Interference with lysosomal biogenesis by *Salmonella* Typhimurium

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Abstract

Salmonella enterica is an intracellular bacterial pathogen that replicates within membrane-bound vacuoles by delivering virulence (effector) proteins across the vacuolar membrane by the SPI-2 type III secretion system (T3SS). In this thesis I show that through the action of this T3SS, *Salmonella* selectively interferes with a subset of endosome-to-*trans*-Golgi network (TGN) traffic that is involved in mannose-6-phosphate receptor (MPR) recycling. Two pathways of endosome-to-TGN traffic have been described: one involves the Q-SNARE syntaxin 6; the other is defined by the Q-SNARE syntaxin 10 and involves retrograde transport of the MPRs. *Salmonella* specifically disrupted syntaxin 10-dependent endosome-to-TGN traffic and this was accompanied by redistribution of MPR from the TGN, misrouting of newly synthesized lysosomal enzymes and attenuation of lysosome function. The SPI-2 T3SS effector SifA is an important virulence determinant that is known to interact with the endolysosomal system. SifA was shown to be required and sufficient to mediate the effects on lysosome biogenesis during infection or following transfection of host cells. By contrast, a variant of SifA with a single amino acid substitution that prevents interaction with its host protein target, SKIP, failed to redistribute MPR or to reduce lysosomal function. SKIP was found to contribute to lysosomal biogenesis in uninfected cells. Inhibition of lysosomal enzyme function with a lysosomal protease inhibitor or by depleting cells of syntaxin 10 enhanced intracellular bacterial replication. I conclude that SifA attenuates lysosomal activity through its interaction with SKIP and that this facilitates intracellular bacterial replication.

Declaration

Data presented in this thesis are solely my own work with the exception of experiments described in Fig. 5.8 and Fig. 5.9 which were undertaken in conjunction with Dr. Teresa Thurston.

Publications

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List of Abbreviations

AP	adapter protein
Arf	ADP-ribosylation factor
BFA	brefelden A
BMM	bone-marrow macrophages
CCV	clatherin-coated vesicles
CD	cation dependent
CI	cation independent
COPI	coat protein complex I
COPII	coat protein complex II
CTxB	cholera toxin B subunit
DC	dendritic cell
EE	early endosome
EEA1	early endosomal antigen 1
ER	endoplasmic reticulum
ERC	early endosomal recycling compartment

ESCRT	endosomal sorting complex required for transport
FACS	fluorescence-activated cell sorting
GAP	GTPase activating protein
GCAT	glycerophospholipid-choesterol acyltransferase
GDF	GDI-displacement factor
GDI	GDP dissociation inhibitor
GEF	Guanine nucleotide exchange factor
HOPS	VpsC–homotypic protein sorting
IQGAP1	IQ-motif GTPase activating protein
Lamp	lysosomal associated membrane proteins
LE	late endosome
LE/MVB	late endosome/multi-vesicular body
lgps	lysosomal membrane glycoproteins
LPS	lipopolysacharide
MPR	mannose-6-phosphate receptor
MTOC	microtubule-organising centre
NTS	non-typhoidal *Salmonella*
PAI	pathogenicity island
PBS	phosphate buffered saline
PH	pleckstrin homology

pi	post-invasion
PI(x)P	phosphatidylinositol x-phosphate
PLEKHM1	pleckstrin homology domain containing, family M (with RUN domain) member 1
PLEKHM2	pleckstrin homology domain containing, family M (with RUN domain) member 2
Rab11FIP	Rab11 family-interacting protein
REP	Rab escort protein
RT	room temperature
SCV	*Salmonella*-containing-vacuole
Sifs	*Salmonella*-induced filaments
SKIP	SifA and kinesin-interacting protein
SNAREs	N-ethylmaleimide-sensitive factor attachment protein receptors
SNX	sorting nexin
SPI	*Salmonella* pathogenecity island
spv	*Salmonella* virulence plasmid
Syn	Syntaxin
T3SS	type three secretion system
TGN	*trans*-Golgi network
Tip47	tail interacting protein of 47kD

TPR	tetratricopeptide repeat
TrfR	transferrin receptor
TRIP6	filamin and thyroid receptor protein 6

Chapter 1

Introduction

1.1 *Salmonella* - General information

1.1.1 Taxonomy

Salmonella are rod-shaped, Gram-negative, motile bacteria that belong to the family Entereobacteriaceae. The genus *Salmonella* is divided into two species, *Salmonella bongori* and *Salmonella enterica,* which itself is sub-divided on the basis of sequence variation and biochemical properties, into a further 6 sub-species: *S. enterica* (I), *S. salamae* (II), *S. arizonae* (IIIa), *S. diarizonea* (IIIb), *S. houtenae* (IV), and *S. indica* (VI) (Boyd *et al.*, 1996; McQuiston *et al.*, 2008). Further classification of these sub-species based on specific surface antigens, the O antigen from lipopolysaccharide structures and the H antigen associated with variations in flagellin (a component of the flagellum), has resulted in approximately 2500 serovars being identified (Brenner *et al.*, 2000; Lan *et al.*, 2009). The huge variety of subspecies and serovars of *S. enterica* is indicative of the broad range of host and environmental niches occupied by this organism.

The vast majority of serotypes associated with disease in humans and warm-blooded animals belong to sub-species I, *S. enterica* sub-species *enterica,* hereafter referred to as either*, S. enterica* or *Salmonella* (Brenner *et al.*, 2000). Serotypes of *S. enterica* show significant differences with respect to host adaptation, clinical presentation and biological

diversity (Kingsley & Bäumler, 2000). For example some serotypes are restricted in their host range, such as *S.* Typhi and *S.* Paratyphi for humans, or *S.* Pullorum for fowl, and are unable to cause disease in other hosts. Other serovars show a preference for certain hosts but are not restricted and are said to be host-adapted, as is the case with porcine-adapted *S.* Dublin and *S.* Choleraesuis, which can occasionally be isolated from the blood of humans (Kingsley & Bäumler, 2000). In contrast to the more restricted serovars there are those, such as *S.* Typhimurium, that cause a broad range of diseases in a variety of hosts including rodents, sheep, humans, cattle, horses, poultry and pigs (Kingsley & Bäumler, 2000; Ohl & Miller, 2001; Lan *et al.*, 2009).

1.1.2 Diseases in humans

Salmonella enterica is the causative agent of several diseases in humans which can be broadly divided into three categories based on their clinical manifestations and causative serovars: typhoid fever, a systemic infection, caused by *S.* Typhi or *S.* Paratyphi; gastroenteritis, predominantly caused by *S.* Typhimurium and *S.* Enteritidis; and non-typhoidal *Salmonella* (NTS) bacteremia caused by *S. enterica* serotypes other than *S.* Typhi or *S.* Paratyphi, predominantly *S.* Typhimurium and *S.* Enteritidis.

1.1.2.1 Disease incidence in humans

Salmonella infection is a major economic and public health burden. A survey on the frequency and morbidity of typhoid fever estimated the annual incidence in excess of 21.5 million cases with over 200,000 associated deaths (Crump *et al.*, 2004). Similarly, there is a significant impact of NTS disease globally, with over 94 million cases recorded annually, and approximately 155,000 deaths (Majowicz *et al.*, 2010). Furthermore, NTS serovars which cause invasive disease are increasingly linked with a high morbidity rate, especially when complicating factors such as co-infection with HIV or malnutrition are considered (Gordon & Graham, 2008; Gordon, 2008).

1.1.2.2 Gastroenteritis

Gastroenteritis in humans is most frequently caused by *S.* Typhimurium and *S.* Enteritidis, although some other NTS serovars can also cause a similar disease. The predominant mode of transmission is through the oral-fecal route with contamination of food or water being the usual source of infection. Symptoms of the disease first appear after an incubation period of between 10 and 72 h after exposure. These include vomiting, abdominal cramps, nausea, fever, chills and inflammatory diarrhoea. Initially bacteria invade and colonise the ileum, Peyer's patches and mesenteric lymph nodes. There is a resultant localised immune response which involves massive infiltration of neutrophils (Hapfelmeier & Hardt, 2005). Diarrhoeal symptoms are believed to be caused by the neutrophil-rich exudate. The resultant risk of dehydration is one of the greatest factors contributing to morbidity in otherwise healthy patients. In the vast majority of cases gastroenteritis is an acute but self-limited disease that does not require pharmacological intervention, resolving in 7 to 10 days. Management primarily involves adequate rehydration with water and electrolytes to negate the risk of dehydration (Zhang *et al.*, 2003; Raffatellu *et al.*, 2008).

1.1.2.3 Typhoid fever

If left untreated, typhoid fever can lead to death in approximately 10% of cases (Raffatellu *et al.*, 2008). As with gastroenteritis, transmission is through the oral-fecal route with contaminated food and water being the predominant sources of infection. The pathology of the illness differs greatly from gastroenteritis. *S.* Typhi and *S.* Paratyphi both infect Peyer's patches of the intestinal ileum. However they induce only a mild intestinal inflammatory response, localised to the Peyer's patches (Parry *et al.*, 2002). Once translocated across the intestinal epithelium the bacteria begin to replicate in the lamina propria and mesenteric lymph nodes. Here the bacteria gain access to the bloodstream which they use as a conduit to colonise the spleen, liver and bone-marrow. After phagocytosis by monocytic cells at these sites *Salmonella* undergoes a second period of extensive intracellular replication and spread within these regions. The bacteria can also colonise the gall bladder from which

they can re-seed the gut. Secondary exposure of the intestinal tissue to the bacteria frequently causes hemorrhagic necrosis of the gut epithelium. This can result in perforation of the intestinal wall leading to infected peritonitis. Much of the morbidity associated with typhoid fever is a consequence of infected peritonitis (Everest *et al.*, 2001).

Symptoms at the earlier stages of infection include prolonged fever and mild inflammation of the small intestinal lymphoid tissue. Later, following reseeding of the gut from the gall bladder, the disease is characterised by a high fever, headaches, and strong abdominal pain. Secondary symptoms include malaise, constipation, anorexia and in some cases diarrhoea (Raffatellu *et al.*, 2008).

Approximately 1–5% of patients resolve acute typhoid fever but go on to develop a chronic asymptomatic infection. In these individuals *S.* Typhi is continually shed in urine or stools (Bhan *et al.*, 2005). Chronicity in the population is also thought to be brought about by subclinical infections (Mohan & Mohan, 2006). Together this population of chronically infected individuals is thought to be an important reservoir of transmission in the community. Furthermore, there is evidence to suggest that long term carriage of *S.* Typhi gives rise to an increased risk of developing gall bladder carcinomas placing chronically infected individuals in a high risk category (Nath *et al.*, 2008).

1.1.2.4 Invasive bacteremia

Less than 5% of patients with NTS-associated gastroenteritis go on to develop non-typhoidal bacteremia, largely caused by *S.* Typhimurium and *S.* Enteritidis (Hohmann, 2001). This is most prevalent in young and immunocompromised individuals especially when secondary factors of malnutrition, malaria or anemia are present (Gordon *et al.*, 2002; Gordon & Graham, 2008; Gordon, 2008). In sub-saharan Africa, where the incidence of HIV infection is high, invasive bacteremia, associated with NTS, is much more prevalent and is a major cause of death (Gordon, 2008). The pathology of this disease differs to typhoidal *Salmonella* infection, in that it is largely a disease of the blood. In immunocompromised individuals with invasive NTS a factor contributing to susceptibility is a dysregulation of the adaptive

immune response. In immunocompetent individuals infection would normally be cleared from the blood through complement and antibody mediated killing (MacLennan *et al.*, 2008; Gondwe *et al.*, 2010; MacLennan *et al.*, 2010). In contrast in immunocompromised individuals the blood contains large numbers of antibodies against LPS which obstruct or otherwise prevent the bactericidal activity of antibodies that recruit complement to the bacterial surface (Moir & Fauci, 2010).

1.1.3 Experimental models of infection

To study the different virulence mechanisms of *Salmonella,* as well as host responses, several model systems have been developed.

