade and persist during infection.

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PREFACE TO CHAPTER I

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CHAPTER I: Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a Gram-negative, facultative, intracellular anaerobe that causes severe inflammation of the intestinal mucosal epithelium resulting in gastroenteritis. *S.* Typhimurium causes disease through its primary virulence mechanism, the type III secretion system (T3SS). There are two T3SSs that are encoded by two regions of the bacterial chromosome called *Salmonella* pathogenicity island 1 and *Salmonella* pathogenicity island 2 (SPI-1 and SPI-2). These pathogenicity islands also encode effector proteins that are secreted from the T3SS and translocated into epithelial cells at the mucosal surface of the intestine. Upon contact with the mucosal epithelium, SPI-1 encoded effector proteins are translocated into epithelial cells and promote bacterial entry and inflammation. SPI-2 encoded effector proteins generally function to maintain the intracellular survival of *S.* Typhimurium after the organism has been macropinocytosed by epithelial cells. More recent studies, however, suggest that SPI-1 and SPI-2 effector proteins may not be as functionally compartmentalized as originally thought [1-3].

The architecture of the mucosal epithelium contains several barriers that attempt to prevent or impede infection by pathogenic bacteria. Mechanisms of protection are employed by all of these barriers in order to maintain the integrity of the epithelial cell monolayer and limit inflammation-associated damage (Figure 1.1). *S.* Typhimurium can modulate the signaling pathways that govern these mechanisms, including targeting specific proteins or inducing pathways through

functional mimicry, in order to provide itself with an ecological advantage with its T3SS virulence mechanism. Although *S.* Typhimurium can, in certain instances, bypass the innate immune response, the adaptive inflammatory immune response is in most instances capable of clearing the pathogen, albeit with increased damage to the mucosal epithelium.

Architecture of the Mucosal Epithelium: Barriers Against Infection

Mucus/Mucins

The luminal side of the intestinal epithelium is covered with a thick layer of mucus primarily composed of mucins, the main secretory product of goblet cells (Figure 1.1). Mucins are high molecular weight glycoproteins that aggregate to form a “gel-like” barrier to defend against endogenous or exogenous luminal insults. To date, at least 17 highly conserved mucins have been identified, each with varying specificities for different epithelial tissues [4]. Furthermore, these mucins have been categorized into two major groups: cell surface mucins and secreted mucins (Figure 1.1). Of these two categories, it is the secreted mucins that form the major structural component of the mucosal layer, and out of the known secreted mucin proteins, MUC2, MUC5AC, MUC5B, MUC6, and MUC19 are classified as gel-forming for human mucosal surfaces [5, 6]. The predominant mucin comprising the mucus layer of the intestinal epithelium is MUC2, although

Figure 1.1: Architecture of the mucosal surface

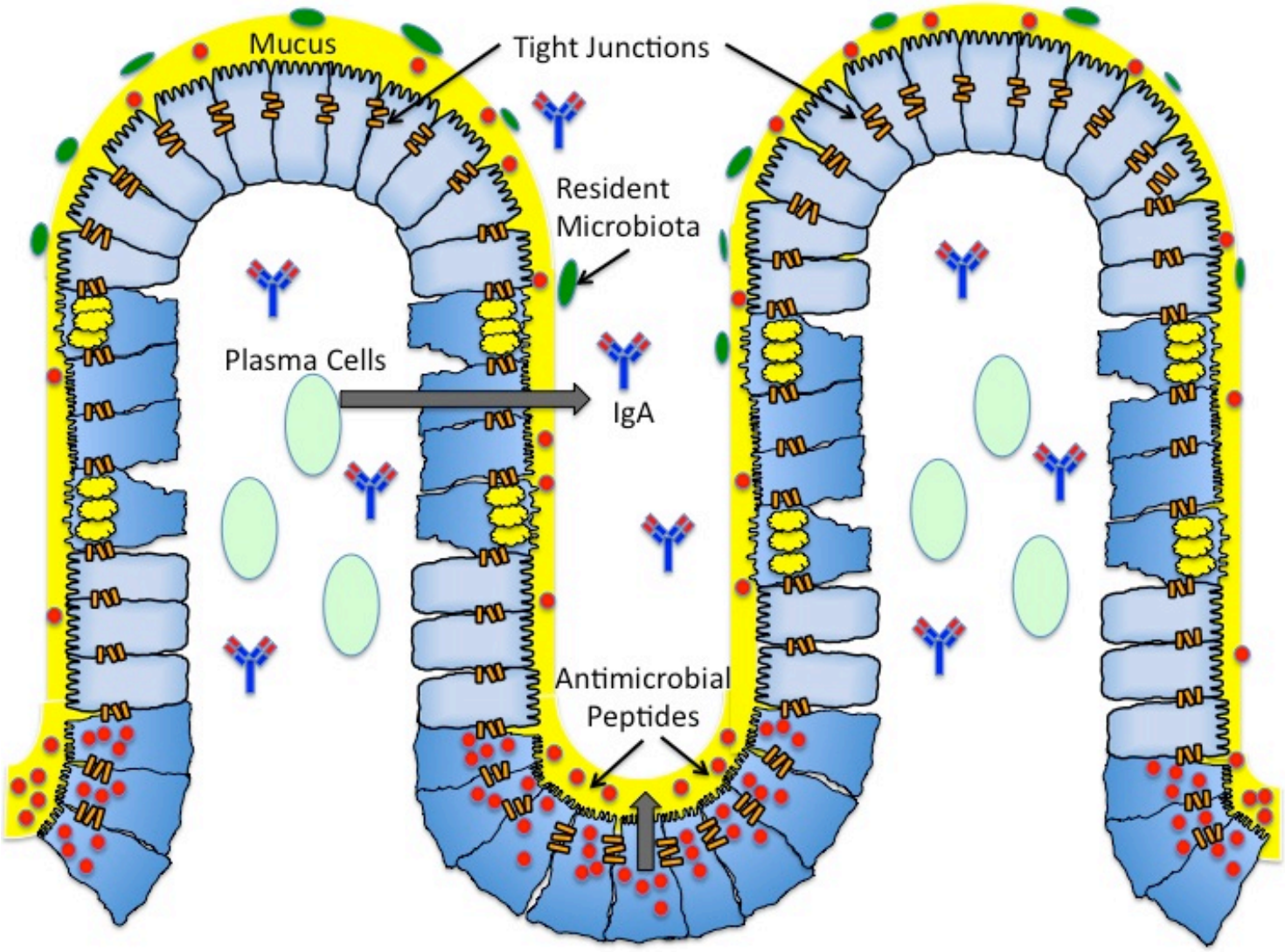

Figure 1.1: Architecture of the mucosal surface. The mucosal surface of the intestine contains a single layer of epithelial cells. The monolayer of epithelial cells is fortified by a layer of mucus (yellow) produced by *Goblet cells (blue cells with yellow granules)*. This thick mucus layer contains membrane bound and secreted mucins. The antimicrobial peptides (red) secreted by *Paneth cells (blue cells with red granules)* reside in the thick mucus layer, providing another form of protection against both pathogenic and commensal bacteria. Antimicrobial peptides include defensins, cathelicidins, and histatins. *Plasma B cells (light green)* reside in the subepithelial region and produce *secretory IgA (blue and red antibody)*. Secreted IgA is found in the subepithelial region and the lumen. *Resident microbiota (green)* reside in the outer mucus layer, providing yet another barrier to pathogenic infection. The majority of resident microbiota belong to two phyla - *Firmicutes* and *Cytophaga-Flavobacterium-Bacteroidetes*. The seal between epithelial cells is maintained by *tight junctions (orange bars)*. Tight junctions are dynamic structures composed of zonula occludens and junctional adhesion molecules.

MUC5AC has been shown to be expressed in the mucus layer of the fetal intestine [7].

The mucosal layer consists of an inner layer of mucus that is firmly adherent to the intestinal epithelial cells (mainly comprised of cell surface mucins) and a looser outer layer of mucus (mainly comprised of secreted mucins) [8]. For quite some time, the mucus layer of epithelial surfaces was thought to solely serve the purpose of providing a physical barrier, preventing access of pathogenic bacteria or resident microbiota to the epithelial cells.

However, this is only the case for the inner, attached mucosal layer of the large intestine, which prevents bacterial entry due to their size [9]. In the small intestine, the mucosal layer is thinner and has been shown to have a higher degree of detachment than in the large intestine [10, 11]. The detachment and thinness of the mucosal layer in the small intestine not only facilitates the removal of both pathogenic bacteria and resident microbiota attached to the mucus, but also allows for a higher degree of bacterial penetration.

It has also been increasingly realized that the mucins in the outer sublayer of the mucosal barrier also provide an energy source for both resident microbiota and pathogenic microoganisms capable of adhering to the mucus layer. This layer provides both commensal and pathogenic microorganisms with a niche in which to grow and colonize the intestine [12, 13] The inner layer of the mucosal surface is considered “sterile”, largely due to the presence of antimicrobial peptides secreted by Paneth Cells (Figure 1.1, discussed later), thereby limiting bacterial colonization to the outer mucus layer [8, 14, 15].

Certain cell surface mucins in the inner mucus layer also directly play a role in protecting against bacterial colonization on the epithelial surface by acting as pathogen-binding decoys. For example, epithelial cells can release Muc1 (called mucin shedding) in response to *H. pylori* infection, and Muc1 will bind the bacteria, preventing its adhesion to the intestinal epithelium [8]. Furthermore, it has been shown that approximately 5-fold more *H. pylori* colonize the intestinal epithelium of *Muc1*$^{-/-}$ mice than wild-type mice [16]. Although the thick mucus layer provides protection in the form of a physical barrier, it is significant to note that the necessity to maintain healthy intestinal microflora does provide pathogenic bacteria with the same energy source and corresponding growth advantage as well. This advantage has allowed certain pathogenic bacteria to develop mechanisms to circumvent the protection provided by the mucus sublayers and infect the underlying epithelial cells. As an example, certain pathogenic *Escherichia coli* (*E. coli*) secrete mucinolytic proteins, thus allowing them to persist and colonize within the mucus layer [17, 18]. Unlike *E. coli*, *S.* Typhimurium doesn't enzymatically degrade mucus in order to colonize the mucosal epithelium. Rather, mucins have actually been shown to be the binding sites for *S.* Typhimurium, and in particular a 250 kDA neutral mucin has been implicated as a receptor for *S.* Typhimurium [13].

Resident Microbiota

The mammalian intestinal microflora contains approximately 10^{14} resident bacteria, comprising approximately 1,000 species, and they reside in the outer

sublayer of the mucosal barrier on the luminal side of the intestinal epithelium (Figure 1.1). The vast majority (~90%) of the commensal bacteria in humans and mice belong to two phyla: *Firmicutes* and *Cytophaga-Flavobacterium-Bacteroidetes*.

Similar to the difference in the mucosal layer architecture, the composition of the resident microbiota differs between the large and small intestine. The small intestine exhibits an increase in *Bacteroidetes* and a decrease in *Firmicutes*, with an overall reduction in species diversity in comparison to the large intestine [19]. Additionally, the resident microbiota are present in reduced numbers, which could be caused by the consistent removal of the detached mucosal layer containing pathogenic bacteria and resident microbiota in the small intestine, though further studies would be required to address this question.

Though much of the resident microbiota are of the same two phyla, there are differences in intestinal floral composition of individuals that arise at the species level [20]. Diversity of the intestinal microflora is susceptible to change due to environmental factors such as nutrition, and there is variation (increases/decreases in quantity of certain species of bacteria or increases/decreases in diversity of a particular genus of bacteria) in microbiota populations within different age groups [21, 22].

The resident microbiota promote resistance to infection by pathogenic microorganisms in several ways. First, they serve as a microbial barrier by competing with pathogens for resources at the outer mucosal sublayer, thereby limiting pathogenic bacterial colonization [8]. Additionally, end products of

metabolic pathways of individual species of bacteria have been shown to prevent pathogenic infection. For example, *Bifidobacteria* carbohydrate metabolism produces high concentrations of acetate, which has been shown to prevent release of Shiga toxin during infection with enterohemmorhagic (EHEC) infection, thereby decreasing the risk of toxin gaining access to the blood stream from where it can otherwise cause lethal damage to target organs such as the kidneys [23]. Along the same lines, it is becoming more appreciated that the composition of the intestinal microbiota may be just as important in defending against infection as the quantity of the commensal bacteria. For instance, selective reduction of *Lactobacilli* and *Enterococci*/group D streptococci groups of bacteria through the use of low concentrations of antibiotics has been shown to make mice more susceptible to colonization of the epithelial surface with *S.* Typhimurium without drastically affecting the overall numbers of commensal bacteria [20]. Further investigation is needed to determine exactly which resident microbiota are necessary to prevent other pathogens from colonizing the intestinal epithelium, especially since certain enteric pathogens have developed mechanisms to subvert this microbial form of protection.

Nevertheless, an emerging concept is that inflammation of the mucosal epithelium plays a role in the bacterial fitness of *S.* Typhimurium. One of the more basic advantages of *S.* Typhimurium-induced inflammation is that the clinical manifestation of diarrhea facilitates the spread of bacteria. Additionally, it has been shown that unlike avirulent strains, wild-type *S.* Typhimurium is capable of out-competing commensal microbiota in re-colonization experiments after

treatment with antibiotics. Furthermore, *S.* Typhimuirum exploits inflammation to promote its own colonization. In this instance, *S.* Typhimurium has been shown to out-compete the resident microbiota in a mouse colitis model [24]. One explanation for this phenomenon is that inflammation provides *S*. Typhimurium with a respiratory electron acceptor that members of the resident microbiota are unable to utilize. In particular, reactive oxygen species generated by neutrophils (PMNs) during inflammation can react with endogenous thiosulfate to form tetrathionate, a respiratory electron acceptor [25]. The ability to respire tetrathionate has been mapped to the *ttrRSBCA* locus, which is located in SPI-2 [26]. Under anaerobic conditions in which thiosulphate was oxidized to tetrathionate, *S.* Typhimurium displays a growth advantage in comparison to resident microbiota under the same conditions [25].

Both resident microbiota and *S.* Typhimurium compete for resources available for fermentation at the mucosal layer; however, resident microbiota are incapable of using the fermentation end products [25]. By reducing the tetrathionate made available by the inflammatory response to infection, *S.* Typhimurium is capable of respiring the fermentation end products in anaerobic conditions, thereby providing it with an advantage over the resident microbiota [25]. Remarkably, the growth benefit is conferred to *S.* Typhimurium only in the presence of inflammation, and it has been suggested that a reason *S.* Typhimurium has evolutionarily maintained its inflammation-inducing virulence mechanisms could be to provide it with an ecological advantage at the mucosal surface of the intestine [25].

Paneth Cells

Paneth cells are specialized epithelial cells located at the base of crypts of Lieberkuhn that generate and secrete antimicrobial peptides of approximately 20 to 40 amino acids in length (Figure 1.1). There are four families of antimicrobial peptides: defensins, cathelicidins, histatins and lactoferrin (Figure 1.1, Table 1.1). Defensins are positively charged and directly interact with the negatively charged membrane of pathogenic microorganisms resulting in membrane destabilization and pore formation. Cathelicidins are also positively charged, and they function in binding and neutralizing lipopolysaccharides (LPS), ultimately resulting in pore formation. Unlike defensins and cathelicidins, histatins do not interact with the membranes of pathogenic bacteria. Instead, histatins are ingested by the bacteria, inhibit mitochondrial respiration, and kill the microorganism by generating reactive oxygen species [27]. Lactoferrin is a cationic protein that sequesters iron, an essential nutrient for pathogenic microorganisms.

Table 1.1. Summary of antimicrobial peptides

Antimicrobial Peptides	Function
Defensins (i.e. HD-5, HD-6)	Destabilization of bacterial membranes
Cathelicidins (i.e. CRAMP, LL-37)	Neutralization of LPS
Histatins	Generation of reactive oxygen species
Lactoferrins	Sequestration of iron and destabilization of bacterial membranes

Additionally, lactoferrin can bind LPS and destabilize bacterial membranes similar to defensins and cathelicidins [27, 28]. The antimicrobial activities of these peptides are nonspecific, as their activity provides a first line of defense against both Gram-positive and Gram-negative bacteria, fungi, and enveloped viruses.

All antimicrobial peptides are produced in an inactive, prepropeptide form and must be processed (i.e. enzymatically) either intracellularly or extracellularly to become active [12]. For example, the alpha-defensin HD-5 is stored in Paneth cells in an inactive, pre-propeptide form and is processed by trypsin into its active form [29]. Antimicrobial peptide production has been shown to be upregulated in response to bacteria [30]. However, pathogenic microorganisms have developed methods to counteract the effectiveness of the antimicrobial peptides. Examples of these methods include covalently modifying the bacterial cell membrane to reduce its net negative charge, using bacterial proteases to catalytically inactivate the antimicrobial peptides, and using ATP-driven pumps to physically remove the antimicrobial peptides from the bacterial cytoplasm [31]. Certain pathogens have developed resistance to the antimicrobial activities of the peptides secreted by Paneth cells in order to promote their intracellular survival.

Antimicrobial peptides that provide protection against *S.* Typhimurium infection have been identified using transgenic mouse models. Alpha-defensin HD-5 transgenic mice were shown to consistently have a significant reduction in the *S.* Typhimurium burden in the distal intestine and spleen in comparison to

wild-type mice that do not express this antimicrobial peptide, indicating the antimicrobial activity of HD-5 conferred the transgenic mice with an enhanced ability to kill *S.* Typhimurium in the intestinal lumen [32]. Another alpha-defensin shown to provide increased defense against *S.* Typhimurium infection is HD-6, which binds bacterial membrane proteins, thereby inhibiting contact of *S.* Typhimurium with epithelial cells [33]. Since HD-6 doesn't kill *S.* Typhimurium, HD-6 transgenic mice do not display the decrease in bacterial burden seen with HD-5 transgenic mice; however, HD-6 transgenic mice display a profound increase in survival rate in comparison to wild-type mice that do not express this antimicrobial peptide, indicating the antimicrobial activity of HD-6 must act in concert with another defense mechanisms at the mucosal barrier to eliminate *S.* Typhimurium [33].

In addition to defensins, mouse models have also identified the significance of cathelicidins and lactoferrin. The sole murine cathelicidin called cathelin-related antimicrobial peptide (CRAMP) has been shown to impair intracellular replication of *S.* Typhimurium *in vivo* and *in vitro* [34]. Additionally, *S.* Typhimurium displayed enhanced survival in macrophages derived from CRAMP-deficient mice [34]. CRAMP is similar in structure and antimicrobial properties to the only human cathelicidin called LL-37, which has been shown to display a broad spectrum of activity against bacteria including *S.* Typhimurium [35]. A recent study identified the *in vivo* effect of lactoferrin on *S.* Typhimurium. In this study, mice treated with bovine lactoferrin displayed a reduction in

severity, mortality, and inflammation during infection, indicating the antimicrobial properties of lactoferrin are significant for defense against *S.* Typhimurium [28].

