Long-term Imaging Methods to Determine the Role of Salmonella Effector Proteins in Vacuole Formation During Infection

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Long-term imaging methods to determine the role of *Salmonella* effector proteins in vacuole formation during infection

by

Sarah Elizabeth McQuate

B.S., University of Puget Sound, 2007

A thesis submitted to the

Faculty of the Graduate School of the

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of the requirement for the degree of

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Department of Chemistry and Biochemistry

2014
This thesis entitled:
Long-term imaging methods to determine the role of *Salmonella* effector proteins in vacuole formation during infection
written by Sarah Elizabeth McQuate
has been approved for the Department of Chemistry and Biochemistry

_____________________________________________
Amy E. Palmer, Ph.D.

_____________________________________________
Natalie G. Ahn, Ph.D.

Date_________________

The final copy of this thesis has been examined by the signatories, and we Find that both the content and the form meet acceptable presentation standards Of scholarly work in the above mentioned discipline.
McQuate, Sarah Elizabeth (Ph.D., Biochemistry)

Long-term imaging methods to determine the role of Salmonella effector proteins in vacuole formation during infection

Thesis directed by Professor Amy E. Palmer, Ph.D.

Salmonella species are Gram-negative bacteria responsible for causing a variety of diseases in multiple hosts across the plant and animal kingdom. In humans, Salmonella are most known for causing typhoid fever and gastroenteritis. In both of these diseases, Salmonella use effector proteins to generate a niche within host cells where the bacteria can survive and/or replicate. The niche, which is composed of a membrane-bound vacuole called the Salmonella containing vacuole or SCV, is trafficked and matured throughout infection. This process requires the work of both effector proteins and host cell proteins, which are recruited to the SCV. Although the sequence of events behind SCV formation and maturation is known, the mechanism behind SCV development is poorly understood. Here we discuss two novel imaging methods and subsequent quantitative analysis techniques we created to study the roles of the specific effector proteins SteA and SseG in the proper establishment and maturation of the SCV during infection of single cells in both gastroenteritis and typhoid fever models.
Acknowledgements

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A huge thank you to my SMART family, especially Vincent Basile, Matt Montemore, Alia Lubers, Cliff Bridges, Lila Saade, Leigh Cooper, Ruth López, Norma Sanchez Gonzalez, Miguel Gonzalez, Marty Baylor, Kristin Lopez, and Barbara Kraus for giving me a community during my time in Boulder. Also I especially appreciate the Colorado Diversity Initiative for helping to support me financially during my first year of graduate school and my trip the Pasteur Institute. Thank you to Carone Lowrey (my trombone buddy), Elin Maki (the first person I met in college), Michael Saxton (my musicals friend), and my crafty friends Jennette Warner and Liz Myers for being supportive and awesome people. Finally thank you to the great BSAC soccer team for letting me play my favorite sport with some very talented scientists/soccer players.

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Chapter 1: Introduction

1.1 BACKGROUND AND SIGNIFICANCE

The *Salmonella enterica* species consists of Gram negative pathogenic bacteria that can infect most organisms within the plant and animal kingdom (1, 2). This species includes over 2600 different serovars, which differ in antigen presentation, host preference, and the particular diseases they cause (3). Two of the most well known *Salmonella enterica* serovars are Typhi and Typhimurium. *S. Typhi* is restricted to human hosts and is responsible for causing typhoid fever. Typhoid fever is a life-threatening systemic disease with more than 27 million cases, resulting in over 200,000 deaths worldwide per year (3). *S. Typhimurium*, on the other hand, causes gastroenteritis, a disease with more than 94 million cases, which result in over 155,000 deaths worldwide per year (4). *S. Typhimurium* is not restricted to human hosts and can also cause a typhoid-like systemic disease in mice (5). Because infections with *Salmonella enterica* serovars result in large numbers of deaths per year and because these bacteria are becoming increasingly resistant to antibiotic treatments (6), it is imperative to better understand the mechanism behind infection in order to identify new ways of mitigating infection. As *S. Typhimurium* can cause both gastroenteritis and a typhoid-like disease, it is the ideal model strain to study mechanisms behind both gastroenteritis and typhoid fever. In this work, we will solely be studying *S. Typhimurium*, and thus will refer to this serovar as “*Salmonella*.”

*Salmonella* infections begin with oral ingestion of contaminated food or water (1, 4). Once consumed, *Salmonella* migrate through the digestive tract to the small intestine and have the ability to invade nonphagocytic intestinal epithelial cells and microfold (M) cells (Figure 1.1A) (1). This invasion allows for *Salmonella* uptake and colonization of host cells and triggers an immune inflammatory response thought to be the cause of many gastroenteritis symptoms (7, 8). In the case of systemic *Salmonella* infections, *Salmonella* can breach the epithelial layer of the intestine and be engulfed by macrophages where *Salmonella* can persist and travel to
Figure 1.1. Overview of *Salmonella* infection. (A) *Salmonella* infection is caused by consumption of *Salmonella*. Once ingested, *Salmonella* travel to the small intestine where they can infect two types of host cells: the epithelial cells that line the small intestine and macrophages found beneath the epithelial layer. (B) *Salmonella* use two Type III secretion systems to infect host cells. For the invasion of epithelial cells, *Salmonella* use T3SS1 to secrete effector proteins that cause membrane ruffling and uptake into the host cell. For invasion of macrophages, *Salmonella* are phagocytosed. Upon uptake into the host cell, *Salmonella* reside within a membrane bound vacuole called the *Salmonella* containing vacuole or SCV, which is trafficked to the perinuclear region of the host cell. Once it reaches its destination, T3SS2 secretes a second set of effector proteins that allow for the continuation of *Salmonella* infection.
new sites of infection for colonization, such as the spleen or the liver (1, 9). In order to understand how *Salmonella* can invade and colonize epithelial cells as well as persist in macrophages, one must examine the mechanism *Salmonella* uses for these processes.

To invade and colonize host cells, *Salmonella* use a series of large protein complexes called type III secretion systems (T3SSs) to secrete bacterial effector proteins into the host cells (10, 11). These effector proteins work in concert to allow for successful *Salmonella* uptake and colonization of host cells (12-14). For epithelial cell invasions, *Salmonella* use the first T3SS (T3SS1) to translocate effector proteins (called SPI1 effector proteins for their ability to be secreted through T3SS1) across the epithelial cell membrane, where they induce actin cytoskeleton reorganization and cause *Salmonella* uptake into the host cell (Figure 1.1B) (5, 14, 15). For macrophage infections, *Salmonella* are phagocytosed by the host cells and T3SS1 is not required for invasion although its presence has been linked to higher *Salmonella* survival rates and persistence (10, 16) (Figure 1.1B). Once inside either epithelial or macrophage host cells, *Salmonella* reside in a membrane bound vacuole and use a second T3SS (T3SS2) to translocate a new set of effector proteins (called SPI2 effector proteins for their ability to be secreted through T3SS2) through the vacuolar membrane into the cytosol, allowing for maturation of the vacuole and the continuation of *Salmonella* infection (5, 10, 14, 15, 17). As proper establishment and maintenance of the *Salmonella* containing vacuole (SCV) is key to successful *Salmonella* replication or persistence (18), many effector proteins are hypothesized to play a role in the formation and maturation of the SCV. However, most of the mechanisms used by effector proteins to promote SCV maturation are not clearly understood. By determining the roles of these proteins in creating a niche for *Salmonella* infection, we can better understand the mechanism behind *Salmonella* virulence.

**1.2 SPI1 EFFECCTOR PROTEINS ARE INVOLVED IN VACUOLE ESTABLISHMENT**

Because the epithelial cells that line the small intestine are non-phagocytic, *Salmonella* must use SPI1 effector proteins to mediate invasion (*Table 1.1, Figure 1.2*). Upon entry in the
Table 1.1 - SPI1 effector proteins involved in vacuole establishment (modified from refs (5, 14, 15, 19))

<table>
<thead>
<tr>
<th>Effector Protein</th>
<th>Molecular Activity</th>
<th>Function in Vacuole Establishment</th>
<th>Host Cell Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>SipA (SspA)</td>
<td>Stabilizes actin polymerization</td>
<td>Causes membrane ruffling during invasion of epithelial cells and proper positioning of the vacuole*</td>
<td>Caspase 3, F-actin, T plastin</td>
</tr>
<tr>
<td>SipC (SspC)</td>
<td>Actin bundling</td>
<td>Causes membrane ruffling during invasion of epithelial cells and aids in vacuole maturation</td>
<td>F-actin, syntaxin6</td>
</tr>
<tr>
<td>SopA</td>
<td>E3 ubiquitin ligase</td>
<td>Allows for <em>Salmonella</em> to escape from the vacuole*</td>
<td>Caspase 3, HsRMA1, UbcH7</td>
</tr>
<tr>
<td>SopB (SigD)</td>
<td>Phosphoinositide phosphatase, Guanine nucleotide-dissociation inhibitor: inhibits activation of GTPases</td>
<td>Allows for <em>Salmonella</em> invasion of epithelial cells and proper vacuole formation and localization*</td>
<td>Inositol phosphates, Cdc24, Cdc42</td>
</tr>
<tr>
<td>SopE</td>
<td>Guanine-nucleotide exchange factor: causes activation of GTPases.</td>
<td>Causes membrane ruffling during invasion of epithelial cells and recruits early endosome fusion with the vacuole*</td>
<td>Cdc42, Rab5, Rac1</td>
</tr>
<tr>
<td>SopE2</td>
<td>Guanine-nucleotide exchange factor: causes activation of GTPases.</td>
<td>Causes membrane ruffling during invasion of epithelial cells*</td>
<td>Cdc42, Rac1</td>
</tr>
<tr>
<td>SptP</td>
<td>GTPase-activating protein: causes inactivation of GTPases, tyrosine phosphatase</td>
<td>Reduces membrane ruffling after invasion of epithelial cells and mediates membrane integrity of the vacuole*</td>
<td>Cdc42, Rac1, VCP, vimentin</td>
</tr>
</tbody>
</table>

* indicates that these effector proteins have other functions in infection not related to vacuole establishment (often in modulating host immune response).
Figure 1.2. Overview of the roles of SPI1 effector proteins in *Salmonella* invasion of epithelial cells. SPI1 effector proteins work cooperatively to allow for uptake into the host cell (via reorganization of the actin cytoskeleton and induction of macropinocytosis) as well as the establishment of the early and intermediate stages of SCV maturation. Host cell proteins associated with both early and intermediate stages are listed in *italics*. Host cell proteins that are known to be recruited by specific effector proteins are listed in italics next to their respective effector proteins. Abbreviations: EEA-1: early endosomal antigen 1, SNX-1: sorting nexin-1, VCP: AAA+ ATPase VCP (p97), LAMP1: lysosomal-associated membrane protein-1.
gut lumen mucosa, *Salmonella* induce the expression of T3SS1 and the SPI1 effector proteins so all SPI1 effector proteins can be translocated across the host cell membrane upon contact (20, 21). Factors that cause the induction of the T3SS1 system include the low oxygen concentration, high osmolarity, and near neutral pH of the small intestine (20, 22). Once inside the host cell, SPI1 effector proteins SipA, SipC, SopE and SopE2 are required for reorganizing the actin cytoskeleton and promoting membrane ruffling around the extracellular bacterium (23). Whereas SipA and SipC actively promote actin polymerization, SopE and SopE2 are responsible for activating Rho GTPases that can drive actin assembly (13, 24-27). Formation of the vacuole around the bacteria is mediated by SopB, which is an inositol phosphatase that contributes to cytoskeleton reorganization and manipulation of lipid dynamics on the cellular membrane (19, 28, 29). Once the bacteria are successfully within the cell, the host cell cytoskeleton is returned to its resting state through SptP, which acts as a GTPase-activating protein (GAP) for activated Rho GTPases by stimulating the conversion of GTP into GDP and therefore deactivating the GTPases (30).

Inside the host cell, the nascent *Salmonella* containing vacuole matures and is trafficked toward the nucleus. This process is mediated by SPI1 effector proteins that recruit and manipulate host cell markers associated with the endo-lyososomal pathway (5, 31). The specific roles of these effector proteins are described in more detail below. SopE and SopB recruit many host cell factors, such as Rab5, sorting nexin-1, and early endosomal antigen 1 to the SCV (31). Within 40 minutes of this recruitment, these early endosomal markers are replaced with late endosomal markers such as v-ATPases, LAMP1, and Rab7 (5). SipA is thought to be responsible for recruiting F-actin to the final resting site of the SCV and controlling proper tethering of the SCV to actin (32). SptP recruits and dephosphorylates an AAA+ ATPase vasolin-containing protein (VCP, also known as p97 or Cdc48) (33, 34). This process is thought to allow VCP to increase membrane fusion events, thus promoting membrane integrity of the SCV (34). Finally, the effector protein SopA is thought to play a role in disrupting vacuolar
integrity and allowing for *Salmonella* to escape into the cytosol. Recently, it has been established that a population of cells have *Salmonella* that escape the SCV and hyperreplicate in the cytosol (35-37). Although T3SS1 and SPI1 effector proteins have been implicated in this phenotype, the role of SopA has yet to be investigated (37). The SPI1 effector proteins are not required for invasion of macrophages but it has been shown that the phagocytosis of *Salmonella* by macrophages induces the expression of SPI1 effector proteins, thus explaining the mechanism behind proper vacuole formation in the macrophage infection model (38). T3SS1 also secretes a number of other effector proteins, which are involved in roles other than vacuole establishment during *Salmonella* infection – such as mediating the host immune response to infection.

### 1.3 SPI2 EFFECTOR PROTEINS ARE INVOLVED IN VACUOLE ESTABLISHMENT AND MAINTENANCE

Inside host cells, SPI2 effector proteins play a role in further controlling vacuole development ([Table 1.2, Figure 1.3](#)). The induction of T3SS2 and SPI2 protein expression is caused by a Mg$^{2+}$ shortage within the SCV, though the host cell transporter responsible for the Mg$^{2+}$ shortage is still currently unknown (10, 39-41). In addition, the recruitment of v-ATPases to the vacuole causes the intravacuolar pH to drop significantly (pH < 4.5) (5, 38, 42). This pH drop induces the assembly of T3SS2 which, upon sensing the neutral environment of the cytosol, allows for the translocation of SPI2 effector proteins through T3SS2 (10). This process is in part due to the involvement of the SPI2 effector protein SpiC, which is also important for preventing SCV fusion with the lysosome (43-46). Many SPI2 effector and host cell proteins have been found to localize to the SCV and membrane tubules, which emanate from the SCV towards the periphery of the host cell. The precise role of these tubules during *Salmonella* infection is still being investigated, though they are hypothesized to play a role in membrane or nutrient gathering, SCV stabilization, or allowing *Salmonella* to spread to other cells (47, 48). Mutant *Salmonella* strains that lack effector proteins known to localize to the tubules are less
<table>
<thead>
<tr>
<th>Effector Protein</th>
<th>Molecular Activity</th>
<th>Function in Vacuole Establishment/Maintenance</th>
<th>Host Cell Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>SseF</td>
<td>unknown</td>
<td>promotes tubule formation and proper vacuole positioning, forms microtubule bundles at the vacuole, redirects late endosomal and exocytic traffic to the vacuole, redirects dynein to the vacuole</td>
<td>TIP60</td>
</tr>
<tr>
<td>SseG</td>
<td>unknown</td>
<td>promotes tubule formation and proper vacuole positioning, forms microtubule bundles at the vacuole, redirects late endosomal and exocytic traffic to the vacuole, redirects dynein to the vacuole</td>
<td>unknown</td>
</tr>
<tr>
<td>SopD2</td>
<td>unknown</td>
<td>mediates tubule formation*</td>
<td>unknown</td>
</tr>
<tr>
<td>SifA</td>
<td>Guanine-nucleotide exchange factor: causes activation of GTPases.</td>
<td>Required for tubule formation and vacuolar membrane integrity, contributes to vacuolar maintenance</td>
<td>Rab7, Rab9, RhoA, SKIP</td>
</tr>
<tr>
<td>SifB</td>
<td>Guanine-nucleotide exchange factor: causes activation of GTPases.</td>
<td>Localizes to the vacuole and tubules</td>
<td>unknown</td>
</tr>
<tr>
<td>SpiC (SsaB)</td>
<td>unknown</td>
<td>Regulates secretion of SPI2 effector proteins. Does not allow the vacuole to fuse with lysosomes</td>
<td>Hook3, TassC</td>
</tr>
<tr>
<td>SseJ</td>
<td>acyltransferase, deacylase, phospholipase A1</td>
<td>Regulates vacuolar integrity and tubules</td>
<td>Cholesterol, RhoA, RhoC</td>
</tr>
<tr>
<td>SpvB</td>
<td>Actin ribosyltransferase</td>
<td>Inhibits vacuole associated actin polymerization and tubule formation</td>
<td>Actin</td>
</tr>
</tbody>
</table>
Table 1.2. (continued)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Localisation</th>
<th>Other Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SteC</td>
<td>Serine/threonine kinase</td>
<td>Required for the vacuole associated actin polymerization</td>
<td>MEK</td>
</tr>
<tr>
<td>PipB</td>
<td>unknown</td>
<td>Localizes to the vacuole and tubules</td>
<td>unknown</td>
</tr>
<tr>
<td>SspH2</td>
<td>E3 ubiquitin ligase</td>
<td>Localizes to vacuole associated actin polymerization</td>
<td>14-3-3γ, AIP, BAG2, Bub3, Filamin A, Profilin-1, Sgt1, UbcH5-Ub</td>
</tr>
</tbody>
</table>

* indicates that these effector proteins have other functions in infection not related to vacuole establishment (often in modulating host immune response).
Figure 1.3. Overview of the roles of SPI2 effector proteins in maintaining the SCV during infection of host cells. SPI2 effector proteins work in concert to allow for SCV membrane integrity, the formation of membrane tubules that emanate from the SCV, and proper SCV tethering within the host cell during the late stage of SCV maturation. Host cell proteins associated with this stage are listed in *italics*. Host cell proteins known to interact with specific effector proteins are listed in italics next to their respective effector proteins. Abbreviations: MTOC: microtubule-organizing center, LAMP1: lysosomal-associated membrane protein-1.
virulent in mouse infection models (47). Therefore it has been suggested that these tubules must play an important role in supporting successful *Salmonella* infection. The roles of some SPI2 effector proteins in SCV maintenance are well characterized (see below). In contrast, other proteins, such as PipB and SifB are known to localize to the SCV and tubules but have no known functions (19). For the effector proteins with well-defined roles in SCV maintenance, their functions can be divided up into two categories, SCV membrane integrity and cytoskeleton manipulation.

Numerous SPI2 effector proteins have been found to promote recruitment of host cell factors to the SCV, leading to manipulation of SCV membrane integrity. SseG and SseF have been shown to recruit a glycoprotein from the vesicular stomatitis virus, VSVG, suggesting that they enable diversion of exocytic traffic to the SCV, as well as lysosomal-associated membrane proteins-1, 2, and 3 (LAMP1, 2, and 3) to tubules, indicating they also recruit endosomal traffic (50, 51). In addition, SseG and SseF recruit the molecular motor dynein to the vacuole (51). Another *Salmonella* effector protein SifA also regulates membrane integrity of the SCV and tubules (52-54) and recruits the host cell protein SKIP (55, 56). SKIP activates kinesin-1, which is recruited to the SCV by other means (see PipB2, below) (55, 56). Consistent with this role in membrane integrity, a deletion mutant of SifA causes *Salmonella* to escape from the SCV and hyperreplicate within the cytosol (57-59). SseJ acts with SifA in regulating SCV and tubule membrane composition (56, 58, 60, 61) and recruits active GTPase RhoA to the SCV (62). GTP-bound RhoA increases the ability of SseJ to control the levels of cholesterol associated with the SCV through esterification of cholesterol (56, 62-65). Cholesterol recruitment to the SCV has been suggested to be important for the recruitment of glycoproteins, which may be important in allowing for nutrient uptake and therefore successful *Salmonella* replication (66). Finally, SopD2 is thought to be an agonist of vacuole integrity by disrupting vesicle transport from the SCV, tubule formation, and membrane integrity of the SCV (67, 68). SpvB may also be involved in the negative regulation of tubule formation (60). Other host cell proteins (such as
Rab11, SCAMP3, and Arl8b) localize to the SCV, although the mechanism behind their recruitment is currently unknown (48, 54, 69, 70).