1.1.3.1 Animal models of human gastroenteritis

S. Typhimurium and *S.* Dublin are natural pathogens of calves and have both been used to model human gastroenteritis in this host. Infection of calves with *S.* Typhimurium is generally believed to provide a more representative system with which to study the disease, as it produces a diarrhoeal disease which more closely resembles the human illness (Zhang *et al.*, 2003). After inoculation, *S.* Typhimurium invades the intestinal epithelium at the Peyer's patches, leading to a large inflammatory response predominantly associated with neutrophil infiltration (Tsolis *et al.*, 1999). Clinical symptoms of the disease include diarrhoea, fever, dehydration and prostration and the intestinal pathologies are similar to those found in human enteritis caused by *S.* Typhimurium (Zhang *et al.*, 2003). Streptomycin-treated mice have also been utilised to study gastroenteritis as a more cost effective animal model (Barthel *et al.*, 2003). Infection using this system produces an illness that resembles many aspects of the human infection, including epithelial ulceration, edema, and large infiltration of neutrophils. In addition to the advantage of cost, the use of mice also allows for greater investigation into host pathogen interactions due to the availability of an ever increasing panel of genetic knock-out mice.

1.1.3.2 Animal models of typhoid fever

The host restriction of *S.* Typhi has made it difficult to study virulence mechanisms. Although higher primates and rhesus monkeys can be infected with *S.* Typhi to cause an illness similar to typhoid fever, the restricted availability and cost associated with using these animals has severely limited their use (Gaines *et al.*, 1968a,b; Rout *et al.*, 1974). The more widely adopted model for the study of systemic *Salmonella* infection is susceptible mice infected with *S.* Typhimurium. This combination produces a systemic illness with many of the features of human typhoid fever and has provided a great deal of information concerning both virulence and host defence mechanisms. However, differences in the pathologies and host tropism between *S.* Typhi and *S.* Typhimurium indicate distinct virulence mechanisms, some of which are not replicated in the mouse model. A clear example of differences in the pathology of *S.* Typhi in humans and *S.* Typhimurium in mice is the ability of *S.* Typhi to re-infect the intestine from the gall bladder (Everest *et al.*, 2001). Although *S.* Typhimurium can be detected in the mouse gall bladder, significant re-seeding of the gut does not occur via this route (Menendez *et al.*, 2009). Furthermore, there are important differences in the virulence determinants of these strains. For example, the virulence plasmid (*spv)* operon of *S.* Typhimurium is absent from *S.* Typhi and the Vi capsule antigen of *S.* Typhi is absent from *S.* Typhimurium. The *spv* operon has been shown to be important for virulence in mice whereas the Vi capsule antigen has anti-inflammatory activity and a role in resistance to complement-mediated killing during the development of typhoid fever in humans (Bäumler, 1997; Raffatellu *et al.*, 2006).

S. Typhimurium can gain access to the spleen and liver when susceptible mice are inoculated orally, intraperitonealy or intravenously. Oral inoculation represents the most physiological route of infection, with bacteria traversing the low pH of the stomach to colonise the small intestine (Carter & Collins, 1974). Here they traverse the epithelium through direct invasion of M-cells in the Peyer's patches, and/or enterocytes or through phagocytosis by $CD18^{+}$cells, likely to be dendritic cells (Vazquez-Torres *et al.*, 1999). After traversing the intestinal barrier the bacteria migrate via the reticuloendothelial system

to colonise the liver and the spleen (Carter & Collins, 1974; Fields *et al.*, 1986). The proliferative phase of infection largely takes place in these organs in which the bacteria are predominately located within macrophages and Kupffer cells (Richter-Dahlfors *et al.*, 1997; Salcedo *et al.*, 2001). Unrestricted bacterial proliferation at these sites of infection leads to bacteremia and eventual death due to lipopolysacharide (LPS)-induced septic shock (Khan *et al.*, 1998; Santos *et al.*, 2001).

1.1.3.3 *In vitro* models

An essential component of *Salmonella* virulence is its ability to survive and replicate within host cells. Much of our understanding of how it achieves this at the cellular level is derived through the use of cultured epithelial and macrophage cells. Infection using cells has yielded much greater insight into the molecular basis of invasion (Patel & Galán, 2006), *Salmonella*-containing-vacuole (SCV) maturation (Méresse *et al.*, 1999a) and other host-vesicular trafficking interactions (Garcia-del Portillo *et al.*, 1993; Garcia-del Portillo & Finlay, 1995; Knodler & Steele-Mortimer, 2003). The importance of replication in macrophages is evident by the finding that many mutants that are defective for growth in macrophages are avirulent in mice (Fields *et al.*, 1986). A variety of cell lines are commonly used, including those from both human and mouse origin, such as mouse RAW 264.7 and J774 macrophage-like cell lines, and human HeLa epithelial cells. Primary bone marrow derived mouse macrophages and elicited peritoneal macrophages are also useful cell types with which interactions with *Salmonella* can be studied in a more physiological and restrictive environment.

1.2 Molecular determinants of pathogenesis

S. enterica employs a broad range of molecular factors that help it to subvert host function in a manner that allows for successful colonisation and infection. Some factors are present in all serovars whereas others are unique to specific serovars which determines the types of

disease caused.

1.2.1 *Salmonella* pathogenicity islands

Many virulence genes of *S. enterica* have been acquired via horizontal gene transfer and are located in genetic elements such as pathogenicity islands (PAI), prophages and plasmids (Groisman & Ochman, 1997). Genetic screens have shown that pathogenicity islands (SPI) are present in the genome and encode many virulence factors that contribute to pathogenesis (Galan & Curtiss, 1989; Shea *et al.*, 1996). Some PAIs are present throughout the entire genus whereas others are only present in certain serovars and are believed to confer to host specificity (Marcus *et al.*, 2000; Hansen-Wester & Hensel, 2001; Hensel, 2004). *S.* Typhimurium, which is extensively used in mice as a model system for the study of typhoid fever, shares 11 pathogenicity islands with *S.* Typhi, though their genetic makeup differs (Sabbagh *et al.*, 2010). Distinct aspects of *Salmonella* pathogenesis have been linked to specific PAIs. These include its ability to invade and replicate within host cells. These actions have been attributed to the activity of two type three secretion systems (T3SS) encoded by SPI-1 and SPI-2. The T3SS (discussed in more detail below) translocate a range of bacterial proteins into the host cell that interfere with host cell processes to promote infection.

1.2.2 Fimbriae

Salmonella attaches to the mucosal surfaces of the small intestine and subsequently invades epithelial cells. Fimbriae are hair-like surface appendages of approximately 8 nm that enable *Salmonella* to adhere to the intestinal epithelium (Bäumler *et al.*, 1996; Humphries *et al.*, 2001). Furthermore, fimbrial adhesins are believed to confer a tropism towards Peyer's patches of the small intestine, which is a predominate site of intestinal colonisation by *Salmonella* (Bäumler *et al.*, 1997). It has also been suggested that fimbrial adhesins can target *Salmonella* for direct uptake by dendritic cells (DC)(Guo *et al.*, 2007). DCs have been visualised taking up *Salmonella* directly from the gut lumen through the intestinal

epithelium (Niess *et al.*, 2005; Chieppa *et al.*, 2006). Thus DCs represent an alternative route through which the intestinal barrier can be traversed.

1.2.3 Flagella

Flagella are surface-localised long helical filaments with a diameter of approximately 20 nm. The rotational movement of the flagella provides motility to *Salmonella* and this in turn contributes to virulence by increasing invasiveness (Schmitt *et al.*, 2001). Additionally, flagellin subunits of the flagella have been shown to be translocated by the SPI-1 T3SS into the cytosol of infected macrophages. This is believed to induce caspase-1-mediated cell death through activation of the inflammasome (Ren *et al.*, 2006; Miao *et al.*, 2007; Sun *et al.*, 2007b). Once inside the cell flagella are believed to be coordinately down-regulated together with the SPI-1 T3SS (Eriksson *et al.*, 2003).

1.2.4 Two component regulatory systems

An essential aspect of *Salmonella* virulence is its ability to respond to environmental molecules and coordinately regulate expression of specific genes required for pathogenesis. The two-component regulatory system PhoP/PhoQ is a major regulator in this regard, co-ordinating the expression of over 40 genes (Groisman *et al.*, 1989; Miller, 1991). The PhoP/PhoQ system responds to environmental signals of low pH, Mg^{2+}concentration and antimicrobial peptides (AMP)s (Alpuche Aranda *et al.*, 1992; García Véscovi *et al.*, 1996; Bader *et al.*, 2005). PhoQ is an inner membrane-bound kinase that senses low pH, Mg^{2+}concentration and/or antimicrobial peptides and phosphorylates its cognate response regulator, PhoP, which can then function as a transcriptional regulator of many genes (Groisman, 2001). Genes positively regulated by PhoP/PhoQ (*pags*) include those encoding a family of proteins involved in Mg^{2+} uptake and transport and others involved in LPS modification (Ernst *et al.*, 2001). The PhoP/PhoQ system has also been shown to be important to repress expression of certain genes (*prgs*). These include regulators of the SPI-1 T3SS (Bijlsma & Groisman, 2005). It has been suggested that PhoP/PhoQ

might also regulate SPI-2 T3SS expression as mutations in the *phoP* gene led to reduced expression of the SPI-2 T3SS *in vitro* (Deiwick, 1999; Lee *et al.*, 2000; Worley *et al.*, 2000). This conclusion has been disputed though as the SPI-2 T3SS expression in cultured infected macrophages is largely independent of PhoP-PhoQ (Valdivia & Falkow, 1997). In addition, during competitive infection experiments in mice these two systems were found to contribute independently to virulence (Beuzón *et al.*, 2001).

A second two component regulatory system important for the pathogenesis of *Salmonella* is OmpR/EnvZ which responds to changes in osmolarity and pH (Bang *et al.*, 2000, 2002). Expression of the SPI-2 T3SS is indirectly regulated by this system through its effect on the SsrA-SsrB two component system encoded by SPI-2. This directly controls the expression of the SPI-2 T3SS (Cirillo *et al.*, 1998; Garmendia *et al.*, 2003).

1.2.5 Type III secretion systems

T3SSs are likened to 'molecular syringes' that deliver bacterial virulence proteins, called effectors, directly from the bacterial cytoplasm into the host cell (Mota & Cornelis, 2005; Galán & Wolf-Watz, 2006). These effectors manipulate cellular processes, frequently by acting as mimics of host proteins. In *Salmonella*, the SPI-1 and SPI-2 T3SSs are regulated at different stages of infection. The SPI-1 T3SS is essential for bacterial invasion (Galan & Curtiss, 1989), whereas the SPI-2 T3SS, expressed intracellularly post-invasion, is required for replication within host cells (Ochman *et al.*, 1996; Hensel *et al.*, 1998).

1.2.5.1 SPI-1 T3SS, invasion and SCV biogenesis

Salmonella translocates approximately 15 effectors via the SPI-1 T3SS into the host cell (McGhie *et al.*, 2009). SopE/SopE2, SopB, SptP and SipA, are SPI-1 T3SS effectors which cooperate to induce actin rearrangements which allow invasion of non-phagocytic host cells (Zhou & Galán, 2001; Patel & Galán, 2005). This occurs via extensive membrane ruffling caused by protrusion of actin fibers at the cell surface. The ruffles trap bacteria, leading to their internalisation. SopE/SopE2 function as guanine nucleotide exchange factors (GEF)s

for Cdc42 and Rac1 and stimulate F-actin assembly by the Arp2/3 complex (Friebel *et al.*, 2001; Unsworth *et al.*, 2004). Once inside the cell *Salmonella* resides in a membrane bound compartment known as a SCV. SptP is a GTPase activating protein (GAP) and functions to down regulate ruffle formation post-invasion (pi) (Fu & Galan, 1999). In addition, it dephosphorylates AAA^+ ATPase VCP modifying recruitment of cellular proteins to the SCV (Humphreys *et al.*, 2009) and together with some other SPI-1 effectors has been implicated in early SCV biogenesis. SipA enhances actin polymerisation which contributes to membrane ruffling facilitating bacterial internalisation. SipA functions to mechanically stabilise the filament by linking actin subunits in opposing strands (Lilic *et al.*, 2003). SipA is also believed to co-operate with the SPI-2 effector SifA by influencing juxtanuclear positioning and morphology of SCVs (Brawn *et al.*, 2007). SopB is a inositol phosphatase which modifies the phosphoinositide content of the SCV membrane (Patel & Galán, 2006). The phosphatase activity has a range of effects and is responsible for activating SGEF which acts on RhoG to promote actin rearrangement facilitating invasion. It is also implicated in efficient sealing of the nascent SCV membrane. Furthermore, SopB modifies the charge composition of the SCV membrane, which has been reported to influence SCV maturation by reducing fusion with lysosomes (Terebiznik *et al.*, 2002; Knodler *et al.*, 2005; Patel & Galán, 2006; Bakowski *et al.*, 2010). Other phenotypes associated with the role of the SPI-1 T3SS include macrophage cytotoxicity and inflammatory responses in the intestinal mucosa (Hersh *et al.*, 1999; Hapfelmeier *et al.*, 2004; Bruno *et al.*, 2009).