The Epithelial Barrier

In addition to mucosal defenses described above, interactions between cells of the epithelial cells in the monolayer also provide a barrier against bacterial entry. Tight junctions are dynamic structures composed of zonula occludens (ZO) and junctional adhesion molecules that effectively adhere the cells of the epithelial monolayer to each other (Figure 1.1) [8]. The integrity of this seal is maintained by the interaction of tight junction components with the actin cytoskeleton. However, the permeability of this seal is regulated by physiological conditions, and it therefore can be manipulated. For example, treating epithelial monolayers with inflammatory cytokines, such as IL-1β, increases the permeability of the tight junctions [36]. The increase in tight junction permeability can facilitate the translocation of bacteria from the lumen to the subepithelial region, making them a target for pathogenic manipulation. Pathogenic microorganisms can accomplish the manipulation of tight junctions by usurping signaling pathways, such as the Rho GTPase pathway, which regulates actin cytoskeleton rearrangement [8].

S. Typhimurium infection has been shown to regulate certain tight junction proteins, which ultimately promotes translocation of the bacteria through the epithelial cell monolayer [37]. Upon infection with *S.* Typhimurium, occludin becomes dephosphorylated and subsequently removed from epithelial tight

junctions [37]. Additionally, ZO-2 is recruited from the cytosol to membrane, indicating *S.* Typhimurium alters the intracellular distribution of this tight junction protein [37]. Suprisingly, ZO-1, which is normally regulated by pathogens in a similar manner to ZO-2, appears to be degraded during *S.* Typhimurium infection [37]. Manipulation of tight junction proteins serves to disrupt the epithelial barrier by increasing its permeability, thereby allowing *S.* Typhimurium to more effectively invade the basolateral side of the epithelial cell monolayer.

In order to mount a successful infection, *S.* Typhimurium must disrupt some aspects of the protective mechanisms employed by the mucosal epithelium. As mentioned previously, the two T3SS and the secreted bacterial effector proteins promote entry, inflammation, and intracellular survival. In addition, in order to subvert the action of antimicrobial peptides, *S.* Typhimurium uses the two-component system PhoQ/PhoP, which regulates the expression of SPI-2 encoded genes as well. Specifically, PhoP/PhoQ regulators promote remodeling of the bacterial envelope, resulting in increased resistance to antimicrobial peptides that recognize LPS. Furthermore, the PhoP/PhoQ regulators repress transcription of genes for the T3SS, in attempt to avoid detection, and induce protective mechanisms against hydrogen peroxide [12]. In addition to rearranging the actin cytoskeleton and targeting specific tight junction proteins, S. Typhimurium also manipulates tight junctions via the action of SipA, SopE, SopE2, and SopB [8]. These effector proteins induce Rho-GTPase activation, and inhibition of this effector-induced Rho-GTPase activation prevents tight junction disruption [8].

The manipulation of tight junctions has also recently been shown to facilitate the transmigration of PMNs across the epithelial cell monolayer [37]. The primary mechanism of PMN migration in *S.* Typhimurium infection involves the recruitment of neutrophils into the subepithelium and the formation of a chemoattractant gradient that directs the neutrophils into the lumen (Figure 1.2, discussed in detail later). However, recent research implicates the disruption of tight junctions in facilitating PMN migration even in the absence of the chemoattractant gradient [37]. Thus, *S.* Typhimurium not only modulates the release of neutrophil chemoattractants that induce PMN migration, but also directly influences the tight junctions that maintain the fidelity of the epithelial cell monolayer in order to promote bacterial translocation and PMN transepithelial migration.

Figure 1.2: Mechanism of PMN recruitment and PMN transmigration

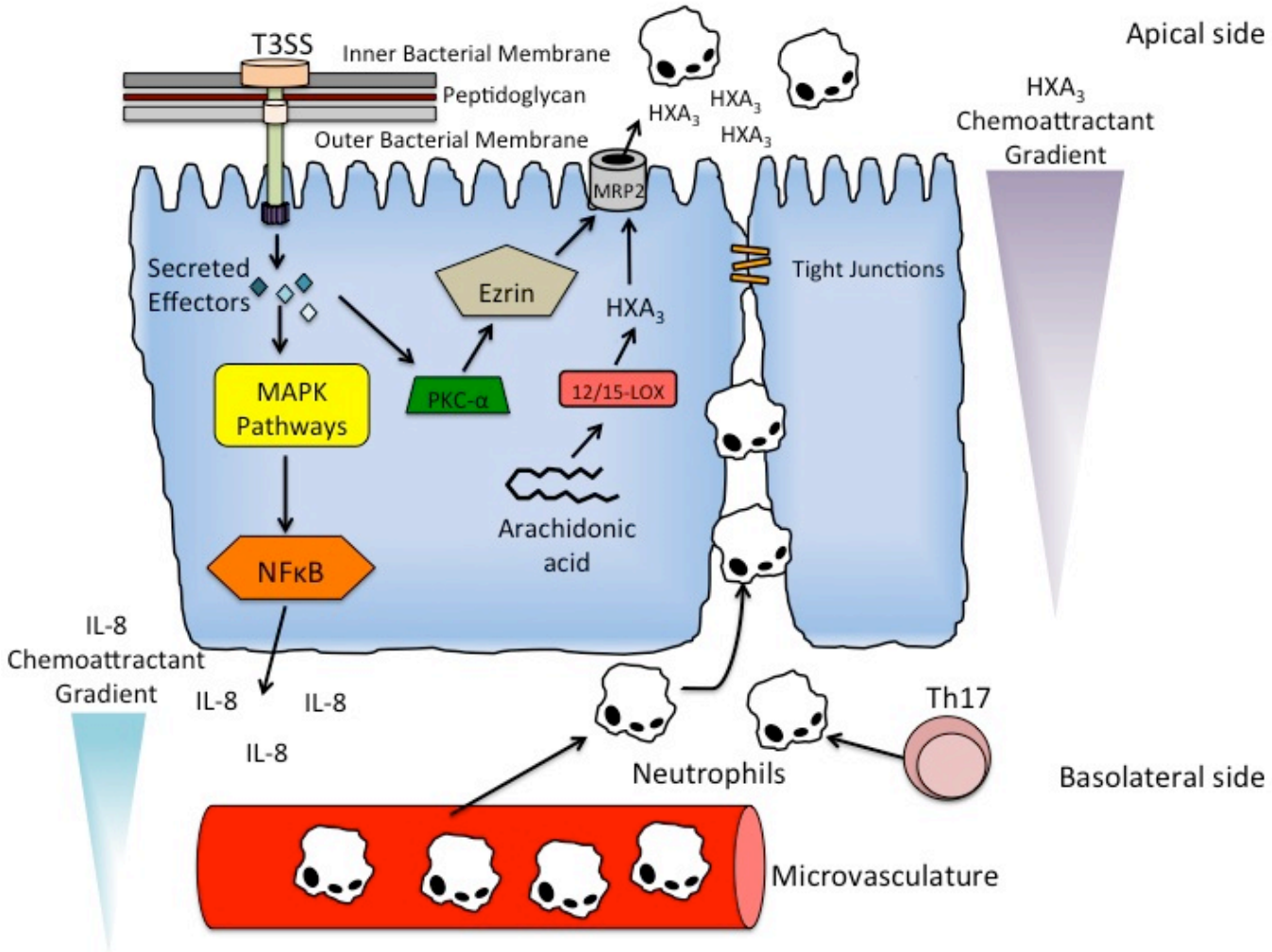

Figure 1.2: Mechanism of PMN recruitment and PMN transmigration. *S.* Typhimurium utilizes its T3SS to secrete effector proteins into epithelial cells to activate inflammatory signaling pathways. In particular, the activation of Rho-GTPases by SopE, SopE2, and SopB result in the induction of mitogen-activated protein kinase (MAPK) pathways. The stimulated pathways include ERK, JNK, and p38, resulting in the terminal activation of major inflammatory regulator NF-κB. Activation of NF-κB results in the basolateral secretion of IL-8 producing a chemoattractant gradient that recruits neutrophils to the subepithelial region from the underlying microvasculature. Th17 cells are also present in the subepithelial region, and function to recruit and activate neutrophils in the subepithelium. PMN transmigration is facilitated by another chemattractant HXA_3. HXA_3 is a bioactive eicosanoid that is synthesized from arachidonic acid via the 12/15-lipoxygenase pathway in epithelial cells. It is secreted into the lumen via the action of an ATP-binding cassette transporter called MRP2. *S.* Typhimurium effector protein SipA stimulates the recruitment of PKC-α to the apical membrane, which in addition to the ERM protein ezrin, modulate the localization of MRP2 to the apical membrane, thereby allowing secretion of HXA_3 into the lumen and production of the chemoattractant gradient that induces PMN transmigration.

Lamina Propria

The lamina propria is the connective tissue underlying the epithelial cell monolayer. It contains multiple immune effector cells including B cells, T cells, dendritic cells, natural killer cells, macrophages, eosinophils, and mast cells. If enteric pathogens are capable of surmounting the barriers described above and penetrate the intestinal epithelium, a coordinated immune response utilizing these immune effector cells is activated. Sampling of luminal antigens occurs in specialized cells called M cells, which transport the antigens to a subepithelial region where the antigen comes in contact with dendritic cells. Dendritic cells bound to antigen then migrate to the mesenteric lymph node to present the luminal antigens to naïve T cells and B cells. These naïve lymphocytes then differentiate into several effector cells including CD8 cytotoxic T cells, CD4 helper T cells, regulatory T cells, and antibody secreting B cells.

Although this marks the beginning of a coordinated immune response to pathogenic bacteria, the resting lamina propria does have protective functions that provide an added layer of defense prior to the full activation of the mucosal immune system.

The most abundant B cell found in the lamina propria is the IgA-secreting B cell (Figure 1.1). Secreted IgA is also the primary secreted immunoglobulin found in the thick mucus layer. One of the main roles of secreted IgA is a process called immune exclusion, which includes prevention of pathogens from adhering to the mucosal surface on the luminal side of the intestinal epithelium and removal of antigens from the basoateral side of the intestinal epithelium. On the

luminal side, secreted IgA primarily interferes with microbial adhesins, whereas on the basolateral side secreted IgA functions in an export mechanism by binding antigens and shuttling them back across the epithelial monolayer into the lumen [38]. Secreted IgA does not activate an inflammatory immune response when it neutralizes pathogenic microorganisms, thereby upholding the integrity while preventing inflammation-induced damage of the mucosal epithelium [39].

Secreted IgA also has a direct effect on the virulence mechanisms of certain pathogens. In *S.* Typhimurium infection, it has been shown that a monoclonal, polymeric IgA antibody Sal4 binds the O-antigen (O-Ag) component of LPS on the bacterial membrane, resulting in its destabilization [40, 41]. Recent evidence indicates that the bacterial membrane destabilization results in impaired T3SS translocon formation, decrease in effector protein delivery and decrease in flagellum-based motility [40]. *S.* Typhimurium responds to the binding of Sal4 to O-Ag by triggering exopolysaccharide (EPS) production and biofilm formation, though this response renders the bacteria noninvasive and avirulent [42]. The mechanism of EPS production and biofilm formation has been attributed to the activation of a cyclic dimeric guanosine monophosphate-dependent pathway via an inner membrane diguanylate cyclase YeaJ [42]. Furthermore, it has been suggested that the triggering of this pathway by *S.* Typhimurium could be a mechanism to restore membrane stability, as EPS production could serve to shed IgA antibody or increase resistance to other luminal insults [42].

The resting lamina propria contains a heterogeneous population of CD8 cytotoxic T cells, CD4 helper T cells, and regulator T cells even in the absence of

pathogenic infection. The number of effector T cells in the resting lamina propria would in any other tissue indicate an inflammatory response; however, the amount of T cells present in the mucosal tissue of the gut is more indicative of constant immune surveillance and recognition than chronic inflammation. The cytokines produced by these T cells maintain the mutualistic response to resident microbiota, stimulate production of IgA, induce secretion of antimicrobial peptides, and promote epithelial repair. Additionally, in the absence of pro-inflammatory cytokines (which are usually produced by innate immune cells in the presence of pathogen) the dendritic cells in the resting lamina propria contribute to maintaining tolerance to non-pathogenic antigens by promoting the production of CD4 regulatory T cells. Regulatory T cells produce immunosuppressive cytokines that inhibit T cell proliferation and dendritic cell differentiation, which prevents unnecessary immune response to innocuous antigens [39].

In addition to a heterogeneous population of T cells, the macrophages in the resting lamina propria play a key role in host defense against *S.* Typhimurium infection, as well. The macrophages of the resting lamina propria can be divided into two different classes: M1 (“classically activated”), and M2 (“alternatively activated”) polarized macrophages. M1 polarized macrophages are pro-inflammatory and display high phagocytic and antimicrobial activity, whereas M2 polarized macrophages are anti-inflammatory and display low phagocytic and antimicrobial activity. Hence, with this type of opposing macrophage regulation it is considered that M1 macrophages function in the clearance of infection versus

M2 macrophages that assist in wound healing and suppression of T-cell function. Manipulation of macrophage polarization by *S.* Typhimurium has become increasingly realized as a defense mechanism against bacterial clearance [43, 44]. A recent study demonstrated that the SPI-1 T3SS enables *S.* Typhimurium to guide macrophage towards the M2 polarization [44]. This type of control permits *S.* Typhimurium to escape the more hostile environment of M1 polarized macrophages, resulting in a macrophage-specific decrease in pro-inflammatory signaling [44].

The mechanism of immunity to invasive *Salmonella* is still disputed, specifically the relevance of cell-mediated versus humoral immunity. The debate is complicated by attempts to compare different experimental models, which vary in route of *Salmonella* administration and/or susceptibility of mouse strains to *Salmonella*. In terms of cellular immunity**,** mice deficient for TCR α/β, MHC class II or interferon- γ (IFN-γ) receptor fail to clear a primary *Salmonella* infection that can be resolved in normal mice [45, 46]. Recently, it has also been shown that Thy1+ natural killer (NK) cells are essential for the early production of IFN-γ during control of *Salmonella* infection [47]. CD8+ T cells seem to also play a role in *Salmonella* clearance [48]. It has also been documented that *Salmonella* infection promotes the expansion of intestinal intraepithelial lymphocytes (iIELs) and the activation of particularly, $CD8^+$ $TCR\gamma\delta^+$ iIELs, which in turn trigger cytolytic activity against *Salmonella*-infected epithelial cells [48].

The role of antibody-producing immune cells or B cells against *Salmonella* is still controversial [49-51]. Some reports have shown the importance of

antibody production and T cell activation for protection from virulent *Salmonella* [49, 50]. However, another study reported that the protective immunity provided by an attenuated *S.* Typhimurium strain required B cells independently of antibody production, proposing that they confer protective immunity by presenting antigen to T cells and acting as a source of inflammatory cytokines [51]. It has also been demonstrated that transfer of immune serum into B cell deficient mice can partially but not completely provide protective immunity [52].

The Type III Secretion System: Co-opting host pathways to promote entry and immune evasion

Upon contact of *S*. Typhimurium with the epithelial cell monolayer, the SPI-1 effector proteins SopE, SopE2, and SopB initiate the process of bacterial entry by activating host cell Rho GTPases resulting in actin rearrangements [53, 54]. SipA is another SPI-1 effector protein that antagonizes actin depolymerizing agents and tethers actin monomers together to form membrane ruffles, which promotes bacterial internalization [55]. *S.* Typhimurium is engulfed by epithelial cells through a macropinocytosis event termed bacterial mediated endocytosis, and is ultimately contained within in a membrane-bound vesicle called a macropinosome (more commonly termed the *Salmonella* Containing Vacuole [SCV]). Although prior studies thought that SopB was the sole mediator of macropinosome formation, a cooperative interaction regulated by the phosphatase activity of SopB has implicated SopD as another mediator of this process [53].

Salmonella Typhimurium also targets antigen-sampling microfold (M) cells to translocate across the gut epithelium. M cells constitute a small subset of highly specialized follicle-associated epithelium (FAE) enterocytes overlying lymphoid follicles in the gut, and are characterized by an irregular brush border, a reduced glycocalyx and lysosomal apparatus, and are programmed to efficiently transcytose a wide variety of macromolecules and micro-organisms from the gut lumen to the underlying immune inductive Peyer's patches (PPs) [56]. Recent evidence shows the S. Typhimurium type III effector protein SopB also induces an epithelial-mesenchymal transition (EMT) of the FAE into M cells. This cellular transdifferentiation is a result of SopB-dependent activation of Wnt/β-catenin signaling leading to induction of both receptor activator of NF-κB ligand (RANKL) and its receptor RANK. The autocrine activation of RelB-expressing FAE enterocytes by RANKL/RANK induces the EMT-regulating transcription factor Slug that marks epithelial transdifferentiation into M cells. Thus, S. Typhimurium may also transform primed epithelial cells into M cells to promote host colonization and invasion [56, 57].

Following bacterial entry of mucosal epithelia, *S.* Typhimurium employs a second set of SPI-1 effector proteins to ensure repair of the actin cytoskeleton. SptP is one such effector that is directly responsible for reversing the affects of SopE and SopE2. SptP promotes restoration of the epithelial cell membrane by functioning as a GTPase-activating protein for the Rho GTPase proteins Rac-1 and Cdc42 [58]. Similarly, as several of the early SPI-1 effectors induce inflammation of the mucosal epithelium, there are effector proteins that have an

anti-inflammatory function, providing *S*. Typhimurium with a form of regulatory control over the inflammatory state of the mucosal tissue (inflammation induced by *S.* Typhimurium will be discussed later).

After successful entry into epithelial cells and restoration of the epithelial cell membrane, *S.* Typhimurium relies primarily on the T3SS encoded by SPI-2 to survive and replicate intracellularly by translocating SPI-2 effector proteins across the membrane of the SCV into the epithelial cell cytoplasm. SPI-2 effector proteins that appear to be necessary for survival and virulence of *S.* Typhimurium inside the SCV are SifA, SseJ, SseF, SseG, SopD2, and PipB2 [54, 59, 60]. SifA has been shown to promote tubulation of the SCV through correlation with another effector protein SseJ [61]. SCV tubulation in conjunction with the effects of SseF and SseG localize the SCV to the perinuclear region in close proximity of the Golgi apparatus [62]. The localization of the SCV is important for intracellular survival because vesicular trafficking through the Golgi network allows for the acquisition of nutrients, thereby allowing the establishment of a replication niche for *Salmonella* [62, 63].