SPI2 effector proteins are also responsible for causing a reorganization of cytoskeleton components around the SCV as well as tethering the SCV to its perinuclear location. In addition to their role in modifying SCV membrane composition, SseG and SseF play a role in restricting the SCV to a perinuclear location near the Golgi (71) and have also been shown to cause microtubule bundling near the SCV (59). The SseF/SseG control of SCV localization to the Golgi is thought to either occur by the manipulation of molecular motors such as dynein, by interacting with Golgi proteins to tether the SCV to the Golgi, or by some combination of both methods (51, 72). SPI2 effector proteins also manipulate actin around the SCV. Although the SPI1 effector protein SipA targets F-actin to the SCV, the SPI2 effector protein SteC is also involved in actin manipulation during infection. SteC phosphorylates the MAP kinase MEK, which allows for the reorganization of vacuole associated F-actin or “actin nests” that surround the SCV (49, 73). It is hypothesized that SteC may be involved in regulating *Salmonella* growth by constraining the actin nests around the SCV (49). SpvB may also be involved in actin reorganization over the course of infection as it is hypothesized to negatively regulate the formation of actin nests (74). SspH2 is also known to localize to these actin nests during infection and can bind actin (74). In addition, SspH2 acts as an E3 ubiquitin ligase and is hypothesized to be responsible for tagging host cell proteins for proteasomal degradation (75, 76). As above, many of these and other SPI2 effector proteins also play roles in modulating the host’s inflammatory immune response.

**1.4 EFFECTOR PROTEINS SECRETED THROUGH BOTH T3SS1 AND T3SS2 ALSO INFLUENCE VACUOLE MATURATION**

Of the ~40 known *Salmonella* effector proteins, nine are hypothesized to be secreted by both T3SSs (Table 1.3). Of these nine proteins, four are known to play a role in vacuole maturation or have been found to localize to the vacuole (19). SopD is hypothesized to work
Table 1.3 – SPI1/2 effector proteins involved in vacuole establishment/maintenance (modified from refs (5, 14, 15, 19))

<table>
<thead>
<tr>
<th>Effector Protein</th>
<th>Molecular Activity</th>
<th>Function in Vacuole Establishment/Maintenance</th>
<th>Host Cell Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>GtgE (Not expressed in Typhi or Paratyphi)</td>
<td>protease</td>
<td>Prevents recruitment of Rab29 to the vacuole</td>
<td>Rab29</td>
</tr>
<tr>
<td>PipB2</td>
<td>unknown</td>
<td>Promotes tubulation and vacuole dynamics</td>
<td>Kinesin-1</td>
</tr>
<tr>
<td>SopD</td>
<td>unknown</td>
<td>Vacuole formation</td>
<td>unknown</td>
</tr>
<tr>
<td>SteA</td>
<td>unknown</td>
<td>Localizes to the vacuole and tubules</td>
<td>unknown</td>
</tr>
</tbody>
</table>

* indicates that these effector proteins have other functions in infection not related to vacuole establishment (often in modulating host immune response).
with SopB in manipulating lipid dynamics and allowing for successful vacuole formation (23, 77).

The newly discovered effector protein GtgE is only expressed in S. Typhimurium (not S. Typhi) and prevents the recruitment of Rab29 to the S. Typhimurium SCV (78). Although there is no known biological role for Rab29, its recruitment to the S. Typhi SCV is associated with decreased replication (78). Therefore, GtgE is important for allowing successful replication in S. Typhimurium infections. PipB2 is largely established as a SPI2 effector protein that promotes tubule formation and recruits kinesin-1 to the SCV (79-81). However, it has recently been suggested that PipB2 may also be secreted by the T3SS1 (82), though its role early in infection has not been established. Finally, the effector protein SteA is known to localize to the SCV and tubules but its function in Salmonella infection has not yet been established, though it has been hypothesized to manipulate SCV membrane dynamics (83, 84). As these proteins have the potential to be secreted both early and late during infection, they may play an especially important role (or series of roles) in SCV maturation over the course of infection. However, many current established methods for studying the cellular roles of these effector proteins lack the sensitivity needed to address direct questions regarding the mechanisms used by effector proteins to modify the SCV over the course of infection.

1.5 CURRENT METHODS USED TO STUDY THE ROLE OF EFFECTOR PROTEINS DURING INFECTION

There are many methods to determine how effector proteins mediate infection, but in general, studies aim to establish the biochemical function of individual effector proteins, their role at the cellular level, and how effector proteins influence acute and chronic infection in animal models. A common method approach for gaining insight into the function of a new protein is to compare its sequence to the sequence of proteins with known functions, using algorithms such as BLAST, in the hopes that homology might signify a similar function. However, many effector proteins share little to no amino acid sequence similarity to host cell proteins (85, 86) so these methods are often not useful in determining potential functions for
effector proteins. Structural techniques such as X-ray crystallography or NMR can provide insight into effector protein functions, as effector protein structures often mimic structures of well characterized host cell proteins (56, 87). However, structural studies can be time consuming and challenging as proteins must be stable and amenable to purification. Thus other methods needed to be developed to probe effector protein function during infection.

When an effector protein is hypothesized to play a role in establishing Salmonella infection but has no known biochemical function, a common approach is to infect cells or model organisms with strains of Salmonella lacking the effector protein and compare the result to infection with wild type (wt) bacteria. Such studies seek to define and determine changes to a phenotype (such as invasiveness or replication) or features of infection (including perturbation of cellular organelles, location of the SCV or bacteria, inflammatory response, spread of infection from the small intestine, etc.) in order to gain insight into an effector protein’s function during infection.

Because one of the hallmarks of Salmonella infection of mice is a persistent infection that breaches the small intestine, one of the main methods to examine the role of an effector protein during infection is to determine whether or not it is important for causing “virulence” or spread to other organs. To examine the ability of wt versus a deletion strain to spread through a mouse model of infection, a competitive index (CI) assay is typically used. In a CI assay, wt and deletion strains are pitted against each other in the infection of live mice (i.e. mice are infected with both the wt and deletion strain simultaneously) (88). Infected mice are sacrificed at 2-4 days post infection and organs often affected by Salmonella spread, such as the liver and spleen, are examined for Salmonella content. The organ lysate is plated on agar plates containing appropriate antibiotics specifically for the wt or deletion strains. After bacterial growth, the number of wt or deletion colonies on their respective plates is indicative of which strain fared better within the mouse (some examples of studies that use CI assays: (44, 49, 89-92)). This assay is instrumental in providing a starting point for understanding the role of an
effector protein during infection. For example, a CI assay was used to determine that SifA is imperative for successful *Salmonella* infection (52).

Once it is established that an effector protein is important for *Salmonella* virulence, it is important to then determine what role it plays within the context of infection. Some possible roles that would be important for virulence include promoting invasion or replication, as both of these mechanisms allow for the increase of bacterial load within cells. In order to study changes in *Salmonella* invasion or replication, the most commonly used assay is a colony forming unit (CFU) assay. CFU assays are batch assays in which cells are infected with the wt or deletion strain and then lysed at certain time points post infection (1 to 2 hours for invasion, 6 to 22 hours for replication (examples of studies that use CFUs for to measure *Salmonella* invasion: (81, 93-95) or replication: (58, 71, 79, 91, 96)). The cell lysate is plated on agar plates with appropriate antibiotics for each strain and incubated for bacterial growth. The number of colonies on each plate is proportional to the bacterial load at a particular time point and is indicative of each strain’s invasion or replication ability (97, 98). However, because the CFU assay fails to show invasion or replication on the single cell level, other methods are also used to examine invasiveness or replication. For example, a differential “inside/outside” staining method can be used to determine invasive ability at the single cell level (some examples from the literature: (27, 99-101)). For this assay, cells are fixed at discrete time points post infection (often 15 minutes to 1 hour). Then extracellular bacteria are stained before the host cell is permeabilized and all bacteria are stained with a different color probe. Thus, upon visualization of cells, extracellular bacteria are clearly differentiated from intracellular bacteria and the invasiveness of wt versus mutant strains can be scored.

Assays involving fixed and stained infections at discrete time points post infection are also used to determine other roles for effector proteins within the context of infection. Because these assays allow for visualization of the SCV or *Salmonella* localization, host cell protein recruitment to the SCV, or cell-to-cell spread, they provide quantifiable information about
effector protein functions during infection (see below for specific examples of these assays in the literature). In these studies, host cells or animals are infected with either wt or mutant *Salmonella*. At discrete time points post infection cells are killed or animals are sacrificed and cells/tissues are fixed and prepared for visualization via electron microscopy or immunofluorescence microscopy. These methods have been essential for defining the involvement of specific effector proteins in established *Salmonella* infection phenotypes such as the roles of SopB in the recruitment of sorting nexin-1 to the SCV (102), SifA, SseJ, SseG, and SseF in tubule formation (52, 59, 60), the role of SptP in allowing *Salmonella* spread to other organs within the mouse (103), the role of SPI1 (but not SPI2) in promoting escape from the SCV (35), and the roles of SifA, SseJ, and SopD2 in SCV membrane integrity (56, 67). However, these assays often yield a variety of phenotypes and researchers are faced with the challenge of choosing the “correct” phenotype to quantify (i.e. counting only infected cells with LAMP1 localized to tubules or with dispersed bacteria) while ignoring the other phenotypes.

It is becoming increasingly recognized that *Salmonella* infections within a single population of cells are quite heterogeneous. In some epithelial cells, *Salmonella* can escape from the SCV and hyperreplicate within the cytosol while in other cells, *Salmonella* continue to replicate inside SCVs (35-37). In addition, it has been discovered that within a single population of infected macrophages, some macrophages can kill vacuolar *Salmonella* while others allow for persistence (16, 104, 105). The existing analytical methods discussed above (CI, CFU, and imaging of fixed and stained infections) are poorly equipped to monitor the roles of effector proteins in causing infection heterogeneity. First, CI and CFU assays are batch assays that do not have the ability to distinguish between different phenotypes within a population of cells. Second, while imaging of fixed and stained infections allows for the visualization of heterogeneity within a population, these assays provide no temporal information about potential causes of diverse phenotypes. Thus, in order to unravel the combination of events that lead to heterogeneity in infection (such as replication in the cytosol versus in the SCV or persistence
versus death), a new set of assays must be developed to further probe the complex nature of
Salmonella infection.

In light of the recently acknowledged heterogeneity in Salmonella infection, many
researchers have developed new assays to allow for the detection of effector proteins involved
in heterogeneous phenotypes with the goal of determining factors that cause Salmonella to
“choose” between hyperreplication versus SCV replication or Salmonella survival versus death.
For example, to further probe for effector proteins that allow for Salmonella to escape the SCV,
Knodler and coworkers designed an assay that selectively killed SCV bacteria but not cytosolic
bacteria (37). In this way, they could perform CFU assays to examine how deletion mutants
specifically affected the fraction of the Salmonella population that escaped from the SCV.
Helaine et al. developed a FACS-based single cell assay to identify effector proteins important
for Salmonella persistence in macrophages. This assay employed a Salmonella strain that
constitutively expressed one fluorescent protein but, upon the uptake of arabinose, could induce
the expression of a second different colored fluorescent protein (104). To determine the
metabolic state of a population of persistent Salmonella within infected macrophages,
Salmonella were harvested from macrophages, grown in LB (to reduce the arabinose driven
expression of a fluorescent protein in metabolically active bacteria) and sorted using flow
cytometry (105). Thus, using this method, Helaine et al. could examine the effects of effector
protein deletion mutants on the ability to influence the persistence phenotype. Both the assays
presented by Knodler et al. and Helaine et al. permit more direct investigation of effector
proteins’ roles in causing Salmonella heterogeneity. However, as these methods are performed
at discrete time points post infection, they lack the ability to examine the evolution of
phenotypes over the course of infection.

Long term live cell imaging experiments allow researchers to monitor the dynamics of
infection in single within cells, in order to preserve the full spatial and temporal complexity of an
infected cell. The ability to monitor changes in dynamics over time is analogous to trying to
learn the rules of football while watching one football game from start to finish because it provides the viewer with a basic understanding of the rules of football (106). Using the same analogy, studying phenotypes solely at distinct time points post infection is akin to trying to learn the rules of football by studying pictures from different football games. Without any information about the timing of events in the pictures, a viewer might find it hard to determine an overall timeline of the game, let alone the rules. Just as watching one football game allows for a more in depth understanding of football, studying phenotype evolution over time in single cells can allow for a more accurate depiction of events that cause different phenotypes during infection and can portray a better picture of specific roles of *Salmonella* effector proteins in eliciting these phenotypes.

### 1.6 DEVELOPMENT OF LONG-TERM IMAGING METHODS TO DETERMINE THE ROLE OF STEA IN SCV MATURATION

To overcome the limitations of existing assays and preserve the spatial and temporal information inherent in a dynamic system, in this thesis work I sought to develop new assays to track important infection phenotypes over long periods of time (> 15 hours) in living cells by fluorescence microscopy. My goal was to then explore the role of specific effector proteins in defining or perturbing these infection phenotypes. Specifically, I set out to explore the role of the effector protein SteA during *Salmonella* infection. SteA deletion causes a decrease in *Salmonella* virulence and persistence in the mouse models of gastroenteritis and typhoid fever, respectively (16, 90). These results suggest that SteA is important during *Salmonella* infection. Furthermore, as SteA is secreted by both T3SS1 and T3SS2 and is known to localize to the vacuole and tubules during infection (83), it may be important in establishing and/or maintaining the SCV during infection. However, the precise cellular role of SteA has not been established. Recently, SteA has been hypothesized to be involved in manipulating membrane composition on the SCV (84). In order to investigate this hypothesis, I designed new long-term imaging methods to directly probe the role of SteA in modifying SCV integrity and in recruiting factors to
the SCV. While designing these assays, I found that each assay needed a corresponding quantitative analysis method that allowed for automated, objective determination of phenotype evolution over the course of infection. These image processing methods are described in each of the Chapters described below.

In Chapter Two, we sought to develop methods that allowed us to monitor the role of effector proteins in modulating SCV integrity specifically within the context of *Salmonella* replication in epithelial cell infection or *Salmonella* survival in macrophage infection. This method builds on a technique previously described by Malik-Kale et al. (36). Briefly, by labeling the SCV with a green fluorescent dye and *Salmonella* with a complementary red fluorescent protein, we were able to track vacuolar *Salmonella* survival and replication over time. We used this technique to develop new automated quantitative image analysis methods using the program ICY (107), and to determine the roles of effector proteins SteA and SseG during *Salmonella* replication and survival.

In Chapter Three, we set out to create an assay that would allow us to determine the role of an effector protein in recruitment of host cell factors to the SCV. We developed a long term imaging assay that quantitatively determines the effects of a deletion mutant of *Salmonella* on host cell and effector protein localization to the SCV over the course of infection. This method involves using a three-color assay to track bacteria, host cell proteins, and effector proteins simultaneously during the experiment. In addition we developed new quantitative analysis methods using the image analysis programs CellProfiler (108, 109) and ICY (107) to allow for objective measurements of changes in localization upon effector protein knockout. Specifically, we developed this new assay to study the effector protein SteA and its role in SCV maturation.

Overall, the long term imaging methods combined with quantitative analysis methods discussed in this work allowed for new insight into the role of SteA in SCV maturation as well as its potential interplay with SseG. In addition to providing new information about SteA in the
context of *Salmonella* infection, these methods proved to be more sensitive over traditional methods used in the field and promise to allow for direct quantification of future effector protein involvement with the SCV maturation process.
Chapter 2: Live cell imaging of *Salmonella* reveals new roles for SteA and SseG in infection of epithelial and macrophage cells

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2.1 ABSTRACT

*Salmonella* Typhimurium is a bacterial pathogen that causes gastroenteritis in humans and a systemic typhoid fever-like disease in mice. Gastroenteritis is caused by infection of and subsequent replication inside epithelial cells that line the small intestine, while typhoid fever is caused by a persistent infection within resident macrophages. Once inside host cells, *Salmonella* often reside within a membrane-bound compartment (called the *Salmonella* containing vacuole or SCV). The SCV allows for replication and/or persistence within macrophages, but in epithelial cells the bacteria escape the SCV and replicate to high levels in the cytoplasm. SCV integrity is controlled by *Salmonella* effector proteins, which are secreted into the host cell and manipulate host cell components. Here we use a long-term live cell imaging assay to follow vacuolar replication or survival in single epithelial cells or bone marrow derived macrophages, respectively, and to examine the roles of the effector proteins SteA and SseG in modulating SCV integrity during these infection processes. \(\Delta\text{steA}\), \(\Delta\text{sseG}\), and \(\Delta\text{steA}/\Delta\text{sseG}\) *Salmonella* strains replicated significantly less than wild type strains within epithelial cells but the effects of \(\Delta\text{steA}\) on replication were more subtle. Furthermore, the integrity of the vacuolar membrane was decreased upon infection with \(\Delta\text{sseG}\) and \(\Delta\text{steA}/\Delta\text{sseG}\), but not with \(\Delta\text{steA}\), mutant strains, suggesting that SseG, but not SteA, plays a key role in stabilizing the SCV membrane. Finally, in bone marrow derived macrophages, we found that SteA is needed to promote *Salmonella* survival in the SCV. Our results show that SseG promotes vacuolar membrane integrity during infection and that SteA is more important for supporting SCV integrity within macrophages than in epithelial cells.
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2.2 INTRODUCTION

Salmonella enterica subsp. enterica are intracellular bacterial pathogens capable of infecting a wide range of host organisms. There are over 2500 serovars, with serovars Typhimurium and Typhi contributing to > 98 million of cases of human disease each year (3, 4). S. Typhi is responsible for causing a systemic typhoid fever disease whereas S. Typhimurium causes gastroenteritis upon infection of the epithelial cells that line the small intestine (1). In mice, S. Typhimurium can breach the epithelial cell layer and infect resident macrophages, causing a systemic typhoid-like disease (1, 9, 110, 111). To establish infection, S. Typhimurium uses two type III secretion systems (T3SSs) to translocate bacterial effector proteins into the host cell (11, 112). In the gastroenteritis model of infection, the first secretion system (T3SS1) delivers effector proteins into the host cell to cause bacterial uptake by the host cell, whereas in the systemic model of infection, bacteria are phagocytosed by resident macrophages (10, 14, 113). Once inside either cell type, S. Typhimurium uses another secretion system (T3SS2) to secrete a second set of effector proteins that work in concert to establish a replicative niche within the cell (12, 13, 26, 113). One aspect of successful Salmonella colonization is the formation of a membrane-bound vacuole, in which the bacteria reside (18). T3SS2 effector proteins mediate the establishment and maintenance of this vacuole by manipulating the host cell cytoskeleton, recruiting and activating Rab GTPases, regulating membrane lipids, and altering membrane trafficking pathways (5, 18, 70, 114-116). An important step in defining the
host-pathogen interface is elucidating how effector proteins contribute to the development of this niche that permits survival of *Salmonella* within the host.