1.2.5.2 SPI-2 T3SS and associated effectors

The SPI-2 T3SS is required for virulence in mice (Hensel *et al.*, 1995; Shea *et al.*, 1996) and replication within macrophages (Ochman *et al.*, 1996; Hensel *et al.*, 1998). Approximately 30 effectors are translocated in a tightly regulated process via this secretion system and are responsible for a several intracellular phenotypes including SCV positioning in the infected cell, SCV membrane dynamics, host cell migration and immune modulation. The secretion apparatus is expressed in response to low pH, nutrient deprivation, Mg^{2+} concentration

and AMPs encountered in the lumen of the SCV (Yoon *et al.*, 2009). It is then assembled in a hierarchical manner, initially with the formation of a secretion machinery spanning the inner and outer membrane of the bacterial cell envelope. This is used to secrete three classes of proteins in an ordered fashion. First, protein subunits are secreted to form a hollow needle-like structure which protrudes from the cell surface. Then translocon proteins are delivered through the needle and insert into the SCV membrane to form a pore. Finally, the neutral pH of the host cell cytosol is sensed. This signal precipitates the disassembly in the bacterial cell of a secretion system-associated regulatory complex composed of SpiC, SsaM and SsaL. This then allows for the secretion and translocation of the effector proteins (Yu *et al.*, 2010).

After invasion of epithelial cells, SCVs migrate along microtubules to a perinuclear region that coincides with the microtubule-organising centre (MTOC) and the Golgi network (Salcedo & Holden, 2003; Harrison *et al.*, 2004; Ramsden *et al.*, 2007). Here, *Salmonella* undergoes replication, forming intracellular microcolonies comprising bacteria contained within discrete vacuoles that are maintained in proximity to the Golgi network and MTOC. SseF and SseG are two functionally interdependent effectors that interact to maintain the SCV at the MTOC (Kuhle & Hensel, 2002; Salcedo & Holden, 2003; Deiwick *et al.*, 2006; Ramsden *et al.*, 2007). It is currently unclear how this is achieved mechanistically.

The Golgi network is a point of convergence for secretory and endosomal traffic in the cell. The ability of *Salmonella* to establish and maintain a microcolony juxtaposed to the MTOC/Golgi network could increase SCV-endosomal or SCV-Golgi interactions to enable nutrient and membrane acquisition (Salcedo & Holden, 2003; Ramsden *et al.*, 2007; Mota *et al.*, 2009). This hypothesis is supported by the observation that *Salmonella* can alter post Golgi traffic and in some cases redirects traffic to the SCV in a SPI-2 dependent fashion (Kuhle *et al.*, 2006; Mota *et al.*, 2009).

A characteristic of intracellular *Salmonella* is its ability to induce the formation of dramatic tubular membranous structures known as *Salmonella*-induced filaments (Sifs) (Garcia-del Portillo *et al.*, 1993). The SPI-2 T3SS effector, SifA, is essential for Sif for-

mation as mutant strains lacking SifA do not produce Sifs (Stein *et al.*, 1996). SifA is also critical in the maintaining the integrity of the SCV membrane, as *sifA* mutant strains are released from their vacuole into the host cell cytosol several hours after internalisation (Beuzón *et al.*, 2000). Intriguingly, SPI-2 T3SS null mutants, that fail to translocate any SPI-2 effectors maintain an intact vacuolar membrane, implicating other SPI-2 effectors in the destabilisation of the SCV observed in a *sifA* mutant (Beuzón *et al.*, 2000). The SPI-2 effectors SseJ, PipB2 and SopD2 have been shown to contribute to the membrane instability of the vacuolar membrane around the *sifA* mutant strain (Ruiz-Albert *et al.*, 2002; Schroeder *et al.*, 2010).

SifA mediates its function through binding SKIP (SifA and kinesin-interacting protein) also known as PLEKHM2 (pleckstrin homology domain containing, family M (with RUN domain) member 2) (Boucrot *et al.*, 2005). SifA functions through SKIP to maintain SCV integrity as in cells depleted of SKIP wild-type bacterial cells are released into the cytosol, phenocopying a *sifA* mutant strain (Boucrot *et al.*, 2005). SKIP contains a RUN domain within its N-terminal region that interacts with the (TPR) domain of kinesin light chain to promote anterograde transport on microtubules (Diacovich *et al.*, 2009). The C-terminal region of SKIP contains a pleckstrin homology (PH) domain to which SifA binds (Jackson *et al.*, 2008a; Ohlson *et al.*, 2008; Diacovich *et al.*, 2009). Upon translocation, SifA is prenylated by a host cell geranylgeranyl transferase allowing it to insert into the SCV membrane (Reinicke *et al.*, 2005). Here SifA interacts with SKIP, cross-linking the SCV with kinesin-microtubule network to promote anterograde movement of membrane, generating Sif formation (Dumont *et al.*, 2010; Schroeder *et al.*, 2011).

SifA also affects cellular GTPases and has been reported to bind to Rab7 and to act as an exchange factor for the RhoA (Harrison *et al.*, 2004; Boucrot *et al.*, 2005; Ohlson *et al.*, 2008). As well as SifA, SKIP also binds the Rab9 (Jackson *et al.*, 2008a). Rab9 is involved with late endosome (LE)/lysosome function (Riederer *et al.*, 1994a; Ganley *et al.*, 2004) and is reported to be antagonised by the interaction of SifA with SKIP (Jackson *et al.*, 2008a).

As mentioned above, PipB2, SseJ and SopD2 are three SPI-2 T3SS effectors that are functionally linked with SifA through the impact they have on Sif formation and vacuolar stability. A *pipB2* mutant strain displays impaired Sif formation (Knodler & Steele-Mortimer, 2005). A *sifA* mutant strain has increased kinesin labelling associated with the SCV prior to rupture (Boucrot *et al.*, 2005). In a *sifA pipB2* double mutant strain kinesin recruitment is no longer enhanced, suggesting that PipB2 recruits kinesin to the SCV (Henry *et al.*, 2006). Furthermore, the same study showed direct binding between PipB2 and kinesin-1.

SseJ, a glycerophospholipid-cholesterol acyltransferase (GCAT) that modifies the SCV membrane, is activated by RhoA which is recruited and activated by SifA (Lossi *et al.*, 2008; Ohlson *et al.*, 2008). Together, the combination of SifA, SseJ, RhoA and SKIP increases membrane tubulation.

Though the function of SopD2 remains unclear, cells infected with a *sopD2* mutant strain of *Salmonella* have fewer Sifs than those infected with wild-type bacteria suggesting a function in Sif formation (Schroeder *et al.*, 2010). Furthermore, in *sifA sseJ* and *sifA sopD2* double mutant strains, defects in vacuolar stability associated with the single *sifA* mutant strain are partially rescued. This provides further evidence of functional interactions between these proteins (Ruiz-Albert *et al.*, 2002; Schroeder *et al.*, 2010).

Recently an hypothesis of how these proteins could be functionally interlinked has been suggested. It is proposed that inactive kinesin-1 is recruited to the SCV by PipB2. Kinesin-1 is then activated indirectly by SifA through SKIP. Together with RhoA, SseJ and SopD2, anterograde transport along microtubules is extended from the SCV to drive Sif formation (Schroeder *et al.*, 2011). The molecular mechanisms of how these proteins are related remains unknown and future work is required to enable a more coherent understanding of the function and interaction between these bacterial and host proteins.

The host cell ubiquitin system is also a target for modulation by effectors of the SPI-2 T3SS. There are four effectors that interact with the ubiquitin pathway (Rytkönen *et al.*, 2007; Bernal-Bayard & Ramos-Morales, 2009; Quezada *et al.*, 2009). SspH1 and SspH2 are

E3 ligases with unknown physiological activity (Quezada *et al.*, 2009). SlrP is also an E3 ligase that has been reported to increase host cell death by ubiquitinating thioredoxin to reduce its activity (Bernal-Bayard & Ramos-Morales, 2009). SseL is an effector that has deubiquitinase activity. It lacks a known cellular target but has been reported to induce host cell cytotoxicity and to have a preference for lysine 63-linked chains *in vitro* (Rytkönen *et al.*, 2007).

The actin cytoskeleton is rearranged extensively during *Salmonella* invasion of non-phagocytic cells. This is dependent on several SPI-1 T3SS effectors (see above). These effects are transient and shortly after invasion the cortical cytoskeleton resumes its normal morphology. However, beginning approximately four hours post-uptake a dense F-actin meshwork accumulates in proximity to the SCVs (Méresse *et al.*, 2001; Unsworth *et al.*, 2004). This is dependent on the SPI-2 T3SS effector SteC, which is a kinase (Poh *et al.*, 2008). In addition, unpublished data from our laboratory has identified MEK1 as a target of phosphorylation by SteC. This leads to the activation of myosin-II, an actin motor protein, which facilitates F-actin bundling and a formation of a meshwork around the microcolony (Odendall and Holden, unpublished). This appears to regulate the replication rate of intracellular bacteria, as mutants lacking SteC display increased intracellular growth when compared to wild-type bacteria (Odendall and Holden, unpublished).

The SPI-2 T3SS effector protein SseI is thought to influence host cell motility, although there is disagreement as to the effects. One study reported that it interacts with filamin and thyroid receptor protein 6 (TRIP6) to enhance motility (Worley *et al.*, 2006), whereas another investigation identified a different cellular target of SseI; IQ-motif GTPase activating protein (IQGAP1). Here the effects reported are of an inhibition of cell migration (McLaughlin *et al.*, 2009). Clearly further work is required to resolve these contradictory results.

Other SPI-2 T3SS effectors are thought to have a function in immune modulation. *Salmonella* infection leads to polyubiquitination of MHC class II molecules in infected antigen-presenting cells. This results in the removal of mature, peptide loaded molecules

from the cell surface and is likely to be a immune evasion mechanism. Furthermore, this was shown to be dependent on the activity of the SPI-2 T3SS though the effector(s) mediating this effect remains unknown (Lapaque *et al.*, 2009). SpvC is a SPI-2 T3SS effector that is encoded by the *spv* operon (discussed further below). It is a member of a family of effectors having phosphothreonine lyase activity on MAPKs involved in immune signaling (Li *et al.*, 2007; Mazurkiewicz *et al.*, 2008). Phosphothreonine lyases remove the phosphate group and adjacent oxygen atom from phosphothreonine in an irreversible process known as eliminylation (Li *et al.*, 2007; Brennan & Barford, 2009). In macrophages infected with bacteria over-expressing SpvC there were reduced levels of secreted TNFα and IL-8 which correlated with effects on the cellular MAP kinases ERK and JNK (Mazurkiewicz *et al.*, 2008).

There have been several other SPI-2 effectors identified. These include SifB, PipB, GogB, SteA, SteB, SseK1, SseK2, SseK3, GtgE, GtgA, SpvD, SteD, SteE and CigR (Niemann *et al.*, 2011). The host targets, mechanisms and physiological effects of these proteins are not known.

1.2.6 *spv* operon

Some *Salmonella* serovars carry virulence plasmids (Boyd & Hartl, 1998) whose phenotypes are largely attributed to the action of the *spv* operon (Gulig *et al.*, 1993; Chu & Chiu, 2006). In mouse models of infection the *spv* genes of *S.* Typhimurium are required for systemic growth in the spleen and liver (Gulig & Doyle, 1993). The expression of *spvR*, *A*, *B*, *C* and *D*, is up-regulated *in vivo* and in tissue culture models of infection as well as *in vitro* where conditions mimic those of the SCV (Wilson *et al.*, 1997; Wilson & Gulig, 1998; Ygberg *et al.*, 2006). SpvR is a transcriptional regulator controlling the expression of *spvA*, *B*, *C* and *D*. The functions of *spvA* and *spvD* remain unknown (Roudier *et al.*, 1992; Matsui *et al.*, 2001b). *spvB* and *spvC* encode proteins translocated by the SPI-2 T3SS into the host cell cytosol (Libby *et al.*, 2000; Matsui *et al.*, 2001a; Mazurkiewicz *et al.*, 2008). *spvB* encodes an actin-ribosyltransferase that prevents F-actin filaments forming, resulting in

delayed cytotoxicity of infected cells (Libby *et al.*, 2000; Otto *et al.*, 2000; Lesnick *et al.*, 2001; Tezcan-Merdol *et al.*, 2005). SpvC is a phosphothreonine lyase which is proposed to have a role in the modulation of the immune response (see above) (Mazurkiewicz *et al.*, 2008) similar to a homolog found in *Shigella flexneri* known as OspF (Li *et al.*, 2007).