An additional means by which SPI-2 promotes intracellular survival of *S.* Typhimurium is by encoding factors that mediate the evasion of immune responses. SPI-2 promotes protection from reactive oxygen intermediates produced by macrophages, specifically nitric oxide (NO) and NADPH oxidase [64-66]. *S.* Typhimurium has been shown to evade NO-mediated killing in macrophages by inhibiting IFN-γ-induced NO production in a SPI-2 dependent manner [65]. SPI-2 is also involved in avoiding NADPH oxidase-dependent killing

by interfering with the trafficking of NADPH oxidase [66]. Although the specific SPI-2 effector proteins involved in the evasion of both NO-dependent and NADPH oxidase-dependent killing of *S.* Typhimurium have yet to be identified, the established role of SPI-2 in evasion of both immune responses suggests a possible role for one or more encoded effector proteins in promoting resistance to reactive oxygen intermediates in macrophages.

***Salmonella*-Induced Inflammation**

Immune Recognition

Pathogen-associated molecular patterns (PAMPs) are recognized by pattern-recognition receptors (PRRs), namely Toll-like receptors (TLRs), located on inflammatory cells and epithelial cells. TLRs can recognize a wide range of PAMPs, though some TLRs do show some specificity for particular PAMPs. For example, TLR4 is mostly involved in recognition of LPS and TLR5 is mostly involved in the recognition of bacterial flagellin. TLRs in epithelial cells are localized to the basolateral or apical membrane, as well as in intracellular vesicles. Thus, TLRs can recognize pathogens on either side of the epithelial cell monolayer and endocytosed extracellular pathogens. Additionally, inflammatory cells, such as macrophages, expressing TLRs can also recognize PAMPs. The importance of some TLRs, specifically TLR4 and TLR5, in *S.* Typhimurium infection have been established, as mutating them has been shown to increase susceptibility to infection and inflammation [39]. Intracellular recognition of bacteria or their products in the cytoplasm is also mediated by nucleotide-binding

oligomerization domain proteins NOD1 and NOD2. NOD1 recognizes peptides containing diaminopimelic acid, which is a component of Gram-negative bacterial cell walls, whereas NOD2 recognizes a muramyl dipeptide present in the peptidoclycan layers of both Gram-positive and Gram-negative bacteria. Similar to TLRs, mutations in NOD1 and NOD2 proteins increase susceptibility to disease and infection caused by intracellular bacteria [67, 68].

Recruitment of immune cells

The host immune system also activates inflammatory pathways in response to infection with *S.* Typhimurium. The binding of TLRs and NOD1/NOD2 proteins to their respective ligands activates the NF-kB pathway leading to production of pro-inflammatory cytokines and chemokines. Basolateral secretion of the cytokine IL-8 recruits neutrophils and is necessary for PMN migration into the subepithelium. Additional chemokines, such as CCL20, play a role in attracting immature dendritic cells, which upon exposure to antigen, can mature and present antigenic peptides to naïve B and T cells in the mesenteric lymph nodes [39]. *S.* Typhimurium can also react with TLRs on macrophages in the subepithelial region after being transcytosed through M cells, thereby activating and inducing them to also produce cytokines and chemokines. Cytokines produced by these activated macrophages include IL-1, IL-6, and IL-23, all of which drive the differentiation of T_H17 cells whose primary function in the subepithelium is recruiting and activating neutrophils (Figure 1.2) [39, 69]. Other cytokines produced by these activated macrophages include IL-18 and IL-

12, both of which drive the IFN-gamma-dependent production of antigen-specific T_H1 cells [39].

Mechanism of neutrophil recruitment

A hallmark of *S.* Typhimurium-induced inflammation is the recruitment of PMNs from the underlying microvasculature to the subepithelial region of the epithelial cell monolayer (Figure 1.2). The neutrophils then migrate across the monolayer into the lumen, resulting in the inflammatory pathology of Salmonellosis. New information is shedding light on the molecular mechanisms and signaling pathways involved in neutrophil recruitment across the intestinal epithelium. As discussed above, it is becoming increasingly appreciated how inflammation induced by *S.* Typhimurium increases its pathogenic bacterial fitness.

In addition to promoting bacterial entry, many effector proteins encoded by SPI-1 also activate inflammatory signaling pathways. The activation of Rho-GTPases by SopE, SopE2, and SopB result in the induction of mitogen-activated protein kinase (MAPK) pathways (Figure 1.2). In particular, the ERK, JNK, and p38 pathways are stimulated, resulting in the terminal activation of inflammatory regulators AP-1 and NF-κB (Figure 1.2) [54, 70]. Furthermore, the activation of NF-κB and AP-1 stimulates the secretion of the cytokine IL-8 on the serosal side of the epithelial cell monolayer, a requirement for the recruitment of neutrophils to the subepithelial region (Figure 1.2) [71]. Although IL-8 is necessary for PMN

migration into the lumen, it has been shown that IL-8 alone is not sufficient enough to drive the migration across the epithelial cell monolayer [71-73].

The migration of neutrophils from the basolateral side to the luminal side of the epithelial cell monolayer is driven by another PMN chemoattractant, hepoxilin A_3 (HXA_3) (Figure 1.2) [72, 74]. HXA_3 is a bioactive eicosanoid that is synthesized from arachidonic acid via the 12/15-lipoxygenase pathway in epithelial cells (Figure 1.2) [74]. After synthesis, HXA_3 is secreted from the apical surface of epithelial cells by an ATP-binding cassette transporter called multidrug resistant protein 2 (MRP2) (Figure 1.2) [75]. Secretion of HXA_3 into the lumen forms a chemoattractant gradient that causes neutrophils to migrate from the region underlying the epithelial cell monolayer into the lumen (Figure 1.2) [74].

Activation of the effector protein SipA has been shown to be necessary for induction of HXA_3 synthesis and the resulting PMN migration (Figure 1.2) [76]. Remarkably, the mechanism for activating SipA was recently shown to require processing by the host enzyme caspase-3 at a particular cleavage site, resulting in two distinct effector domains [77]. Furthermore, the two domains were shown to be functionally different. The ability to promote PMN migration is confined to the SipA N-terminal domain, whereas the C-terminal domain has been shown to be involved in actin rearrangement [76, 77]. The current understanding of the mechanism of SipA-dependent synthesis of HXA_3 is that SipA induces the recruitment of ADP-ribosylation factor 6 (ARF6) to the apical membrane of the epithelial cells. ARF6 activates phospholipase D, which generates phosphatidic acid. Phosphatidic acid is then converted to diacylglycerol (DAG), which recruits

protein kinase C-α (PKC-α) to the apical membrane (Figure 1.2). PKC-α, in addition to an ERM protein ezrin, modulate the localization of MRP2 to the apical membrane of epithelial cells, thereby allowing the secretion of HXA_3 into the lumen and production of the chemattractant gradient that induces neutrophil transmigration (Figure 1.2) [76, 78, 79].

Conclusion

The architecture of the mucosal immune system, including mucins, antimicrobial peptides, resident microbiota, paracellular junctions, and effector cells of the lamina propia, functions to prevent pathogenic bacteria from disrupting the epithelial cell monolayer and causing disease. If enteric pathogens are able to penetrate these barriers, then it results in a host inflammatory response and eventually activation of an adaptive immune response, designed to eradicate the intruding pathogen. However, *S.* Typhimurium has evolved systems, namely the SPI-1 an SPI-2 T3SS, to manipulate the defensive mechanisms of the mucosal immune system in order to develop a replication niche in the mucosal epithelium. Additionally, the ability of *S.* Typhimurium to exploit inflammation allows it to penetrate the epithelial barriers, a condition in which activation of the adaptive immune response would be required for pathogenic clearance. Investigating how *S.* Typhimurium exploits host cell signaling pathways will allow for increased understanding in its pathogenesis, and consequently provide further insight into how inflammation can seemingly result in both increased bacterial fitness and increased pathogenic clearance.

Thesis Objectives

Given the numerous barriers against *Salmonella* infection including host immune response, Salmonella relies on the functions of the SPI-1 and SPI-2 encoded effector proteins in order to evade immune response and promote it's own pathogenesis. Although the functions of several effector proteins have been identified, the mechanism of activation for each effector protein has been much less appreciated.

The combined observations from [55], [76], and [77] demonstrated that SipA has different functional domains, each domain can perform its indicated function independently, and that caspase-3 processing (also referred to as caspase-3 cleavage) is required for SipA function. However, all of the aforementioned studies focused primarily on the function and activation of SPI-1 effector proteins (SipA and SopA). As such, we chose to take a closer look at SifA, a SPI-2 effector protein identified as having a caspase-3 processing site [77]. Determining the mechanism of SifA activation not only will broaden our understanding of the effector proteins encoded by *Salmonella*, but will also allow us to determine if caspase-3 processing is a general activation paradigm used by effector proteins to allow *Salmonella* to invade and persist during infection.

Specific aims of this thesis are outlined below:

Aim 1: Determine if SifA function is dependent upon caspase-3 cleavage

A. Is the caspase-3 cleavage site in SifA functionally active?

B. Is proteolytic cleavage relevant for SifA function?

Aim 2: Determine the role of caspase-3 processing in the subcellular localization of SifA

A. Does caspase-3 colocalize with SifA during an infection?
B. Are the subcellular locations of each domain dependent on caspase-3 cleavage?
    a. What are the subcellular locations of the functional domains?

Appendix A: Define the evolutionary relationship between caspase cleavage and their respective hosts by tracking effector evolution as an indicator of host adaptation

A. What is the identity of cleavage sites in different strains of *Salmonella*?
    a. Is the identity of the cleavage site related to host specificity?
B. When do we first see the emergence of cleavage sites?

PREFACE TO CHAPTER II

The data within this chapter has been accepted for publication and is found in the following paper:

Patel, S., Wall, D.M., Castillo, A., & McCormick, B.A. Caspase-3 cleavage of *Salmonella* Type III secreted effector protein SifA is required for localization of functional domains and bacterial dissemination. Accepted for publication in *Gut Microbes.* July 2018.

DESCRIPTION OF AUTHOR CONTRIBUTIONS

Study conception and design: B.A.M., D.M.W., S.P.

Acquisition of data: S.P., A.C.

Analysis and interpretation of data: B.A.M., D.M.W, S.P., A.C.,

Drafting of manuscript: B.A.M., D.M.W., S.P.

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CHAPTER II: Caspase-3 cleavage of *Salmonella* Type III secreted effector protein SifA is required for localization of functional domains and bacterial dissemination

Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a Gram-negative, facultative, intracellular anaerobe that causes gastroenteritis. In the United States, *S.* Typhimurium is responsible for nearly 25% of all food-borne infections and continues to be a major public health and economic burden [80]. *S.* Typhimurium infection in humans is typically acquired by ingestion of contaminated food or water leading to acute gastroenteritis, and can also lead to severe complications or death in persons at risk [81]. *Salmonella* species, like other Gram-negative pathogens, have 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 aimed at reprogramming eukaryotic cell functions [82-84]. *S. enterica* use two distinct type III T3SSs encoded on *Salmonella* pathogenicity islands (SPIs)-1 and-2 to inject their arsenal of effectors. In general, the SPI-1-encoded T3SS is primarily required for the invasion of non-phagocytic cells, where expression is induced by the intestinal microenvironment, enabling *Salmonella* to cross the epithelial gut barrier and promote intestinal inflammation. Subsequently, the SPI-2-encoded T3SS mediates intracellular bacterial replication and is necessary for the establishment of systemic disease.

The type III secreted effectors (T3SEs) constitute a large and diverse group of virulence proteins that mimic eukaryotic proteins in structure and function. In fact, over 30 different effector proteins are delivered into host cells by *S*. Typhimurium through both SPI-1 and SPI-2 T3SSs [85]. A prominent feature shared by bacterial effectors is their modular architecture, which is often comprised of well-defined regions that confer a subversive function. Strikingly, the distinct modules within an effector often mediate very different, unrelated functions, strongly suggesting that they evolved independently of each other and subsequently combined to form a chimeric protein [86, 87]. Chimerization is a common theme shared by many effectors [88], and this forms the basis of a provocative hypothesis termed 'terminal reassortment', proposed by Guttman and colleagues to explain the diversity of bacterial effectors [87]. The terminal reassorment tenet is substantiated by their finding that 32% of all T3SE families contain chimeric effectors, far greater than any other analyzed protein family. Additionally, other studies suggest that terminal reassortment is important for the evolution of these virulence proteins [86, 87]. In keeping with this theory, we previously observed that many T3SEs harbor a functional caspase-3 cleavage motif (DxxD) uniquely positioned at the junction separating the two distinct functional domains [77]. This finding revealed that T3SEs had evolved to use the host defense system in a manner pivotal to the pathogenicity of the organism [77]. More specifically, *S.* Typhimurium appears to have evolved a mechanism to deliver effector proteins in a precursor form to the host cell where they are processed into independent functionally active domains.

Although much attention has been given to identifying the functions of T3SEs, mechanisms underlying host mediated effector processing (e.g., cleavage) are far less appreciated. To further our understanding in this regard, we had previously examined at two SPI-1 effector proteins, SipA and SopA, and found that caspase-3 processing was necessary for the function of these T3SEs [77]. In this report, we chose to take a closer look at SPI-2, which mediates the translocation across the vacuolar membrane of a set of bacterial effector proteins that support collectively the intra-vacuolar replication. One such SPI-2 effector protein SifA [60] plays a significant role in *Salmonella* virulence by maintaining the integrity of the *Salmonella*-containing vacuole (SCV) [89], and hence promoting the formation of tubular membranous structures connected to SCVs that are named *Salmonella*-induced tubules (also referred to as Sif filaments) [61]. Unique to this effector protein, the N-terminal domain of SifA interacts with the pleckstrin homology domain of the host kinesin-binding protein SKIP, and the C-terminal domain harbors a WxxxE motif that mimics the active the form of Rho-GTPases. Cooperatively these functional domains, along with another SPI-2 T3SE SseJ, promote host membrane tubulation [61].

The resolution of the crystal structure and domain function analysis of SifA has shown that the protein is divided into two distinct major domains [61, 90] separated by a potential caspase-3 cleavage site [77, 91], suggesting the two domains of SifA might act independently of each other upon cleavage. Herein we characterize the structural features of the caspase-3 cleavage motif in SifA, and describe a new method of tracking T3SEs to show that cleavage of SifA by

caspase-3 is indeed critical for proper localization of its functional domains, and is essential for bacterial dissemination. Understanding how T3SEs are functionally controlled through caspase-3 processing, will shed new light concerning the co-evolutionary interplay between *S*. Typhimurium and its host.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

Wild type *S*. Typhimurium SL1344, and isogenic mutants thereof (Table 2.1), were used throughout this study. All cloning was carried out in *Escherichia coli* strain BL21 DE3 (Invitrogen). All bacterial strains were constructed and cultured in LB as previously described [92], unless otherwise specified. Primers used for construction of bacterial plasmids are listed in Table 2.2.

Table 2.1. Bacterial Strains and Plasmid Constructs

GST-SifA	This Paper based on Ohlson 2008
GST-SifAcsm	This Paper
∆SifA	This Paper
∆SifA/pGST-SifA and ∆SifA/pGST-SifAcsm	This Paper
∆SifA/SifA-phiLOV	This Paper
V5-SifA-HA, V5-SifA, SifA-HA, V5-SifAcsm-HA	This Paper

Table 2.2. Primers used for construction of bacterial plasmids (5'→ 3')

SP1	CCGATTACTATAGGGAATGGTTTTTTAAAAAGTGAAATCCTTACCAACTCGTGTAGGCTGGAGCTGCTTCG
SP2	AAAACAACATAAACAGCCGCTTTGTTGTTCTGAGCGAACGTGTAGCGTGGCATATGAATATCCTCCTTAGT
SP3	ATCCGCGGTAGTCCTTCTTT
SP4	CAGAGGATGGGGTGCTTTTA
SP5	GCGCGCGAATTCCCGATTACTATAGGGAATGGTTT
SP6	GCGCGCCTCGAGTTATAAAAAACAACATAAACAGCCGC
SP7	GCGCGCGAGCTCATGCCGATTACTATAGGGAATGGTTT
SP8	GCGCGCCTCGAGTAAAAAACAACATAAACAGCCGC
SP9	CGATCCCGCGAAATTAATACGACTC
SP10	CAAAAAACCCCTCAAGACCCGTTTA

-GST-SifA

SifA was PCR-amplified from wild-type (WT) *S*. Typhimurium (SL1344) using primers SP5 and SP6. The PCR product was then digested, purified, and ligated to the GST-containing vector, pGEX-6p-1 (GE Healthcare), as described previously [61].

-GST-SifAcsm

SifAcsm was synthetically generated and cloned into pUCIDT-AMP by Integrated DNA Technologies (IDT) using the same cloning sites as GST-SifA. *sifA*csm was then cloned into pGEX-6p-1 and sequences were verified using the same methods used for GST-SifA.

-ΔSifA

The ΔSifA mutant was constructed from SL1344 by deletion mutagenesis using a chloramphenicol cassette as described by Datsenko and Wanner [93]. Briefly, a chloramphenicol resistance cassette was PCR amplified from the pKD3 plasmid using primers SP1 and SP2. The pKD3 PCR product was then concentrated and transformed into electrocompetent SL1344 expressing the pKD46 plasmid. The *sifA* deletion was sequence verified using SP3 and SP4 primers.

-ΔSifA/pGST-SifA and ΔSifA/pGST-SifAcsm

The ΔSifA/pGST-SifA and ΔSifA/pGST-SifAcsm complemented strains were made using the ΔSifA mutant. pGST-SifA and pGST-SifAcsm plasmid DNA were transformed into the electrocompetent ΔSifA strain of *Salmonella*. Transformants were sequence verified using SP3 and SP4 primers.

-ΔSifA/SifA-phiLOV

SifA-phiLOV was made using pUC57-SipA-phiLOV. *sif*A was PCR amplified from SL1344 using SP7 and SP8 primers. These primers added a *Sac*I and *Xho*I site on the 5' and 3' ends of *sif*A, respectively. Since *sip*A was cloned into pUC57-phiLOV using the same cloning sites, the pUC57-SipA-phiLOV plasmid was digested with *Sac*I and *Xho*I (NEB) to remove *sip*A, and then gel purified [94]. The *sif*A PCR product was similarly digested and purified and ligated to pUC57-phiLOV before being transformed in the same manner as pGST-SifA. Plasmid DNA was then isolated from transformants that had ampicillin resistance and transformed into electrocompetent ΔSifA.

-Single/Dual Tagged SifA Constructs for Lentiviral Transduction

V5-SifA-HA, V5-SifA, SifA-HA, and V5-SifAcsm-HA constructs were generated and cloned into pUC57 with *Age*I and *Eco*RI cloning sites by Genscript. Both V5 and HA are small ectopic tags that can be visualized using fluorescent antibodies. Plasmids were then digested with *Age*I and *Eco*RI, and cloned into pLVX-TetOne-Puromycin and pLVX-TetOne-Blasticidin using the same restriction sites.