A common approach for defining whether an effector protein plays an important role in establishing and maintaining the intracellular niche is to examine whether a mutant strain in which the effector protein has been deleted is defective for intracellular survival and replication. This experiment is widely accomplished by quantifying the net bacterial load at discrete time points post infection through enumeration of colony forming units (CFUs) upon plating (97, 98). However, it is becoming increasingly evident that there can be substantial heterogeneity in infection, survival, and replication in pathogen populations, and this heterogeneity is masked by bulk assays like the CFU assay. For example, *Salmonella* has been traditionally described as a vacuolar pathogen, but there are now numerous reports that it can escape from the vacuole and replicate in the cytosol of epithelial cells (18, 36, 37). This phenomenon may play an important role in extrusion of cells from the gut epithelium and transmission in gastroenteritis (35). Moreover, single cell studies have revealed that cytosolic replication constitutes a significant fraction of the total bacterial population in epithelial cells (36, 37), and effector proteins that influence vacuole integrity can promote cytosolic replication (52, 57-59). In the systemic model of infection, where bacteria infect macrophages and must reside within a vacuole to survive (58), bacterial load reflects a combination of resistance to macrophage-induced killing and replication. T3SS2 effector proteins may therefore be important for different functions in macrophages, such as promoting survival or enabling replication. In this study, we sought to distinguish different infection phenotypes by utilizing a live cell imaging assay, similar to that employed by Malik-Kale et al. (36), that we have adapted for automated analysis. By tracking *Salmonella* replication in epithelial cells and survival in bone marrow derived macrophages at the single cell level, we reveal new contributions of effector proteins in establishment of the intracellular niche.
We were particularly interested in investigating T3SS2 effector proteins that mediate maturation of the *Salmonella* containing vacuole (SCV) and whether these effector proteins play distinct roles in epithelial cells versus macrophages. SCV maturation is a complex process involving positioning of the SCV, interception of membrane trafficking pathways, and control of membrane dynamics (5). Numerous effector proteins have been implicated in this process, including SifA, PipB2, SopD2, SseF, SseG, and SseJ (14, 15, 19). More recently it has been suggested that SteA may also contribute to SCV membrane dynamics (84). However, the precise cellular role of SteA is not well characterized and how it contributes to vacuolar replication in epithelial cells or survival within the SCV of macrophages has not been defined. It was recently suggested that in infected epithelial cells SteA may be functionally linked with another effector protein involved in SCV maturation, SseG (84). SseG has been shown to be important for localization of the SCV near the Golgi by controlling molecular motors, as well as for SCV maintenance by recruiting host cell membrane components and causing microtubule bundling at the SCV (50, 51, 59, 71, 72, 117). In this study we reveal that SteA is important for *Salmonella* replication in epithelial cells and survival in macrophages. Additionally, we demonstrate that SseG plays a role in SCV integrity.

### 2.3 MATERIALS AND METHODS

#### *Salmonella* strains.

All mCherry *Salmonella* strains used in these studies were *Salmonella enterica* serovar Typhimurium SL1344 expressing a plasmid (pAmCh plasmid, parent pACYC177) for constitutive expression of mCherry under the PrpsM ribosomal promoter. ΔsteA and ΔsseG mCherry *Salmonella* strains were made as described previously (83, 118, 119). Briefly, this technique uses a 14028S *Salmonella* strain (based off of the LT2 *Salmonella* strain) which harbors a recombination system to facilitate the replacement of the endogenous SteA or SseG genes with a kanamycin resistance gene. This knockout was then transferred to wild type SL1344 strains using P22 phage derived from the resistance free strain SL1290. To make the
ΔsteA/ΔsseG strain, the endogenous SseG and SteA genes were replaced with a kanamycin and chloramphenicol resistance gene, respectively. To make the ΔsseG and wt SteA-GFP11 mTagBFP2 Salmonella strain, the mCherry gene in the pAmCh plasmid was replaced with the mTagBFP2 gene (obtained from Vladislav Verkhusha, Albert Einstein College of Medicine) using the In-Fusion cloning kit (Clontech). The resulting pAmT plasmid was modified further to make mTagBFP2 Salmonella that expressed SteA-GFP11 using the Split GFP system designed in (83). Briefly, the SteA promoter, the SteA gene, a flexible linker (GSSGGSSG), and GFP β-strand 11 (GFP11: RDHMVLHEYVNAAGIT) were cloned between two HindIII restriction sites in the pAmT plasmid in an orientation trans to the mTagBFP2 gene. For the wt SteA-GFP11 mTagBFP2 Salmonella strain, the endogenous SteA gene was replaced with a kanamycin resistance gene. For the ΔsseG SteA-GFP11 mTagBFP2 Salmonella strain, the endogenous SteA and SseG genes were replaced with chloramphenicol and kanamycin resistance genes, respectively. To prepare strains for infection of HeLa cells, Salmonella strains were grown in LB (EMD) supplemented with 300 mM NaCl (Fisher Scientific) and 25 mM MOPS (Sigma) at pH 7.6 and appropriate antibiotics (wt mCherry SL1344 strain: 50 μg/mL ampicillin and 50 μg/mL streptomycin; ΔsteA and ΔsseG mCherry strains or SteA-GFP11 mTagBFP2 strains: 50 μg/mL ampicillin, 50 μg/mL streptomycin, and 50 μg/mL kanamycin; and ΔsteA/ΔsseG mCherry or ΔsseG SteA-GFP11 mTagBFP2 strains: 50 μg/mL ampicillin, 50 μg/mL streptomycin, 50 μg/mL kanamycin, and 10 μg/mL chloramphenicol) at 37°C without shaking. To prepare for infection of primary Bone Marrow Derived Macrophages (BMDMs), bacteria were grown in LB with appropriate antibiotics at 37°C with shaking. Prior to infection of BMDMs, bacteria were opsonized in a 1:1 solution of mouse serum (Sigma) and cell culture media (Gibco) for 20 minutes at room temperature.

Cell Culture and Salmonella infection.

HeLa cells were maintained in DMEM (Gibco) supplemented with 10% FBS (Gibco), 100 Units/mL penicillin G sodium (Gibco), and 100 μg/mL streptomycin sulfate (Gibco) at 37°C with
5% CO₂. For infections, HeLa cells were plated in 35 mm glass-bottom dishes. Primary BMDMs were isolated as previously described (120). Briefly, marrow was flushed from the femurs, tibias, and humeri of 2- to 4-month-old SV129S6 mice (Taconic Laboratories, Hudson, NY). Cells were resuspended in Dulbecco modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with fetal bovine serum (10%), l-glutamine (2 mM), sodium pyruvate (1 mM), beta-mercaptoethanol (50 µM), HEPES (10 mM), and penicillin-streptomycin (50 IU/ml penicillin and 50 µg/ml streptomycin). Cells were overlaid onto an equal volume of Histopaque-1083 (Sigma-Aldrich, St. Louis, MO) and centrifuged at 500 × g for 15 min. Monocytes at the interface were harvested and incubated for 6 to 7 days at 37°C in 5% CO₂ in supplemented DMEM that also contained 30% MCSF (macrophage colony stimulating factor) obtained from NIH/3T3 cells (acquired from Jeffery Cox, University of California, San Francisco) to promote monocyte differentiation into macrophages. For infections, BMDMs were plated in 96 well glass bottom plates coated with poly-L-lysine (Sigma).

For HeLa cells to be infected with wt or ΔsseG SteA-GFP11 mTagBFP2 Salmonella, cells were transiently transfected twenty-four hours after plating with 2.5 µg pCMV-mGFP1-10 using the TransIT LT1 system (MirusBio). The pCMV-mGFP1-10 plasmid, which encodes the GFP β-strands 1-10 fragment with mammalian optimized codons was purchased from Theranostech (83). Twenty-four to forty-eight hours post transfection, cells were infected with Salmonella. Immediately prior to Salmonella infection of either HeLa cells of BMDMs, the medium was exchanged for antibiotic- and phenol red- free DMEM (Gibco). For cells to be infected with mCherry bacteria, AlexaFluor 488 labeled Dextran (10,000 MW, Molecular Probes) was added to HeLa cells or BMDMs to a final concentration of 200 µg/mL in order to label the Salmonella containing vacuole. HeLa cells were infected at a multiplicity of infection (MOI) of 30 while BMDMs were infected at an MOI of 10. To account for potential differences in harvested primary BMDMs, wt and ΔsteA infections for these cells were performed and imaged simultaneously (in different wells of the 96 well plate). Infections were allowed to proceed for 30
minutes at 37°C and 5% CO₂ before the *Salmonella*-containing media was exchanged with phenol red free DMEM containing 100 µg/mL gentamicin to kill any extracellular bacteria. After incubating for 45 minutes at 37°C and 5% CO₂, the media was replaced with phenol red free DMEM containing 10 µg/mL gentamicin, in which cells remained for the entirety of the imaging experiment.

**Colony Forming Unit (CFU) assays.**

HeLa cells were seeded into 12 well cell culture plates maintained in antibiotic free DMEM supplemented with 10% FBS and were held at 37°C with 5% CO₂ throughout the infection process. Infections with *Salmonella* strains were carried out as described above. At 3 and 17 hours post infection, infected cells were rinsed twice with PBS and incubated with 0.1% Triton in PBS at room temperature for 5 min. A series of dilutions in PBS were generated and plated in quadruplicate on appropriate antibiotic containing LB-Agar plates. The colony forming units calculated for each infection were normalized to the wild type strain. Statistical significance was measured using a one-way ANOVA test.

**Imaging of infected cells.**

All infections were performed in duplicate or triplicate and imaged on either a Nikon Ti-E widefield or a Nikon A1R laser scanning confocal microscope. Both microscopes were equipped with the Nikon Elements software platform, Ti-E Perfect Focus system, a motorized XY stage with a Ti Z drive, and an environmental chamber (Pathology Devices) to maintain cells at 37°C, 5% CO₂ and 70% humidity. The motorized XY stage enabled us to select and store the locations of multiple fields of view in order to follow the fates of many infected cells over the course of the experiment. The Z drive was used to create z stacks that encompassed the entirety of the cells within each field of view, thus ensuring the complete detection of any bacteria present. Images for all fields of view and Z planes were collected every 15 minutes from 2 hours to 17 hours post infection. Each field of view was split into an individual file and z stacks were flattened using the Extended Depth of Field algorithm within the Nikon Elements.
software platform. Then each field of view was exported as a series of Tiff files separated into each channel for further analysis in the bioimaging analysis software ICY (107).

Images acquired on the widefield microscope used a 60x oil objective (numerical aperture 1.40), an iXon3 897 EMCCD camera (Andor), and a xenon-arc lamp to image mCherry Salmonella (excitation: 560/40 nm, emission: 630/75 nm, dichroic: 585 nm) and AlexaFluor 488 labeled Dextran (excitation: 472/30 nm, emission: 520/40 nm, dichroic: 490 nm). All confocal imaging experiments used a 40x oil objective (numerical aperture 1.30) on a Nikon A1R laser scanning confocal microscope equipped with the capacity image red (561 nm laser line, gain: 110, emission filter: 600/50 nm), green (488 nm laser line, gain: 90, emission filter: 525/50 nm), and bright field (using a Differential Interference Contrast system) simultaneously. Thus, this system allowed us to track mCherry Salmonella, AlexaFluor 488 labeled Dextran, and cell membranes/cell health concurrently over the course of the experiment. All fields of view were imaged with a pixel dwell time of 2 µs. To maximize the XY resolution of BMDM infections, an additional optical zoom of 1.7x was used.

For mTagBFP2 Salmonella experiments, we imaged Split GFP-labeled SteA (488 nm laser line, gain: 110, emission filter: 525/50 nm) every 15 minutes and mTagBFP2-labeled Salmonella (405 nm laser line, gain: 110, emission filter: 450/50 nm) every hour with an additional optical zoom of 1.2x. Infections were imaged from 2-17 hours post infection.

ICY Analysis of Salmonella replication in HeLa cells.

To streamline image analysis, a semi-automated protocol for quantifying the change in fluorescence pixel area was developed for ICY. First, ImageJ (NIH) was used to generate a separate Tiff file for each infected cell containing bacteria colocalized with Dextran (indicating bacteria within a vacuole). The mCherry bacterial channel was imported into ICY and the Thresholder plugin was used to remove noise. Pixels corresponding to fluorescent bacteria were detected automatically using the Connected Components plugin, and a script was written to count the number of pixels for each time point. Data were exported to Excel and used to
generate a bacterial growth curve for each infected cell, where the total pixel area was used to represent the amount of bacteria. In order to analyze bacterial replication, individual growth curves were generated for all infections imaged by confocal microscopy. For fitting growth curves, we limited analysis to those infections with a starting pixel area less than 50, because we observed a correlation between initial pixel area which was proportional to the initial bacteria count, and the growth rate (see Supplementary Information for details). A MatLab script was developed to automatically extract pixels in the bacterial channel for each infected cell at each time point, fit these curves to an exponential function \(Y = Ae^{mt}\); where \(Y\) is the number of pixels, \(A\) is a constant, \(m\) is the growth rate, and \(t\) is the time in hours (121), and only accept fits with \(R^2 > 0.7\). For the wt growth curves, 98% met the fit criterion, suggesting that the majority of growth curves for the wt strain fell within the logarithmic phase of bacterial growth. For mutant strains, 94% of \(\Delta steA\) growth curves, 83% of \(\Delta sseG\) growth curves, and 35% of \(\Delta steA/\Delta sseG\) growth curves fit this criterion. For \(\Delta sseG\) and \(\Delta steA/\Delta sseG\) fewer curves met the \(R^2 > 0.7\) fit criterion because these mutants had increased numbers of growth curves with pronounced lag phases, stationary phases, or no growth. For those curves that were characterized by logarithmic growth, the growth rate \((m)\) was extracted from the exponential fit. Statistical significance was measured using a one-way ANOVA test.

**Analysis of Salmonella survival in BMDMs.**

For each field of view, fluorescence channels corresponding to mCherry bacteria, green Dextran, and bright field were imported into ICY where noise was removed using Thresholder plugin. The channels were merged into one image and the Spot Detector plugin was used to find bacteria (i.e. mCherry positive pixels) at each time point. The Spot Tracking plugin was used to track bacteria over time. Tracks were exported into the Track Manager plugin, and were organized using the Intensity Profiler, which displays the track duration as well as colocalization of the mCherry bacteria and green Dextran channel. Bacterial survival was
assessed by determining whether an mCherry track persisted throughout the experiment. Statistical significance was measured using a one-way ANOVA test.

2.4 RESULTS

*Live cell imaging allows for direct visualization of vacuolar Salmonella replication within infected epithelial cells*

Characterizing and quantifying the survival and replication of bacterial pathogens within vacuoles is an important step in defining the intracellular niche and identifying factors that perturb this niche. Here we establish a quantitative approach for tracking bacterial replication in individual host cells over long time periods (> 17 hours) to examine the role of effector proteins in regulating this niche. To identify the SCV, HeLa cells were incubated with a green fluorescently-tagged Dextran (AlexaFluor 488 Dextran) immediately prior to infection with *Salmonella enterica* serovar Typhimurium. Upon infection, Dextran was taken up into the vacuole and was routinely visible for up to 17 hours (Figure 2.1). Infection with a *Salmonella* strain constitutively expressing the mCherry fluorescent protein enabled simultaneous tracking of the fate of *Salmonella* and the vacuole over time (Figure 2.2A), allowing for clear differentiation of vacuolar and cytosolic bacteria (Figure 2.3). At each time point, the number of pixels corresponding to fluorescently labeled bacteria were extracted using the bioimaging analysis program ICY, and the change in pixel area was used to obtain a growth curve for each infected cell (Figure 2.2B and Figure 2.4). Bacteria that colocalized with Dextran throughout the experiment and did not hyperreplicate were defined as vacuolar. Under our experimental
Figure 2.2. Live cell imaging reveals *Salmonella* curves in infected cells and distinguishes cytosolic hyperreplication from vacuolar replication. (A) Representative fluorescence images of wt mCherry *Salmonella*, AlexaFluor 488 Dextran and an overlay (*Salmonella* in red, Dextran in green, and colocalization in yellow). Images are from 13.5 hrs post infection. (B) Representative images showing ICY analysis of the bacterial channel at three different time points. Green boxes mark the detected bacterial areas at each time point. Images were processed using a Threshold plugin and fluorescent pixels were quantified at each time point to generate a growth curve. (C) Fraction of infections characterized by cytosolic hyperreplication for wt *Salmonella*, ΔsteA, ΔsseG, and ΔsteA/ΔsseG *Salmonella* infected cells. ΔsseG causes a decrease in cytosolic replication events. Values are mean ± the standard error of the mean (wt n= 182 cells, ΔsteA n= 166 cells, ΔsseG n=128 cells, ΔsteA/ΔsseG n=165 cells) and are a combination of two (for ΔsseG) or three independent experiments (all other strains). p values labeled with *** are <0.0001. All p values were calculated using a one-way ANOVA test.
Figure 2.3. Comparison of Dextran labeling of vacuolar versus cytosolic *Salmonella* replication with widefield and confocal fluorescence microscopy. Vacuolar *Salmonella* (Ai and Bi) are coated in Dextran whereas cytosolic hyperreplicating *Salmonella* (Aii and Bii) are not. (A) Representative widefield fluorescence images of mCherry *Salmonella* and AlexaFluor 488 Dextran at 3, 6, 9, and 12 hours post infection (*Salmonella* in red, Dextran in green, colocalization in yellow). (B) Representative confocal fluorescence images of mCherry *Salmonella* and AlexaFluor 488 Dextran at 3, 6, and 10 hours post infection (*Salmonella* in red, Dextran in green, colocalization in yellow).
Figure 2.4. Validation of the ICY replication assay. The number of bacteria for a particular replication event were tabulated by eye at 6 different time points post infection (top panel) and compared to the readouts from the ICY replication assay (bottom panel). The resulting data points can be fit to a straight line with an $R^2$ value of 0.99, suggesting that the ICY replication assay produces results that are comparable to the number of bacteria present.
conditions, 57% of cells infected with wild type (wt) Salmonella remained in the vacuole during replication (Figure 2.2C), while the remainder escaped into the cytosol.

**SseG influences the proportion of infected cells exhibiting cytosolic hyperreplication**

We next examined whether specific effector proteins influence the distribution of cytosolic hyperreplication versus vacuolar replication. These experiments focused on two Salmonella effector proteins: SteA and SseG, which localize to the SCV upon translocation (59, 83), play a role in promoting this intracellular niche, and have been shown to be functionally linked (84). To explore the role of SteA and SseG in replication we developed ΔsteA, ΔsseG, and ΔsteA/ΔsseG mutant strains in Salmonella enterica serovar Typhimurium SL1344 that constitutively expresses mCherry (118, 119). There was no significant difference in the overnight growth of these strains in LB media (Figure 2.5). HeLa cells were infected with the different strains as described above and growth curves were obtained for each infected cell in which bacteria colocalized with Dextran (n ~ 100 cells for each condition). Cells containing cytosolic hyperreplicating bacteria corresponded to 43%, 42%, 19%, and 12% of wt, ΔsteA, ΔsseG, and ΔsteA/ΔsseG infected cells, respectively (Figure 2.2C). Intriguingly, it appears that SseG, but not SteA, influences the proportion of infections exhibiting hyperreplication in the cytosol, as deletion of SseG significantly decreases the number of cells characterized by cytosolic hyperreplication.

**SseG affects vacuole membrane integrity during infection of epithelial cells**

Establishing an intracellular niche is essential for efficient Salmonella replication (5, 18, 115), and effector proteins are key to this process due to their ability to modify the nature of the SCV. Examination of phenotypes such as appearance, location, and dynamics of bacteria in infected cells has led to identification of important features of the vacuolar environment and the role of effector proteins in directing this niche (32, 72, 117). Comparison of wt and ΔsteA infections revealed no substantial difference in the appearance and localization of the SCV (Figure 2.6), except for late time points (>16 hours) where the vacuole containing ΔsteA
Figure 2.5. Growth curves of *Salmonella* mutants used in this study. *Salmonella* mutants have no significant growth defects compared to wt *Salmonella* in LB. *Salmonella* strains were grown overnight in LB and then diluted 1:100 into fresh LB prior to measuring the OD$_{600}$ value at time point 0. Values are the mean ± the standard error of the mean (n= 3 for all four different strains at each time point).
**Figure 2.6.** Replication for wt versus ΔsteA strains. Representative fluorescence images showing wt mCherry *Salmonella* (top) and ΔsteA mCherry *Salmonella* (bottom) at 3 hrs, 12 hrs, and 17 hrs post infection and the individual growth curve generated using ICY to measure the area of bacterial pixels for each infection. Arrowheads on the replication curve denote the vacuole size for each image.
Salmonella appeared more compact, consistent with previously reported immunofluorescence results at 14 hours post infection (84).

For wt and ΔsteA strains, Dextran colocalized with non-hyperreplicating bacteria in 74% and 82% of cells, respectively (Figures 2.7 and 2.8). For the ΔsseG and ΔsteA/ΔsseG infections, Dextran colocalized with non-hyperreplicating *Salmonella* in only 44% and 12% of infections, respectively (Figures 2.7 and 2.8). Interestingly infections with all four strains produced some fraction of non-hyperreplicating bacteria that were not labeled with Dextran. We hypothesized that these bacteria were still replicating in a membrane-enclosed compartment but that it might be damaged so that Dextran, but not bacteria, could leak out. Indeed, our preliminary experiments using the Split GFP system (83) to monitor SteA localization in the context of an ΔsseG infection show colocalization of SteA, which localizes to the SCV, with ΔsseG bacteria at all time points post infection (Figure 2.9). These results further suggest that deletion of SseG may alter the membrane integrity of the SCV, causing the Dextran-labeled dye to leak out.