1.3 Host cell vesicular traffic

The spatial and temporal organisation of membrane compartments inside the eukaryotic cell is essential for life. Many vital physiological processes take place enclosed in, on or across membranes. These include the synthesis of ATP, biogenesis of organelles of specialised function, protein secretion/uptake and cellular signaling. Intracellular membrane systems create compositionally and morphologically distinct compartments that allow the cell to regulate different activities through spatial separation. An example of one such compartment is the separation of hydrolytic enzymes into lysosomes where through their hydrolytic activity they breakdown macromolecules such as proteins, lipids and carbohydrates. Membrane enclosed transport vesicles and tubules (referred to as tubular-vesicular transport) are utilised as a means by which the cell transfers material between membranous organelles (Mellman & Warren, 2000). For the purposes of this thesis I will deal primarily with the vesicular transport of proteinaceous cargo.

1.3.1 Cytoskeleton

The eukaryotic cell is provided with structure and shape by an internal filamentous cytoskeleton (Frixione, 2000). It comprises three primary protein networks of filaments: actin filaments, intermediate filaments and microtubules. In addition to structural functions the cytoskeleton plays an essential role in vesicular traffic, where it acts as a support network for the movement of transport vesicles between compartments (Doherty & McMahon, 2008).

1.3.1.1 Actin filaments

Actin is 42-kDa globular protein found in eukaryotic cells which is known as globular actin (G-actin) in its monomeric form. It can polymerise to form linear actin filaments known as F-actin. Two parallel F-actin filaments can intertwine to create double helix structures of approximately 7 nm in diameter with a helix repeat every 37 nm. This creates the double helix microfilament structure of the cytoskeleton. Actin filaments are polar in nature and contain dynamic barbed polymerising (plus) ends and less active pointed (minus) ends. Actin is involved in a range of cellular processes including muscle contraction, cell division, cell signaling and cell motility. It is also important for the formation and maintenance of cell junctions and cell shape (Doherty & McMahon, 2008). Furthermore, the actin cytoskeleton functions as a support network on which myosin motor proteins mediate short range movement of vesicle carriers (Kjeken *et al.*, 2004). Filament turnover involving nucleation, elongation, and depolymerisation is controlled by actin-binding proteins including ARP2/3, WASP, WAVE, WHAMM, WASH and JMY (Campellone & Welch, 2010).

1.3.1.2 Intermediate filaments

Intermediate filaments are made up from a large family of related proteins that share common structural and sequence characteristics (Herrmann *et al.*, 2007). There are approximately 70 different intermediate filament proteins including lamins, which are nuclear structural components; vimentin, which support cellular membranes and maintain the spatial distribution of some organelles within the cytoplasm; keratins, which function in acidic and basic pairs to form filaments for a variety of structures like hair, nails and in epithelial cell structural components (Herrmann *et al.*, 2007). The majority are polymers of around 10 nm in diameter. The subunit proteins consist of globular domain at the N and C-termini linked by an alpha-helical rod domain. The proteins form dimers consisting of a coiled coil between the alpha-helical rod domains (Qin *et al.*, 2009). Dimer protein complexes come together to form multimeric filamentous structures. They help maintain the cell shape by bearing tension and act as mechanical stress absorbers (Herrmann *et al.*, 2007). In addition

the internal distribution of some organelles are maintained by anchoring to intermediate filaments (Herrmann *et al.*, 2007). Unlike actin or microtubule filaments they are non-polar with respect to their polymerisation (Herrmann *et al.*, 2007).

1.3.1.3 Microtubules

Microtubules are composed of α- and β-tubulin subunit dimers that form in a head to tail arrangement to form protofilaments. The protofilaments arrange themselves in a helical formation of 13 filaments to create a hollow cylinders about 23 nm in diameter. The dimer subunits exhibit a bent conformation when bound to GDP and a straight conformation when bound to GTP. Tubulin polymerises end to end with the α subunit of one tubulin dimer contacting the β subunit of the next. Accordingly, the protofilament has an exposed α subunit at one end and β subunit at the other, which confers polarity. Protofilaments bundle parallel to one another to form microtubules so polarity is extended to the microtubule. The end that has exposed α subunits is designated the plus end and the β exposed end being designated minus end. There is a cap at the minus end, which is normally anchored at the microtubule organising centre and was recently identified to be composed of a protein called Patronin (Howard & Hyman, 2007; O'Rourke & Sharp, 2011). Microtubules have a very dynamic behavior. Elongation occurs in the plus direction which, like the minus end, is also capped to prevent depolymerisation. The subunit dimers bind GTP straightening them to facilitate incorporation into the elongating plus end. After incorporation the GTP is hydrolysed to GDP but the dimer subunits are kept in their straightened conformation by the tubulin lattice and are prevented from reverting to their bent shape. The resultant 'bending energy' is stored within the microtubule and released upon plus end cap dissociation. It is the driving force for microtubule depolymerisation (Nogales & Wang, 2006; Howard & Hyman, 2007). Microtubules are biologically important for many cell processes including cell motility, meiosis and mitosis. They also contribute to cell structure by resisting compressive force. In addition, they play key roles in intracellular vesicular transport with the network acting as a substrate along which motor proteins

move tubular-vesicular traffic. There are two major classes of motor proteins, kinesins and dyneins, which generally move along the microtubule towards the plus end and the minus end respectively (Vale, 2003).

1.3.1.4 Anterograde traffic

Anterograde vesicular traffic is generally associated with kinesin dependent vesicular movement along microtubules towards their plus ends (Vale, 2003). Within eukaryotes it is carried out among highly organised, directional, and compartmentalised routes. Post-synthesis in the endoplasmic reticulum (ER), cargo is packaged in vesicular carriers and ferried from the ER exit sites to Golgi network. After arriving at the Golgi, cargo is sorted into specific post-Golgi carriers that move through the cytoplasm to target organelles in the endosomal network or directly to the cell membrane. The directional anterograde movement of membrane away from the ER and Golgi network is balanced by retrograde endocytic and retrieval pathways.

1.3.1.5 Retrograde traffic

Retrograde traffic occurs in a dynein-mediated process along microtubules towards their minus ends. Cargo proteins can be targeted to terminal degradative endocytic compartments or to the Golgi network and subsequently to the ER (Pfeffer, 2007; Johannes & Popoff, 2008). Often, retrograde pathways are exploited by pathogens to facilitate toxin transport to the cell cytoplasm (Duclos & Desjardins, 2000; Watson & Spooner, 2006).

1.3.2 Major trafficking machinery

1.3.2.1 SNARES

In eukaryote cells soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are involved in almost all membrane fusion events (Behnia & Munro, 2005; Hong, 2005; Jahn & Scheller, 2006; Leabu, 2006). They function in distinct groups of polypeptides (from 3 or 4 proteins) to form an active SNARE complex. Vesicles, containing

a specific R type SNARE subunit, fuse with vesicles or membranes containing 2 or 3 partner specific Q-SNAREs. The Q-SNAREs can be subdivided into three categories, Qa, Qb, and Qc. Each category, (R, Qa, Qb and Qc) contribute 1 sub-domain, categorised according to their conserved motifs, to form the active SNARE complex. Once the R SNARE binds to the QaQbQc SNARE sub domains on the target membrane an extended four helical bundle is formed. The trans-complex functions to pull the two membranes closer together to overcome the fusion energy barrier and the two membranes fuse. Specific complexes of SNARE family proteins govern vesicular trafficking pathways, as well as their intermediary steps.

1.3.2.2 Rabs

Rab GTPases are molecular switches that control much of the trafficking machinery within the cell to regulate the spatio-temporal activity of vesicular traffic. They are synthesised as cytosolic proteins and post-translationally modified by prenylation which allows them to associate with and insert into target membranes. Localisation to specific organelles is believed to be initially undertaken by Rab escort proteins (REP)s (Ali & Seabra, 2005).

They alternate between two conformational states of a GTP-bound active form and a GDP-bound inactive form. A GEF converts the inactive GDP to GTP-bound forms, whereas GAP induce a GTP active form conversion to a GDP inactive form (Stenmark, 2009). In their active form Rabs undergo a conformational change that allows them to bind proteins known as Rab effectors. It is through these effectors and the functions they mediate that Rab proteins exert influence on cellular traffic. In this way Rab GTPases specify membrane identity through the proteins they recruit (Ali & Seabra, 2005; Stenmark, 2009). An example of the cascading regulation of Rab proteins is illustrated in their binding of tethering factors, which themselves interact with SNAREs enabling their assembly (Epp *et al.*, 2011).

Rab proteins can be extracted from membranes by Rab GDP dissociation inhibitor (GDI) when they are no longer needed. They remain in a GDI bound state in cytosol until

required. GDI proteins can be displaced by Rab GDI-displacement factors (GDF)s which allows retargeting to the membrane and reactivation of the Rab proteins (Ali & Seabra, 2005).

Specific Rab proteins function on cell organelles that have unique characteristics. This spatial regulation is thought to be due in a large part to the lipid composition of the membrane, most especially the phosphoinositide composition. Targeting of Rabs and effectors to restricted membranes can be accomplished in two ways. Firstly, certain Rab effector proteins have domains that can recognise unique phosphoinositide content which is believed to enhance their recruitment to membranes with this phosphoinositide composition, thereby increasing the frequency of interaction with their cognate Rab. Furthermore, Rab protein association with the membrane can be stabilised in the presence of a bound effector, as can be seen with Rab9 and tail interacting protein of 47kD (Tip47) (Ganley *et al.*, 2004; Aivazian *et al.*, 2006). A second method is associated with an interesting aspect of Rab GTPases effector activity. Some Rab effectors can function to modify the phosphoinositide composition facilitating the recruitment of downstream effectors. Rab5 functions in this regard by recruiting a class III PI3K complex to produce phosphatidylinositol 3-phosphate (PI(3)P) on endosome membranes. Interestingly, the Rab5 effectors early endosomal antigen 1 (EEA1), rabenosyn 5 and rabankyrin 5 contain PI(3)P recognition domains suggesting they may be recruited after PI3K (Stenmark, 2009).

1.3.2.3 Retromer

A component of cellular machinery that is of particular importance during retrograde recycling is the retromer complex (Bonifacino & Rojas, 2006; Bujny *et al.*, 2007). It functions to sort and package cargo into tubular vesicular carriers derived from early endosome/endocytic recycling compartments (EE/ERC) that are destined for the *trans*-Golgi network (TGN) or late-endosome/multi-vesicular body (LE/MVB) compartments (Bonifacino & Hurley, 2008; Pfeffer, 2009). The complex is composed of two sub-complexes which include a multimeric cargo recognition complex of Vps26-Vps35-Vps29 and tubulating com-

plex of sorting nexins (SNX) proteins (Seaman, 2005; Rojas *et al.*, 2007). The retromer has been shown to be a critical component in endosome-to-TGN pathways affecting the traffic of a broad variety of cargo including mannose-6-phosphate receptors (MPR)s (Arighi *et al.*, 2004; Seaman, 2004; Bonifacino & Hurley, 2008), wntless, Shiga toxin, and polymeric immunoglobulin receptors (Vergés *et al.*, 2004; Bujny *et al.*, 2007; Belenkaya *et al.*, 2008).

1.3.2.4 ESCRT complex

Endosomal sorting complex required for transport (ESCRT) refers to the cellular machinery that sorts ubiquitin-modified cargo proteins targeted for degradation in the lysosome into multivesicular bodies (MVBs). The complex is composed of 4 sub-complexes (ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III) that act sequentially by recognising a mono ubiquitin modification on the cytosolic side of a membrane associated target protein. In this way cells modify ubiquitinated protein to target them for degradation in the lysosome. Through acting in concert the complexes invaginate and pinch off the membrane internalising the ubiquitinated protein on the luminal side of a vesicle contained in a MVB. These vesicles and the cargo they contain are then degraded after subsequent fusion with lysosomes (Hurley & Emr, 2006; Hurley, 2008).