-SifA Purification

A 10 mL overnight culture of *Escherichia coli* BL21 expressing pGST-SifA in LB/Ampicillin media was back diluted 1:100 into a 1 L LB/Ampicillin. The culture was grown at 37°C for 3.5 hr and then induced with 1 mM IPTG at 22°C for 3 hr.

The bacteria were resuspended in GST Lysis Buffer (25 mM Tris pH 8, 150 mM NaCl, 3 mM DTT, and 1 mM PMSF) and then sonicated for 30 sec intervals 4 times. The lysate was then clarified at 14,000 rpm for 1 hr at 4 degrees Celsius. The clarified lysate was then run through glutathione sepharose beads in a column (GE Healthcare), washed with 1 X phosphate buffered solution (PBS), and then eluted using reduced glutathione (GE Healthcare).

Caspase-3 Cleavage Assay

Purified GST-SifA and GST-SifAcsm were incubated with 10U and 20U of active recombinant human caspase-3 (BioVision) for 1 hr at 37°C. The resulting products were then Western blotted using Anti-GST antibody (GE Healthcare). Densitometry analysis was performed using the free online software FIJI.

Mouse Dissemination and Colonization Experiments (Both oral and tail vein infection)

For intestinal colonization, mice were treated with 40 uL of 100 mg/mL streptomycin 24 hrs prior to infection, as described previously [76]. Mice were then infected with 1 x 10^7 colony forming units (CFUs) of each bacterial strain by oral gavage. At 48 hr post-infection (hpi) the liver and proximal colon were harvested for dissemination analysis. Dissemination analysis was completed by homogenizing the tissues and serial dilution plating. For tail vein injection, mice were injected with 1 x 10^6 bacteria and dissemination analysis was again carried

out but at 24 hpi. Statistical analyses were completed using an unpaired Student's *t* test.

Generation of Caspase-3 KO HeLa cells

The caspase-3 KO HeLa cells were generated according to the Caspase-3 CRISPR/Cas9 construct manufacturer's instructions (Santa Cruz Biotechnology). Briefly, 1µg of the Caspase-3 CRISPR/Cas9 plasmid and 1µg of the HDR plasmid (contains puromycin resistance marker for selection) were incubated with Plasmid Transfection Medium (Santa Cruz Biotechnology). Ten µL of the UltraCruz Transfection Reagent (Santa Cruz Biotechnology) was incubated with Plasmid Transfection Medium. The solution containing the plasmids and the solution containing the UltraCruz Transfection Reagent were then combined and incubated at room temperature for 20 min. After incubation, the combined solution was added to HeLa cells in fresh H1 media. H1 media was replaced 24 hr later, and selection with H1 media containing 2 µg/mL puromycin was started 72 hr post-transfection. Cells were sub-cultured for 2 weeks before use in experiments.

Lentiviral transduction of WT and Caspase-3 KO HeLa cells

Six µg of each construct was cloned into pLVX-TetOne-Puro (for WT HeLa cells) or pLVX-TetOne-Blasticidin (for Caspase-3 KO HeLa cells), 4µg of a packaging plasmid psPAX2 and 2ug of an envelope plasmid pMD2.G were added to Opti-MEM media (Difco). Thirty-six µL of TransIT-293 transfection reagent was added

to the Opti-MEM mixture and incubated at room temperature for 30 min. The transfection mixture was then added dropwise to individual 10 cm dishes of HEK293T cells. HEK293T cells were plated at 6-8 x 10^6 in 8 mLs of D10 media (made using 500 mL DMEM, 10 % FBS, 5 mL Pen/Strep, 10 mL L- glutamine, and 50 µL plasmocin, Invivogen).

After 48 hr, the supernatant containing the virus from each plate was collected and added dropwise to WT HeLa cells (constructs in pLVX-TetOne-Puro) or caspase-3 KO HeLa cells (constructs in pLVX-TetOne-Blasticidin) that were >60% confluent. Both WT and caspase-3 KO HeLa cells were incubated with virus overnight, and then selected using puromycin or blasticidin, respectively.

In vitro infection experiments

A 3 mL overnight LB culture of SL1344 or LB/Ampicillin cultures of ∆SifA/pSifA-phiLOV were back-diluted 1:5 into 10 mL LB broth or LB/Ampicillin/1 mM IPTG, respectively, and grown at 37°C for 1hr. Fifty µL of this culture was then added to 1mL H1 media, and 200 µL per well was used for infection of WT and caspase-3 KO HeLa cells. Following a 1 hr infection, the cells were incubated with H1 media containing 50 µg/mL gentamicin for 1 hr, before incubation with H1 media containing 10 µg/mL gentamicin until 1, 2, 4, 8, and/or 10 hpi. Cells were fixed and permeabilized in 1 % bovine serum albumin (BSA)/0.2 % saponin then stained using Image-iT LIVE Caspase-3 Detection Kit (ThermoFisher) for SifA-phiLOV/Caspase-3 colocalization experiements. For dual-tagged SifA constructs,

cells were stained using anti-V5 (Abcam) and anti-HA primary antibodies (Santa Cruz), and goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 568 secondary antibodies (Invitrogen). Cells were then visualized using a 63X objective lens on a confocal microscope Leica SP5 confocal microscope. Colocalization data was determined using the Coloc 2 tool in the free online software FIJI. The Mander's coefficient was the selected output, which is a pixel intensity spatial correlation analysis.

Results

Structural features of the caspase-3 *motif in S. Typhimurium T3SE SifA*

Given that the resolution of the crystal structure and domain function analysis of SifA has shown that the protein is divided into two distinct major domains [61, 90] separated by a potential caspase-3 cleavage site [77, 91], we investigated the structural features of the caspase-3 cleavage motifs by *in silico* modeling of the crystallized caspase-3 substrate [61, 91]. Protein sequences were retrieved from the NCBI protein database, screened for caspase-3 motifs, and then analyzed in the web server Phyre2 for the identification of structural homologues. We found that the caspase-3 motif is surrounded by hydrophilic residues, exposed at the surface, and localized in coiled regions of proteins (Figure 2.1). Additionally, we studied the frequency of the DxxD site by analyzing the residue downstream of the caspase-3 cutting site (DxxD-**x**) in 33 known caspase-3 substrates [95]. Of these residues, serine, glycine, and asparagine (all hydrophilic/polar residues) are most frequently located downstream of the

caspase-3 cleavage site; serine was found 13 times, glycine 10 times, and asparagine 4 times and is consistent with our observation of the CASP3 motif being surrounded by hydrophilic residues.

Figure 2.1 The structural characteristics of the caspase-3 motif

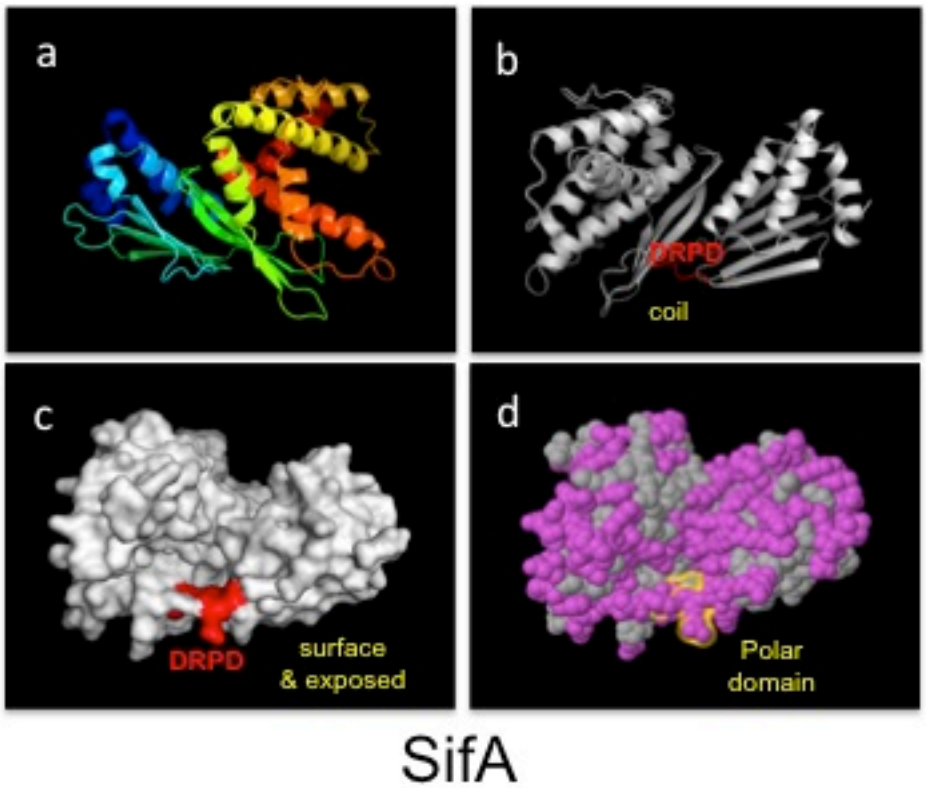
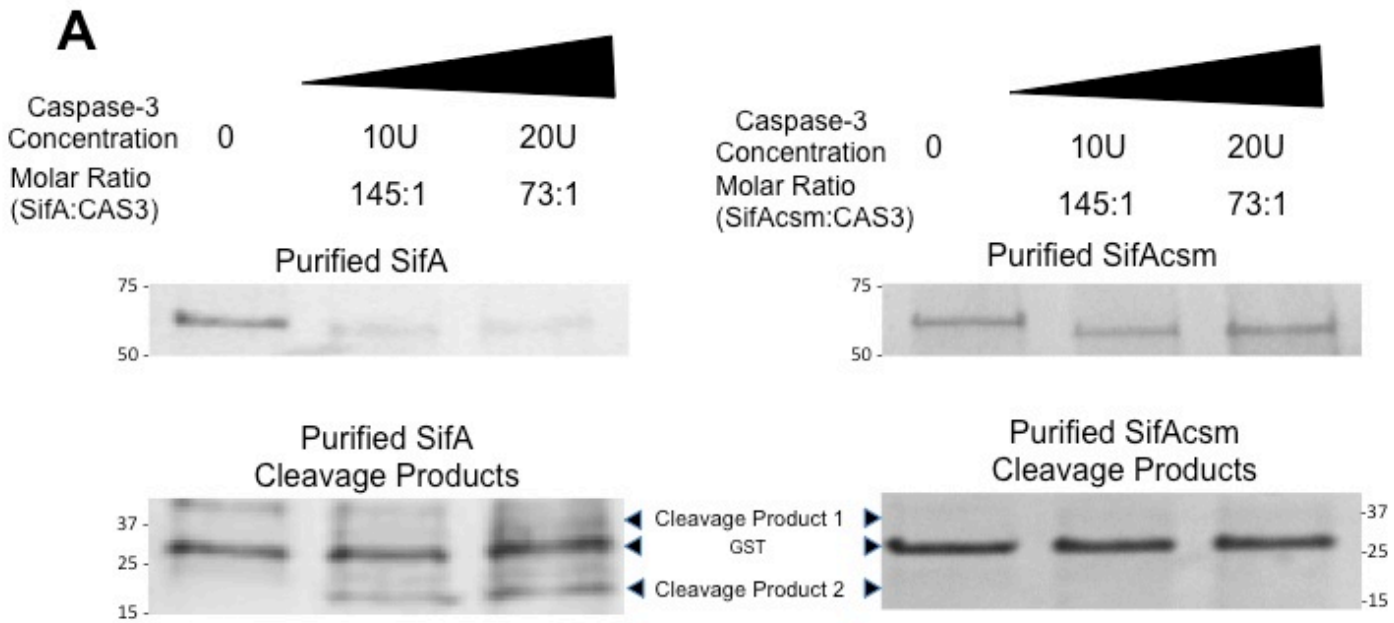


Figure 2.1 The structural characteristics of the caspase-3 motif. The structural features of the caspase-3 motif found in the *Salmonella* effector protein SifA (PDB ID 3CXB). Protein structures are displayed as cartoon (**Panels a and b**), surface (**Panel c**), and spheres (**Panel c**). **Panel (a)** shows protein structures colored with the spectrum color set of PyMOL to display the different protein domains, α-helixes, β-sheets, and coil regions. In **Panels (b)** and **(c)**, the location of the caspase-3 motif is colored in red, whereas in **Panel (d)** in yellow. In Panel **(d)**, polar residues are colored in pink and hydrophobic in grey. DRPD, DVHD, and DGQD are putative caspase-3 motifs. PDB; Protein data bank. SifA: Confidence 100%; Identity 79%; Coverage 90%.

SifA Harbors a Functionally Active Caspase-3 Cleavage Site

SifA is a SPI-2 *S*. Typhimurium effector protein necessary for intracellular survival. Based on the structural data as shown in Figure 2.1, as well as the observation that each of the SifA domains function independently [61, 91], we next sought to determine whether the caspase-3 motif of SifA (DRPD) is functionally active. To do this we PCR amplified SifA from SL1344 and cloned it into pGEX-6p-1 (GE Healthcare), which adds an N-terminal GST tag to SifA that has been previously used to purify SifA protein using a glutathione sepharose matrix [61]. We found that that exogenous addition of purified fractions of SifA (5µg) with commercially obtained active caspase-3 enzyme (10U and 20U) (Biovision) resulted in the cleavage of this effector protein at the expected site as evidenced by a decrease in the amount of SifA protein and concomitant increase in degradation products (Figure 2.2A, 2.2B)**.** Additionally, a single amino acid substitution in the caspase-3 recognition site is known to render substrates insensitive to caspase-3. Thus, we altered the caspase-3 site (DRPD) by one amino acid to DRPA (refer to methods). The plasmid bearing the mutant clone was sequence confirmed and named pSifAcsm (for SifA caspase site mutant). Such mutation of the caspase-3 site by one amino acid to alanine rendered SifA insensitive to caspase-3 cleavage (Figure 2.2A, 2.2B).

Figure 2.2 SifA harbors a functionally active caspase-3 cleavage site



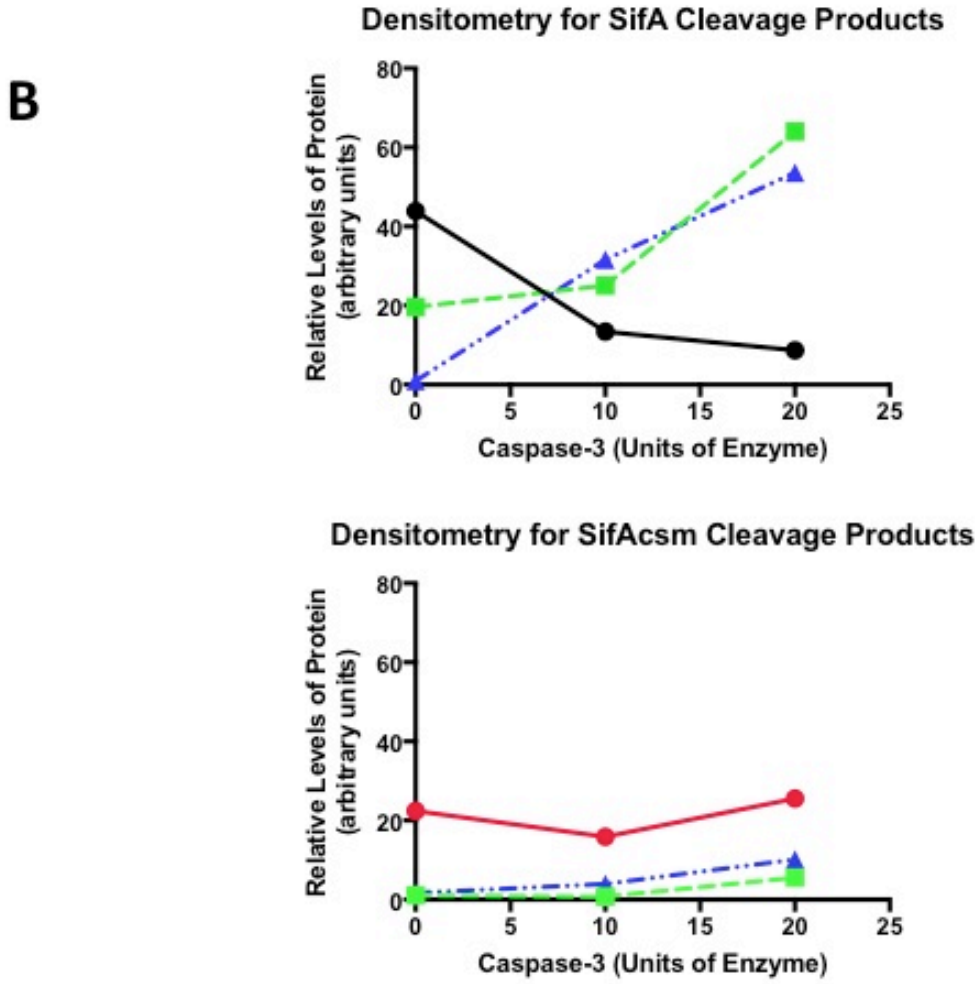


Figure 2.2 SifA harbors a functionally active caspase-3 cleavage site. A.) Purified fractions of SifA and SifAcsm (5μg) were incubated with 10 units or 20 units of human recombinant caspase-3 (Biovision) for 1 hr at 37 ºC. Each sample was then Western blotted using an Anti-GST primary antibody (GE Healthcare) at 1:20,000 and Donkey anti-Goat IgG HRP secondary antibody (Santa Cruz) at 1:5,000. The cleavage products for both SifA and SifAcsm were visualized using coomassie blue staining (BioRad). SifA protein, but not SifAcsm protein, was cleaved by caspase-3 in a concentration dependent manner. B.) Densitometry of Western Blot in (A), SifA (Black), SifAcsm (Red), Cleavage Product 1 (Green), and Cleavage Product 2 (Blue). SifA, but not SifAcsm, displays a caspase-3 concentration dependent decrease, which coincides with an increase in both Cleavage Product 1 and Cleavage Product 2.

SifA Colocalizes with Caspase-3 during infection

To more deeply understand how the processing of SifA occurs through caspase-3 cleavage, we used the small fluorescent phiLOV tag to determine the extent to which we can observe caspase-3 and SifA colocalizing during an *in vitro* infection. This method does not interfere with secretion kinetics of the effector through the T3SS and can be used to examine *de novo* expression of T3SEs *in vitro* and *in vivo* [94, 96, 97]. As shown in Figure 2.3A, following infection of HeLa cells with *Salmonella*, SifA-phiLOV colocalizes with caspase-3 beginning at 2 hpi (seen by yellow), with colocalization continuing through at 4 hpi After 4 hr, colocalization was found to decrease (Figure 2.3A and 2.3B). Our prior studies found that increased caspase-3 activation occurs in SL1344-infected intestinal epithelial cells over the first 5 hr of infection, with the effector protein SipA playing a central role [77]; thus such co-localization of SifA with caspase-3 is consistent with the timing of these events. Moreover, consistent with our previous findings [77], we show that early after *Salmonella* infection of epithelial cells this pathogen activates caspase-3, without inducing apoptosis as determined by a Western blot for activated caspase-3 (Figure 2.3C) and Annexin V staining (Figure 2.3D); as shown, infection with *Salmonella* expressing SifA-phiLOV results in an increase in activated caspase-3 expression (via the function of SipA), but no significant difference in Annexin V positive cells in comparison to uninfected HeLa cells.