**SseG influences SCV localization during infection of epithelial cells**

Previous studies have revealed that knock out of SseG alters the directional movement of the SCV and leads to more dispersed, non-Golgi localized bacteria (117). For example, only 30% of SCVs were found to be associated with the Golgi marker Giantin by 8 hours post infection (117). Combined with the observation that ΔsseG strains exhibited decreased replication, it was concluded that Golgi localization must be important for replication (117). Intriguingly, we found that both ΔsseG and ΔsteA/ΔsseG strains exhibited three different localization phenotypes (Figure 2.10 and Figure 2.11). In 50% of cells infected with ΔsseG or ΔsteA/ΔsseG *Salmonella*, bacteria localized together in a compact structure, similar to the SCV in wt and ΔsteA *Salmonella* infections. In the other 50% of infected cells, bacteria started out localized together but then spread out across the cell over the course of the experiment,
Figure 2.7. Comparison of Dextran colocalization with wt and mutant *Salmonella* strains at 17 hours post infection. Dextran leaks out of ΔsseG and ΔsteA/ΔsseG vacuoles more frequently than ΔsteA and wt vacuoles. The fraction of vacuoles examined that contain Dextran at 17 hrs post infection was tabulated by visual inspection for Dextran localization to each *Salmonella* replication event where the *Salmonella* were not hyperreplicating. Values are mean ± the standard error of the mean (wt n= 104 cells, ΔsteA n= 96 cells, ΔsseG n= 104 cells, ΔsteA/ΔsseG n= 145 cells). The p values labeled with *** are <0.0001. The difference that is not statistically significant has a p value of 0.17. All p values were calculated using a one-way ANOVA test.
Figure 2.8. Time lapse fluorescence images of a cell exhibiting cytosolic hyperreplicating Salmonella and a cell infected with ΔsteA/ΔsseG. Although some Salmonella replication events appear to lose their Dextran markers (visualized by a loss of Dextran fluorescence colocalized with the bacteria), they replicate differently than the hyperreplicating Salmonella in the cytosol. Representative fluorescence images for hyperreplicating ΔsteA mCherry Salmonella (top) and ΔsteA/ΔsseG mCherry Salmonella (bottom) replication at 3, 10, and 17 hrs post infection. The right most panel for shows a trace of each respective replication curve generated using ICY to measure the area of bacterial pixels for each infection. Arrowheads on the replication curve denote the bacterial pixel area for each time point.
**Figure 2.9.** Representative fluorescence images of SteA-GFP\textsubscript{comp} and mTagBFP2 \textit{Salmonella} in wt (top) or ΔsseG (bottom) infected cells at 8.6, 12.3, and 16.1 hours post infection (\textit{Salmonella} in red, SteA in green, and colocalization in yellow). Regardless of labeling with Dextran, non-hyperreplicating ΔsseG bacteria are in a membrane-enclosed compartment that is labeled by SteA throughout the infection process.
**Figure 2.10.** Evolution of replication phenotypes in epithelial cells for ΔsseG strains. Representative fluorescence images of the three different ΔsseG replication phenotypes at 3 hrs, 12 hrs, and 17 hrs post infection and the respective growth curves generated using ICY to measure the area of bacterial pixels for each infection. Arrowheads on the replication curve denote the vacuole size for each image. Phenotypes were tabulated by visual inspection of every replication event.
Figure 2.11. Evolution of replication phenotypes in epithelial cells for ΔsteA/ΔsseG strains. ΔsteA/ΔsseG Salmonella have three different replication phenotypes in epithelial cells. Representative fluorescence images showing the three different ΔsteA/ΔsseG mCherry Salmonella replication phenotypes at 3 hrs, 12 hrs, and 17 hrs post infection and the individual replication curves generated using ICY to measure the area of bacterial pixels for each infection. Arrowheads on the replication curve denote the vacuole size for each image. Phenotypes were tabulated by visual inspection of every replication event.
reminiscent of the dispersed phenotype first described by Salcedo and Holden (117). Surprisingly, for ΔsseG infections, in 20% of cells with dispersed *Salmonella*, the bacteria coalesced into a compact structure by the end of the experiment, while in 30% of cells *Salmonella* remained dispersed (Figure 2.10). The ΔsteA/ΔsseG strains showed a similar trend with 18% cells exhibiting *Salmonella* dispersion followed by coalescence, while 32% of cells had *Salmonella* that remained dispersed (Figure 2.11). Interestingly, in many infections dispersed bacteria were capable of undergoing replication, as the total bacterial area increased over time (Figure 2.8, bottom panel). This result suggests that localization to Golgi is not an absolute requirement for replication. However, dispersed bacteria didn’t replicate at the same rate or to the same degree as wt bacteria in an SCV close to the Golgi (see below).

Although we saw no difference in the fraction of ΔsseG and ΔsteA/ΔsseG infections displaying the dispersed phenotype, we wondered if ΔsteA altered the timing of dispersal or coalescence. To address this idea, we noted the time of dispersal and coalescence upon infection with ΔsseG or ΔsteA/ΔsseG bacteria and found that the timing of bacterial dispersion was indeed different for ΔsseG and ΔsteA/ΔsseG, with the double mutant spreading out on average 1.6 hours later than the single mutant. However, this difference depended on whether or not bacteria coalesced later in infection (Figure 2.12A). Finally, there was no difference in the timing of coalescence (Figure 2.12B). These results suggest that bacterial dispersion and coalescence may occur through two independent effector protein-controlled pathways and that SteA is only important in the dispersal.

**SteA and SseG contribute to vacuolar replication in infected epithelial cells**

To compare the extent of vacuolar replication between wt *Salmonella* and the three different deletion strains in HeLa cells, the number of pixels corresponding to bacterial fluorescence at discrete time points was divided by the number of pixels at 3 hours post infection. This value was referred to as the “fold replication.” For wt and ΔsteA infections, analysis was limited to infections in which bacteria colocalized with Dextran, indicative of
Figure 2.12. Timing of Salmonella dispersion and coalescence during infection with ΔsseG or ΔsteA/ΔsseG strains. (A) Box and whisker plot showing the mean (black dot), the median (black line), and any outliers (gray dots) for ΔsseG dispersed (n= 29 cells), ΔsseG dispersed/coalesce (n= 20 cells), ΔsteA/ΔsseG dispersed (n= 38 cells), and ΔsteA/ΔsseG dispersed/coalesce (n= 21 cells). * denotes p < 0.05, ** denotes p = 0.007, and n.s signifies p > 0.05. (B) Box and whisker plot showing the mean (black dot) of coalescence times for ΔsseG (n= 20 cells) and ΔsteA/ΔsseG (n= 21 cells). All p values were calculated using a one-way ANOVA test.
replication with the SCV. The fold replication of ΔsteA Salmonella tracks with wt until late time points (> 16 hours) where there is a significant (21%) decrease in fold replication compared to wt (Figure 2.13A). Our results are consistent with a previous study using a CFU assay that found no defect in replication in HeLa or Caco-2 cells at 6 hours post-infection, although this study did not examine replication at longer time points (96). Interestingly, our results differ from previous reports that deletion of SteA didn’t significantly impair replication in a model mouse macrophage cell line (Raw264.7 cells) (84) or bone marrow derived macrophage (122) at 16 or 17 hours post infection, respectively. As discussed below, this difference could result from the use of different cell types for these experiments, the ability of our assay to track replication within individual cells, or the fact that our assay enables us to explicitly define replication within the SCV, excluding the potentially confounding contribution of cytosolic replication.

Deletion of SseG led to a profound replication defect. Because fewer ΔsseG and ΔsteA/ΔsseG replication events were consistently labeled with Dextran (Figure 2.7), all non-hyperreplicating bacteria were included in the analysis. For the ΔsseG strain, decreased replication was observed at all time points, resulting in a 48% decrease in fold replication compared to wt at 17 hours post infection (Figure 2.13B), consistent with previous results using a CFU assay to quantify replication between 2 and 16 hours post infection (117). The ΔsteA/ΔsseG also had a replication defect throughout the experiment resulting in a 68% decrease in fold replication compared to wt at 17 hours post infection (Figure 2.13C). The fold replication for the ΔsseG and ΔsteA/ΔsseG strains were significantly different by 17 hours post infection, with deletion of SteA causing an additional 20% decrease compared to deletion of SseG alone. The contributions of SteA and SseG to vacuolar replication appear to be additive, further suggesting that SteA indeed contributes to vacuolar replication of Salmonella within epithelial cells.

We next compared the fold replication of infections at the single cell level with a CFU assay at 3 and 17 hours post infection (Figure 2.13D). Not surprisingly, the CFU assay yielded
All p values were calculated using a one-way ANOVA test. (n.s. signifies p=0.07).

Fold change in bacterial load for infections with ΔsteA, ΔsseG, and ΔsteA/ΔsseG strains compared to wt Salmonella. Results shown here are either from single cell long term imaging assays (A-C) or CFU assays (D-E). For the single cell imaging assays, replication events in single cells were monitored from 2-17 hours post infection and the bacterial area in pixels at each discrete time point was calculated using ICY. The Fold replication for the single cell imaging assays was calculated by diving the bacterial area at discrete time points by the bacterial area at 3 hours post infection. (A) ΔsteA exhibits decreased fold replication compared to wt infections at late time points post infection (wt n= 100 cells, ΔsteA n= 99 cells, 3 independent experiments, * indicates p <0.02). (B) ΔsseG exhibits a decreased fold replication compared to wt infections at all time points (ΔsseG n= 98 cells, 2 independent experiments, *** indicates p <0.0002). C) ΔsteA/ΔsseG exhibits a decreased fold replication that is additive between the two single knock out strains (ΔsteA/ΔsseG n= 116 cells, 3 independent experiments, *** indicates p <0.0003). For the single cell assay in A, B, and C, bacterial load was defined by the change in pixel area of the mCherry bacterial signal. D) Fold replication as measured by a CFU assay (n = 3 independent experiments, n.s. signifies p =0.09). The Fold replication for the CFU assay was calculated by dividing the CFUs counted at 17 hours post infection by the CFUs counted at 3 hours post infection. E) CFUs at 17 hours post infection show decreased bacterial load for ΔsseG and ΔsteA/ΔsseG (n=3 independent experiments, *** signifies p<0.0003, n.s. signifies p= 0.07). Values are mean ± the standard error of the mean. All p values were calculated using a one-way ANOVA test.
much higher values for fold replication likely because it combines cytosolic hyperreplication with vacuolar replication. In addition, our method may be underestimating bacterial load at later time points due to the fact that we measure area and not volume of the bacterial channel. While both methods show similar trends, the results from the CFU assay show no statistically significant difference between wt and the three mutant strains (Figure 2.13D). We hypothesized that this lack of significance could be due to the very large heterogeneity in CFU assays, particularly at the 3 hour time point, resulting in large error bars for the assay. Indeed when we only compare the CFUs at 17 hours, we find that ΔsseG and ΔsteA/ΔsseG strains exhibit significantly fewer CFUs than wt Salmonella (66% and 79% decrease, respectively, Figure 2.13E). The 13% decrease in fold replication between the ΔsseG and ΔsteA/ΔsseG strains further indicates that SteA plays a role in promoting vacuolar replication. Overall, the single cell assay yields results that are comparable to the more commonly used CFU assay but with the advantage of allowing us to strictly quantify vacuolar replication and discern subtle differences in replication.

**SteA and SseG influence both the shape of the growth curve and growth rate**

Tracking bacterial load in individual infected cells enabled us to generate full in situ growth curves to monitor trends associated with *Salmonella* replication kinetics. Bacterial growth *in vitro* is typically characterized by a lag phase, followed by exponential growth, and finally a stationary phase (123). A defect in the fold change in bacterial load within a given time period could result from a more pronounced lag phase, a change in the exponential growth rate, or increased bacterial death. Our in situ growth curves for wt and mutant strains of *Salmonella* generally exhibited features similar to in vitro growth curves (Figure 2.14). However, we observed substantial heterogeneity in the shapes of these curves, including steady growth, a pronounced lag phase followed by exponential increase, an increase followed by a plateau, a flat line (i.e. no growth), or a complex behavior that included both an increase and a decrease in bacterial area. Figure 2.14A presents the percentage of infections in each of these categories
Figure 2.14. Comparison of replication curves for ΔsteA, ΔsseG, and ΔsteA/ΔsseG strains compared to wt Salmonella. Replication events in single cells were monitored from 2-17 hours post infection and the bacterial area in pixels at each discrete time point was calculated using ICY. Graphing the change in area versus time for each infected cell yielded a replication curve. (A) Replication curve phenotypes and the frequency for each strain. Steady Growth: a curve containing solely exponential or linear growth, Lag phase: a curve containing a substantial lag phase, Plateau phase: a curve containing a substantial stationary phase, No growth: a curve that is flat or has a decrease in area, Complex: a curve with abnormal features. All curves were generated using ICY to calculate bacterial area at each time point imaged for each infected cell and were tabulated here by visual inspection. wt n= 103 cells, ΔsteA n= 99 cells, ΔsseG n= 103 cells, and ΔsteA/ΔsseG n= 116 cells. (B) Representative “Steady Growth” replication curves for wt (black), ΔsteA (red), ΔsseG (blue), and ΔsteA/ΔsseG (green) strains. (C) Representative “ Pronounced Lag” replication curves for wt (black), ΔsteA (red), ΔsseG (blue), and ΔsteA/ΔsseG (green) strains. (D) Growth rates for infections that had replication curves categorized as exponential growth for ΔsteA, ΔsseG, and ΔsteA/ΔsseG strains compared to wt Salmonella. ΔsteA, ΔsseG, and ΔsteA/ΔsseG infections have slower growth rates compared to wt Salmonella within infected epithelial cells (wt n= 78 cells, ΔsteA n= 69 cells, ΔsseG n= 73 cells, and ΔsteA/ΔsseG n= 35 cells). Rates were calculated by fitting the replication curves to an exponential function Y=Ae^{mt} where m represents the rate. Only infections that had 50 or less pixels in the thresholded bacterial channel at the initial time point were analyzed. Results include only rates from fits with R^2 > 0.7 and are graphed in a box and whisker plot showing the mean (black dot), the median (black line), and any outliers (gray dots) for each data set. *** signifies p<0.0001 and ** signifies p= 0.004. All p values were calculated using a one-way ANOVA test.
for the different strains. The wt and $\Delta steA$ strains show a similar distribution of growth curves, whereas $\Delta sseG$ shows a marked reduction in the number of curves with steady exponential growth and an increase in infections with a pronounced lag phase prior to exponential growth. Finally $\Delta steA/\Delta sseG$ infections had a substantial number of curves with no growth and an increase in complex behavior. The increase in complex behavior, marked by both increases and decreases in bacterial area, upon deletion of both SteA and SseG could indicate increased bacterial cell death, though more studies would be required to confirm this hypothesis. Representative growth curves categorized as steady growth or a pronounced lag reveal different exponential growth rates, with wt > $\Delta steA$ > $\Delta sseG$ > $\Delta steA/\Delta sseG$ (Figure 2.14B), and a longer lag in $\Delta steA/\Delta sseG$ than the other strains (~ 12 hours versus 8 hours, Figure 2.14C).

In order to directly compare in situ growth rates for the different *Salmonella* strains all curves that fit into the steady growth category were fit to an exponential function in MatLab ($Y = Ae^{mt}$, Figure 2.15). Wild type *Salmonella* had the fastest growth rate (0.15 hr$^{-1}$), followed by $\Delta steA$ (0.13 hr$^{-1}$), $\Delta sseG$ (0.12 hr$^{-1}$), and $\Delta steA/\Delta sseG$ (0.11 hr$^{-1}$) (Figure 2.14D). These results indicate that the decreased bacterial load (Figure 2.13) is not solely due to a lack of replication for the mutant strains. Rather, the decreased fold replication seen for all three mutant strains is in part due to the fact that these strains have slower growth rates compared to the wt strain.

**Direct visualization of the requirement for SCV integrity for *Salmonella* survival within macrophages**

In the murine typhoid model of *S. Typhimurium* infection, *Salmonella* infect macrophages as well as epithelial cells (1). The SCV is necessary for survival within macrophages because *Salmonella* that escape into the cytosol are killed (58, 124). To study the role of effector proteins in promoting survival in a macrophage infection model, we adapted the long term imaging assay to monitor *Salmonella* survival within a Dextran-enclosed compartment (i.e. the vacuole) in primary bone marrow derived macrophages (BMDMs) from SV129S6 mice.
Figure 2.15 A and B
Figure 2.15. Comparison of growth rates between wt *Salmonella* and ΔsteA, ΔsseG, and ΔsteA/ΔsseG *Salmonella* infections. Replication events in single cells were monitored from 2-17 hours post infection and the bacterial area in pixels at each discrete time point was calculated using ICY. Graphing the change in area versus time for each infected cell yielded a replication curve. Individual growth curves categorized as “exponential growth” were fitted to an exponential function \( Y = Ae^{mt} \), where \( m \) represents the replication rate. (A) Representative growth curves and respective fits are shown for wt *Salmonella* (blue circles: data, blue lines: fits), ΔsteA *Salmonella* (red squares: data, red lines: fits), ΔsseG *Salmonella* (green diamonds: data, green lines: fits), and ΔsteA/ΔsseG *Salmonella* (black triangles: data, black lines: fits). (B) In order to directly visualize the growth rates for our four *Salmonella* strains, the fits from (A) are graphed with a log y-axis so that the slope of the linear plot represents the growth rate for each curve. (C) Curves from infections with all three mutants have slower growth rates compared to those from wt infections. Values are mean ± the standard error of the mean (wt n= 81 cells, ΔsteA n= 69 cells, ΔsseG n= 73 cells, and ΔsteA/ΔsseG n= 35 cells). *** signifies p<0.0001 and ** signifies p= 0.004. All p values were calculated using a one-way ANOVA test.
Macrophages derived from these mice express the metal transport protein NRAMP1 (Slc11a1) which confers macrophage resistance to pathogens that reside within vesicles by restricting pathogen access to iron, an essential nutrient (120, 125-127). Nramp1<sup>+</sup> mice allow *Salmonella* to persist within macrophages in a chronic infection for up to one year (128, 129). Upon infection of BMDMs with wt *Salmonella*, 50% of internalized bacteria colocalized with fluorescently-tagged Dextran at 2 hrs post infection, suggesting enclosure within an SCV ([Figure 2.16A](#)). To monitor the fate of bacteria over time, ICY was used to track both the bacterial and Dextran fluorescent signal throughout the experiment ([Figure 2.16B](#)). As illustrated in [Figure 2.16B](#), most bacteria not colocalized with Dextran disappeared during the experiment. We interpreted the abrupt disappearance of red fluorescence as an indicator of bacterial death and degradation by the host cell. For wt *Salmonella*, we analyzed 190 infected cells and found that 21% of internalized bacteria were still visible at 16 hours post-infection, suggesting survival at least until this time point ([Figure 2.17A](#)). Analysis of visible bacteria at 16 hours post-infection revealed that 96% were colocalized with Dextran, suggesting that survival is strongly enhanced by encapsulation within the SCV ([Figure 2.17B](#)). Perhaps the cells in which the remaining 4% of bacteria that survived and were never colocalized with Dextran resided had less antimicrobial activity, thus allowing for the survival of unprotected *Salmonella*. Regardless, these results vividly demonstrate that the macrophage cytosol is antibacterial and that therefore SCV integrity in macrophages is essential for *Salmonella* survival.