1.3.2.5 Coat proteins and adapter proteins

The self-assembling molecular cytosolic coat scaffolds play an important role in vesicular trafficking between sub-cellular organelles. They are directly recruited from the cytosol to the membrane by small GTPases of the Arf1/Sar1 family where they help to deform flat membranes into round buds (Springer *et al.*, 1999). There are three known coat protein complexes described to date in mammalian cells. These are clathrin, coat protein complex I (COPI) and coat protein complex II (COPII) (Stagg *et al.*, 2007). These coats all contain an inner layer of adaptor protein (AP) complexes that link different types of cargo and aid in the selective incorporation of cargo into specific vesicle carriers for specific trafficking pathways (Cai *et al.*, 2007).

Clathrin forms a triskelion shape composed of three clathrin heavy chains and three light chains. Triskelions interact to form a polyhedral lattice to enclose a vesicle (Stagg *et al.*, 2007). Clathrin-coated vesicles (CCV) selectively function in several membrane trafficking pathways in post-Golgi traffic throughout the endosomal network and at the cell membrane. APs such as the Epsin family of proteins (Hirst *et al.*, 2004; Miller *et al.*, 2007) and AP180 (Ford *et al.*, 2001) are important for self-assembly and recruitment of the lattice as well as the correct selection of cargo.

COPI is a protein complex consisting of 7 subunits in a heptameric complex. COPI vesicles traffic cargo from the *cis-Golgi* to the ER and between Golgi stacks (Beck *et al.*, 2009; Hsu & Yang, 2009; Pinot *et al.*, 2010). COPI vesicle formation initiates with coat self-assembly followed by membrane deformation and fission of the coated vesicle and finally coat disassembly for recycling. Cargo containing the sorting motif KKXX interact with COPI to form carriers which are transported from the *cis-Golgi* to the rough ER (Beck *et al.*, 2009; Pinot *et al.*, 2010).

COPII is the coat protein involved in traffic from the ER to the Golgi. The COPII protein complex comprises two protein heterodimers Sec23/Sec24 and Sec13/Sec31 which are recruited to the site of budding on the ER membrane by Sar1 (Lee & Miller, 2007). GTP bound Sar1 initially recruits Sec23/Sec24. Sec23 activates the GTPase activity of Sar1 and Sec24 facilitates the selection cargo for incorporation into the nascent vesicle by recognising IxM, LxxLE and DxE class motifs on target proteins (Mancias & Goldberg, 2008). Sec13/Sec31 are then recruited to the membrane to complete the outer layer of the COPII coat which buds off and is transported to the Golgi (Lee & Miller, 2007).

1.3.2.6 Tethers

Molecular tethers are a class of large proteins that function in the initial interaction between a vesicle and its target membrane. The tether guides the vesicle into position to allow the pairing of transmembrane SNAREs on apposing membranes to facilitate fusion. Tethers have been shown to interact at long range with the coat proteins of incoming vesicles (Ford

et al., 2001). Together with Rabs, SNAREs and coat proteins they play an important role in the specificity of vesicle targeting (Cai *et al.*, 2007).

1.3.3 Organelles of the vesicular trafficking network

Cellular organelles are composed of proteins and lipids with distinct biochemical properties and processes. They maintain these characteristics in spite of a large flux of transiting cargoes.

1.3.3.1 The endoplasmic reticulum

The ER is a large cellular organelle that forms an interconnected network of tubules, vesicles, and cisternae. There are two types of ER in the cell. Rough ER are intimately associated with ribosomes and are the sites of protein synthesis within the cell whereas smooth ER are the sites within the cell where synthesis of lipids, steroids and the regulation calcium concentration occurs (McMaster, 2001; Hegde & Ploegh, 2010).

Proteins that are destined for secretion or transport to cellular organelles carry a signal sequence of between 5-15 hydrophobic amino acids preceded by a positively charged amino acid. When they are being synthesised by the ribosome the signal is recognised by the signal recognition protein on the ER membrane. This enables the ribosome to associate with the ER membrane and to translocate the emerging polypeptide into the lumen of the ER. In the ER lumen chaperone proteins function to ensure that newly synthesised proteins are correctly folded. This is also where proteins are modified through N-linked glycosylation (Matlack *et al.*, 1998; Baumann & Walz, 2001; Lee *et al.*, 2004; Hegde & Ploegh, 2010).

Vesicular carriers traffic continuously between the ER and the *cis*-Golgi ferrying proteins and lipids between these compartments. Proteins within the ER containing IxM, LxxLE and DxE class motifs are incorporated in COPII coated vesicles that leave the ER exit sites and traffic to the *cis*-Golgi. Then COPI coated vesicular carries traffic from the *cis*-Golgi back to the ER retrieving ER resident proteins and vesicle machinery (Lee &

Miller, 2007; Hsu & Yang, 2009). Soluble ER resident proteins that exit the ER and traffic to the *cis*-Golgi in vesicular carriers contain a specific KDEL motif that is recognised by KDEL receptor. At the *cis*-Golgi the KDEL receptor, which itself contains a KKXX COPI recognition sequence, binds the KDEL motif. The receptor and bound ligand are then transported from the *cis*-Golgi back to the ER in a retrograde manner in COPI coated vesicles (Pfeffer, 2007; Beck *et al.*, 2009).

1.3.3.2 The Golgi network

Synthesis of macromolecules in a cell is a continuous process which is essential for the viability of the cell. The Golgi network functions as a staging post within the cell for newly synthesised material and is integral in modifying, sorting and packaging them into carrier vesicles for anterograde traffic as well as receiving incoming retrograde traffic.

The Golgi network is composed of a series of cisternal membrane structures that are arranged in closely associated stacks. The stacks are arranged end to end in animal cells forming a ribbon-like structure (De Matteis & Luini, 2008). The stack is polarised and contains three substructure networks known as the *cis*-Golgi network, *medial*-Golgi network and the TGN. The *cis*-Golgi network exchanges proteins and lipids with the ER, whereas the TGN exchanges proteins and lipids with the plasma membrane and compartments of the endosomal network (Shorter & Warren, 2002; Wei & Seemann, 2010). Secretory material from the ER traverses through the Golgi network in a *cis* to *trans* direction. During this process proteins are post-translationally modified (e.g. O-glyosylation and N-glycosylation modification) in a hierarchical fashion (Wei & Seemann, 2010).

1.3.3.3 The *trans*-Golgi network

The TGN is a tubular network that sorts newly synthesised proteins that arrive from earlier Golgi network compartments into carrier vesicles for onward traffic as well as receiving incoming vesicular traffic from the endosomal network (De Matteis & Luini, 2008). TGN associated sorting motifs and signals are present in many of the proteins to allow

for the correct incorporation into carrier specific carrier vesicles. Examples include NPXY or YXXØ and di-leucine [DE]XXXL[LI] or DXXLL consensus motifs which are recognised as signals for endosomal-directed proteins. Post-translational modifications of cargo including O-glycosylation and N-glycosylation and ubiquitylation can also affect its sorting (De Matteis & Luini, 2008). Ubiquitylation can signal endosomal targeting in cooperation with ARF-binding proteins and Golgi-localised-ear-containing (GGA) proteins (Scott *et al.*, 2004).

Cytosolic adapter-protein (AP) complexes that recognise cargo sorting motifs are recruited to the TGN face in an ARF1 dependent fashion and mediate the packaging of cargo into emerging coated vesicles. AP proteins can also bind clathrin and function as adaptors between cargo proteins and the clathrin coat of the budding CCVs. Different AP proteins function in different trafficking pathways such as AP1A and AP3 complexes which mediate endosomal sorting (Robinson, 2004).

Despite being highly dynamic, the Golgi network maintains its structural organisation through a mixture of Golgi-resident matrix proteins and a balance between incoming and outgoing traffic (Short *et al.*, 2005).

1.3.3.4 The endosomal network

Endosomes are membrane-bound compartments within the cell which are components of the endocytic membrane transport pathway from the plasma membrane to the lysosome. There are three main classes of cellular endosomes, early endosomes (EE), late endosomes (LE), and recycling endosomes (RE). They differ in their biochemical and molecular properties and the cargo they are associated with (Mukherjee *et al.*, 1997; Mellman & Warren, 2000). These compartments and the endocytic trafficking pathway are discussed in more detail below.

Lysosomes are membrane bound compartments filled with acid hydrolase enzymes that digest materials, including microorganisms and cellular debris. The lumen of the lysosome is approximately pH 4.8. It is maintained by the pumping of H^+ ions by vacuolar-type H^+-

ATPase (v-ATPase) proton pump from the cytosol across the membrane into the lumen. Enzymes within the lysosome are pH sensitive and have an optimum activity around pH 4.5. This helps to restrict their activity to the lysosomal and late endosomal compartments as they are not active at the neutral pH in other cellular compartments. Lysosomes function by fusing with other membrane bound vacuoles, digesting their contents by exposing them to the degradative enzymes within their lumen (Luzio *et al.*, 2007). Lysosomal biogenesis and maintenance is discussed in greater detail below.

1.3.4 Major vesicular trafficking pathways

Major vesicular trafficking pathways of the cell are illustrated in Fig. 1.1.

1.3.4.1 The secretory pathway

The secretory membrane system allows cells to regulate delivery of newly synthesised proteins, carbohydrates, and lipids to the cell surface. Secretory cargo is synthesised in the ER and, once correctly folded, is transported from the ER to the Golgi network in a COPII mediated vesicular trafficking process (Palmer & Stephens, 2004). The cargo then traverses through the cisternae of the Golgi network, where it can be enzymatically modified by processes such as glycosylation. Eventually it arrives at the TGN where it is sorted and packaged into appropriate carrier vesicles for onward traffic. Apical and basolateral secretary proteins are sorted into their relevant vesicular trafficking routes based on specific signal sequences or glycosylation motifs (De Matteis & Luini, 2008). Secretory protein traffic can travel directly or via trans-endosomal routes to reach the plasma membrane (De Matteis & Luini, 2008).

1.3.4.2 Endocytic pathway

Endocytosis of extracellular molecules taken up at the plasma membrane can be clathrin dependent or independent (Maxfield & McGraw, 2004; Jones *et al.*, 2006). Following internalisation and formation of vesicles, material traverses the endocytic network of organelles

to be delivered to its final destination. The endocytic network consists of individual organelles with specific characteristics denoted by their protein and lipid content, their pH and morphology, all of which enable the correct sorting of cargo traveling in a retrograde manner (Johannes & Popoff, 2008).

Following internalisation, the newly formed vesicle moves through the endocytic network in a progressive manner. Initially, endocytic vesicles fuse with EE, denoted by the presence of PI(3)P, mild pH, Rab5 and Rab5 effector proteins such as EEA1 and PI3K. From here cargo can be targeted for degradation in the lysosome, recycled to the plasma membrane or targeted to the Golgi network. In the case of cargo destined for degradation in lysosomal compartments their carrier EE continues to mature, transitioning from an EE Rab5 positive compartment to late endosome/multi-vesicular body (LE/MVB) Rab7 positive compartment. These events are marked by a remodeling of the EE by the ESCRT machinery, resulting in the creation of intralumenal vesicles, a marked drop in luminal pH and Rab5 and its associated effectors being replaced by Rab7 and its effectors (Rink *et al.*, 2005; Poteryaev *et al.*, 2010; Wollert & Hurley, 2010). Finally the intralumenal vesicles of the LE/MVB are delivered to the lysosome, in a Rab7 dependent manner, for hydrolysis (Saftig & Klumperman, 2009; Cabrera & Ungermann, 2010).

1.3.4.3 Endocytic recycling to the plasma membrane

Endocytosis is a continuous process that removes membrane and protein from the plasma membrane. This is replenished by a process of endocytic recycling pathways, efficiently returning proteins and lipids to the plasma membrane. Plasma membrane composition is specified and maintained by balancing uptake and recycling. As such, endocytic recycling pathways are important factors in cellular activities, such as nutrient uptake, cell migration, cell adhesion and junction formation, cell cycle, signal transduction, and cell polarity (Maxfield & McGraw, 2004).