Figure 2.3. SifA colocalizes with caspase-3 during infection

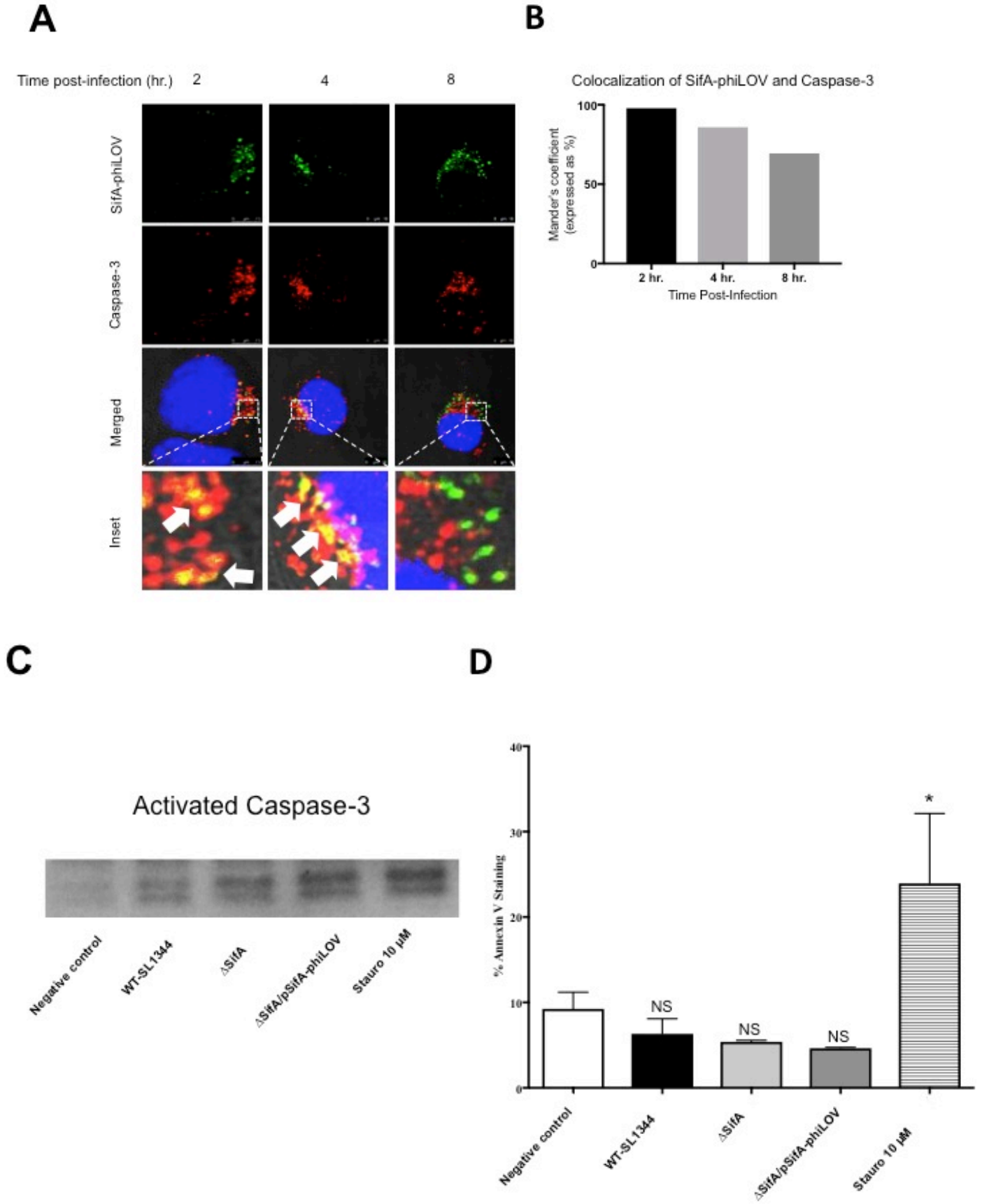


Figure 2.3. SifA colocalizes with caspase-3 during infection. A.) HeLa cells were infected with Δ SifA/pSifA-phiLOV, fixed at 2, 4, and 8 hrs post-infection, stained with Image-iT LIVE Caspase-3 Detection Kit (ThermoFisher) and visualized using fluorescent confocal microscopy. Caspase-3 (Red) and SifA-phiLOV (Green) can be seen colocalizing at 2 hrs post-infection (Yellow; bottom, left panel), with colocalization decreasing through 8 hrs. post-infection (bottom, bottom middle, and bottom right panels). B.) Colocalization of SifA-phiLOV and caspase-3 quantified using FIJI Software. C.) Western Blot for activated-Caspase-3. HeLa cells were infected with indicated strains, lysed, and analyzed for activated caspase-3 expression. Protein concentration was determined using a Bradford Assay to ensure equal amounts of protein were loaded into each well D.) Annexin V Staining Assay. HeLa cells were infected with indicated strains, trypsinized, and stained with Annexin V. Flow cytometric analysis was then completed on a MACSQuant Analyzer to determine the number of Annexin positive cells. In comparison to the negative control (uninfected), HeLa cells only showed a significant increase in Annexin V positive cells with the use of 10μM of staurosporine. HeLa cells infected with WT-SL1344, ΔSifA, and ΔSifA/pSifA-phiLOV all do not exhibit a significant change in Annexin V positive cells in comparison to negative control (uninfected). *P* Values (statistics calculated using unpaired Student's *t* test): NS, not significant; $*P < 0.05$. Experiments were performed at least three times using cells of different passage.

Caspase-3 cleavage of SifA is necessary for dissemination

SifA plays a key role in maintaining the integrity of the *Salmonella*-containing vacuole (SCV) [89], through the formation of tubular membranous structures connected to SCVs named *Salmonella*-induced tubules. Because the formation of such tubules is an essential virulence requirement for intracellular replication in macrophages, and hence contributes to the ability of *Salmonella* to successfully disseminate to extraintestinal organs (i.e., liver and spleen), we next sought to determine whether caspase-3 cleavage of SifA is necessary for *Salmonella* dissemination. Using the murine model of *Salmonella*-induced enteritis, as described in the Methods, we found that consistent with prior reports [98], deletion of the *sif*A gene from *S.* Typhimurium results in a 1 log decrease in dissemination of bacteria to the liver (Figure 2.4A).

Next, we complemented this SifA deletion strain of *Salmonella* with two plasmids; one expressing SifA and the other SifAcsm. We reasoned that if cleavage of SifA at the caspase-3 cleavage site was a critical event required for dissemination, we would expect to see the SifAcsm phenocopy the SifA deletion strain. As shown in Figure 2.4A, we found that when the caspase-3 cleavage site is mutated, we indeed observed a similar 1 log decrease in dissemination to the liver. However, when the SifA deletion strain is complemented with a plasmid containing SifA, there was a rescue of the phenotype to near wild-type levels of dissemination to the liver.

While all strains colonized the large intestine to similar levels (Figure 2.4B), we needed to rule out the possibility that the decrease in dissemination

observed for the SifAcsm mutant strain resulted from a defect in the ability to disseminate via the blood to the liver. We therefore delivered the *Salmonella* strains systemically through tail vein injection, and found that both the ΔSifA strain as well as the SifAcsm strain, showed similar levels of bacterial load in the liver as compared to the wild type and SifA-complemented strain (Figure 2.4C). Given that the SifAcsm strain does not demonstrate an impairment in either the ability to colonize the intestine or reach the liver when delivered systemically suggests that the defect observed results from a failure to disseminate from the intestine.

Figure 2.4. Caspase-3 cleavage of SifA is necessary for dissemination

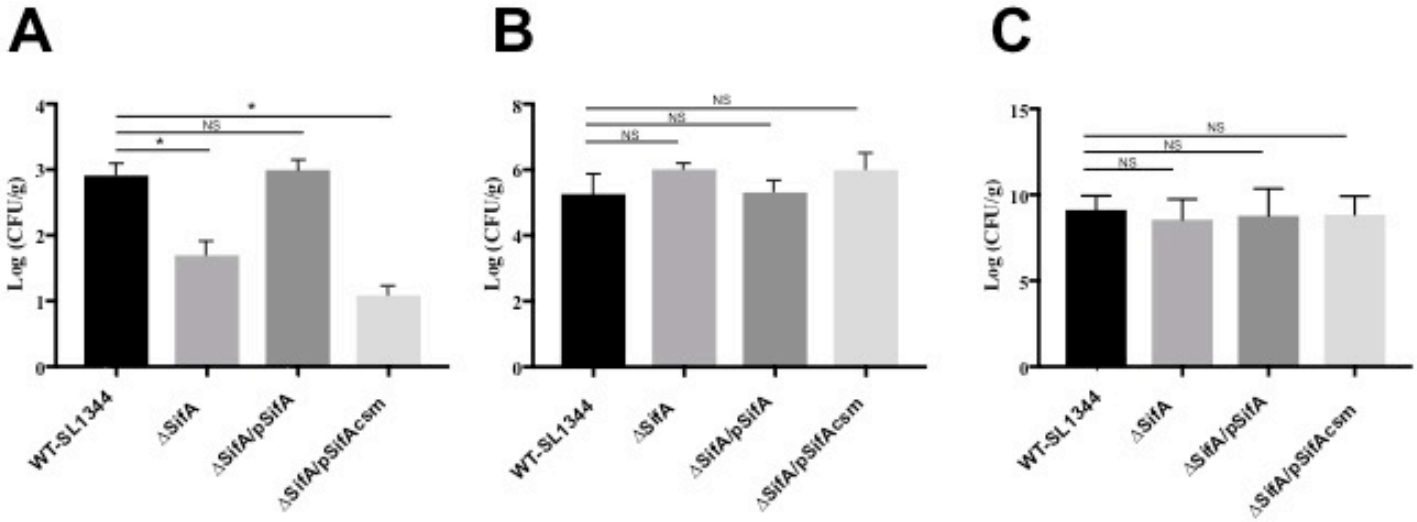


Figure 2.4. Caspase-3 cleavage of SifA is necessary for dissemination. A.) Mice were infected by oral gavage and livers were harvested 48 hrs. post-infection. Bacterial burdens were determined by homogenizing the livers and serial dilution plating. ∆SifA and ∆SifA/pSifAcsm exhibit over 1 log decrease in bacterial burden in the liver. This decrease is restored upon complementation with pSifA. Results are averages of 6 mice per group +/- SD. B.) Mice were infected by oral gavage and proximal colon samples were taken 48 hr post-infection. Bacterial burdens were determined similarly to (A). Results are averages of 6 mice per group +/- SD. ∆SifA, ∆SifA/pSifA, and ∆SifA/pSifAcsm do not exhibit any significant defects in colonization of the proximal colon in comparison to WT-SL1344. C.) Mice were infected via the tail vein, and livers were harvested 24 hr post-infection. Bacterial burden was determined similar to (A). In comparison to WT-SL1344, ∆SifA, ∆SifA/pSifA, and ∆SifA/pSifAcsm all show similar levels of bacteria in the liver. *P* Values (statistics calculated using unpaired Student's *t* test): NS, not significant; $*P < 0.05$. Data shown are representative of at least three independent experiments.

Subcellular localization of SifA functional domains is dependent upon caspase-3 cleavage

Although we have been able to link SifA function to cleavage at the caspase-3 cleavage site, we next sought to determine whether the each of the SifA functional domains could be sub-cellularly tracked following caspase-3 cleavage. SifA is ideal for addressing this question given that the N-terminal domain of SifA interacts with the pleckstrin homology domain of the host kinesin-binding protein SKIP, and the C-terminal domain harbors a WxxxE motif that mimics the active the form of Rho-GTPases. If caspase-3 cleavage is necessary for SifA domain sub-cellular localization, then each of the functional domains would remain in the perinuclear region of the cell, where the SCV resides, until after its interaction with caspase-3. Then following caspase-3 cleavage, the individual functional domains would move to distinct subcellular locations to perform their functions. To this end, we designed dual-tagged V5-SifA-HA and dual-tagged V5-SifAcsm-HA (Figure 2.5A), allowing both red (V5) and green (HA) probes to be displayed on a single effector. In addition to the full-length protein, we also designed the individual domains (N and C terminal domain) with a single tag; V5-SifA (green) and SifA-HA (red).

The benefit of using these constructs is that they permit us to directly investigate the involvement of the caspase-3 motif in SifA domain localization. The V5-SifA-HA and V5-SifAcsm-HA constructs can therefore be used to determine where each domain localizes during infection, and if the domain localization is dependent on caspase-3 cleavage. Additionally, the V5-SifA and

SifA-HA individual domain constructs can be used in combination with caspase-3 KO HeLa cells to determine if we restore domain localization when we use individual domains that resemble their post-caspase-3 cleavage form. Two of these constructs (V5-SifA-HA and V5-SifAcsm-HA) were transduced into wild-type Hela cells using lentiviral transduction. The plasmids used also had an inducible promoter, which allowed us to control expression of all four constructs.

In order to stimulate caspase-3 activation typical of *Salmonella* infection, we infected the HeLa cells transduced with all four constructs with wild-type *Salmonella*, and then looked for the redistribution of each of the functional domains at 1 and 10 hpi using immunofluorescent confocal microscopy. Maximum Sif filament formation has been observed previously between 8 and 10 hpi ([59, 99]; Supplementary Figure 2.1). Since we found that SifA colocalizes with caspase-3 at 2 hpi (Figure 2.2), we included this earlier point (1 hr) to determine the extent to which subcellular localizations change following the interaction of the SifA with caspase-3.

At 1 hpi (prior to caspase-3 activation), we observe both SifA domains located in the perinuclear region for both V5-SifA-HA and V5-SifAcsm-HA (Figure 2.5B, top left panel, Figure 2.5C). However, following interaction with caspase-3 at 2 hpi, the N-terminal domain of V5-SifA-HA begins to branch out from the perinuclear region towards the extremities of the cell and continues to spread throughout the cell at 10 hpi. The role of caspase-3 processing in the domain localization of V5-SifA-HA domains is further supported by the profound decrease in colocalization of the SifA domains at 10 hpi (Figure 2.5B, 2.5C).

Additionally, we observed a significant increase in the relative distance of the N-terminal domain from the nucleus for V5-SifA-HA at 10 hpi relative to 1 hpi (Figure 2.5D). We also observed this distance to be markedly larger when the caspase-3 site was intact, as evidenced by the significant difference at 10 hpi between V5-SifA-HA and V5-SifAcsm-HA (Figure 2.5D). This result is consistent with current literature indicating that the N-terminal domain of SifA binds to a protein called SKIP, and then forms Sif filaments via an interaction with kinesin [61]. Such Sif filaments are tubular membrane extensions that emanate from the SCV, which is located in the perinuclear region, towards the outer part of the cell. Considering the localization of the N-terminal domain for V5-SifA-HA, our results are in line with current understanding of SifA's role in Sif filament formation.

Since the C-terminal domain of SifA mimics host cell GTPases, and these host cell GTPases function in a variety of roles, including cellular division and actin cytoskeletal rearrangements, it is difficult to predict where the C-terminal domain of SifA would localize during *Salmonella* infection [100-102]. Furthermore, the C-terminal domain of SifA also contains a CaaX motif. The CaaX motif is prenylated by the geranylgeranyl transferase PGGT-1, which is a process that has been shown to facilitate attachment to cellular membranes [103]. On the balance of these reports, it would be feasible to observe the C-terminal domain in the perinuclear region at the SCV membrane, the outer cell membrane, or both. However, as shown in Figure 2.5B and Figure 2.6A, the C terminal of SifA appears to remain primarily in the perinuclear region even after

interaction with caspase-3 at the 2 hr time point, indicating the C-terminal domain of SifA likely remains at the SCV membrane through the 10 hpi time point.

We next determined the extent to which the caspase-3 site in SifA plays a role in the localization events we observed with V5-SifA-HA. To do this we used the V5-SifAcsm-HA construct and found that both of the functional domains of this strain remained in the perinuclear region for all time points following infection (Figure 2.5B). Additionally, we did not observe a similar decrease in V5-SifAcsm-HA domain colocalization as observed with V5-SifA-HA (Figure 2.5C). This result suggests that processing of SifA at the caspase-3 site is essential for proper localization of each of the SifA domains, and further infers that the C-terminal domain appears to perform its “GTPase mimicry” function in the perinuclear region after it’s interaction with caspase-3. Nonetheless, further studies are required to discern whether the C-terminal domain of SifA is indeed functionally active at this location.