**SteA contributes to the ability of the SCV to promote *Salmonella* survival within bone marrow derived macrophages**

SteA has been found to be important for promoting persistence during model typhoid infections (16) and for manipulating membrane dynamics of the SCV (84). As such, we hypothesized that deletion of SteA might compromise vacuolar integrity and therefore negatively affect the ability of the vacuole to protect *Salmonella* within the context of macrophage infection. To test this hypothesis, we used the live cell imaging assay described above to compare
Figure 2.16. Monitoring vacuolar *Salmonella* survival in single primary bone marrow derived macrophages (BMDMs). (A) Representative fluorescence images of wt mCherry *Salmonella*, AlexaFluor 488 Dextran and an overlay with bright field (*Salmonella* in red, Dextran in green, and bright field is in gray). In the merged image, *Salmonella* colocalized with Dextran (encapsulated in an SCV, yellow) are circled in blue while *Salmonella* not colocalized with Dextran (in the cytosol, red) are circled in purple. The Dextran is added to BMDMs immediately prior to infection with *Salmonella*. Images presented here are from 1.9 hrs post infection. (B) Representative images showing ICY analysis of the bacterial and Dextran channels at 1.9, 8.9, and 15.7 hours post infection. The red circles mark the bacteria that ICY detected and tracked at each time point. ICY also determined whether the bacteria colocalize with Dextran. These tracks were used to determine the fraction of survivors (and whether or not they colocalize with Dextran) for each infected cell.
Figure 2.17. Comparison of vacuolar wt and ΔsteA *Salmonella* survival in BMDMs. (A) Deletion of SteA causes a small decrease in the fraction of *Salmonella* that survive infection of BMDMs over 16 hours of infection. Vacular *Salmonella* were monitored from 2 to 16 hours post infection in single BMDMs. The ability of the *Salmonella* to survive was then monitored using ICY. The fraction of survivors was calculated by dividing the number of surviving bacteria by the total initial bacteria for each infected cell. Values are mean ± the standard error of the mean (wt n= 190 cells, ΔsteA n= 126 cells). The p value marked in the panel by * is 0.05. (B) Both wt and ΔsteA *Salmonella* require colocalization with Dextran (i.e. a membrane-enclosed compartment) to survive the macrophage infection process. The fraction of green survivors was calculated by dividing the number of green survivors by the total number of survivors for each infected cell. Values are mean ± the standard error of the mean (wt n= 130 cells, ΔsteA n= 63 cells). (C) SteA is important for vacuolar integrity and *Salmonella* survival within BMDMs. The fraction of *Salmonella* survivors was calculated by determining how many of the bacteria that initially colocalized with Dextran survived until the end of the experiment (~16 hrs post infection). Values are mean ± the standard error of the mean (wt n= 147 cells, ΔsteA n= 133 cells). The p value marked in the panel by *** is <0.0001. (D) Deletion of SteA causes an increase in the fraction of bacteria initially colocalized with Dextran. The fraction of bacteria initially colocalized with Dextran was calculated by dividing the number of colocalized bacteria by the total number of bacteria at 2 hours post infection for each infected cell. The p value marked in the panel by *** is <0.0001. All p values were calculated by a one-way ANOVA test and results are a combination of three independent experiments.
survival of wt and ΔsteA Salmonella and the role of the SCV in promoting survival. We examined 136 cells infected with the ΔsteA strain and found that 16% of internalized bacteria survived until the end of the experiment, a 23% decrease compared to wt Salmonella (Figure 2.17A). Similar to wt Salmonella, analysis of visible bacteria at 16 hours post-infection revealed that 99% were colocalized with Dextran (Figure 2.17B), reiterating that survival is strongly enhanced by encapsulation within the SCV. One of our key questions was whether SteA might influence the SCV environment and hence selective survival of bacteria within the vacuole. Therefore, we compared the fraction of wt or ΔsteA Salmonella that were initially colocalized with Dextran that survived for 16 hours. We found that 41% of wt Salmonella initially within a vacuole survived for 16 hours (Figure 2.17C). The remaining 59% of bacteria either were killed within the vacuole (i.e. both Dextran and Salmonella signal disappeared simultaneously) or escaped into the cytosol and then were killed by the macrophage (i.e. Dextran signal disappeared first followed by the disappearance of Salmonella signal). However, for ΔsteA infections, only 18% of Salmonella initially coated in Dextran survived until the end of the experiment. To better understand why we observed such a dramatic decrease in the survival of vacuolar bacteria (56% decrease compared to wt) but only a modest decrease overall (23% decrease compared to wt), we analyzed the % of bacteria that colocalized with Dextran at our initial time point (~ 2 hrs post infection). Surprisingly, deletion of SteA resulted in a significant increase in the percent of bacteria that colocalized with Dextran, suggesting a higher proportion of bacteria encapsulated within an SCV (90% versus 50% for wt, Figure 2.17D). Overall, our results reveal that SteA is important for Salmonella survival within the context of bone marrow derived macrophage infections, and in particular that the presence of SteA is important for promoting survival of bacteria within the SCV.

2.5 DISCUSSION

Infections are by their very nature dynamic, and this dynamism is often key to understanding cause and effect. Live cell imaging permits visualization and quantification of
these dynamics and evolution of complex phenotypes over time. Moreover, given the
heterogeneity in infection, live cell imaging can help discern whether a particular observation is
deterministic of downstream events. In this study, we established live cell imaging assays and
subsequent data analysis methods, applied these methods to examine replication and survival
of wt and mutant *Salmonella* strains in both epithelial cells and BMDMs, and determined
whether effector proteins may play different roles in these model systems. Our results
uncovered new features of infection and the roles of effector proteins in shaping these
phenotypes. For example we observed for the first time that dispersed bacteria can coalesce
back into an aggregate resembling a canonical vacuole, that SseG may play a role in promoting
cytosolic hyperreplication in epithelial cell infections, and that SteA is important for vacuole
integrity in the macrophage model of infection.

Monitoring bacterial replication in epithelial cells at the single cell level revealed a rich
variety of replication phenotypes that were differentially impacted by effector proteins. The
absence of SseG increased the percent of infections that exhibited a pronounced lag phase and
decreased the rate of exponential growth (*Figure 2.14*). These results suggest that ΔsseG
causes a defect in the SCV development even at early time points and indicate that SseG is
required for effective establishment of the intracellular niche. This observation is consistent with
previous reports suggesting that SseG is important for replication in epithelial cells (117). On
the other hand, deletion of SteA had a more subtle effect, showing only a decreased rate of
exponential growth (*Figure 2.14*), suggesting that perhaps SteA is not required for establishing
the SCV but rather it plays a role in maintaining the SCV environment over time. Our findings
lead us to speculate that perhaps loss of effector proteins that help set up the SCV environment
lead to substantially delayed replication and an overall decrease in growth rate. These gross
defects give rise to pronounced decrease in total bacterial load even at early time points. Such
effector proteins are more likely to be identified in single time point assays using fold replication.
However, effector proteins that play a role at later stages maintaining the SCV environment can
still impact the rate of replication, but loss of these effector proteins yields a subtle defect that is only manifested at late stages of infection and captured in single cell assays. This phenomenon could explain why SteA was not previously identified as influencing replication in epithelial cells (96).

The mechanisms by which SseG and SteA affect replication are not yet known. Tracking infections over a long time period enabled us to capture previously unidentified features of infection with mutant Salmonella strains. For example, the leakage of AlexaFluor-488 Dextran in the absence of SseG observed for both ΔsseG and ΔsseG/ΔsteA (Figure 2.7), suggests that SseG plays a role in maintaining integrity of the vacuolar membrane. Combined with the observation that loss of SseG led to a pronounced lag and decreased bacterial growth, it is possible that leakage of SCV contents results in decreased nutrients required for replication and that in the absence of SseG, it takes much longer for the SCV to attain the chemical environment necessary for bacterial growth. Previous studies have suggested that SseG plays a role in vacuole construction and placement within epithelial cells, either by physically tethering the SCV to the Golgi or by manipulating molecular motors (51, 59, 71, 72, 117). Consequently, loss of SseG results in dispersion of bacteria away from the Golgi (117). We observed dispersion of SCVs containing ΔsseG bacteria in ~50% of infections (Figure 2.10), consistent with previous observations. However, much to our surprise, in 20% of infections the dispersed SCV coalesced into what appeared to be a normal SCV in the perinuclear region. These observations suggest a mechanism for manipulating molecular motors to promote both the centrifugal and centripetal movement of bacteria. Bacterial dispersion could occur as a result of the activation of microtubule (+)-end directed motors such as kinesin or inhibition of (-)-end motors such as dynein while coalescence could occur through the opposite mechanism (i.e. inhibition of kinesin or activation of dynein). Intriguingly, that it has been suggested that SteA may manipulate motors although data were inconclusive as to whether it activated kinesin or
inhibited dynein (84). Using the Split GFP system, we found that SteA colocalized with dispersed SCVs in ΔsseG infections (Figure 2.9). While deletion of SteA did not substantially alter the propensity of bacteria to disperse or disperse and then coalesce, it did delay the timing of dispersal (Figure 2.12), consistent with a putative role in influencing centrifugal movement, i.e. direct or indirect inhibition of dynein or activation of kinesin. However, SCV dispersal still occurs in ΔsseG/ΔsteA infections, suggesting that SteA is not the only protein responsible for promoting centrifugal movement. Finally, deletion of SteA didn't influence the propensity or timing of SCV coalescence and we speculate that this phenotype may be regulated by another yet to be identified effector protein.

A final unexpected observation from our epithelial cell study was that the absence of SseG led to a substantial increase in the proportion of infections that exhibited vacuolar replication and a decrease in the number of infections in which bacteria hyperreplicated in the cytosol (Figure 2.2C). This result suggests that either SseG plays a role in promoting escape from the vacuole or that it facilitates survival and replication in the cytosol of epithelial cells. This finding was unanticipated as previous work using a GFP reporter system demonstrated a lack of expression of T3SS2 effector proteins (such SseG) in cytosolic bacteria at 8 hrs post invasion (35). More recently, Knodler and colleagues showed that a ΔssaR mutant, defective for the translocation of T3SS2 effector proteins, did not give rise to a significant change in the percentage of cytosolic bacteria in Caco-2 C2Bbe1 cells, as determined by a chloroquine resistance CFU assay at 2, 4, and 7 hrs post-invasion, suggesting that deletion of the T3SS2 translocon does not alter vacuolar escape (37). One possible explanation to reconcile these observations is that T3SS2 effector proteins that are translocated early in infection, while bacteria are still contained within the SCV, may differentially influence vacuolar escape and/or cytosolic replication, such that deletion of the translocon yields no net effect, but deletion of individual effector proteins may reveal some effector proteins that promote or inhibit these
processes. Indeed, using the Split GFP system to monitor SteA at early time points post infection (~6 hours post infection), we observed SteA expression even in cells containing hyperreplicating bacteria (Figure 2.18), demonstrating that T3SS2 effector proteins can be detected in the cytosol of infected cells dominated by hyperreplication. As the infection progresses and hyperreplicating bacteria take over the cell, SteA signal decreases, consistent with the observation that SteA and other T3SS2 effector proteins are not expressed by cytosolic bacteria (35). However these results raise the intriguing idea that T3SS2 effector proteins could influence the propensity for hyperreplication in the cytosol or replication within the SCV.

Figure 2.18. Representative fluorescence images of SteA-GFP<sub>comp</sub> and mTagBFP2 <i>Salmonella</i> in wt infected cells at 6.5, 9.0, 11.5, 14, and 16.5 hours post infection (<i>Salmonella</i> in red, SteA in green, and colocalization in yellow). SteA signal is present at early time points in cells containing hyperreplicating cytosolic bacteria.

SteA is an intriguing effector protein because a ΔsteA mutant shows decreased virulence and persistence in mouse models of gastroenteritis and systemic infection, respectively (16, 90), yet the mechanism by which SteA promotes virulence remains elusive. Moreover, studies have yielded confounding results with respect to the cellular role of SteA. For example, ΔsteA <i>Salmonella</i> did not show a replication defect in a model macrophage cell line RAW 264.7 or BMDMs from CL57 BL/6 mice, both of which lack a functional NRAMP1 (84, 122), and although we find a replication defect in epithelial cells, the effect is subtle and hard to reconcile with the documented virulence defect for ΔsteA. Therefore we examined the role of the SteA in promoting survival in a typhoid fever model of infection. This model involves infection of BMDMs from SV129S6 mice, which possess an intact NRAMP1 (128). As the NRAMP1 gene restricts <i>Salmonella</i> replication in BMDMs during the first week of infection, the
presence of this gene allows for the establishment of a persistent typhoid fever infection model as opposed to a gastroenteritis model in which the *Salmonella* have no replication restrictions (130). In this study we directly visualized the ability of the vacuole to support survival in single infected BMDMs, and role of SteA in this process. Our results reveal that SteA plays a major role in proper vacuole formation in the typhoid model of infection, as $\Delta$steA causes a 56% decrease in the ability of the vacuole to promote survival (*Figure 2.17C*). These results are particularly intriguing in light of the subtle defect in replication caused by $\Delta$steA in infected epithelial cells (*Figure 2.13*) and suggest that effector proteins may play different roles in infection depending on cell type, and in different infection models (acute versus persistent infection).

Although long term imaging of single infected cells provides in depth information about the roles effector proteins play during *Salmonella* replication or survival in infected cells, these experiments are challenging to design and perform. Two inherent challenges that arise are the need to 1) collect enough data on individual cells to provide statistical power, and 2) automate analysis to enable systematic quantification of important parameters in order to streamline data analysis. The ability to determine significant subtle differences between replication or survival phenotypes is directly related to the size of the data set (131). Therefore we acquired at least 100 single cells for each data set. Because there is a limited number of infected cells per field of view and cells can move out of view over the course of the experiment, it was necessary to image multiple fields of view in each experiment to maximize the total number of infections that could be analyzed. However, analysis of these data sets is also challenging because large data sets are unwieldy to analyze in a non automatic fashion and require efficient quantification in order to perform rigorous statistical validation (132). Image analysis programs such as ICY allow for the development of pipeline protocols that yield quantitative information about each infection and allow users to conduct analysis on entire datasets at least semi-automatically.
Thus while these challenges for single cell imaging experiments are significant, they are not insurmountable and can be addressed through experimental design.

**2.6 SUPPLEMENTARY NOTE**

When we compared the extent of vacuolar replication between wt and mutant *Salmonella* strains, we limited analysis to cells in which bacteria replicate within a vacuole. To calculate the extent of replication, or fold replication, we divided the number of pixels at discrete time points post infection by the number of pixels at 3 hours post infection. We found that there was still substantial heterogeneity in the calculated fold replications for each data set. This result is at least in part due to the fact that fold replication depends not only on the rate of bacterial multiplication, but also on the initial number of internalized bacteria. To illustrate this trend, we examined the fold replication as a function of pixel count in the bacterial fluorescence channel at the first time point (~ 2-3 hours) and observed a strong correlation, where the cells exhibiting the lowest initial pixel counts gave rise to the highest fold replication and those with a high number of pixel counts yielded the lowest fold replication (**Figure 2.19**). The wide range of calculated fold replication at 17 hours illustrates the substantial cell-to-cell heterogeneity and reinforces the value of single cell measurements.

Using the fold replication data above, we established a cutoff of starting pixels for further data analysis. Replication events with the initial number of pixels >50 behaved differently than those with initial number of pixels <50 (**Figure 2.19**). Therefore, to only compare replication events with similar phenotypes, we decided to only use the events with initial number pixels <50 when we calculated the growth rate for the replication events that had steady growth curves (**Figure 2.14**). Within that data set, however, we determined that the growth rate still depended on the starting pixel count. For this analysis, we split our data into two data sets: those with starting pixels between 0 and 9 (~1-2 *Salmonella*) and those with starting pixels between 10 and 50 (~3-10 *Salmonella*) (**Figure 2.20A**). Although it appears that starting with fewer bacteria causes increased growth rates for all four *Salmonella* strains, the trend between the strains
remains the same – with wt *Salmonella* curves having the fastest growth rate and ΔsteA/ΔsseG *Salmonella* having the slowest growth rate (Figure 2.20B and Figure 2.14D).

**Figure 2.19.** Fold replication versus initial pixel area for the *Salmonella* strains in this study. Each dot represents an individual infected cell. The different colors/shapes represent different independent experiments demonstrating that a correlation between fold replication and initial pixel area was reproducible. Note that the fold replication numbers reported in this Figure are higher than in Figure 2 because here the final pixel area at 17 hrs was divided by the initial pixel area of the first time point (between 2 and 3 hrs depending on the experiment), whereas in Figure 2 the final pixel area was divided by the pixel area at 3 hrs.
Figure 2.20. Comparison of growth rates between wt and ΔsteA, ΔsseG, and ΔsteA/ΔsseG Salmonella infections based on initial number of pixels. Replication events in single cells were monitored from 2-17 hours post infection and the bacterial area in pixels at each discrete time point was calculated using ICY. Graphing the change in area versus time for each infected cell yielded a replication curve. Individual growth curves categorized as “exponential growth” were fitted to an exponential function $Y=Ae^{mt}$, where $m$ represents the replication rate. Growth rates are dependent on the number of pixels in the initial time point (~starting number of Salmonella).

(A) Representative fluorescence images of wt mCherry Salmonella that have been thresholded in ICY. The left panel shows an example of a starting image with less than 10 pixels (4 pixels ~ 2 Salmonella) and the right panel shows an example of a starting image with between 10 and 50 pixels (20 pixels ~ 7 Salmonella). (B) Infections containing fewer Salmonella at early time points yield curves that have faster growth rates. Growth rates for each infected cell were categorized according to the starting number of starting pixels: less than 10 starting pixels (dark gray) and between 10 and 50 starting pixels (light gray). Values are mean ± the standard error of the mean (wt less than 10: n= 24 cells, wt between 10 and 50: n= 54 cells, ΔsteA less than 10: n= 26 cells, ΔsteA between 10 and 50: n=43 cells, ΔsseG less than 10: n= 21 cells, ΔsseG between 10 and 50: n=52 cells, ΔsteA/ΔsseG less than 10: n= 18 cells, ΔsteA/ΔsseG between 10 and 50: n= 17 cells. The p value labeled with ** is 0.003 and the p values labeled with * are <0.01. All p values were calculated using a one-way ANOVA test.
The fact that vacuolar replication rate is in part inversely dependent on the initial number of bacteria present could occur for multiple reasons. This phenomenon could be due either to nutrient limitation when more bacteria are present (133), a quorum sensing mechanism that regulates the bacterial population size (134-137), or both. These mechanisms will have to be examined more deeply in future studies.

2.7 ACKNOWLEDGEMENTS

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Chapter 3: Development of a long-term imaging method to monitor changes in protein localization to the *Salmonella* containing vacuole upon deletion of effector proteins

3.1 ABSTRACT

*Salmonella enterica* serovar Typhimurium is a pathogenic bacterium that causes gastroenteritis in humans by infection of epithelial cells that line the small intestine. Inside host cells, *Salmonella* often reside within a membrane-bound compartment (called the *Salmonella* containing vacuole or SCV), which allows for replication within host cells. In order to promote successful replication within the host cell, the SCV is established through the action of *Salmonella* effector proteins, which target host cell and effector proteins to the SCV. While many effector and host cell proteins recruited to the SCV play important roles in establishing membrane integrity of the SCV as well as the environment within the vacuole, little is known about the mechanism most effector proteins use to target other effector and host cell proteins to the SCV. Here we develop a long term imaging assay to study changes in localization for effector and host cell proteins that are known to localize to the SCV upon infection with effector protein deletion strains in order to determine the roles of specific effector proteins in establishing the SCV. This assay allows for the direct visualization of effector protein, host cell protein, and bacteria localization within the cell during infection. Using this method, we aim to determine the role of the effector protein SteA in directing the localization other effector proteins such as PipB2 and trans-Golgi host cell proteins over the course of infection.

3.2 INTRODUCTION

The Gram negative pathogen *Salmonella enterica* serovar Typhimurium is a major cause of gastroenteritis worldwide (3, 4). Gastroenteritis results from *Salmonella* infection of the epithelial cells that line the human gut (1). In order to successfully infect epithelial cells, *Salmonella* use a series of type III secretion systems to secrete bacterial effector proteins into the host cell (11, 112). These effector proteins work in concert to allow for *Salmonella* uptake
into the host cell (12-14, 113). Once inside the host cell, *Salmonella* reside within a membrane-bound vacuole called the *Salmonella* containing vacuole (SCV), which is localized near the Golgi (18). Effector proteins also cause the formation and maturation of the SCV by hijacking membrane trafficking pathways and recruiting host cell proteins such as molecular motors, cytoskeleton proteins, and Rab GTPases (5, 114-116, 138). During the vacuole maturation process, membrane tubules emanate from the vacuole toward the cell periphery (47). These tubules are dynamic over the course of infection (139, 140). Although the function of these tubules has not been established role during infection, they have been hypothesized to play a role in vacuole maintenance, nutrient and membrane uptake, and successful *Salmonella* spread to uninfected cells (47, 48). Indeed, *Salmonella* strains lacking effector proteins known contribute to tubule formation are less virulent than wild type *Salmonella* strains (47). Because effector proteins are largely responsible for establishing the SCV, causing tubule formation, and allowing for successful *Salmonella* infection, it is important to define the roles of specific effector proteins in order to further understand and perturb this host-pathogen interaction.