After internalisation vesicles quickly adopt the characteristics of EEs, recruiting Rab5 and EEA1 (Poteryaev *et al.*, 2010). At this stage a proportion of the endocytosed cargo

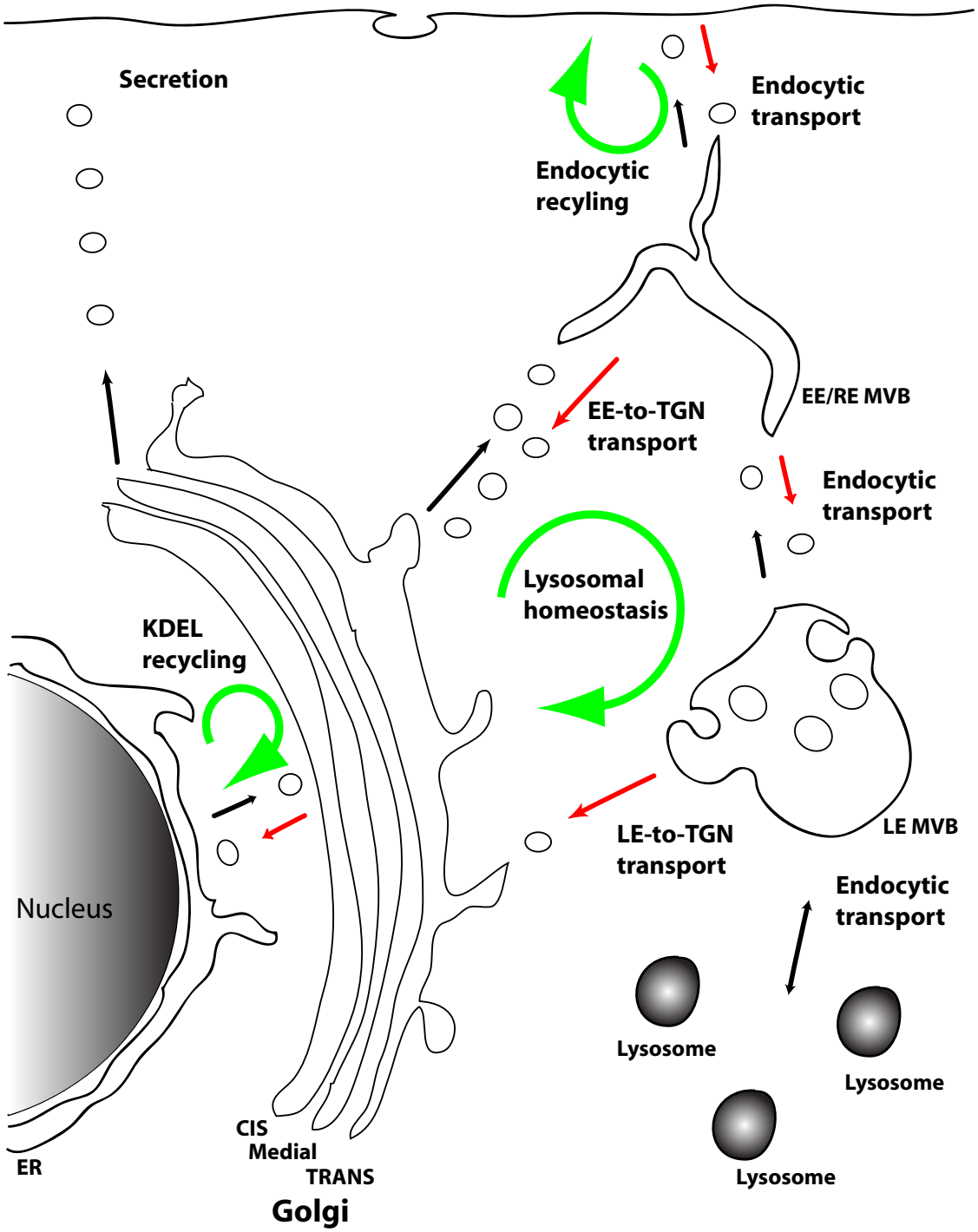


Figure 1.1 : ***Major vesicular trafficking routes.*** *Retrograde trafficking routes are denoted by red arrows and anterograde trafficking routes are denoted by black arrows. Recycling trafficking processes are denoted by green arrows and involve both anterograde and retrograde transport. EE, early endosome; RE, recycling endosome; MVB, multivesicular body; ER, endoplasmic reticulum.*

can be rapidly recycled back to the cell surface in a Rab4 and Rab35 dependent fashion. A slower recycling pathway to the plasma membrane involves the migration of cargo from EEs to the tubular early endosome recycling compartment (ERC) in a manner that requires SNX4 , Rab22A, Rab10, Rab11, dynein and Rab11 family-interacting protein (Rab11FIP)2/3/5 (Grant & Donaldson, 2009). From the ERC cargo can be packaged into tubular vesicular carriers and recycled to the plasma membrane, dependent on the function of Rab10, Rab11, Rab22A, Rab35, CDC42, ADP-ribosylation factor (Arf) 6 and cortical actin (Maxfield & McGraw, 2004; Grant & Donaldson, 2009).

An example of a cellular protein that undergoes recycling to the plasma membrane through the endocytic recycling pathway is the transferrin receptor (TrfR). It is a carrier protein for transferrin which is required to import of iron into the cell. It is regulated in response to intracellular iron concentration, internalising the transferrin-iron complex through endocytosis at the plasma membrane. It is transported to the ERC where the mild acidic environment causes iron cations to be released from transferrin and pass into the cytoplasm. The receptor, still bound to empty transferrin, is then transported back to the cell surface through the endocytic recycling pathways (Grant & Donaldson, 2009).

1.3.4.4 Endosome-to-TGN pathways

Anterograde traffic leaving the Golgi network is balanced by retrograde and retrograde recycling pathways. Cargo molecules that have been targeted to endosomes from the Golgi network or through the endocytic pathway can be transported to the TGN, other Golgi membranes and/or on to the ER via retrograde and retrograde recycling pathways (Bonifacino & Rojas, 2006; Johannes & Popoff, 2008).

Certain cellular proteins need to be recycled after they have carried out their function in the cell. Continuous cycling between endosomes and the TGN is a means by which the cell can maintain homeostasis between compartments. One of the most widely studied retrograde recycling pathways is that of the MPRs that function in the delivery of hydrolytic enzymes to lysosomes. Aspects of this trafficking pathway are discussed in section 1.3.4.6.

Additional cargoes trafficked in a retrograde manner include a protein of unknown function named TGN46/TGN38 and Shiga, Cholera and Ricin toxins that are transported to the Golgi network and then on to the ER before being translocated into the cytosol (Watson & Spooner, 2006; Ganley *et al.*, 2008; Johannes & Popoff, 2008; Stechmann *et al.*, 2010).

Much of the cellular machinery utilised in endosome-to-TGN pathways is common to all retrograde vesicular traffic to the Golgi and includes, among others, the multi-protein retromer complex and a clathrin adaptor protein known as EpsinR (Saint-Pol *et al.*, 2004; Bujny *et al.*, 2007; Bonifacino & Hurley, 2008). Two distinct retrograde trafficking routes that differ in the cargo trafficked and in the Q SNAREs that govern their specificity have recently been described by Ganley *et al.* (2008). MPRs are recycled to the TGN in a Syntaxin (Syn)10-dependent fashion whereas TGN46 and Cholera toxin B subunit require Syn6 (Ganley *et al.*, 2008). It is also likely that certain Rab GTPases and their effector proteins are restricted in the retrograde trafficking pathways that they function in. For example Rab9 and Tip47 are believed to function in retrograde recycling of MPRs from the LE/MVB compartment to the TGN but have little effect on Shiga toxin traffic from EE/ERC to the TGN (Johannes & Popoff, 2008; Pfeffer, 2009).

1.3.4.5 Endolysosomal homeostasis

Lysosomal organelles are maintained in the cell by a tightly regulated biogenesis pathway that requires constant delivery of synthesised lysosomal proteins to ensure homeostasis of the compartment. This is primarily undertaken by two MPRs, the cation-independent MPR (CI-MPR) and the cation dependent MPR (CD-MPR), that cycle between the TGN and the endocytic compartment (Ghosh *et al.*, 2003).

Most lysosomal hydrolases acquire a mannose-6-phosphate glycosylation tag during transport through the Golgi network, which is recognised by MPRs in the TGN (Ghosh *et al.*, 2003). The trafficking of the two MPR receptors has been studied extensively (Ghosh *et al.*, 2003; Johannes & Popoff, 2008). Although there is some debate as to the

finer details of the receptor trafficking, it is widely accepted that they bind their cargo of immature lysosomal enzymes in the Golgi network and traffic within nascent clathrin-coated-vesicles (CCV)s from the TGN to early endosomes. In the predominant pathway of MPR trafficking, the receptors remain with the maturing EE until they develop into LE/MVB compartments, an event characterised by increasing acidity of the luminal environment. In this acidic environment, the lysosomal enzymes dissociate from the receptors and mature, via peptide cleavage, into active lysosomal enzymes. After cargo dissociation, the receptors are sequestered to specific regions of the LE/MVB membrane and then recycled back to Golgi network to engage in another round of lysosomal enzyme binding and ferrying. In the case of the CI-MPR, a small proportion traffics from the TGN to the plasma membrane from where it is internalised into the endocytic pathway to traffic to be cycled back to the TGN (Ghosh *et al.*, 2003). Retrograde trafficking of MPRs has been shown to be dependent on a range of proteins and protein complexes including EpsinR, the retromer complex and SNX1 and 2, Rab9 and its effector Tip47 as well as the SNARE protein Syn10 (Riederer *et al.*, 1994a; Hanna *et al.*, 2002; Arighi *et al.*, 2004; Burguete *et al.*, 2004; Saint-Pol *et al.*, 2004; Ganley *et al.*, 2008; Mari *et al.*, 2008). Interference with any of these proteins by siRNA or dominant-negative proteins results in reduced Golgi-localisation of MPRs in HeLa cells. In the case of Rab9, Tip47 and Syn10, this caused increased secretion of lysosomal enzymes via the default secretory pathway (Riederer *et al.*, 1994a; Díaz & Pfeffer, 1998; Ganley *et al.*, 2008). Furthermore, it has been shown through investigations of CLN3, a Golgi network resident protein necessary for exit of MPR from the TGN, that a blockade in MPR traffic can directly affect lysosomal function (Metcalf *et al.*, 2008).

There is also evidence to suggest that there is a degree of MPR independent targeting of lysosomal enzymes. This conclusion is primarily based on observations of fibroblasts from patients with I-cell disease which are unable to add the mannose-6-phosphate modification to newly synthesised lysosomal enzymes. The predicted phenotype of enhanced secretion of lysosomal enzymes was observed for most but not all enzymes suggesting that some

were retained and trafficked independently of the MPRs (Neufeld, 1991). In agreement, LIMP-2 is a protein that has been reported to target beta-glucocerebrosidase to lysosomes in a mannose-6-phosphate-independent manner (Reczek *et al.*, 2007).

1.4 Interactions between bacterial pathogens and vesicular pathways

1.4.1 Phagosomal maturation

Phagocytosis is a form of endocytosis that allows cells to take up large particles, including bacteria, and is an essential innate immune function. Once the particles are internalised they are enclosed in a membrane bound compartment known as a phagosome. The phagosome matures in a process similar to endocytosis through sequential interactions with the endosomal network culminating in fusion with lysosomes (Vieira *et al.*, 2002).

The early phagosome recruits Rab5 and EEA1, fuses with EE/ERC and but not lysosomes. It has a neutral pH and very little hydrolytic activity (Mukherjee *et al.*, 1997; Flannagan *et al.*, 2009). The Rab5 effector PI3K is also recruited which generates PI3P on the membrane similar to EE membranes (Vieira *et al.*, 2001). As the early phagosome matures, EEA1 is lost from the intermediate phagosome but Rab5 is retained (Flannagan *et al.*, 2009). This maturation process continues with Rab5 being supplanted by Rab7 and its effectors on the late phagosomal membrane in a process probably dependent on the VpsC–homotypic protein sorting (HOPS) complex (Rink *et al.*, 2005). Additionally, Lamp1/2/3,CD63 and the v-ATPase, which reduces the luminal pH to 5.5 are recruited to the late phagosomal membrane (Desjardins *et al.*, 1994; Flannagan *et al.*, 2009). Finally the late phagosome fuses with lysosomes in a process dependent on Rab7 and Rab interacting lysosomal protein (RILP), and the late endocytic SNAREs Vamp8 and Vti1b to form a highly acidic, hydrolytic and microbicidal compartment known as a phagolysosome (Vieira *et al.*, 2002; Haas, 2007; Smith *et al.*, 2007; Furuta *et al.*, 2010).