Figure 2.5. Subcellular localization of SifA functional domains is dependent upon caspase-3 cleavage

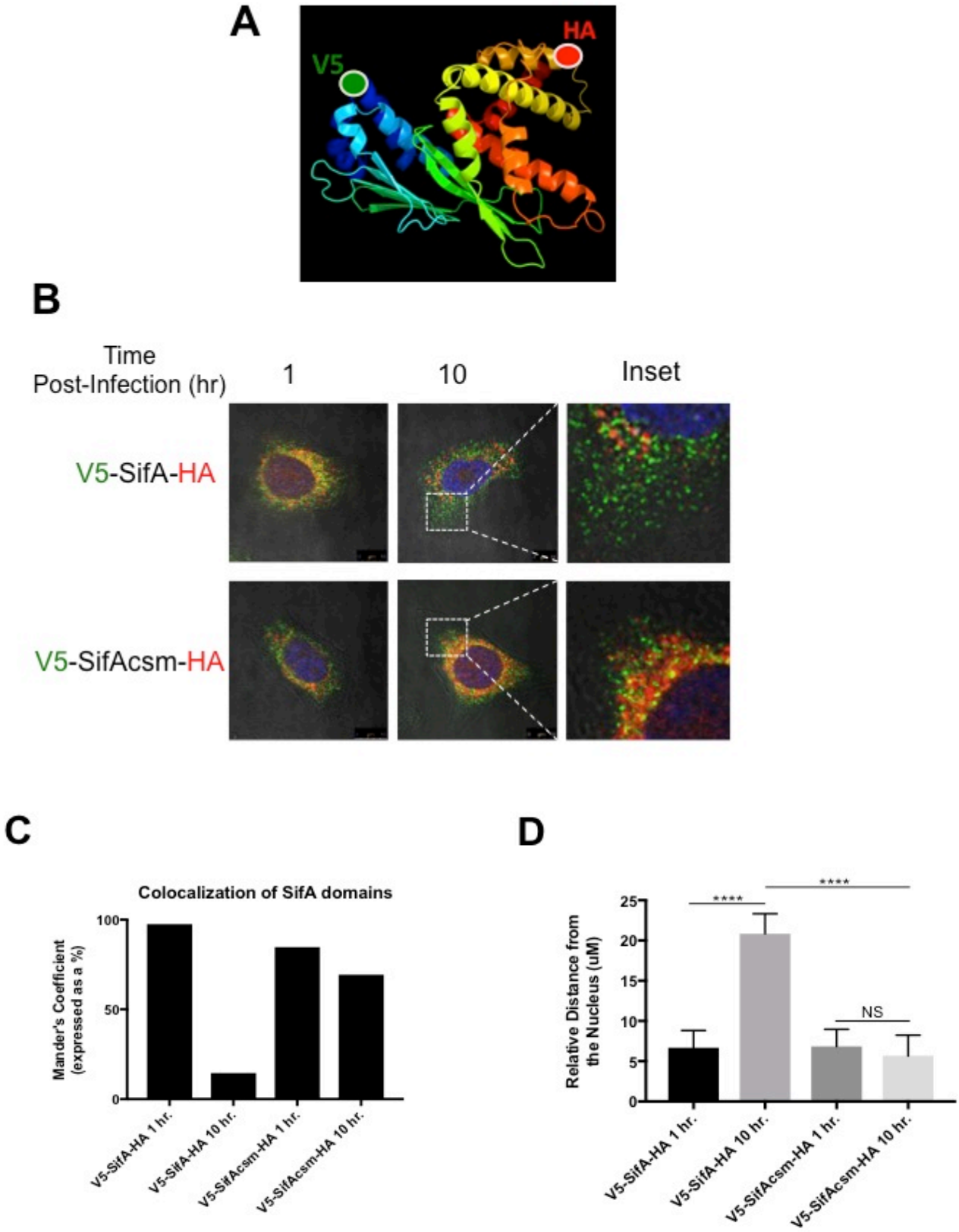


Figure 2.5. Subcellular localization of SifA functional domains is dependent upon caspase-3 cleavage. A.) Schematic depicting the dual-tagging strategy for SifA. The V5 tag (green) is used on the N-terminus and the HA tag (red) is used on the C-terminus. B.) Wild-type HeLa cells containing either V5-SifA-HA or V5-SifAcsm-HA were infected with WT-SL1344. The cells were fixed and stained (refer to methods) at 1 and 10 hr post-infection. The N-terminus of SifA begins to branch out following interaction with caspase-3 at 2 hr post-infection (follow localization of green V5 tag from 1 – 10 hr post-infection), and the C-terminus of SifA remains in the perinuclear region for all time points following infection (red). When the caspase-3 cleavage site is mutated (V5-SifAcsm-HA), both domains (red and green) are observed in the perinuclear region at all time points following infection. Experiments were performed at least three times using cells of different passage. C.) Colocalization of the SifA N-terminal and C-terminal domains following caspase-3 cleavage quantified using FIJI Software. D.) Relative distance (measured using a micron scale) of the distance travelled by the N-terminal domain in both V5-SifA-HA and V5-SifAcsm-HA at 1 and 10 hrs. post-infection. *P* Values (statistics calculated using unpaired Student's *t* test): NS, not significant; **** $P < 0.0001$

Activated individual SifA domains restore SifA domain localization in Caspase-3 KO Cells

To further confirm the role of caspase-3 in the localization of the SifA functional domains, we generated caspase-3 KO HeLa cells using CRISPR/Cas9 (refer to Methods). Additionally, we designed the V5-SifA and SifA-HA constructs containing individual domains (without the caspase-3 site) to assess whether we could restore localization each SifA functional domain by using a form of each domain that resembles their post-caspase-3 cleavage structure. V5-SifA-HA, V5-SifA and SifA-HA were transduced into caspase-3 KO HeLa cells in the exact same manner as they were transduced into wild-type HeLa cells. As shown in Figure 2.6A, both domains of V5-SifA-HA remain in the perinuclear region in the caspase-3 KO cells for all time points following infection, phenocopying what we see in V5-SifAcsm-HA in wild-type HeLa cells. When we use the post-caspase-3 cleavage form of each functional domain (V5-SifA and SifA-HA), we restore the domain localization we observed for V5-SifA-HA in wild-type HeLa cells, further implying that caspase-3 plays a pivotal role in the functional domain localization of SifA (Figure 2.6A). Although SifA-HA resembles the post-caspase-3 cleavage form of the SifA C-terminal domain, the relative distance from the nucleus does not change following infection as it does for V5-SifA (Figure 2.6B). These results agree with what is observed in Figure 5 for V5-SifA-HA in wild-type HeLa cells, indicating the perinuclear region of the HeLa cell is where the C-terminal domain of SifA is inferred to perform its function.

Figure 2.6. Activated individual SifA domains restore SifA domain localization in caspase-3 KO Cells

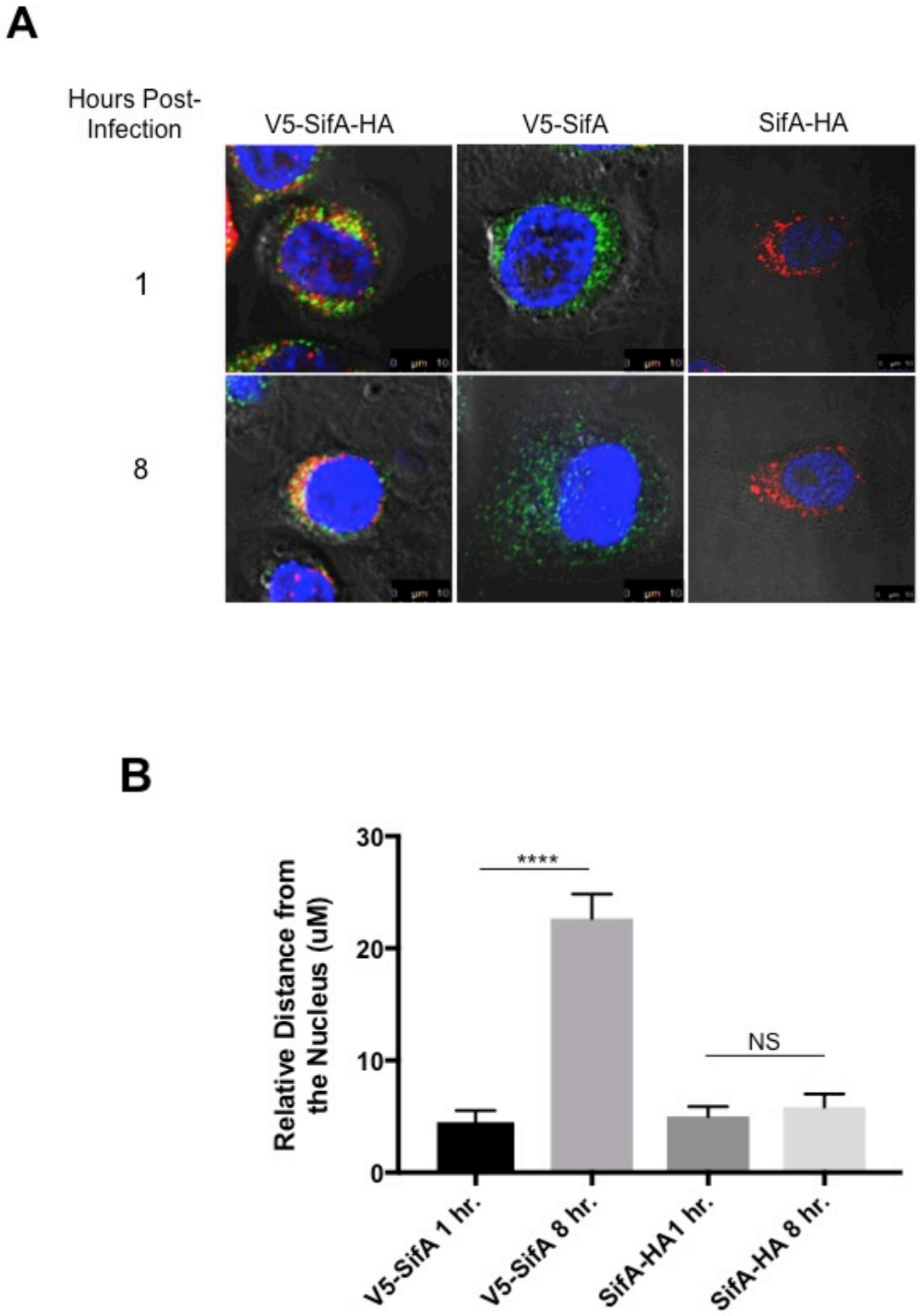


Figure 2.6. Activated individual SifA domains restore SifA domain localization in caspase-3 KO Cells. Caspase-3 KO HeLa cells containing either V5-SifA-HA, V5-SifA or SifA-HA were infected with WT-SL1344. The cells were fixed and stained (refer to methods) at 1 and 8 hr post-infection. Without caspase-3, both domains of V5-SifA-HA remain in the perinuclear region at all time points following infection (top and bottom left panels), suggesting caspase-3 cleavage is important for domain localization (compare to top panels in Fig. 5). When using V5-SifA, which resembles the post-caspase-3 cleavage form of the SifA N-terminal domain, we restore domain localization (top and bottom middle panels). Experiments were performed at least three times using cells of different passage. B.) Relative distance (measured using a micron scale) of the distance travelled by the N-terminal and C-terminal in both V5-SifA and SifA-HA at 1 and 8 hrs. post-infection. *P* Values (statistics calculated using unpaired Student's *t* test): NS, not significant; **** $P < 0.0001$

Discussion

In silico modeling of the SifA caspase-3 motif at the structural level revealed that this motif is surrounded by hydrophilic residues, exposed at the surface, and localized in coiled regions of proteins. This finding is consistent with two other crystallized structural homolog proteins of *Salmonella* effectors (SopA and SptP) that we examined (Supplementary Figure 2.2, and 2.3). This information coupled to the known structural data of SifA [61], suggested that cleavage of the caspase-3 motif in this effector divides this protein into an interacting partner for the host protein SKIP (N-terminal) and a member of the WxxxE family of proteins with GTPase mimicry function (C-terminal). We further report that cleavage of SifA by caspase-3 is indeed critical for proper localization of its functional domains, and is essential for bacterial dissemination from the intestine. Such data are consistent with prior evidence that each of these domains can function independently [61], and is also in step with recent findings that SifA can be split into two functional domains [91]. Our demonstration that caspase-3 cleavage of SifA is required for bacterial dissemination, adds to the emerging concept that caspase-3 sites are present and functionally active in T3SE. Such processing has been described for *Salmonella* invasion (SipA) [77], ubiquitination (SopA) [77], and now in this report for SCV maintenance and bacterial dissemination (SifA), implying that caspase-3 cleavage may have a broad impact on the bacteria's ability to both invade and persist during infection.

Although we have previously shown that the *Salmonella* T3SE, SipA, induces increased caspase-3 activity upon *S*. Typhimurium infection, such

activation remains insufficient to induce the signatures of apoptotic cell death ([77], and this report). Thus, our data suggest that SipA is not only critical in inducing its own proteolytic cleavage but also that of other effectors such as SifA via its role in caspase-3 activation. This idea is consistent with our prior observations that *Salmonella* is significantly less virulent in caspase-3 knockout (CASP3$^{-/-}$) mice and *Salmonella* invasiveness in bone-marrow-derived macrophages from CASP3$^{-/-}$ mice is profoundly reduced [77]. Moreover, in *Salmonella* strains lacking SipA, it has been previously described that SifA and SCVs are incorrectly positioned in the cell [1] and in a manner shown herein, since the function of SifA depends on activation by caspase-3.

Method development for dual-tagging effectors permitted the tracking of effector proteins that also reflects the timeline of effector protein activation and localization during *Salmonella* infection. Unlike other strategies, this method does not rely on the T3SS secretion system for delivery of effector proteins, avoiding any complications associated with effector protein secretion kinetics or lack of secretion signals. The benefit to using this method is that it allows for the simultaneous tracking of domain localization and activation. One potential limitation to this method, however, is that it may be difficult to determine activation of T3SE domains if the localization does not change upon processing. For example, the subcellular localization of the N-terminal domain of SifA changes upon activation, whereas the C-terminal domain remains in the perinuclear region (Figure 2.5 and Figure 2.6). Although we speculate the C-terminal domain is active, additional experimentation will be required to

determine if this is indeed the case. Nevertheless, dual-tagging of SifA demonstrated for the first time that the individual domains of SifA have different subcellular localizations. Additionally, the results from our dual tagging method, in conjunction with the findings of [1] suggest that the C-terminal domain alone may be acting to maintain the SCV in the perinuclear region, though further testing of this hypothesis is required to determine if this is indeed the case. Moreover, as Reinicke and colleagues demonstrated in [103], the process termed prenylation occurs at the *CAAX* motif in the C-terminal domain of SifA, which is a process important for membrane association. Taking into account the localization of the C-terminal domain determined in this study and the perinuclear localization of the SCV determined in [1], the prenylation of the C-terminal domain may be required for interaction with the SCV membrane in the perinuclear region, although further studies would be required to determine if this is true.

Our prior studies revealed that *S*. Typhimurium T3SEs evolved the ability to use host enzymes, such as caspase-3 to activate themselves, displaying a high degree of co-evolution [77]. We speculate that the cleavage motifs of T3SEs have evolved over time and have targeted their cleavage and activation via different host enzymes depending on the host and cell type infected [104]. SipA, the first T3SE delivered into intestinal epithelial cells during infection is an excellent example of the evolution of a T3SE in response to selective pressures at the host-pathogen interface. With type III secretion requiring the delivery of a number of effectors rapidly over a short span of time, the formation of a chimeric

effector with a lone signal sequence and requiring just a single chaperone, greatly reduces the workload at the T3SS interface.

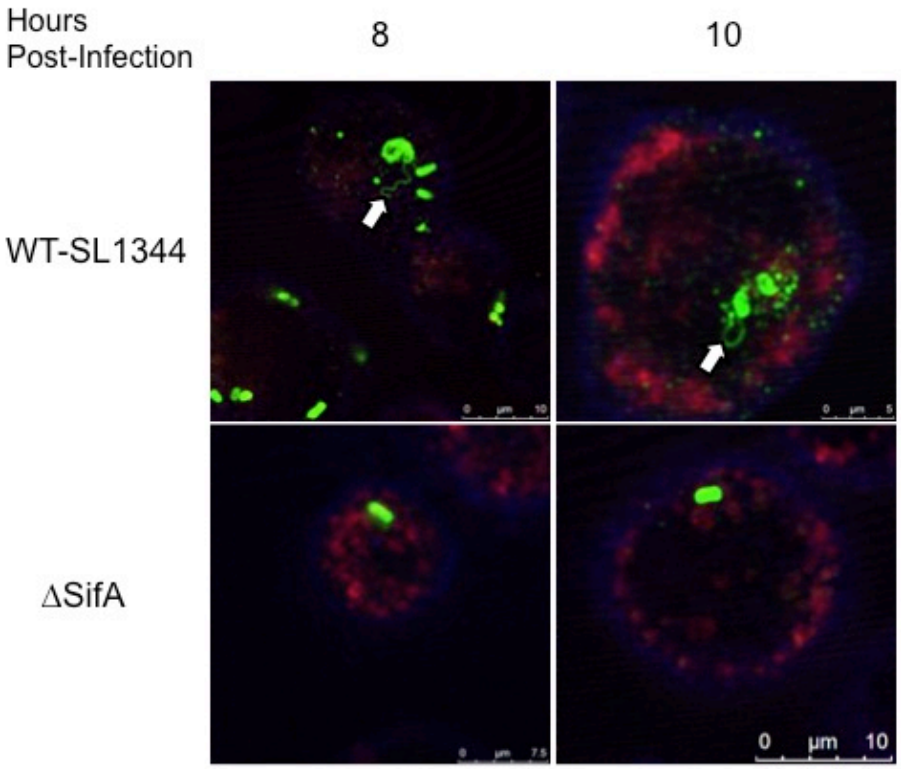
The relationship between bacterial effectors and the activation of caspase-3 is an area of increasing interest. While the outcome of such rearrangements results in independently functional domains coming together as one effector, this also creates a unique challenge in separation of the domains upon host cell delivery to ensure their correct location and function. It is now understood that caspase-3 is constantly present in the cell at levels below those required to induce apoptosis [94], suggesting that caspase-3 processing of effectors likely occurs throughout the cell rather than being dependent on contact merely at the cell surface or within the cytosol. Moreover, the interaction between effector proteins and caspase-3 may play a role in preventing caspase-3 from performing other important functions during infection, as has been described during Yersinia infection [105].

Caspase-3 activation during bacterial infection is also likely a common by-product of the co-evolution of bacterial-host interactions, perhaps precipitated by the stress placed on host cells during invasion [106-109]. In addition to non-specific or indirect activation of caspase-3, bacterial effectors are also able to promote caspase-3 activation through subtle changes within cellular pathways or even through direct interaction with the enzyme. The outcome for the pathogenic intruder, such as *S.* Typhimurium is often an increase in infectivity rather than a clearing of the infection as expected by the conventional understanding of the protective role of apoptosis.

The study herein therefore supports the concept that that caspase-3 cleavage of T3SS secreted effectors represents a common mechanism by which effector functions are regulated in host cells. Understanding of this biological phenomenon as well as advancing methods to interrogate T3SE function inside host cells will allow us to dissect specific aspects of *Salmonella*-host interactions that have yet to be documented, and in general could have broad impact on the field of bacterial pathogenesis.