One method for determining the role an effector protein plays in SCV establishment or maturation is to examine changes in SCV morphology during infection when the effector protein in question is either knocked out or overexpressed. For example, the knockout of the effector protein SipA, which localizes to the SCV, caused the SCV to migrate away from the perinuclear region, mislocalization of effector and host cell proteins that are normally recruited to the SCV, and a defect in *Salmonella* replication (32). On the other hand, overexpression of SipA caused SCV instability, enabling *Salmonella* to escape into the cytosol; an increased recruitment of effector and host cell proteins to the SCV; and increased *Salmonella* replication (32). These results suggest that SipA abundance must be precisely controlled during infection and that it may play a role in the proper trafficking of the SCV to its perinuclear location and thus successful *Salmonella* replication. The roles of other effector proteins known to localize to the SCV have been determined from similar localization studies, see, for example, references (59,
These effector proteins have been found to be important for proper recruitment of host cell factors to the SCV (49, 55, 62, 81), SCV membrane composition and trafficking (64, 67), and tubule formation (56, 83). From these studies, it can be concluded that effector proteins that localize to the SCV may play a role in promoting SCV maintenance.

We are interested in further exploring how effector proteins that are known to localize to the SCV might be involved in mediating maturation of the SCV. Numerous effector proteins (including SifA, PipB2, SopD2, SseG, SseF, and SteA) and host cell proteins (including LAMP1, SCAMP3, Rab7, Arl8b, and GalT) are known to localize to the SCV and the tubules emanating from the SCV throughout infection (14, 15, 19, 47, 48, 54, 67, 113). However, the majority of these effector proteins have unknown functions with respect to Salmonella infection and most host cell proteins are recruited by unknown mechanisms. Recently, the effector protein SteA has been hypothesized to manipulate membrane dynamics on the SCV during infection (84). The goal of this work was to design methods to assess changes in localization of host cell and Salmonella effector proteins known to localize to the SCV upon the deletion of SteA in order to gain insight into possible mechanisms by which SteA may modify the SCV during Salmonella infection.

3.3 MATERIALS AND METHODS

Salmonella strains (Table 3.1).

mCherry Salmonella strains used in these studies were Salmonella enterica serovar Typhimurium SL1344 strains expressing a plasmid (parent pACYC177) for constitutive expression of mCherry (plasmid pAmCh) under the PrpsM ribosomal promoter. For mCherry strains that expressed PipB2-GFP11 or SteA-GFP11, the pAmCh plasmid included a region that coded for the promoter of the effector protein of interest, the effector protein itself (SteA or PipB2), a flexible linker (GSSGGSs), and GFP β-strand 11 (GFP11: RDHMVLHEYVNAAGIT) between two HindIII restriction sites (83). This cloning strategy allows for the GFP11-tagged effector proteins to be expressed in cis or trans with respect to the mCherry protein. However,
Table 3.1. List of *Salmonella* strains used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Strain</th>
<th>Vector</th>
<th>Additional Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt mCherry</td>
<td>SL1344</td>
<td>pAmCh</td>
<td>wt SL1344 strain</td>
</tr>
<tr>
<td>SteA-GFP11 mCherry</td>
<td>SL1344</td>
<td>pAmCh + SteA-GFP11 region</td>
<td>steA::KanR †</td>
</tr>
<tr>
<td>PipB2-GFP11 mCherry</td>
<td>SL1344</td>
<td>pAmCh + PipB2-GFP11 region</td>
<td>pipB2::ChlorR ‡</td>
</tr>
<tr>
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<td>SL1344</td>
<td>pAmCh + PipB2-GFP11 region</td>
<td>pipB2::ChlorR, steA::KanR †</td>
</tr>
<tr>
<td>wt mTagBFP2</td>
<td>SL1344</td>
<td>pAmT</td>
<td>wt SL1344 strain</td>
</tr>
<tr>
<td>SteA-GFP11 mTagBFP2</td>
<td>SL1344</td>
<td>pAmT + SteA-GFP11 region</td>
<td>steA::KanR †</td>
</tr>
<tr>
<td>ΔsseG SteA-GFP11 mTagBFP2</td>
<td>SL1344</td>
<td>pAmT + SteA-GFP11 region</td>
<td>sseG::KanR, steA::ChlorR ‡</td>
</tr>
</tbody>
</table>

*This region encodes for: the promoter of the effector protein of interest, the effector protein itself (SteA or PipB2), a flexible linker (GSSGGSSG), and GFP β-strand 11 (GFP11: RDHMVLHEYVNAAGIT) between two HindIII restriction sites. This region is expressed in trans to the fluorescent protein gene within the vector.

† This nomenclature (ep::yR) indicates that the gene for the endogenous effector protein ep was replaced with the resistance gene for the antibiotic y.

Abbreviations: pAmCh= pACYC177 with constitutive expression of mCherry, pAmT= pACYC177 with constitutive expression of mTagBFP2, Kan= kanamycin, Chlor= chloramphenicol
we specifically chose plasmids that expressed the effector proteins in trans with the mCherry protein to avoid leaky expression of the effector proteins (83). To make mTagBFP2 *Salmonella*, the mCherry gene in the pAmCh plasmid was replaced with the mTagBFP2 gene (obtained from Vladislav Verkhusha, Albert Einstein College of Medicine) using an In-Fusion cloning kit (Clontech). The resulting pAmT plasmid was transformed into SL1344 *Salmonella*. To make mTagBFP2 *Salmonella* that expressed SteA-GFP11, the SteA promoter, SteA, the flexible linker, and GFP11 were cut out of the corresponding pAmCh plasmid using HindIII (NEB) and cloned into the pAmT plasmid. GFP11-tagged strains had the endogenous effector proteins knocked out using a recombination technique described previously (83, 118, 119). Briefly, this technique uses a 14028S *Salmonella* strain (based off of the LT2 *Salmonella* strain) that harbors a recombination system to allow for the endogenous effector gene to be replaced with a kanamycin or chloramphenicol resistance gene. This knockout was then transferred to our mCherry or mTagBFP2 *Salmonella* strains using a P22 phage derived from the resistance free strain SL1290. PipB2-GFP11 expressing strains had endogenous PipB2 knocked out with a chloramphenicol resistance gene (83) and SteA-GFP11 expressing strains had endogenous SteA knocked out with either a kanamycin resistance gene (wt SseG strains) or a chloramphenicol resistance gene (ΔsseG strains). For the ΔsteA PipB2-GFP11 strain, endogenous SteA was replaced with a kanamycin resistance gene. For the ΔsseG SteA-GFP11 mTagBFP2 strain, the endogenous SseG gene was replaced with a kanamycin resistance gene. To prepare strains for infection of HeLa cells, *Salmonella* strains were grown overnight in LB (EMB) supplemented with 300 mM NaCl (Fisher Scientific) and 25 mM MOPS (Sigma) at pH 7.6 and appropriate antibiotics (wt mCherry strain: 50 µg/mL ampicillin and 50 µg/mL streptomycin; SteA-GFP11 mCherry or mTagBFP2 strains: 50 µg/mL ampicillin, 50 µg/mL streptomycin, and 50 µg/mL kanamycin; PipB2-GFP11 mCherry strain: 50 µg/mL ampicillin, 50 µg/mL streptomycin, and 10 µg/mL chloramphenicol; ΔsteA PipB2-GFP11 mCherry strain and ΔsseG SteA-GFP11 mTagBFP2 strain: 50 µg/mL ampicillin, 50 µg/mL
streptomycin, 50 µg/mL kanamycin, and 10 µg/mL chloramphenicol) at 37°C without shaking.

**Plasmids.**

The pCMV-mGFP1-10 plasmid, which encodes the GFP β-strands 1-10 fragment with mammalian optimized codons was purchased from Theranostech (83). The pGalT-mCherry plasmid encodes for amino acids 1-60 of human β1,4-galactosyltransferase followed by mCherry and was acquired from Jennifer Lippincott-Schwartz (US National Institutes of Health). The pGalT-oxCerulean plasmid was made from the pGalT-mCherry plasmid by replacing the mCherry gene with the gene for the oxCerulean fluorescent protein (acquired from Erik Snapp, Albert Einstein College of Medicine) using Agel and Xhol cutsites. pActin-mCherry encodes for human β-actin followed by mCherry and was acquired from Roger Tsien (University of San Diego). pmCherry-Rab6a encodes for mCherry followed by human Rab6a and was acquired from Irina Kaverina (Vanderbilt University). The pSteA-GFP plasmid encodes for the effector protein SteA gene followed by the eGFP gene. PCR was used to remove the SteA gene from wt SL1344 Salmonella strain, delete the stop codon, and add NheI and Agel restriction sites on the 5’ and 3’ ends of SteA, respectively. Then, using NheI and Agel (NEB) enzymes, the SteA gene fragment was cloned into a Clontech N1 eGFP vector.

**Cell Culture and Salmonella infection for live imaging.**

HeLa cells were maintained in DMEM (Gibco) supplemented with 10% FBS (Gibco), 100 Units/mL penicillin G sodium (Gibco), and 100 µg/mL streptomycin sulfate (Gibco) at 37 °C with 5% CO₂. For infections, HeLa cells were plated in 35 mm glass-bottom dishes. Cells were transiently transfected 24 hours later with 500 ng – 1 µg host cell marker DNA (pGalT-mCherry, pmCherry-Rab6, or pGalT-oxCerulean) and 2.5 µg pCMV-mGFP1-10 using the TransIT LT1 system (MirusBio). Twenty-four to forty-eight hours post transfection cells were infected with Salmonella. Immediately prior to Salmonella infection, the cell media was exchanged for antibiotic-free DMEM. For live cell imaging at discrete time points post infection, cells were infected at a multiplicity of infection (MOI) of 50. Infections were allowed to proceed for 30
minutes at 37°C and 5% CO₂ before the *Salmonella*-containing media was exchanged with DMEM containing 100 µg/mL gentamicin to kill any extracellular bacteria. After incubating for 45 minutes at 37°C and 5% CO₂, the media was replaced with DMEM containing 10 µg/mL gentamicin, in which cells remained until live imaging or fixation. Immediately prior to live cell imaging, media was exchanged with Hanks Balanced Salt Solution with 10 mM HEPES, pH 7.4 (HHBSS, Gibco) containing 10 µg/mL gentamicin. In select experiments, live-cell Hoechst 33342 dye (NucBlue Live ReadyProbes, Life Technologies) was added to stain DNA. Two drops of dye per milliliter of media were added to the cells and cells were incubated for 5 minutes at room temperature prior to imaging. For live imaging experiments performed at discrete time points, wt and ΔsteA infections were staggered by two hours to permit acquisition of parallel time points. For long-term imaging experiments, cells were infected at an MOI of 30 and all DMEM used during the infection process was phenol-red free (Gibco). Cells remained in phenol red free DMEM containing 10 µg/mL gentamicin for the entirety of the imaging experiment.

*Fixing and staining of infected cells.*

HeLa cells were plated on glass coverslips (Fisher, 0.15 mm thickness) in 6 well cell culture plates, transfected with pCMV-mGFP1-10, and infected with PipB2-GFP11 or ΔsteA PipB2-GFP11 mCherry *Salmonella* at an MOI of 50, as described above. At 8 and 21 hours post infection, media was removed and cells were washed three times in 1x PBS. Cells were fixed in 3.7% (vol/vol) paraformaldehyde (Electron Microscopy Sciences) for 10 minutes before they were washed twice in 1x PBS. To prevent free radicals caused by paraformaldehyde, cells were incubated in 20 mM NH₄Cl for 5 minutes after paraformaldehyde treatment. Cells were washed twice in 1x PBS and then incubated in 0.1% Saponin (wt/vol) for 10 minutes to permeabilize cell membranes. After two washes with 1x PBS, cells were incubated with 5 Units/mL AlexaFluor 594 conjugated phalloidin for 20 minutes at room temperature to stain actin/cell peripheries. Cells were rinsed three times with 1x PBS and then coverslips were
mounted on slides (VWR, 1 mm thickness) using 10 µL of DAPI mount (Sigma Aldrich) to stain nuclei and bacteria. Slides were stored at 4°C until they were imaged.

**Imaging fixed and stained samples.**

Fixed and stained samples were imaged on a Zeiss Axiovert 200M inverted widefield microscope equipped with a Lambda 10-3 filter changer (Sutter Instruments), a Cascade 512B CCD camera at 5 MHz transfer speed (Roper Scientific), an oil 40x objective (1.4 numerical aperture), a Xenon arc Lamp (XBO 75), and Metaflouur software (Universal Imaging). Using this setup, we imaged DAPI-labeled bacteria and nuclei (excitation: 350/20 nm, emission: 470/40 nm, dichroic: 430 nm), PipB2-GFP<sub>comp</sub> (excitation: 480/20 nm, emission: 520/10 nm, dichroic: 515 nm), and AlexaFluor 594-labeled phalloidin (excitation: 577/20 nm, emission: 630/60 nm, dichroic: 595 nm) using a neutral density filter of 1.0.

**Imaging of live infections at discrete time points post infection.**

Infected cells were imaged on a Nikon Ti-E widefield microscope. This microscope was equipped with the Nikon Elements software, Nikon Ti-E Perfect Focus system, and a motorized XY stage with a Ti Z-drive (Nikon). Images acquired on this microscope used a 60x objective (numerical aperture 1.40), an iXon3 897 EMCCD camera (Andor), and a xenon-arc lamp to image Hoechst 33342-labeled DNA (excitation: 350/50 nm, emission: 460/50 nm, dichroic: 400 nm), oxCerulean-labeled GalT (excitation: 434/17 nm, emission: 474/23 nm, dichroic: 458), GFP-labeled effector proteins (PipB2-GFP<sub>comp</sub>, SteA-GFP<sub>comp</sub>, or SteA-GFP) (excitation: 472/30 nm, emission: 520/40 nm, dichroic: 490 nm), and FM 4-64 dye and mCherry-labeled actin, GalT, or *Salmonella* (excitation: 560/40 nm, emission: 630/75 nm, dichroic: 585 nm).

**Long term imaging of infected cells.**

All long term imaging experiments were performed using a 40x oil objective (numerical aperture 1.30) on a Nikon A1R laser scanning confocal microscope equipped with the Nikon Elements software, Nikon Ti-E Perfect Focus system, a motorized XY stage with a Ti Z-drive (Nikon), and the capacity to image mTagBFP2-labeled *Salmonella* (405 nm laser line, gain: 110,
emission filter: 450/50 nm), GFP-labeled effector proteins (SteA-GFP<sub>comp</sub> or SteA-GFP) (488 nm laser line, gain: 110, emission filter: 525/50 nm), and mCherry-labeled-GalT, Rab6, or Rab7 (561 nm laser line, gain: 90, emission filter: 600/50 nm) simultaneously. The motorized XY stage enabled us to select and store the locations of multiple fields of view within the 35 mm dish in order to follow the fates of infected cells in multiple fields of view over the course of the experiment. The Z drive was used to create z stacks that encompassed the entirety of the cells within each field of view, thus ensuring the complete detection of any bacteria present. All fields of view were imaged with a pixel dwell time of 2 µs. To maximize the XY resolution of SteA-GFP<sub>comp</sub> or PipB2-GFP<sub>comp</sub> localization, an additional optical zoom of 1.2x was used. Red and green channels and Z planes for all fields of view were collected every 15 minutes while the blue channel was collected every hour to minimize the risk of phototoxicity. Infected cells were placed in an environmental chamber (Pathology Devices) which allowed cells to remain at 37°C with 5% CO<sub>2</sub> and 70% humidity throughout the experiment. Infections were imaged from 2-17 hours post infection. After each imaging experiment, each field of view was split into an individual file and z stacks were flattened using the Maximum Intensity Projection algorithm within the Nikon Elements software platform. Each field of view was exported as a series of Tiff files separated into each channel for further analysis in the bioimaging analysis software ICY (107).

*ImageJ analysis of PipB2-GFP<sub>comp</sub> localization in wt and ΔsteA infections.*

To quantify PipB2-GFP<sub>comp</sub> localization at the cell periphery versus on the vacuole in wt and ΔsteA infections we compared the intensity and area of PipB2-GFP<sub>comp</sub> pixels on the vacuole to those within 10 µm of the cell periphery. Briefly, the PipB2-GFP<sub>comp</sub> channel for each cell was opened in ImageJ and thresholded to mask PipB2-GFP<sub>comp</sub> localized to tubules. Regions of interest (ROIs) were drawn around the remaining PipB2-GFP<sub>comp</sub> fluorescence localized to the vacuole and any fluorescence within 10 µm of the cell periphery. The “Analyze Particles” command was then used to determine the minimum, maximum, and mean
fluorescence intensity and size of each ROI. With these parameters we quantified the average ratio of peripheral PipB2-GFP<sub>comp</sub> intensity to vacuole PipB2-GFP<sub>comp</sub> intensity, as well as the ratio of total PipB2 intensity at the periphery compared to vacuolar PipB2-GFP<sub>comp</sub> intensity. We also tabulated by visual inspection the number of PipB2-GFP<sub>comp</sub> ROIs at the cell periphery at different time points. These results were used to develop a quantitative description of wt PipB2-GFP<sub>comp</sub> localization and to determine the effects of ΔsteA on this localization.

**CellProfiler analysis of PipB2-GFP<sub>comp</sub> localization in wt and ΔsteA infections.**

To automate the above analysis method, we used CellProfiler (108, 109) to find and differentiate between vacuolar and peripheral PipB2-GFP<sub>comp</sub>. For this assay, we developed a pipeline within CellProfiler to load and threshold both the bacterial and PipB2-GFP<sub>comp</sub> channels for each cell and generate respective bacterial or PipB2-GFP<sub>comp</sub> ROIs within each channel. CellProfiler then defined PipB2-GFP<sub>comp</sub> ROIs that colocalized with bacterial ROIs as “vacuolar” PipB2-GFP<sub>comp</sub> and PipB2-GFP<sub>comp</sub> ROIs that did not colocalize with bacterial ROIs as “peripheral” PipB2-GFP<sub>comp</sub>. CellProfiler exported the size and max, mean, and min intensities of all PipB2-GFP<sub>comp</sub> ROIs in separate Excel files, depending on their qualification as “vacuolar” or “peripheral.” Similarly to the results gained from the ImageJ protocol, these results could be used to quantitatively measure changes in PipB2-GFP<sub>comp</sub> localization in the absence of SteA. However, this process was completely automated and could be used to quickly analyze an entire set of data.

**ICY analysis of PipB2-GFP<sub>comp</sub> localization in wt and ΔsteA infections.**

To measure PipB2-GFP<sub>comp</sub> distribution across each cell, the PipB2-GFP<sub>comp</sub> channel for each cell was imported into ICY where noise was removed using the Thresholder plugin. Spot Detector was used to find and analyze all PipB2-GFP<sub>comp</sub> ROIs across the cell. With the Spot Detector plugin, ICY automatically saves location, size, and max, mean, and min intensity of each ROI it detects in an Excel spreadsheet for each cell. Similarly to CellProfiler, ICY can analyze an entire batch of images in a completely automated fashion.
Analysis of GalT-oxCerulean bleedthrough.

To quantify the amount of GalT-oxCerulean bleedthrough into the GFP channel, HeLa cells were transiently transfected with 1 µg GalT-OxCerulean DNA using the TransIT LT1 system (MirusBio). Twenty-four to forty-eight hours post transfection cells were imaged on a Nikon widefield microscope. oxCerulean-labeled GalT was imaged in the ox-Cerulean channel (excitation: 434/17 nm, emission: 474/23 nm, dichroic: 458) as well as the GFP channel (excitation: 472/30 nm, emission: 520/40 nm, dichroic: 490 nm) to define the total oxCerulean signal and the bleedthrough into the GFP channel. Multiple cells were imaged for this experiment. Bleedthrough was calculated by subtracting the background from each channel, and then dividing the intensity in the GFP channel by the intensity in the oxCerulean channel.