Although not considered phagocytic cells, epithelial cells have fully functional endolysosomal systems. Bacteria that invade these cells and either do not escape or modify the vacuole, undergo a process of phagolysosomal fusion very similar to that observed in phagocytic cells.

1.4.2 Interference with phagolysosomal maturation

1.4.2.1 Bacterial pathogens other than *Salmonella*

When bacteria enter phagocytic host cells they are contained a membrane-bound vacuolar compartment called the phagosome. Following invasion of non-phagocytic cells, this is referred to as a vacuole. Under normal circumstances this compartment is trafficked sequentially through the endocytic network until fusion with degradative lysosomal compartments occurs (Vieira *et al.*, 2002; Haas, 2007). Many intracellular pathogens target the maturation process at a variety of stages, interfering with fusion steps between EE and LE and lysosomes or they modify the environment within the phagolysosome to allow intracellular survival and/or replication (Méresse *et al.*, 1999b; Duclos & Desjardins, 2000; Haas, 2007). Another class of invasive intracellular pathogen lyses the vacuolar membrane to escape into the host cytosol after entry into cells (Ray *et al.*, 2009).

The intracellular vacuole is lysed by pathogens such as *Shigella flexneri* and *Listeria monocytogenes,* after which they are propelled throughout the cytosol by the formation of an actin tail at one pole of the bacterial cell (Ray *et al.*, 2009). The vacuole integrity is compromised by bacterial proteins that are known virulence factors. In the case of *Shigella*, two T3SS effectors, IpaB and IpaC, that can insert into the membrane, are required for vacuolar rupture though the precise mode of action remains unclear (Blocker *et al.*, 1999; Mounier *et al.*, 2009). Vacuole lysis by *Listeria monocytogenes* is a result of several bacterial proteins including a pore-forming toxin listeriolysin-O (LLO), a phosphatidylinositol-specific phospholipase C (PI-PLC) and a broad-range phosphatidylcholine-specific phospholipase C (PC-PLC) (Smith *et al.*, 1995; Beauregard *et al.*, 1997; Ray *et al.*, 2009). Perforation of the vacuolar membrane by LLO also results in an alteration of pH and calcium ion

concentration which is believed to delay fusion with lysosomal compartments prior to full vacuolar escape (Shaughnessy *et al.*, 2006).

An example of an intracellular pathogen that deviates the phagosome from the endocytic route to create a specific intracellular niche is *Chlamydia*. The specialised membrane-bound compartment that *Chlamydia* resides in is known as an inclusion. After uptake the inclusion traffics to a region in close proximity to the Golgi network which then becomes fragmented, forming mini-stacks around the inclusion that are important for bacterial replication (Hackstadt *et al.*, 1996; Heuer *et al.*, 2009). The inclusion preferentially fuses with sphingomyelin containing exocytic vesicles that facilitate its growth (Hackstadt *et al.*, 1996; van Ooij *et al.*, 2000). The inclusion lacks markers of LE and lysosomes such as lysosomal membrane glycoproteins (lgps). In this manner *Chlamydia* creates a unique compartment that selectively interacts with a subset of host cell vesicular traffic enabling growth and survival (Capmany & Damiani, 2010). Small Rab GTPase proteins and SNAREs are thought to be candidates for subversion by *Chlamydia* effectors translocated across the inclusion membrane by a T3SS to enable specific interaction with cellular traffic (Betts *et al.*, 2009). An interesting effector protein of *Chlamydia* known as IncA contains domains that are similar to mammalian SNAREs and are believed to function as SNARE mimics, facilitating fusion at the inclusion surface with vesicular traffic (Delevoye *et al.*, 2008).

The *Mycobacterium tuberculosis* phagosome is believed to be retarded in its maturation as it retains Rab5, is of a neutral pH and excludes the late phagosomal GTPase Rab7, lgps and V-ATPase (Sturgill-Koszycki *et al.*, 1996; Deretic & Fratti, 1999; Li & Xie, 2011). EEA1, a Rab5 effector protein from the early phagosome is also absent potentially indicating a maturation block at the intermediate phagosome (Flannagan *et al.*, 2009). In this way *Mycobacterium tuberculosis* is believed to negate the harmful effects of lysosomal fusion (Deretic & Fratti, 1999; Li & Xie, 2011). There are several bacterial factors and unique features of the *Mycobacterium tuberculosis* phagosome that are thought to have a role in preventing its maturation. Coronin-1 (also referred to as TACO) is a host protein that has been observed on the *Mycobacterium tuberculosis* phagosome which is absent from

phagosomes containing killed bacteria or phagosomes of activated macrophages. In cells depleted of Coronin-1, *Mycobacteria bovis* is delivered to lysosomes implicating it in having a role in inhibiting maturation (Jayachandran *et al.*, 2008). The mycobacterial lipoamide dehydrogenase C (LpdC) has been shown to bind Coronin-1 and is believed to have a role in its retention on the *Mycobacterium* phagosome (Deghmane *et al.*, 2007). *Mycobacterium* is also believed to disrupt the interaction between Rab7 and its effector RILP, thereby preventing phagolysosomal formation (Sun *et al.*, 2007a). In addition, SapM is a phosphatase of *Mycobacterium tuberculosis* which hydrolyses PI(3)P, depleting it from the phagosomal membrane (Vergne *et al.*, 2005). This is thought to prevent the transition to form the phagolysosome.

Both *Legionella pneumophila* and *Brucella abortus* are retained within vacuoles that segregate from the endocytic pathway and interact extensively with the ER. They also acquire LC3, a protein integral to the autophagic response pathway, suggesting that these compartments also interact with the autophagic membranes (Haas, 2007; Lerena *et al.*, 2010).

Coxiella burnetii is the only intracellular pathogen which appears to survive and replicate in an acidified lysosomal-like compartment (Méresse *et al.*, 1999b). Early on after uptake into macrophage cells, *C. burnetii* recruits the autophagic protein LC3 which is thought to delay fusion with lysosomes (Romano *et al.*, 2007). The delay allows time for the bacteria to enter into its replicative large-cell variant phase of its life cycle (Flannagan *et al.*, 2009). Later on, *C. burnetii* resides in a compartment called the replicative *Coxiella* vacuole that is acidic (pH 4.8) and contains several lysosomal proteins, including the V-ATPase (Maurin *et al.*, 1992). The mechanisms of how *Coxiella burnetii* survives and replicates is such a compartment are not yet known (Flannagan *et al.*, 2009).

1.4.2.2 Interaction between *Salmonella* and endosomal pathways - early events

After uptake into host cells by phagocytosis or invasion, *Salmonella* resides in a membrane bound compartment or SCV. Immediately after entry, the SCV begins to migrate to a

perinuclear location, that in epithelial cells coincides with the Golgi network, to establish an intracellular replicative niche (Salcedo & Holden, 2003; Knodler & Steele-Mortimer, 2003; Abrahams & Hensel, 2006). During this process of migration, the SCV undergoes a variety of changes which resemble that of the normal pathway of endocytic/phagosomal maturation (Méresse *et al.*, 1999b). Within 5-10 minutes following uptake markers such as EEA-1 and the Rab5 GTPase are present on the SCV membrane, indicating association with EE (Steele-Mortimer *et al.*, 1999). As the trafficking of the SCV progresses towards the centre of the cell these early markers are lost and LE/lysosome markers such as Rab7 and the lgps, Lamp1 and Lamp 2 are detectable on the SCV surface (Méresse *et al.*, 1999a; Garcia-del Portillo & Finlay, 1995). However, LE/lysosomes markers such as cathepsin D and the MPRs are reduced on the SCV indicating that *Salmonella* selectively avoids fusion with mature lysosomes within the first two hours of infection (Garcia-del Portillo & Finlay, 1995; Knodler & Steele-Mortimer, 2003; Brumell & Grinstein, 2004; Bakowski *et al.*, 2010).

1.4.2.3 Interaction between *Salmonella* and endosomal pathways - late events

In epithelial cells bacterial replication occurs after SCV migration to the perinuclear region (Ramsden *et al.*, 2007). The onset of bacterial replication coincides with the translocation of SPI-2 T3SS effectors and the appearance of Sifs (Garcia-del Portillo *et al.*, 1993; Stein *et al.*, 1996). In addition, *Salmonella* replication in epithelial cells is characterised by the formation of tight bacterial clusters (microcolonies) in the vicinity of the Golgi network (Salcedo & Holden, 2003). Efficient intracellular bacterial growth, Sif formation, SCV integrity and microcolony location have been shown to be dependent on the SPI-2 T3SS. At later time points the SCV and Sifs are enriched in the lgps Lamp1 and Lamp2 and the Golgi network associated protein SCAMP3 but not with other proteins such as MPRs and cathepsins (Garcia-del Portillo & Finlay, 1995; Smith *et al.*, 2005; Mota *et al.*, 2009). This implies the SCV requires the fusion of certain membranous compartments and the segregation of others. This was believed to be the basis through which *Salmonella* avoided

the detrimental effects associated with lysosomal fusion. Recently this view has been challenged after live cell imaging experiments suggested that at late stages of infection some fusion between the SCV and lysosomal compartments occurs (Drecktrah *et al.*, 2007). This is a view that is corroborated by an older study that shows complete phagolysosomal formation in *Salmonella* infected macrophages (Oh *et al.*, 1996). The mechanisms by which *Salmonella* is able to integrate certain components of the host endomembrane system whilst excluding others is not understood.

1.5 Aims

Little is known about the specific vesicular trafficking pathways that *Salmonella* interacts with, although fusion and fission of vesicles with SCVs can be readily observed by live imaging microscopy (Dumont *et al.*, 2010). It seems likely that through interaction with vesicular traffic in the perinuclear region *Salmonella* acquires the materials necessary for its replication (Drecktrah *et al.*, 2008; Jackson *et al.*, 2008a). The possibility that *Salmonella* manipulates specific host cell machinery to modulate vesicular traffic is highly likely given the observation that the SCV acquires some LE/Lysosome proteins but excludes others (Garcia-del Portillo & Finlay, 1995). However, the roles that specific cellular components may have in the recruitment of membrane for replication and Sif formation during *Salmonella* infection remain unclear. Key cellular candidates that could be involved in the interaction between *Salmonella* and late endocytic compartments are SNARE proteins, especially the LE R-SNAREs, VAMP7 and VAMP8, and Q-SNARES, Syntaxin7 (Syn7), Syntaxin8 (Syn8) and Vti1b as well as late endocytic GTPases that govern LE homeostasis.

During this study I aimed to understand more about the interactions between *Salmonella* and vesicular traffic that intersects with LE compartments. This was to be achieved by:

- undertaking an immunofluorescence microscopy screen in infected HeLa cells infected with *Salmonella* to assess any morphological changes in the distribution of proteins

involved in trafficking pathways of LE compartments.

- analysing, in a quantitative manner, the changes in distribution of any proteins found to have altered labelling in infected cells.
- assessing what role SPI-2 T3SS effector proteins might have in altering the distribution of any candidate proteins.
- understanding what the physiological impact that any altered protein localisation might have on the cellular environment and how this could influence *Salmonella* virulence.

Chapter 2

Materials and Methods

2.1 Bacterial strains and plasmids

The *S.* Typhimurium strains used in this study were wild-type 12023s and its mutant derivatives: *ssaV::aphT* (Deiwick *et al.*, 1998), *aroCpurD* (Ruiz-Albert *et al.*, 2002), *sifAsseJ* (Ruiz-Albert *et al.*, 2002), *sifAsopD2* (Schroeder *et al.*, 2010) and *sseJ* (Ruiz-Albert *et al.*, 2002). All *S.* Typhimurium strains carried the pFPV25.1 plasmid carrying *gfpmut3A* under the control of the *rpsM* constitutive promoter (Valdivia & Falkow, 1997) with the exception of those used during experiments to quantify levels of CD-MPR at the TGN, where detection of $pSifA_{wt}$-2HA, $pSifA_{(L130D)}$-2HA or pSseJ-2HA was required. The plasmids $pSifA_{wt}$-2HA and $pSifA_{(L130D)}$-2HA (Diacovich *et al.*, 2009) and transfection vectors encoding EGFP::SifA and EGFP::SifAL130D were gifts from Dr. Stéphane Méresse (Centre d'Immunologie de Marseille-Luminy; Marseille, France). Plasmid pSseJ-2HA, which is a derivative of pACYC184 carrying the *sseJ-2HA* gene fusion under the control of a constitutive promoter (Chang & Cohen, 1978), was introduced into the *aroCpurD* mutant to detect effector translocation from this strain.