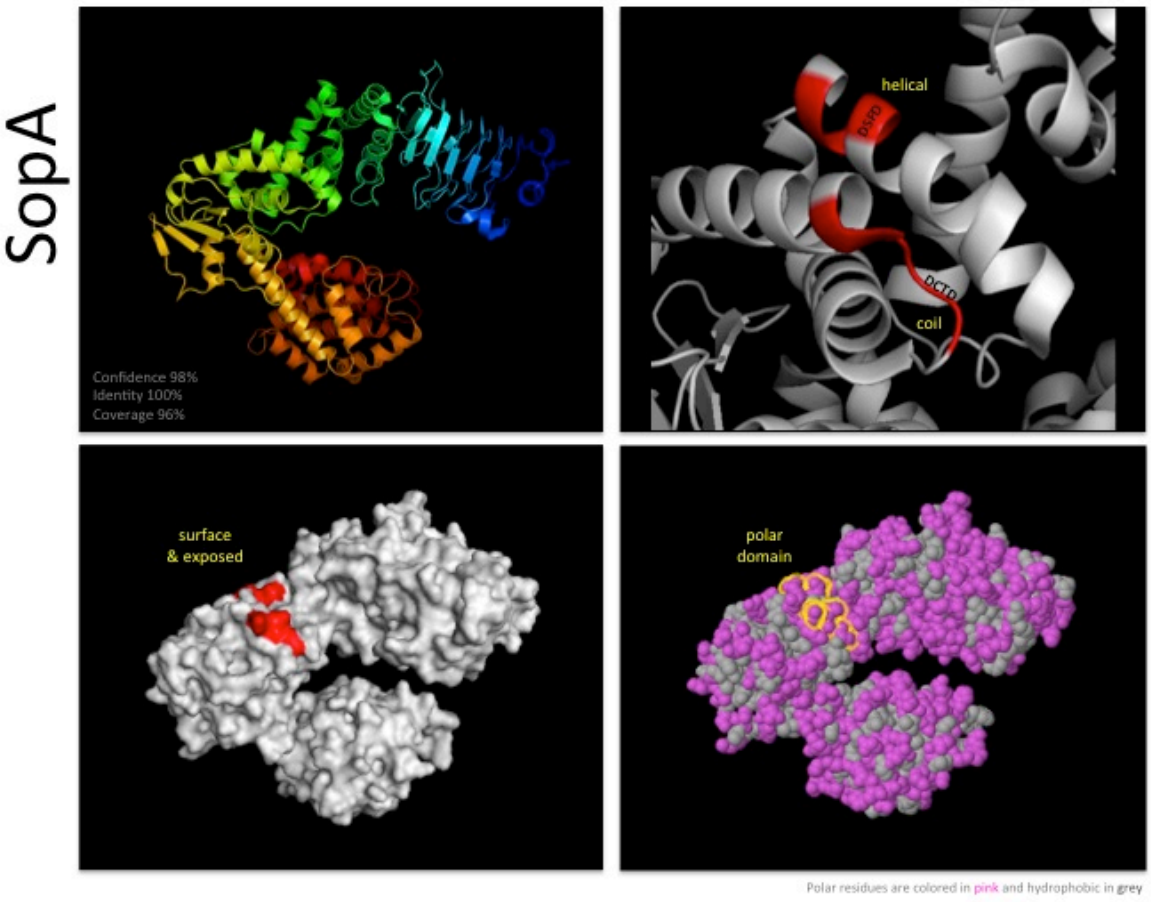
SUPPLEMENTARY FIGURES FOR CHAPTER II

Supplementary Figure 2.1. Sif Filaments form between 8 and 10 hpi



Supplementary Figure 2.1: Sif Filaments form between 8 and 10 hpi. Wild-type HeLa cells were infected with WT-SL1344 or ΔSifA bacteria. Cells were fixed and stained using Anti-*Salmonella* LPS primary antibody (Abcam) and goat anti-rabbit 488 Alexa Fluor secondary antibody (Invitrogen) at 8 and 10 hpi. As shown in the top two panels, Sif filaments are visible at both 8 and 10 hpi (white arrows). Deletion of SifA, however, results in a loss of these Sif filaments. Experiments were performed at least three times using cells of different passage.

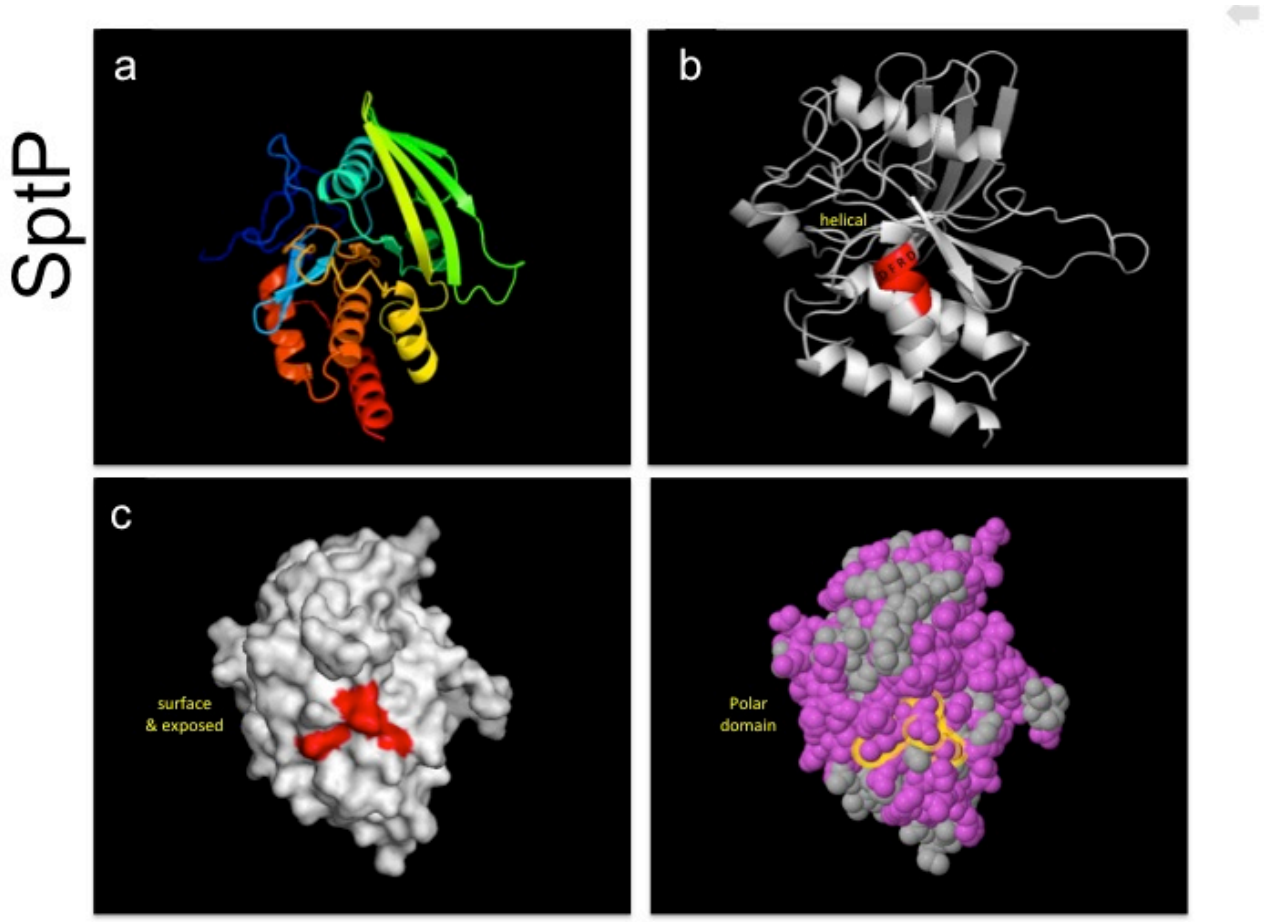
Supplementary Figure 2.2. The structural characteristics of the caspase-3 motif in the Salmonella T3SE SopA



Supplementary Figure 2.2. The structural characteristics of the caspase-3 motif in Salmonella T3SE SopA. The structural features of the caspase-3 motif found in the *Salmonella* effector protein SopA (PDB ID 2QYU). Protein structures are displayed as cartoon (**Panels a and b**), surface (**Panel c**), and spheres (**Panel c**). **Panel (a)** shows protein structures colored with the spectrum color set of PyMOL to display the different protein domains, α-helixes, β-sheets, and coil regions. In **Panels (b)** and **(c)**, the location of the caspase-3 motif is colored in

red, whereas in **Panel (d)** in yellow. In Panel **(d)**, polar residues are colored in pink and hydrophobic in grey. DSFD and DCTD are putative caspase-3 motifs. PDB; Protein data bank. SopA: Confidence 98%; Identity 100%; Coverage 96%.

Supplementary Figure 2.3. The structural characteristics of the caspase-3 motif in Salmonella T3SE SptP



Supplementary Figure 2.3. The structural characteristics of the caspase-3 motif in Salmonella T3SE SptP. The structural features of the caspase-3 motif found in the *Salmonella* effector protein SptP (PDB ID 1G4W). Protein structures are displayed as cartoon (**Panels a and b**), surface (**Panel c**), and spheres (**Panel c**). **Panel (a)** shows protein structures colored with the spectrum color set of PyMOL to display the different protein domains, α-helixes, β-sheets, and coil regions. In **Panels (b)** and **(c)**, the location of the caspase-3 motif is colored in red, whereas in

Panel (d) in yellow. In Panel **(d)**, polar residues are colored in pink and hydrophobic in grey. DFRD is the putative caspase-3 motif. PDB; Protein data bank. SifA: Confidence 100%; Identity 100%; Coverage 44%.

APPENDIX A: Define the evolutionary relationship between caspase cleavage and their respective hosts by tracking effector evolution as an indicator of host adaptation

Although we found that caspase-3 processing of SifA, SipA, and SopA [77] is pivotal for the function of these effector proteins, we also found the presence of other proteolytic cleavage sites in effector proteins belonging to different serovars of *Salmonella enterica*. This finding could potentially be explained from an evolutionary perspective, in that different cleavage sites may have arose to increase the fitness of the bacteria depending on the particular host it was infecting.

In order to explore the possibility of proteolytic cleavage sites being related to host specificity and adaptation, we chose to examine the protein sequences of effector proteins across different serovars of *Salmonella enterica*. Preliminary data comparing SifA from *Salmonella* arizonae (*S.* arizonae) and *S.* Typhimurium revealed that the processing sites found in the SifA for these particular two serovars of *Salmonella enterica* were different. Absent from the SifA in *S.* arizonae is the caspase-3 cleavage site; however, there is a caspase-1 cleavage site. This caspase-1 site is not found in SifA in *S.* Typhimurium (Figure A.1). So the obvious question arises: Why does the same effector protein have a different proteolytic cleavage site?

Figure A.1. Caspase processing sites in *S.* arizonae and *S.* Typhimurium

SifA (*S.* Typhimurium)

```
MPITIGNGFLKSEILTNSPRNTKEAWWKVLWEKIKDFFFSTGKAKADRCLHEMLFAERAPTRERLTEIFF
ELKELACASQRDRFQVHNPHENDATIILRIMDQNEENELLRITQNTDTFSCEVMGNLYFLMKDRPDILKS
HPQMTAMIKRRYSEIVDYPLPSTLCLNPAGAPILSVPLDNIEGYLYTELRKGHLDGWKAQEKATYLAAKI
QSGIEKTTRILHHANISESTQQNAFLETMAMCGLKQLEIPPPHTHIPIEKMVKEVLLADKTFQAFLVTDP
STSQSMLAEIVEAISDQVFHAIFRIDPQAIQKMAEEQLTTLHVRSEQQSGCLCCFL
```

SifA (*S.* arizonae)

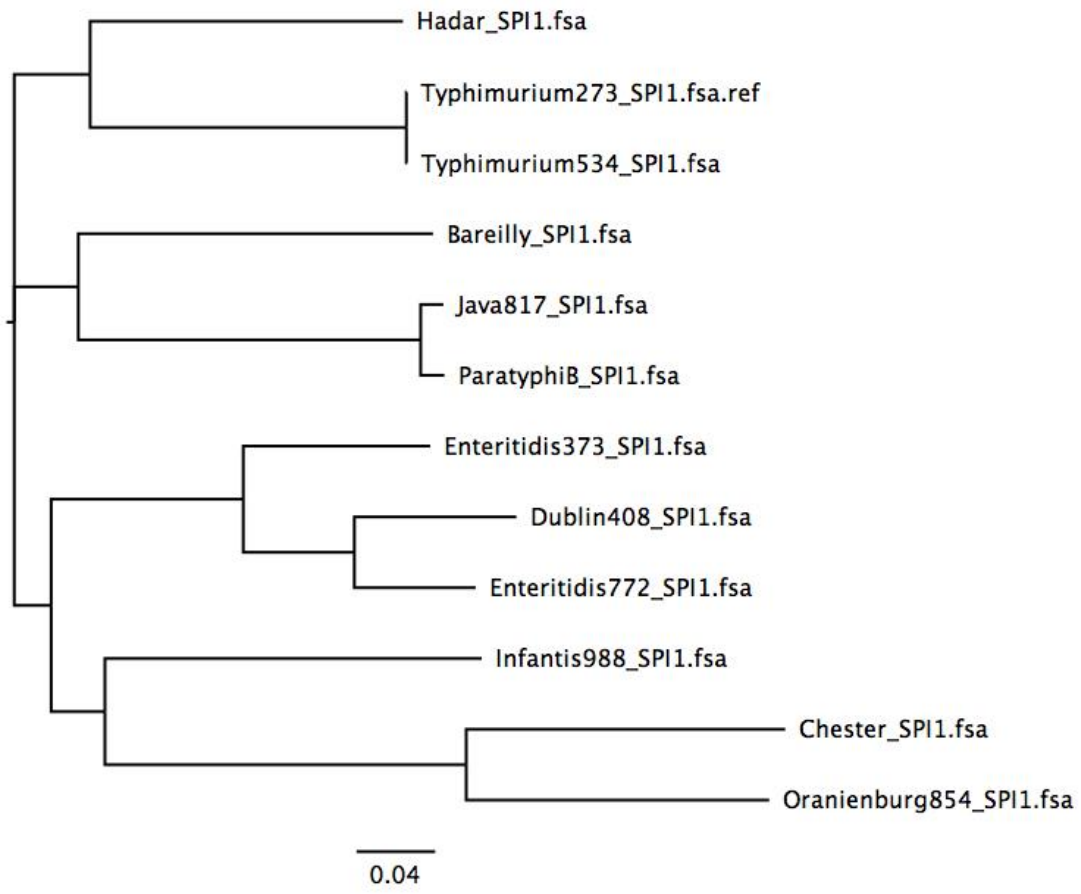
```
MPITIGNGYLKSEIFINAPSKTREPWWKALWEALKDLFFSTGRAKADSYIHEMFFSDPPPTRERLADIFF
ELKGLACPSHKERFQVYNPHEDDSTIIYHILDENGKDELLCIIQNTDTVHCKAMGNSYFAVREQPVCLKS
YPQMTYTVNKKYSEIVESSLPSTLCLKLAGTPFLSVPLNNIVKYLYSELDNRNLDKWKTQEKANYLAEKI
RSGIEKAMRILYHADISESMQQRAFLETMSMCGLKSTETSPPPTHIPIVKIVQEVLLADKKFKRFLATDT
NASQSMLAEIIEIVSDGVFRALFRTDPQAIQKMAEEQLTTLHVRSDQQDGRLGGFL
```

Figure A.1. Caspase Processing Sites in *S.* arizonae and *S.* Typhimurium. By aligning the regions of SifA that contain caspase-3 cleavage sites in *S.* Typhimurium and comparing it to the same region of SifA from *S.* arizonae, we find that the caspase processing sites are different. The protein sequence for the proteolytic cleavage site of SifA in *S.* Typhimurium (highlighted in grey) is DRPD, a caspase-3 cleavage motif. The protein sequence for the proteolytic cleavage site of SifA in *S.* arizonae (highlighted in grey) is EQPV, a caspase-1 cleavage motif. This finding suggests that different serovars of *Salmonella enterica* could express different caspase processing sites, which we speculate may have evolved as a host adaptation mechanism.

In order to expand this inquiry further, we chose to compare the effector protein sequences of SPI-1 and SPI-2. After obtaining full genome sequences of various *Salmonella enterica* serovars that infected different hosts, we developed a phylogenetic tree to determine if we observed any differences that would show a correlation between proteolytic processing sites and host adaptation. The phylogenetic trees shown in Figure A.2A and Figure A.2B indicate that there is an evolutionary difference between the SPI-1 and SPI-2 protein sequences among different serovars of *Salmonella enterica*, which further substantiates our observation of the different cleavage sites in SifA of *S.* arizonae and *S.* Typhimurium. Although it does not entirely demonstrate that there are sequence differences in the individual effector proteins, it does demonstrate that there is an evolutionary difference in the protein sequences of SPI-1 and SPI-2 that relates to host specificity.

Figure A.2. Phylogenetic analysis of the SPI-1 and SPI-2 protein sequences of *Salmonella enterica*

A.



B.

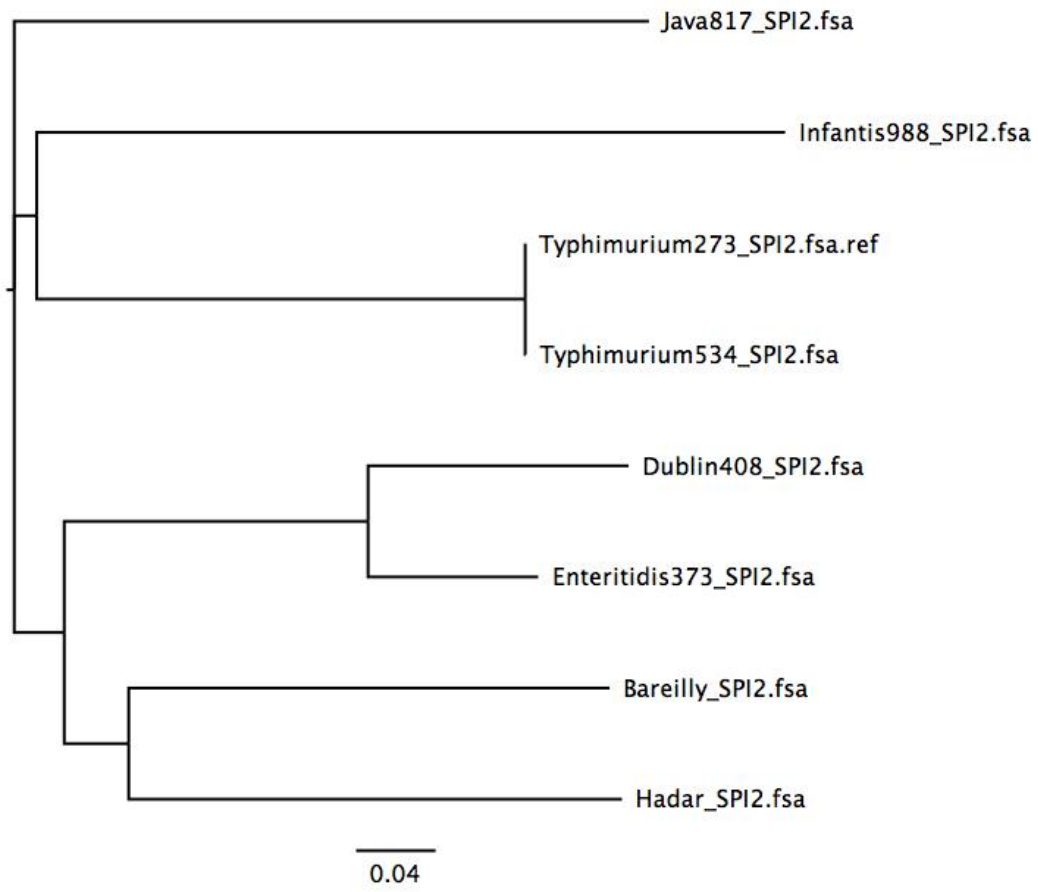

Java817_SPI2.fsa
Infantis988_SPI2.fsa
Typhimurium273_SPI2.fsa.ref
Typhimurium534_SPI2.fsa
Dublin408_SPI2.fsa
Enteritidis373_SPI2.fsa
Bareilly_SPI2.fsa
Hadar_SPI2.fsa
0.04

Figure A.2. Phylogenetic analysis of the SPI-1 and SPI-2 protein sequences of *Salmonella enterica*. Phylogenetic trees for both SPI-1 and SPI-2. A.) and B.) Phylogenetic tree of SPI-1 and SPI-2 protein sequences revealed that in relationship to *S.* Typhimurium, the SPI-1 and SPI-2 protein sequences of several other serovars of *Salmonella enterica* have evolutionary differences. This observation further supports the findings in A.1, namely that SifA in *S.* Typhimurium and SifA in *S.* arizone display different proteolytic cleavage sites. *Labeling*: The labels indicate the serovar of *Salmonella enterica*. Since the full genome sequences were obtained from infected patients, the numbers following some of the serovar names were used to identify the patient. The scale bar at the bottom of each phylogenetic tree indicates distance, which is used to measure the evolutionary similarity between the SPI-1 and SPI-2 protein sequences of each serovar in reference to *S.* Typhimurium.