Analysis of Golgi tubulation in wt and ΔsteA infections.

To quantify changes in Golgi tubulation for wt and ΔsteA infections, we used ICY. Briefly, the GalT-mCherry channel for each cell was imported into ICY and Spot Detector plugin was used to determine ROIs for GalT-mCherry. These ROIs were imported into the Anisotropy Profiler plugin, which calculated the ratio of the area of each ROI to the length of each ROI raised to the fourth power. In this way, elongated or tubular ROIs had much smaller ratios compared to round ROIs. ICY then determine the fraction of total ROIs with ratios less than 0.01 to yield the fraction of tubulated Golgi per cell. This method is called the Modified Morton Criterion. In this way, we could quantitatively compare Golgi tubulation between wt and ΔsteA infected cells at different time points post infection.

3.4 RESULTS

Establishment of the Salmonella containing vacuole is important for Salmonella replication. As effector proteins and host cell proteins are recruited to the SCV to promote maturation, defining the mechanism behind their recruitment and their localization throughout infection is crucial to understanding and potentially perturbing the vacuole maturation process. Here we discuss the development of a quantitative approach for tracking and quantifying host
cell and effector protein localization to the *Salmonella* containing vacuole over the course of infection.

**Quantification of effector protein localization during *Salmonella* infection.**

In order to track effector protein localization over the course of infection, we used the previously established Split GFP system (83) to label the effector protein PipB2 with GFP during infection of live epithelial cells. PipB2 is known to localize to the SCV and tubules (79) and is responsible for recruiting kinesin-1 to the vacuole (81). To image PipB2 using the Split GFP system, we transiently transfected HeLa cells with a plasmid that encodes for GFP β-strands 1-10 (GFP1-10). After 24-48 hours, we infected these cells with an mCherry-expressing *Salmonella enterica* serovar Typhimurium strain that also expresses PipB2 tagged with GFP β-strand 11 (GFP11) under its native promoter (PipB2-GFP11 mCherry *Salmonella*). Upon translocation of PipB2-GFP11 into the host cell, GFP11 complements with GFP1-10 to become fully fluorescent GFPcomp (83, 142). This approach enabled us to monitor bacteria and PipB2 localization over time. We chose to examine PipB2-GFPcomp localization at early and late time points post infection (6-10 hours and 17-19 hours post infection, respectively) to determine whether PipB2 localization changed over the course of infection. We found that PipB2-GFPcomp localized to the SCV, tubules, and in small clusters at the cell periphery at both early and late time points (Figure 3.1). These results are in agreement with previous results using immunofluorescence and the Split GFP system at 10-12 hours post infection (79, 83).

We next examined PipB2-GFPcomp localization in cells infected with a *Salmonella* strain containing a deletion mutant of another effector protein in order to determine the role of other effector proteins in controlling localization of PipB2-GFPcomp to the SCV. These experiments focused on the effector protein SteA, which also localizes to the SCV upon translocation (83) and has been hypothesized to influence membrane dynamics on the SCV as well as manipulate molecular motors such as dynein or kinesin (84). PipB2 also localizes to the SCV and tubules and has been found to recruit kinesin-1 to the SCV. Therefore, we examined the effect of ΔsteA
Figure 3.1. Representative fluorescence images of PipB2-GFP11 mCherry Salmonella and PipB2-GFP<sub>comp</sub> localization at 8.8 and 17 hours post infection (Salmonella in red, PipB2-GFP<sub>comp</sub> in green, and colocalization in yellow). PipB2-GFP<sub>comp</sub> localizes to the SCV, tubules, and clusters around the cell periphery during infection.
on PipB2 localization during infection to determine potential interactions between SteA and PipB2. The ΔsteA mutant was generated in the PipB2-GFP11 mCherry Salmonella strain using a previously described recombination technique (118, 119). HeLa cells were transiently transfected and infected as described above with both the wild type (wt) and the ΔsteA mutant strain and infections were imaged at early (Figure 3.2) and late (Figure 3.3) time points. PipB2-GFP<sub>comp</sub> localization showed heterogeneity upon infection with the ΔsteA strain. In some ΔsteA-infected cells, PipB2-GFP<sub>comp</sub> localization was similar to that in wt infections (Figures 3.2A and 3.3A). However, in other ΔsteA-infected cells, PipB2-GFP<sub>comp</sub> appeared to localize more to the clusters at the periphery of the cell than to the SCV (Figures 3.2B and 3.3B).

In order to compare PipB2-GFP<sub>comp</sub> localization upon infection with wt or ΔsteA Salmonella more quantitatively, we tried two approaches to more accurately define the cell periphery so that we could compare PipB2-GFP<sub>comp</sub> signal associated with the SCV versus the periphery. First we transiently transfected a plasmid encoding β-actin tagged with mCherry. Because actin localizes throughout the cell and accumulates at the SCV (73, 143), we reasoned that this method would allow for visualization of both the SCV and cell periphery. However, we found that actin labeling allowed for cell periphery visualization (Figure 3.4) but it did not always allow for resolution of the SCV. Because the transfected β-actin-mCherry was not sensitive enough to resolve the SCV in all infected cells, we decided to fix infected cells and stain them with phalloidin, a toxin that binds tightly to actin (144). Cells were infected as described above, fixed at 8 or 21 hours post infection and stained with both AlexaFluor 594-labeled phalloidin (to label actin and the cell periphery) and DAPI (to label bacteria and nuclei). Although this method proved successful in labeling bacteria, cell peripheries, and PipB2-GFP<sub>comp</sub> (Figure 3.5), the PipB2-GFP<sub>comp</sub> signal was very dim in fixed cells and was only visible at 21 hours post infection. In addition, we were interested in observing changes in PipB2-GFP<sub>comp</sub> dynamics and localization over time, which isn’t permitted in fixed samples. Results from these methods highlighted the need to simultaneously label bacteria, the cell periphery, and effector proteins.
Figure 3.2. Comparison of PipB2-GFP$_{\text{comp}}$ localization upon infection with wt or ΔsteA Salmonella at early time points post infection. Representative fluorescence images of PipB2-GFP11 mCherry Salmonella (A and B, top panels) or ΔsteA PipB2-GFP11 mCherry Salmonella (A and B, bottom panels) at 6 hours post infection (Salmonella in red, PipB2-GFP$_{\text{comp}}$ in green, and colocalization in yellow). PipB2-GFP$_{\text{comp}}$ localization during ΔsteA infections is heterogeneous with a subset of ΔsteA infections causing similar localization compared to wt (A) and a subset causing different localization compared to wt (B).
Figure 3.3. Comparison of PipB2-GFP\textsubscript{comp} localization upon infection with wt or ΔsteA *Salmonella* at late time points post infection. Representative fluorescence images of PipB2-GFP\textsubscript{11 mCherry Salmonella} (A and B, top panels) or ΔsteA PipB2-GFP\textsubscript{11 mCherry Salmonella} (A and B, bottom panels) at 17 hours post infection (*Salmonella* in red, PipB2-GFP\textsubscript{comp} in green, and colocalization in yellow). PipB2-GFP\textsubscript{comp} localization during ΔsteA infections is heterogeneous with a subset of ΔsteA infections causing similar localization compared to wt (A) and a subset causing different localization compared to wt (B).
**Figure 3.4.** Comparison of PipB2-GFP<sub>comp</sub> localization upon infection with wt or ΔsteA *Salmonella* using β-actin-mCherry to label cell periphery and the SCV. Representative fluorescence images of PipB2-GFP11 mCherry *Salmonella* (A and B, top panels) or ΔsteA PipB2-GFP11 mCherry *Salmonella* (A and B, bottom panels) at 17 hours post infection (β-actin-mCherry in red, PipB2-GFP<sub>comp</sub> in green, and colocalization in yellow). Use of β-actin-mCherry allowed for labeling of the cell periphery. In a subset of infections, β-actin-mCherry also labeled the SCV, as seen by a colocalization of the SCV-like buildup of PipB2-GFP<sub>comp</sub> (labeled SCV) and actin (A, black arrows) but in others it did not, as seen by no colocalization between the SCV-like build up of PipB2-GFP<sub>comp</sub> (labeled SCV) and actin (B).
Figure 3.5. Representative fluorescence images of a HeLa cell infected with PipB2-GFP11 mCherry Salmonella and then fixed and stained for phalloidin (to label the cell periphery) and DAPI (to label nuclei and Salmonella) at 21 hours post infection. Phalloidin staining is in red, PipB2-GFP$_{\text{comp}}$ is in green, and nuclei and Salmonella (SCVs marked by black arrows) are in blue.

during these experiments to allow for quantification of effector protein localization over the course of infection.

Despite the drawbacks of the abovementioned experiments, we used data retrieved from preliminary experiments (no cell periphery labeling) and transient transfection of β-actin-mCherry (no SCV labeling) to design analysis methods to quantify differences in PipB2-GFP$_{\text{comp}}$ localization in wt or ΔsteA infections. We wanted to be able to quantitatively determine the amount of PipB2-GFP$_{\text{comp}}$ localized to the SCV or in clusters along the cell periphery so we decided to compare the abilities of three different image analysis programs, ImageJ (NIH), CellProfiler (Broad Institute) (108, 109), and ICY (Pasteur Institute) (107), in yielding quantitative information regarding PipB2-GFP$_{\text{comp}}$ localization (Table 3.2, Figure 3.6). For ImageJ, we developed an analysis method that could determine the size and mean, minimum, and
<table>
<thead>
<tr>
<th>Image Analysis program</th>
<th>Open Source?</th>
<th>Allows for user-defined plugins?</th>
<th>Allows for pipeline workflows?</th>
<th>Documentation &amp; Ease of use</th>
<th>Can open Nikon microscopy (.nd2)?</th>
<th>Interfaces with ImageJ?</th>
<th>High Throughput Analysis?</th>
<th>Export of Data/Results</th>
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<tr>
<td>ImageJ/Fiji (NIH)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Yes</td>
<td>Yes, Script language: ImageJ Macro Language</td>
<td>Requires user-defined macros</td>
<td>Hard to find documentation for most built in or user-defined macros. &amp; Hard to use for data analysis without proper documentation.</td>
<td>Has a plugin for Windows computers. Cannot open .nd2 files on Macs.</td>
<td>---</td>
<td>Requires user-defined macros</td>
<td>Macros can export data to Excel and save ROIs on images.</td>
</tr>
<tr>
<td>CellProfiler (Broad Institute)&lt;sup&gt;5&lt;/sup&gt;, refs 107-108</td>
<td>Yes</td>
<td>Yes, Script language: Python</td>
<td>Software operates with a pipeline model for loading, analyzing and exporting data.</td>
<td>All modules have online and in-program documentation. Has an online Q&amp;A forum and a downloadable manual. &amp; 1) Very easy to learn how to use. Example datasets and pipelines available. 2) Hard to customize some modules.</td>
<td>&quot;RunImageJ&quot; module opens a file in ImageJ and processes it with any command, script, or macro</td>
<td>Yes</td>
<td></td>
<td>1) &quot;ExportTo Spreadsheet&quot; module can export data to Excel. 2) &quot;SaveImages&quot; module can save images at any point in the analysis process.</td>
</tr>
<tr>
<td>ICY (Pasteur Institute)&lt;sup&gt;6&lt;/sup&gt;, ref 106</td>
<td>Yes</td>
<td>Yes, Script language: Javascript, Python</td>
<td>1) Plugins can upload data into the &quot;Swimming Pool&quot; so that other Plugins have access to it. 2) Protocols allow users to create a data analysis pipeline.</td>
<td>Most plugins, protocols, and scripts have online documentation and examples. Has an online community to answer questions or create personalized scripts as needed. &amp; The &quot;Photoshop of image analysis&quot; GUI looks similar to Adobe Photoshop and treats images in a similar fashion (i.e. ROIs, detections, and tracks are displayed as &quot;layers&quot; on an image).</td>
<td>Yes, preserves metadata</td>
<td>ICY access directly within the ICY GUI.</td>
<td>Yes, using Batch Plugins, Protocols or scripts</td>
<td>1) Many plugins export data to Excel 2) Many plugins save results/ROIs for each image in a &quot;save&quot; folder within the image folder 3) Allows for a snapshot of any image to be taken and saved.</td>
</tr>
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<sup>3</sup>imagej.nih.gov/ij, <sup>5</sup>cellprofiler.org, <sup>6</sup>icy.bioimageanalysis.org
**Figure 3.6. A and B**

(A) Image showing the localization of PipB2-GFPcomp signal in wild-type (wt) and ΔsteA strains.

(B) Graphs showing the average intensity of SCV PipB2-GFPcomp signal and the average ratio of peripheral PipB2-GFPcomp signal to SCV PipB2-GFPcomp signal per cluster in early (8-10 hrs p.i.) and late (17-19 hrs p.i.) time points for wt and ΔsteA strains.
Figure 3.6. Comparison of quantitative analysis methods used to examine PipB2-GFP\textsubscript{comp} localization to the cell periphery versus the SCV after infection with wt or ΔsteA Salmonella. (A) Representative images for PipB2-GFP\textsubscript{comp} localization analysis in ImageJ. ROIs measured in ImageJ are outlined in blue and numbered. The SCV is labeled as number 1. (B) PipB2-GFP\textsubscript{comp} localization to the periphery of the cell versus the SCV at early and late time points post infection. Top panel: absolute values of SCV or cluster mean intensities for wt and ΔsteA infections at early (wt n= 37 clusters and 19 SCVs, ΔsteA n= 21 clusters and 12 SCVs), and late time points (wt n= 178 clusters and 20 SCVs, ΔsteA n= 93 clusters and 15 SCVs). Bottom panel: the average of the ratio of PipB2-GFP\textsubscript{comp} mean intensity for each cluster compared to its respective SCV. Early time points (wt n= 41 (four SCVs had no clusters), ΔsteA n= 21), late time points (wt n= 180 (one SCV had no clusters), ΔsteA n= 93). Values are mean ± the standard error of the mean. ** represents a p value <0.006, * represents a p value= 0.015. n.s. represents a p value of 0.06. All p values were calculated using a one-way ANOVA test. (C) Representative images for PipB2-GFP\textsubscript{comp} localization analysis in CellProfiler and in ICY. CellProfiler could automatically distinguish between PipB2-GFP\textsubscript{comp} localized to SCVs (outlined in blue) and clusters (outlined in green) on the basis of colocalization between the bacterial and PipB2-GFP\textsubscript{comp} channels for each cell. ICY could automatically detect all PipB2-GFP\textsubscript{comp} localization that was not saturated (pixel values= 65536 for a 16-bit image, = 255 for an 8-bit image).
maximum intensities for all PipB2-GFP_{comp} clusters and the SCV (Figure 3.6A). Using this method, we compared the average ratio of PipB2-GFP_{comp} localization for each cluster (as measured by mean intensity of that cluster) to the PipB2-GFP_{comp} localization to the SCV for each cell (Figure 3.6B). We found that at early time points, PipB2-GFP_{comp} was more likely to be localized to a cluster than to the SCV upon infection with the ΔsteA strain. At late time points, PipB2-GFP_{comp} localization was the same in both wt and ΔsteA infections. These results suggest that at early time points, SteA may be involved directing PipB2 localization to the SCV. However, this analysis method was neither automated nor objective (as the user had to set thresholds and determine which PipB2-GFP_{comp} clusters were SCVs by visual inspection). Because single cell assays often require analysis of >100 cells (108), we wanted to design an assay that would allow for quick, objective analysis of large data sets.

CellProfiler and ICY are bioimaging analysis programs developed to allow users to design objective analysis methods for imaging data in an automated fashion (107, 108). To determine each program's ease of use and its ability to quickly and objectively analyze complex datasets, we developed automated methods in both CellProfiler and ICY to analyze PipB2-GFP_{comp} localization during wt and ΔsteA infections. With CellProfiler, we used a pipeline method that detected both bacteria and PipB2-GFP_{comp} within a cell, automatically defined SCVs versus clusters, and determined the size, mean, minimum, and maximum intensities for all PipB2-GFP_{comp} regions (SCVs or clusters) found (Figure 3.6C). CellProfiler could analyze an entire data set and compile the results in Excel spreadsheets. With ICY, we used a plugin to detect all PipB2-GFP_{comp} clusters in a cell. ICY returned the size and mean, minimum, and maximum intensity for each cluster detected (Figure 3.6C). Like CellProfiler, ICY could analyze an entire data set and compile results into Excel spreadsheets although ICY created a new spreadsheet for each cell. However, the plugin in ICY was unable to detect the entirety of large objects, such as the SCV, due to the variance in pixel intensities (see Figure 3.6C) so we were unable to easily design a protocol in ICY that successfully differentiated between the SCV and
PipB2-GFPcomp clusters. Thus, while both ICY and CellProfiler allowed for automated and objective analysis of PipB2-GFPcomp localization in wt or ΔsteA infected cells, CellProfiler may be easier to use for quantifying PipB2-GFPcomp localization over the course of infection.

**Quantification of host cell protein localization during Salmonella infection.**

Upon *Salmonella* infection, effector proteins mediate the trafficking and maturation of the SCV in part by recruiting host cell proteins to the vacuole (5, 14, 15, 19). The recruitment process is dynamic as different host cell proteins are recruited at different times post infection, depending on their role in SCV establishment (5, 115). During SCV trafficking and maturation, effector proteins that localize to the SCV are thought to recruit host cell proteins that allow for proper trafficking of the vacuole to its perinuclear location within the cell (29, 31), establishment of the internal environment of the vacuole (5, 42), and avoidance of the lysosomal degradation pathway (18, 69, 70, 115, 145). However, many host cell proteins that localize to the SCV and tubules emanating from the SCV have no known mechanism for recruitment. For example, host cell proteins known to reside in the trans-Golgi, such as Arl8b and GalT, have been seen to localize to the SCV and tubules (48, 83). While these results suggest that the trans-Golgi is being tubulated during infection, it is not known what effector proteins may play a role in causing this tubulation. The effector protein SteA localizes to the trans-Golgi upon transient transfection (Figure 3.7A and (90)) and translocated SteA localizes to these GalT-containing tubules upon infection (Figure 3.7B and (83)). These results suggest that SteA could be responsible for causing trans-Golgi (and thus GalT) tubulation. In order to determine whether SteA causes GalT tubules, we transiently transfected HeLa cells with a plasmid encoding for GalT fused to the mCherry fluorescent protein and a plasmid that encodes for GFP β-strands 1-10. After 24-48 hours, cells were infected with either SteA-GFP11 or ΔsteA PipB2-GFP11 mCherry *Salmonella*. Because both GalT and the *Salmonella* were labeled with mCherry, we used the presence of PipB2-GFPcomp or SteA-GFPcomp to define infected cells. These infections were imaged at either early or late time points post infection (8-10 hours and 17-19 hours post
infection, respectively) to monitor changes in GalT-mCherry localization over the course of infection. We found that GalT-mCherry localized to tubules in some wt and ΔsteA infections (Figure 3.8A) but not in others (Figure 3.8B). These results suggest that SteA is not required for formation of GalT-mCherry tubules.

Although GalT-mCherry tubulation was not abolished during infection with ΔsteA Salmonella, we determined whether ΔsteA might cause a decrease in the number of cells with GalT-mCherry tubules during infection. As effector proteins often work together in concert (13, 14), the knockout of one effector protein may not completely disrupt a common phenotype. Using our preliminary data, we scored the GalT-mCherry localization in each cell by visual inspection and quantified the fraction of infected cells with tubules containing GalT-mCherry. At both early and late time points, GalT-mCherry was less likely to be localized to tubules during ΔsteA infections (Figure 3.8C). These preliminary results were not statistically significant, possibly due to the fact that these data were analyzed by eye. In addition, we only counted cells as infected if they contained PipB2-GFP_comp or SteA-GFP_comp signal. However, the transient transfection method we used to induce the expression of GFP1-10 in the cells did not ensure that all cells had taken up the GFP1-10 DNA. Thus, some cells could have been infected but not expressing GFP1-10 and therefore would not have been counted.

In order to increase the number of cells counted for each condition, we reasoned that being able to visualize bacteria, GalT, and effector proteins simultaneously would allow us to image a larger proportion of cells within a single experiment. We tried two different methods to image both bacteria and GalT-mCherry simultaneously over the course of infection. One method employed a live-cell Hoechst 33342 stain, which is added to infected cells immediately prior to imaging, to label Salmonella inside infected cells. However, although Hoechst dye is typically used to stain fixed bacteria, this dye failed to label live Salmonella within host cells (Figure 3.9A). Another method involved using GalT tagged with the oxCerulean fluorescent protein instead of mCherry. The oxCerulean fluorescent protein is supposed to be excellent for
Figure 3.7. SteA localization upon transient transfection or translocation. (A) Representative fluorescence images of GalT-mCherry and SteA-GFP transiently transfected into HeLa cells (GalT-mCherry in red, SteA-GFP in green, and colocalization in yellow). SteA-GFP and GalT-mCherry colocalize with a Pearson’s Correlation Coefficient of 0.69 (n= 23 cells). (B) Representative fluorescence images of GalT-mCherry and translocated SteA-GFP\textsubscript{comp} at 21.5 hours post infection (GalT-mCherry in red, SteA-GFP\textsubscript{comp} in green, and colocalization in yellow). In this image, SteA-GFP\textsubscript{comp} and GalT-mCherry colocalize with a Pearson’s Correlation Coefficient of 0.59.
Figure 3.8 A and B
Figure 3.8. Comparison of GalT-mCherry localization upon infection with wt or ΔsteA Salmonella. Representative fluorescence images of SteA-GFP11 mCherry Salmonella (A and B, top panels) or ΔsteA PipB2-GFP11 mCherry Salmonella (A and B, bottom panels) at 17.5 hours post infection (for SteA-GFP11 mCherry Salmonella infections) or 22 hours post infection (for ΔsteA PipB2-GFP11 mCherry Salmonella infections). Salmonella is in red, PipB2-GFPcomp/SteA-GFPcomp is in green, and colocalization in yellow. GalT-mCherry localizes to tubules in some wt or ΔsteA infections (A, black arrows) but not in others (B). (C) The fraction of cells containing GalT-mCherry localization to tubules was tabulated by visual inspection for each cell. Values are mean ± the standard error of the mean (Early time points: wt n= 38 cells, ΔsteA n= 39 cells; Late time points: wt n= 58 cells, ΔsteA n= 63 cells). These data are not statistically significant as calculated by a one-way ANOVA test.
Figure 3.9. Potential new methods to label *Salmonella* or GalT with an alternate fluorescent marker. (A) Representative fluorescence images of mCherry *Salmonella* and a live-cell Hoechst 33342 dye at 8.4 and 20.3 hours post infection (*Salmonella* in red, Hoechst dye is in blue, and merge is purple). The live-cell Hoechst dye failed to label live bacteria within a host cell. (B) Representative fluorescence images of mCherry *Salmonella* and GalT-oxCerulean at 7.3 hours post infection (*Salmonella* in red, GalT-oxCerulean bleedthrough into the GFP channel in green, GalT-oxCerulean in blue, and colocalization in white). oxCerulean had 14% bleedthrough into the GFP channel (n= 17 cells).
tagging Golgi or ER host cell proteins, as it is less likely to misfold and aggregate within these organelles (E. Snapp, personal communication). While this method resolved both mCherry-labeled bacteria and oxCerulean-labeled GalT, the spectral overlap between GFP and oxCerulean prevented dual labeling of GalT-oxCerulean and Split GFP-tagged effector proteins during infection (Figure 3.9B). Finally, because our preliminary results (Figure 3.8C) show such subtle changes in GalT-mCherry tubulation upon infection with ΔsteA bacteria, we wanted to design a long term imaging assay that would allow monitoring of phenotype evolution over time. We wondered if ΔsteA would cause a change in the dynamics of GalT-mCherry tubule formation and this question could only be answered with a long term assay. This assay, which allows us to resolve both bacteria and GalT localization as well as study GalT-mCherry dynamics over the course of infection, is discussed below.

**Quantification effector and host cell protein localization using a long-term imaging assay.**

In order to monitor the evolution of *Salmonella*, GalT, and GFP-labeled effector protein localization over the course of *Salmonella* infection, we developed an imaging assay that allowed for long term imaging and a three-color system in which *Salmonella*, GalT, and effector proteins could simultaneously be monitored. Because there is not an alternative color for the Split FP system, Split GFP-labeled effector proteins cannot be changed to a different color. As described above, GalT-mCherry was more compatible with a GFP-labeled effector or host cell proteins compared to GalT-oxCerulean. Therefore, we decided to create an mTagBFP2 strain of *Salmonella*. The mTagBFP2 fluorescent protein was chosen because it is blue shifted from oxCerulean, is much brighter than oxCerulean, and is more photostable (146-148). After making a suite of *S. enterica* serovar Typhimurium strains that constitutively express mTagBFP2 (Table 3.1), we designed a long term imaging assay to monitor infections from 2 to >17 hours post infection by simultaneously capturing mCherry, mTagBFP2, and GFP fluorescence as well as a brightfield image (to monitor the cell periphery). mCherry, GFP, and
Figure 3.10. Validation of three-color long-term imaging experiment. (A) Representative fluorescence images of Rab6-mCherry, SteA-GFP<sub>comp</sub>, and SteA-GFP<sub>11 mTagBFP2</sub> Salmonella at 21 hours post infection (Rab6-mCherry in red, SteA-GFP<sub>comp</sub> in green, and Salmonella in blue). This three-color method has little to no bleedthrough for each channel. (B) Representative fluorescence images of Rab6-mCherry, SteA-GFP<sub>comp</sub>, and SteA-GFP<sub>11 mTagBFP2</sub> Salmonella at 9.3, 11.8, 14.3, and 16.8 hours post infection. All three colors are visible at all time points post infection.
brightfield images were captured every 15 minutes, while the mTagBFP2 image was only captured every hour to minimize exposure to the 405 nm laser which can induce phototoxicity and cell death. Preliminary experiments with mTagBFP2 *Salmonella* show little to no bleedthrough of mTagBFP2 fluorescence into the GFP channel and allow for resolution of mCherry-labeled host cell proteins (such as GalT and another Golgi protein Rab6, both of which were used in preliminary experiments for this assay), Split GFP-labeled effector proteins, and *Salmonella* at all time points post infection (Figure 3.10). Using this assay, we designed a new analysis method to objectively quantify GalT-mCherry tubulation and to further examine SteA localization over the course of infection.

Preliminary results from our original GalT-mCherry imaging assay (Figure 3.8C) suggested that knockout of SteA might decrease the fraction of cells in which GalT-mCherry was tubulated, but the effects observed weren’t statistically significant. To investigate this finding further, the long-term imaging assay described above was used to track GalT-mCherry tubulation over the course of infection (Figure 3.11A). To analyze these data we developed a method in ICY to quantify GalT-mCherry tubulation using a plugin that can determine fluorescence signal tubulation in an image. Briefly, this plugin finds all GalT-mCherry intensities (Figure 3.11B and C) and determines the fraction of GalT-mCherry signal that is elongated compared to the total GalT-mCherry signal (149). We found that the formation of GalT-mCherry tubules is dynamic over the course of infection (Figure 3.11D). Using this method, we will be able determine the effects, if any, on GalT-mCherry tubulation dynamics upon infection with ΔsteA *Salmonella*.

Previously we found that SteA-GFPcomp and GalT-mCherry colocalize during infection (Figure 3.7B and (83)). To determine if SteA colocalization with the Golgi is important for its role in infection, we wanted to monitor SteA-GFPcomp localization upon infection with a *Salmonella* strain containing a deletion of the effector protein SseG. SseG has been shown to play a role in establishing the SCV and ΔsseG *Salmonella* infections cause *Salmonella* to be
Figure 3.11 A, B, and C
Figure 3.11. Quantification of Golgi tabulation during infection (A) Representative fluorescence images of GalT-mCherry in cells at 4.8, 8.3, 13.8, and 19.5 hours post infection. (B) The images from (A) with ROIs drawn in green around the two cells to be analyzed using this method. The cell on the left is infected while the cell on the right is not. (C) GalT-mCherry intensities that are not saturated (pixel values = 65536 for a 16-bit image, = 255 for an 8-bit image) detected by ICY for each cell within an ROI at all four time points. (D) Fraction of GalT-mCherry signal localized to tubules for both the infected (dark gray) and uninfected (light gray) cells at all time points. A detection is deemed “tubulated” if its area divided by its length to the fourth power is less than 0.01. GalT-mCherry in uninfected cells is not tubulated, whereas in infected cells, GalT-mCherry is tubulated and the degree of tubulation changes over the course of infection.
localized away from the Golgi and dispersed throughout the cell (50, 51, 71, 72, 117). Intriguingly, we found that SteA remained localized with bacteria, even when the bacteria were spread out across the cell (Figure 3.12). These results suggest that although SteA may interact with the Golgi during infection, it must have additional binding partners within the cell that allow it to perform its role during Salmonella infection. However, this assay will need to be repeated using GaIT-mCherry to label the trans-Golgi to further confirm that SteA and bacteria are indeed localized away from the Golgi upon dispersal. Although we only have preliminary data and methods from this assay, it promises to provide us with data sets that we can analyze using the analysis methods developed above. Therefore our long-term imaging assay provides us with a multifunctional tool to answer a variety of questions regarding effector and host cell protein vacuolar localization phenotypes during Salmonella infection.

3.5 DISCUSSION

Infection with Salmonella enterica serovar Typhimurium causes reorganization of host cell proteins to the SCV. While this organization is mainly orchestrated by Salmonella effector proteins, little is known about the mechanisms by which these events happen. One method for studying the role of effector proteins in SCV establishment and maturation is to monitor changes

![Figure 3.12. SteA-GFP comp localization upon knockout of the effector protein SseG. Representative fluorescence images of SteA-GFP comp and ΔsseG SteA-GFP11 mTagBFP2 Salmonella at 5, 8, 12, 16, and 18 hours post infection (SteA-GFP comp in green, Salmonella in red, and colocalization in yellow). SteA-GFP comp continues to colocalize with ΔsseG Salmonella even when they are dispersed throughout the cell, regardless of the time post infection (4.7 and 8.4 hours post infection in the top panel and 8.6, 12.3, and 16.1 hours post infection in the bottom panel).](image-url)
in host cell or effector protein localization to the SCV when an effector protein in question is knocked out. Because many studies have shown a parallel between recruitment to the SCV and function in *Salmonella* infection, proteins known to localize to the SCV often play a role in SCV trafficking or maturation and establishment of *Salmonella* infection (59, 67, 73, 80, 141). However, many proteins are recruited to the SCV through an unknown mechanism upon infection. For this study, we wanted to determine potential roles of the effector protein SteA in recruiting other effector proteins and/or host cell proteins to the SCV during the vacuole maturation process. In pursuing this question, it became apparent that it was necessary to design a method to simultaneously monitor the localization of an effector protein, a host cell protein, and bacteria over the course of infection in single cells. In addition, this imaging method also required quantitative analysis methods to accurately distinguish subtle changes in localization during infection with mutant strains of *Salmonella*.

To determine which parameters we wanted to label with our new long-term imaging assay, we monitored the localization of the effector protein PipB2 and the trans-Golgi marker GalT upon infection with either wt or ΔsteA *Salmonella*. We found that although PipB2-GFP_comp and GalT-mCherry localization phenotypes were not completely abolished under these infection conditions, both proteins showed some possible localization defects compared to wt *Salmonella* infection. PipB2-GFP_comp localization appeared to be clustered more towards the cell periphery at early time points (Figures 3.2 and 3.6B) whereas GalT-mCherry was not tubulated as frequently (Figure 3.8C) during ΔsteA infections. Both changes in phenotypes suggest an intriguing possible role for SteA. PipB2 is known to recruit the molecular motor kinesin-1 to the SCV and interact with another effector protein SifA to activate kinesin-1 and cause tubule formation (55, 81). Upon infection with a ΔpipB2 *Salmonella* strain, tubules show reduced extension towards the cell periphery (80). However, upon overexpression of PipB2, tubule components are seen to cluster on the cell periphery (80). Because we see similar phenotypes upon the knockout of SteA (reduced formation of GalT-mCherry tubules and increased PipB2
clusters on the cell periphery) it is possible that SteA also plays a role in tubule formation. Indeed, SteA has also been hypothesized to either activate a kinesin or inhibit a dynein over the course of infection (84). These results further support the idea that SteA plays a role in tubulation. Furthermore, because transiently transfected SteA-GFP localizes to the trans-Golgi (Figure 3.7A and (90)) and translocated SteA-GFP\textsubscript{comp} colocalizes with GalT-mCherry during infection (Figure 3.7B and (83)), SteA may have a binding partner within the trans-Golgi. However, we see SteA-GFP\textsubscript{comp} remain localized to bacteria even when they are spread out across the cell (Figure 3.12). These results suggest that SteA may have alternative binding partners within the SCV during infection. Although these possibilities are intriguing, all of these results must first be confirmed using the quantitative methods developed throughout this paper before these possible roles can be investigated further.

Throughout the experiments performed in this paper, we found the pressing need to develop both new imaging methods as well as analysis techniques that allowed for an objective and quantitative assay to distinguish subtle differences within infected cells. Recently, it has become apparent that *Salmonella* infections are incredibly heterogeneous. For example, within the same population of cells, *Salmonella* have been seen to escape from the SCV and replicate within the cytosol of some cells, while in other cells *Salmonella* solely replicate in the SCV (35-37). The observations presented in this Chapter have also been heterogeneous; while some of the ΔsteA infections showed significantly different PipB2-GFP\textsubscript{comp} and GalT-mCherry localization phenotypes from wt infection, others showed no difference (Figures 3.2, 3.3 and 3.8). This heterogeneity required us to develop a long-term imaging method. Over the course of experiments described here, we found that single snapshots of phenotypes at discrete time points post infection did not provide information about the dynamics behind those phenotypes. For example, fixed time point images don’t allow us to discern whether PipB2-GFP\textsubscript{comp} clusters form through PipB2-GFP\textsubscript{comp} tubules or whether entire PipB2-GFP\textsubscript{comp} clusters migrate from the SCV to the cell periphery? Being able to monitor and quantify the phenotype dynamics may help
in pinpointing potential differences between PipB2-GFP\textsubscript{comp} and GalT-mCherry localization during $\Delta$steA infections. Finally, in order to perform rigorous statistical tests to compare data sets, we needed analysis methods that could objectively define each localization phenotype to have a method that could objectively find subtle differences between datasets. The analysis methods developed here in CellProfiler and ICY allow for automated detection of various parameters within images (GalT-mCherry localization to tubules and PipB2-GFP\textsubscript{comp} localization to the SCV versus clusters). Both of these programs allow for easy creation of new analysis pipelines and thus can be used to develop new quantitative methods for future imaging experiments. Immediate future experiments include quantification of GalT-mCherry tubulation and PipB2 localization in wt and $\Delta$steA infections. Other future experiments should include further characterization of the $\Delta$steA SCV by examining changes in localization of Rab6, which is also localized to the Golgi and known to form tubules upon disruption of the Golgi fission process, and Rab7, which is known to localize to the SCV and tubules during infection, upon infection with $\Delta$steA Salmonella. Another possible future experiment should involve examining changes in cell shape upon infection with wt and $\Delta$steA Salmonella because it is possible that the localization phenotypes we see in these experiments could be due to changes in cell shape during infection. Finally, further characterization of the $\Delta$sseG SCV should be performed in which GalT localization is monitored to determine if the dispersed bacteria continue to colocalize with the trans-Golgi. Because the imaging and analysis methods designed here can provide for a quick and objective assay that can be applied to all of these future directions, it should be fairly straightforward to determine the role SteA might play in helping to establish the SCV during Salmonella infection of epithelial cells.
Chapter 4: Future Directions

As emphasized throughout this thesis, *Salmonella* infection of host cells is a complex dynamic process. In order to preserve this complexity and maintain the ability to track how cellular perturbations evolve over time, I sought to develop methods that would allow us to probe the roles of specific *Salmonella* effector proteins in manipulating SCV integrity during the infection process. We developed assays that allowed us to monitor the effects of specific effector protein deletion mutants on: 1) processes that are dependent on SCV integrity, such as *Salmonella* replication in epithelial cells and *Salmonella* survival in macrophages (Chapter Two) and 2) the localization of both effector and host cell proteins important for SCV establishment and maintenance (Chapter Three). Using these methods, we determined new roles for the effector proteins SteA and SseG in promoting vacuole integrity. In addition, our results presented intriguing new questions about the role *Salmonella* effector proteins play in vacuole integrity and *Salmonella* infection.

In Chapter Two, we determined that both SteA and SseG played roles in promoting vacuole integrity. SseG seemed to be involved in manipulating membrane integrity and both effector proteins seemed to be important for SCV positioning during infection. These results also showed that effector proteins’ roles in infection are dependent on cell type as deletion of SteA caused only a subtle decrease in *Salmonella* replication during epithelial cell infections but a massive decrease in *Salmonella* survival during macrophage infections. The idea that effector protein function may vary according to cell type is intriguing and suggests that the sole use of epithelial or macrophage cell models to probe for effector protein functions during infection may not be sufficient in teasing apart the distinct roles for each effector protein. With this thought in mind, one future direction for this project is to study the effects of ΔsseG and ΔsseG/ΔsteA on survival within BMDM infections. The fact that Dextran leaks out of these SCVs suggests that these vacuoles are not properly formed and that these mutant *Salmonella* should not survive in
this assay. In addition, we are interested to see if the massive decrease in replication caused
by these two mutant strains in the epithelial infection model will be translated to a significant
decrease in survival in the BMDM model.

Further areas for study on this project include, but are not limited to, the mechanism
through which Salmonella sense and respond to the initial number of bacteria within the cell (i.e.
why are fold replication and replication rate inversely proportional to the initial number of
vacuolar bacteria?) and the mechanism through which SteA promotes vacuolar integrity.

In Chapter Three, we developed a new assay that will allow for us to directly monitor
effects of effector protein deletion on the localization of both effector and host cell proteins to the
SCV. We preliminarily determined that upon the deletion of SteA, the localization of PipB2-
GFP_{comp} and the host cell Golgi marker GalT-mCherry appeared altered (with PipB2-GFP_{comp}
localizing more to clusters at the cell periphery instead of on the SCV and GalT-mCherry being
less likely to be tubulated). Immediate future studies for this project involve quantifying changes
in PipB2-GFP_{comp} and GalT-mCherry localization upon infection with ΔsteA Salmonella using the
imaging and analysis methods discussed in this chapter. Furthermore, these assays can be
repeated with other host cell proteins, such as Rab6, which is Golgi-associated and is known to
localize to tubules upon disruption of the fission process of exocytic traffic (150) or Rab7, which
is known to be recruited in its GTP-bound state to the SCV and tubules (145, 151), to determine
localization changes upon the deletion of SteA. Results from these experiments should provide
insight into possible roles for SteA in manipulating vacuolar integrity.

In addition, we also determined that upon infection with ΔsseG Salmonella, SteA
encircled bacteria, even when they were not localized in the perinuclear region. As SteA is
predicted to be a cytoplasmic protein (90, 152), these results suggest that SteA has a binding
partner within the SCV membrane. Thus, future experiments could determine binding partners
for SteA during the context of infection (perhaps by immunoprecipitation assays followed by mass spectrometry).

In addition to providing insight into the roles of SteA and SseG in promoting vacuole integrity, the long-term imaging live cell assay and appropriate quantitative analysis methods described in Chapter Two proved to be more sensitive than traditional methods currently used to study the role of effector proteins in promoting SCV integrity during *Salmonella* infection. Thus, this method should be redesigned to study other pressing questions within the field that have remained nebulous due to a lack of sensitive quantitative assays. One example is studying effector protein interplay. If we had orthogonal imaging systems for multiple *Salmonella* effector proteins (i.e. if we used the Split GFP system to label one effector protein and another orthogonal tag to label another effector protein), then we could use the long-term imaging assay to track multiple effector proteins over the course of infection. This setup could allow for the differentiation of the functions between two tightly linked effector proteins such as SseG and SseF, which are hypothesized to work together throughout the infection process and have no known distinguishing features (51, 59, 71, 72). However, this long term imaging assay is not limited to studying the roles of effector proteins during infection, nor is it limited to studying *Salmonella* infections. With the right experimental design and analysis methods, this assay can be used to study interactions between any host and any pathogen over the course of infection, thus demonstrating the power of this technique.
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