As such, we chose to look at individual effector protein sequences from the different serovars of *Salmonella enterica* used to develop the phylogenetic tree. The results we obtained were surprisingly inconclusive, primarily due to the fact that we were unable to find any sequence differences in proteolytic cleavage sites among the effector proteins analyzed. This observation, however, does not exclude the possibility that a more comprehensive analysis that includes other species of *Salmonella* could yield results demonstrating differences in proteolytic cleavage sites. At this time, this type of broad analysis is rather challenging due to the fact that many full genomes and effector proteins do not have readily available full sequences that can be used for analysis. Thus, key questions regarding the correlation between proteolytic cleavage sites and host specificity remain, but it is important to acknowledge that the initial results from *S.* arizonae and *S.* Typhimurium suggest host adaptation might potentially play a role in the evolution of proteolytic cleavage sites in effector proteins.

CHAPTER III: Discussion

Thesis Summary

The findings described in this study address the question of whether or not caspase-3 processing of SifA is required for protein function and domain localization. The results from this work have expanded our knowledge of how *S.* Typhimurium utilizes host enzymes during an infection. Most importantly, this study allowed for the development of a potential model of effector protein activation -- proteolytic processing via the use of host enzymes is an activation paradigm used by *S.* Typhimurium to promote it's own pathogenesis.

Previous observations from our lab demonstrated that caspase-3 cleavage of the T3SE SipA is required for activation of protein function and that both functional domains of SipA function independently [55, 76, 77]. Since SipA is involved in bacterial entry via its role in inducing inflammation and actin rearrangement, we sought out to determine whether effector proteins involved in intracellular survival and bacterial dissemination also required caspase-3 processing for activation. As such, we chose to look at SifA, a SPI-2 effector protein containing a caspase-3 cleavage site that functions in inducing endosomal tubulation, which is required for SCV maintenance and bacterial dissemination.

We were able to demonstrate that SifA has a functionally active caspase-3 cleavage site, and that caspase-3 cleavage was required for SifA function using previously established methods [Chapter 2]; however, in order to address the hypothesis that caspase-3 cleavage is important for subcellular localization of

SifA functional domains, we had to develop new methods to track effector proteins during *Salmonella* infection. As shown in Chapter 2, these new tracking methods were utilized to address three questions -- Do SifA and caspase-3 colocalize during infection, is caspase-3 cleavage important for SifA functional domain localization, and is it possible to restore functional domain localization.

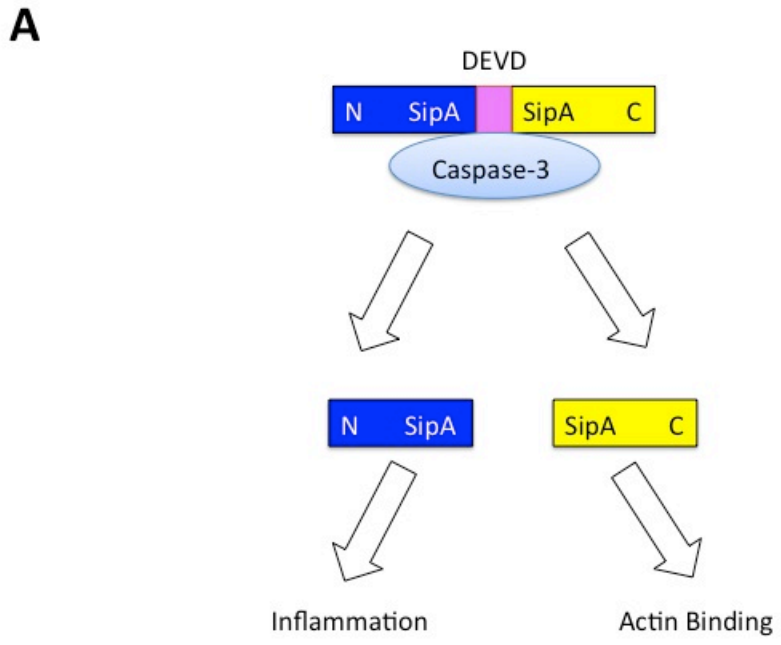
Utilizing the phi-LOV tracking method, we were able to demonstrate that SifA and caspase-3 colocalize during infection. Moreover, we were able to use this method to demonstrate that this colocalization of SifA and caspase-3 occurs as early as 2 hrs. post-infection, and then decreases through 8 hrs. post-infection (Figure 2.3A and 2.3B). It is important to note that these results concur with the timeline of Sif filament formation, in that Sif filaments form between 8 hrs. and 10 hrs. post-infection ([59, 99]; Supplementary Figure 2.1). If caspase-3 processing was required for Sif filament formation, then it would be expected for caspase-3 and SifA to colocalize prior to their formation, which we were able to validate using the phi-LOV tracking method.

Addressing the inquiry of SifA domain localization and restoration of domain localization required the development of a new effector protein tracking method. This method involved tagging SifA in a manner that would allow us to individually track each functional domain [Chapter 2; Figure 2.5 and 2.6]. The strategy we developed was dual-tagging SifA and SifAcsm, using a V5 tag on the N-terminal domain and a HA tag on the C-terminal domain (Figure 2.5A and 2.6). The premise behind this strategy was rooted in the idea that following infection we would be able to track each functional domain to their respective subcellular

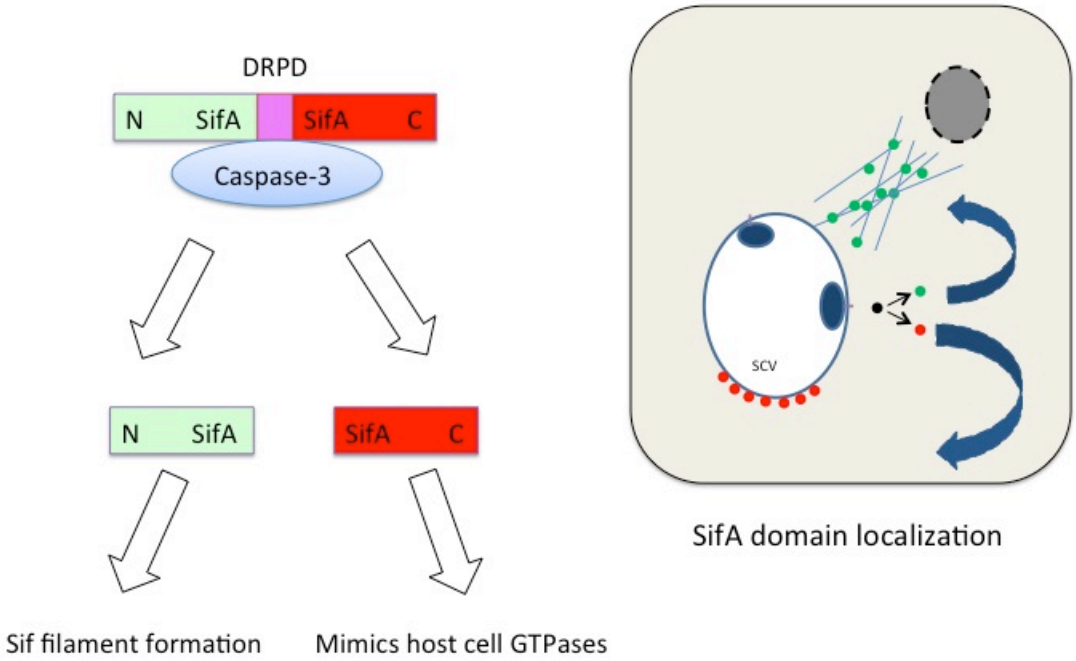
locations. Additionally, our dual-tagging method would allow us to address the relevance of caspase-3 cleavage to SifA functional domain localization.

As discussed in Chapter 2, we were able to demonstrate that caspase-3 cleavage of SifA is required for proper subcellular localization of SifA functional domains utilizing our dual-tagging method (Figure 2.5 and 2.6). In further support of the significance of caspase-3 cleavage in SifA functional domain localization, we were able to show that individually tagged SifA domains that represented their post-cleavage activated forms restored subcellular localization (Figure 2.6). Thus, the use of our dual-tagging method allowed us to expand the role of caspase-3 processing from being pivotal in SifA function to also being essential for intracellular localization.

Figure D.1. Summary of Findings



B


DRPD
N
SifA
SifA
C
Caspase-3
N
SifA
SifA
C
SCV
SifA domain localization
Sif filament formation
Mimics host cell GTPases

C

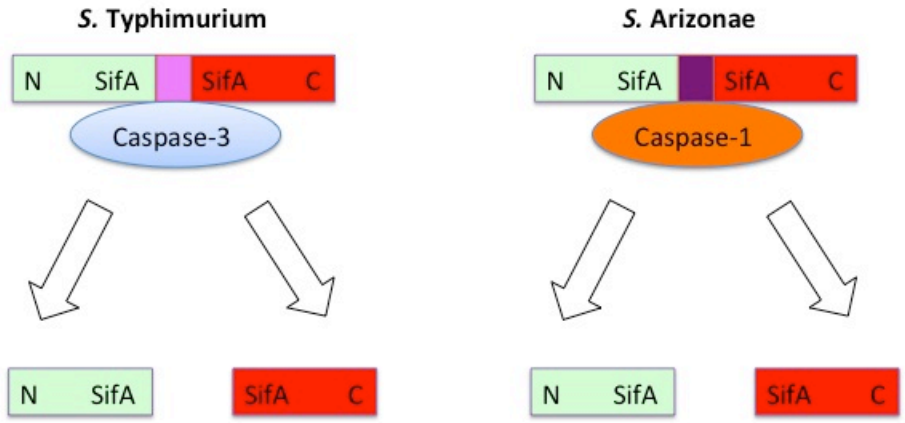

S. Typhimurium
N
SifA
SifA
C
Caspase-3
N
SifA
SifA
C
S. Arizonae
N
SifA
SifA
C
Caspase-1
N
SifA
SifA
C

Figure D.1. Summary of Findings. A.) Prior to the work presented in this thesis, it was known that caspase-3 processing of SPI-1 effector proteins (i.e. SipA) was required for the induction of inflammation and actin binding. B.) Since SPI-1 effector proteins are involved in bacterial entry, we sought to determine if SPI-2 encoded effector proteins (involved in bacterial dissemination and SCV maintenance) also required caspase-3 processing for function and domain localization. We found that SifA not only requires caspase-3 cleavage for function, but that it is also required for proper domain localization. The N-terminal domain of SifA branches out from the SCV, which is consistent with the N-terminal being involved in Sif filament formation. The C-terminal domain localizes to the perinuclear region, which could suggest that it is performing its GTPase mimcry function at the SCV membrane. Additional studies will be required to determine whether or not caspase-3 cleavage is important for individual domain function and interaction with host cell interacting partners C.) An interesting observation made was that the processing sites in different serovars of *Salmonella* appear to have different proteolytic cleavage sites. This suggests that *Salmonela* co-evolved with the hosts that they infect, as *S.* Typhimurium infects humans/poultry and *S.* arizonae infects alligators, though further studies are required to determine if this is indeed the case. Furthermore, it would be interesting to determine if the different “variants” of SifA play an identical role in the hosts that they infect.

Future Directions

The role of caspase-3 in domain function

Although we found that caspase-3 cleavage is required for bacterial dissemination and subcellular localization of SifA functional domains, there are still fundamental questions that remain. Perhaps the biggest question that remains is whether caspase-3 cleavage plays a role in the individual functions of each SifA domain. Specifically, is caspase-3 cleavage necessary for the N-terminal domain of SifA to induce the formation of Sif filaments? Also, is caspase-3 cleavage necessary for the GTPase mimicry function of the C-terminal domain? Since caspase-3 cleavage of SifA is important for both bacterial dissemination and functional domain localization [Chapter 2], it is plausible that caspase-3 cleavage is also important for the functions of the individual domains of SifA. As the formation of Sif filaments via the function of the N-terminal domain is well documented, the involvement of caspase-3 processing in Sif filament formation could be addressed using the caspase-3 cleavage site mutant our lab has developed ([59-61, 99]; this study). However, the GTPase mimicry function of the C-terminal domain is not fully understood, and consequently it is difficult to develop an assay that would allow for a direct analysis of the involvement of caspase-3 processing in the function of this domain at this time. Nevertheless, it would be a worthwhile pursuit to determine the involvement of caspase-3 processing in the functions of each SifA domain.

The role of caspase-3 processing in the interaction of individual domains with host cell interacting partners

An additional question worth exploring is the possibility that proteolytic cleavage by caspase-3 is a prerequisite for interaction of SifA functional domains with their respective host cell interacting partners. Diacovich and colleagues demonstrated that the interaction between the N-terminal of SifA and its host cell interacting partner, SKIP, is essential for *Salmonella* pathogenesis [90]. Since both caspase-3 processing of SifA and SifA's interaction with SKIP are vital for the virulence of *Salmonella*, it is tempting to speculate that caspase-3 processing could play a role in the ability of the N-terminal domain of SifA to interact with SKIP. Since antibodies for many effector proteins have yet to be developed, utilizing the dual-tagging method established in this study makes it possible to begin addressing this question. Unfortunately, a host cell interacting partner for the C-terminal domain has yet to be identified, though our data from Chapter 2 (Figure 2.5 and 2.6) suggests the host cell interacting partner(s) of the C-terminal may be found in the perinuclear region and possibly at the SCV membrane. Nonetheless, if a C-terminal domain interacting partner is uncovered, the dual-tagging method will be a useful tool in determining the relevance of caspase-3 cleavage to the ability of both SifA functional domains to interact with their respective host cell interacting partners.

The role of caspase-3 processing in the functional interaction between effector proteins

As discussed in the two previous paragraphs, it is possible for caspase-3 cleavage to be required for both SifA individual domain function and also for the interaction of SifA domains with their respective host cell interacting partners. One intriguing question that remains is if the interaction between effector proteins also requires caspase-3 processing. Brawn and colleagues have demonstrated that SipA and SifA coordinate not only promote intracellular replication, but also to localize the SCV to the perinuclear region [1]. According to their model, deletion of SipA disrupts the localization of the SCV. Additionally, it is now known that both SipA and SifA require caspase-3 cleavage for function ([77] and this study). As such, it would be interesting to determine whether or not mutating the caspase-3 sites in either SipA or SifA affects the perinuclear localization of the SCV.

Salmonella-induced activation of caspase-3 and apoptosis

It is well documented that the terminal activation of caspase-3 results in apoptosis; however, in the case of *Salmonella* infection, we observe that apoptotic cell death does not occur until 18-20 hpi even though caspase-3 activation is found to be increased during the first 5 hours following *Salmonella* infection [110]. This finding suggests that caspase-3 activation increases during the first 5 hpi because of the role caspase-3 plays in effector protein activation [77, 110]. In further support of this theory, Knodler and colleagues found that the effector protein SopB prevents apoptosis by sustained activation of Akt [110].

Since SopB is one of the first effector proteins translocated into the host cell, it is plausible that SopB plays a pivotal role in the sustained activation of caspase-3 without induction of apoptosis. It would be a worthwhile pursuit to determine if SopB requires caspase-3 processing for this function, as it does contain a caspase-3 cleavage site [77]. This could potentially demonstrate that not only does *Salmonella* activate caspase-3 for effector protein activation, but also has mechanisms based on effector protein function designed to regulate caspase-3 activity.

It still remains to be determined whether or not other effector proteins play a role in the prevention of apoptosis. Furthermore, considering that Rosado and colleagues demonstrated caspase-3 has roles in cellular functions not related to apoptosis, it would be intriguing to determine how *Salmonella*-induced activation of caspase-3 affects its involvement in other cellular functions [111].

Evolution of proteolytic cleavage sites

It is still unclear as to why *S.* Typhimurium selected caspase-3 in order to process effector proteins. Indeed, the data in Appendix A does suggest there is an evolutionary relationship between effector proteins from different serovars of *Salmonella*, their processing sites, and the respective hosts that they infect. However, there are many other proteolytic enzymes that could have been evolutionarily selected in order to increase bacterial fitness in the same host. As such, looking further into the advantages or disadvantages of evolving particular

proteolytic processing sites in effector proteins could help further determine why individual serovars chose particular proteolytic enzymes.

In the case of *S.* Typhimurium, one possibility is that the additional roles caspase-3 plays in cellular function could be required or be a target for yet unidentified effector protein functions. Moreover, the manipulation of the additional roles caspase-3 plays in cellular function by effector proteins may potentially be required for *Salmonella* pathogenesis.

Conclusion

The findings reported in this study broaden our understanding of the role caspase-3 processing plays in the activation, function and localization of the *S.* Typhimurium effector protein SifA. They also demonstrate for the first time a direct correlation between the function and localization of a SPI-2 effector protein and proteolytic cleavage by caspase-3. Our results build upon previous observations made by our lab, namely that the SPI-1 effector proteins SipA and SopA require caspase-3 cleavage for function. Through the development of new tracking methodologies, we were able to further expand the role of caspase-3 from being a critical for effector protein activation to also being critical for effector protein domain localization. Moreover, we demonstrate that the development of new methods to assess effector protein function will allow us to further advance our understanding of effector protein biology, ultimately leading to a more comprehensive understanding of the mechanisms used by *S.* Typhimurium to promote it’s own pathogenesis.

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