2.2 Antibodies

The primary antibodies used in this work are listed in Table 2.1. Working dilutions for each individual applications are also displayed in Table 2.1. Alexa 488- (Invitrogen), Alexa 555- (Invitrogen), Alexa 633- (Invitrogen) conjugated donkey anti-goat, anti-rabbit, anti-mouse, anti-sheep or anti-rat antibodies were used for immunofluorescence at a dilution of 1:200. Goat anti-rabbit (Santa Cruz) and sheep anti-mouse (Amersham Pharmacia Biosciences) were used at a dilution of 1:10,000 for immunoblot analysis.

2.3 siRNA oligos

siRNA oligo duplexes targeted against non-overlapping regions of Vti1b, CD-MPR, Syntaxin 10 or Syntaxin 6 mRNA, were either used singly or in pools of 4 (SMARTpool, Dharmacon Inc., UK). The siRNA oligo duplex targeted against SKIP was custom synthesised using a previously described targeting sequence (Boucrot *et al.*, 2005). The scramble pool of 4 control oligos (Dharmacon Inc., UK) were designed not to target any human mRNA transcript.

2.4 Cell growth

Bacteria were grown in Luria Bertani (LB) medium at 37 °C with shaking and supplemented with ampicillin (50 μg/ml), kanamycin (50 μg/ml), tetracycline (25 μg/ml) or chloramphenicol (34 μg/ml) as appropriate.

HeLa (human epithelial cell line 93021013) cells used in this study were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL) supplemented with 10% foetal calf serum (FCS) (PAA Laboratories) at 37 °C in 5% CO_2. RAW264.7 macrophages were obtained from the European Collection of Cell Cultures (Sigma, U.K.) were maintained in DMEM supplemented with 10% FCS at 37 °C in 5% CO_2.

Primary bone-marrow macrophages (BMM) were obtained from C57BL/6 WT (Charles

River) mice. Cells were grown in RPMI (GIBCO BRL) supplemented with 10% FCS, 2 mM glutamine, 10 mM Hepes, 50 μM β-mercaptoethanol, 100 U/mL penicillin/streptomycin, and L929 cell-conditioned medium 20% (vol/vol; National Institute for Medical Research)(complete medium). After 3 days of culture, further fresh complete medium containing L929 cell–conditioned medium was added to the growing macrophages. On day 7, cells were washed and seeded in complete medium without antibiotic and incubated for 24 h before bacterial challenge.

2.5 Bacterial infection of macrophages

For infection, RAW264.7 macrophages and BMM were grown overnight then infected with *S.* Typhimurium strains as described previously (Beuzón *et al.*, 2000). In brief, all bacteria were grown in LB broth, supplemented with the appropriate antibiotic, overnight at 37 ºC with shaking. For RAW264.7 macrophage infections bacteria were opsonised with DMEM containing 10% of FCS and 10% of normal mouse serum for 20 min. Bacteria were added to the cell monolayers at a multiplicity of infection (MOI) of approximately 5, centrifuged at 110 x g for 5 min at room-temperature (RT) and incubated at 37 ºC, 5% CO_2 for 25 min. The cells were washed 3 times with DMEM containing 10% FCS and 100 μg/ml gentamicin and incubated in the same medium for 1 h. After 1 h cells were washed once with fresh medium and the concentration of gentamicin was decreased to 20 μg/ml for the remainder of the experiment. The infection protocol for BMM was as described for RAW264.7 macrophages with the exceptions that complete medium (without penicillin/streptomycin and LCM) was used in place of DMEM for opsonisation of bacteria prior to infection and subsequent media exchanges.

Antibody, Antigen	**Species Origin**	**Application, Dilution**	**Source**
TGN46	Sheep	IF 1/200	ABD Serotec
C19 Rab6	Rabbit	IF 1/500	Santa Cruz
GM130	Mouse	IF 1/1000	Transduction Laboratories
EpsinR	Rabbit	IF 1/100	Gift from Professor Margaret Robinson, (Cambridge Institute for Medical Research)
Vamp3	Rabbit	IF 1/400	Gift from Doctor Andrew Peden, (Cambridge Institute for Medical Research)
Vamp 4	Rabbit	IF 1/500	Gift from Doctor Andrew Peden
Vamp 7	Rabbit	IF 1/400	Gift from Doctor Andrew Peden
Vamp 8	Rabbit	IF 1/400	Gift from Doctor Andrew Peden
Vti1b	Mouse	IF 1/33	BD Biosciences
		IB 1/250	
		FACS 1/100	
Syntaxin 6	Mouse	IF 1/100	BD Biosciences
Syntaxin 7	Rabbit	IF 1/500	Gift from Doctor Andrew Peden
Syntaxin 10	Rabbit	IF 1/50	Gift from Doctor Andrew Peden
		IB 1/250	

Table 2.1 : ***Antibodies used in this work.*** *IF - Immunofluorescence; IB - Immunoblot; FACS - flow cytometry.*

Antibody, Antigen	Species Origin	Application, Dilution	Source
Syntaxin 8	Rabbit	IF 1/500	Gift from Doctor Andrew Peden
Syntaxin 16	Mouse	IF 1/1000	Gift from Doctor Andrew Peden
H68.4 TrfR	Mouse	IF 1/50	Invitrogen
SNX1	Mouse	IF 1/100	BD Biosciences
SNX2	Mouse	IF 1/100	BD Biosciences
VPS29	Rabbit	IF 1/200	Gift from Doctor Matthew Seaman, (Cambridge Institute for Medical Research)
VPS35	Rabbit	IF 1/200	Gift from Doctor Matthew Seaman
H4A3 Lamp1	Mouse	IF 1/500	Santa Cruz
SKIP	Mouse	IB 1/1000	Gift from Doctor Stéphane Méresse University of Iowa
E7 Tubulin	Mouse	IB 1/1000	Human Hybridoma Bank
Actin	Rabbit	IB 1/5000	Sigma
HA	Mouse	IF 1/200	Roche
22D4 CD-MPR	Mouse	IF 1/100 IB 1/1000 FACS 1/300	Human Hybridoma Bank
2G11 CI-MPR	Mouse	IF 1/5000 FACS 1/5000	Abcam

Table 2.2 : ***Antibodies used in this work.*** *IF - Immunofluorescence; IB - Immunoblot; FACS - flow cytometry.*

2.6 Bacterial infection of HeLa cells

For infection, HeLa cells were grown overnight and then infected with *S.* Typhimurium strains as described previously (Beuzón *et al.*, 2000). In brief, all bacteria were grown in LB broth, supplemented with the appropriate antibiotic, overnight at 37 ºC with shaking. The bacterial cultures were diluted 1:33 in 3 ml of fresh medium, supplemented with appropriate antibiotic, and were grown under the same conditions until the culture reached OD600 of 1.5 – 2.0. Bacteria were diluted in pre-warmed Earl's buffered salt solution (EBSS, Invitrogen) and added to the cells at a MOI of approximately 100 (5 µl of bacterial culture to 5 x 10^4 HeLa cells). Invasion was allowed to proceed for 15 min at 37 ºC, 5% CO_2. After 15 min cells were washed once with DMEM containing 10% FCS and 100 µg/ml gentamicin and incubated in the same medium for 1 h. After 1 h cells were washed once with fresh medium and the concentration of gentamicin was decreased to 20 µg/ml for the remainder of the experiment.

2.7 Preparation and transformation of electrocompetent cells

Bacteria were grown in LB overnight at 37 ºC with shaking. The culture was diluted 1:100 in 5 ml of fresh medium and bacteria were grown under the same conditions until the culture reached OD600 of 0.5–0.6. Bacteria were incubated on ice for 15-30 min, centrifuged at 1000 x g at 4 ºC for 10 min and then the supernatant was discarded. The bacterial pellet was washed three times in 25 ml ice-cold sterile MilliQ water with centrifugation and supernatent removal after each wash. The final centrifugation and supernatent removal was followed by resuspending the bacterial pellet in 0.25 ml ice-cold sterile 10% glycerol. Aliquots of 75 µl were used immediately or stored at -80 ºC.

Bacteria were transformed by electroporation by incubating 75 µl of electrocompetent bacterial cells with approximately 25 ng plasmid DNA in a 2 mm electroporation cuvette

(Molecular Bioproducts) on ice for 5 min. Electroporation was performed at 2.5 kV, 200 Ω, 25 mF for 4-5 milliseconds using a GenePulser II (BioRad). Bacteria were then incubated in 1 ml SOC medium and incubated at 37 ^{o}C with shaking for 1 h. Transformants were selected by plating on LB agar containing the appropriate antibiotic.

2.8 siRNA transfection

HeLa cells were seeded in 12 well plates and grown until they were 70% confluent. siRNA transfection using RNAiMAX (Invitrogen, UK) was carried out according to the manufacturer's protocol with a final concentration of pooled or single siRNA oligos of 10 nM. A scrambled pool of 4 oligo sequences was included in all experiments as a negative control. Cells were collected 24 h after transfection and re-seeded in 12 well plates at a concentration of 1 x 10^5 cells/well. Cells were then transfected a second time as above and 24 h after that were seeded again at appropriate concentrations for analysis.

2.9 DNA transfection

HeLa cells were seeded in 24 well, 12 well or 35 mm glass bottomed dishes (Matek) and grown until they were 70% confluent. DNA transfection procedures were carried out according to the manufacturer's protocol (Lipofectamine™ 2000, Invitrogen, UK) with 400 ng of DNA. Samples were prepared for analysis 24 h after transfection. Only cells with similar levels of expression, as determined from their GFP fluorescence using FACS analysis or fluorescence microscopy, were included for analysis.

2.10 Fixation, fluorescence labelling and microscopy

Cell monolayers were fixed, permeabilised and labelled as described previously (Beuzón *et al.*, 2002). Briefly, samples were fixed using 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 15 min at RT. Remaining free aldehyde was quenched by incu-

bation with 10 mM NH_4Cl for at least 1 h. Samples were permeabilised for 5 min in 0.1% saponin in PBS (saponin-PBS). The antibodies were diluted to the appropriate concentration in 10% horse serum in saponin-PBS. Coverslips were washed twice in saponin-PBS and incubated with primary antibodies for 1 h at RT. Following incubation coverslips were washed twice with saponin-PBS and incubated with secondary antibodies for 30 min. After incubation with secondary antibodies cells were washed twice in saponin-PBS, once in PBS, once in MilliQ H_2O and mounted in Mowiol mounting medium. Fluorescently labelled samples were analyzed using either an epi-fluorescence microscope (BX50; Olympus, Melville, NY) or a confocal laser-scanning microscope (LSM510; Zeiss, Thornwood, NY).

2.11 Qualitative scoring of Vti1b localised to the TGN

HeLa cells were infected with wild-type or *ssaV* mutant *S.* Typhimurium for 14 h. Quantification of the number of cells showing a TGN localisation of Vti1b in *Salmonella* infected and non-infected cells was performed by epi-fluorescent microscopy. Quantification was initially conducted in the fluorescent channels associated with Vti1b and TGN46 labelling. The cells were assessed as to whether there was TGN-associated labelling of Vti1b or not. Next, the fluorescent channel was switched to reveal *Salmonella* and the same cells were assessed according to whether they were infected or not. At least 50 cells were analyzed for each condition. Triplicate experiments were conducted for each condition analyzed. Statistical differences were determined using a two-tailed student t-Test. A probability value of less than 0.05 was used to indicate a statistically significant difference.

2.12 Quantification of proteins localised to the TGN

Confocal three-dimensional projections were acquired for each sample using a slice increment of 0.4 μm and 40 x optical magnification. Individual TGNs from cells were selected automatically using a protocol established in Volocity image analysis software (Perkin Elmer, UK). TGNs were identified as objects within a volume range of 50 to 500 μm^3

according to an arbitrary threshold of fluorescence intensity associated with anti-TGN46 or anti-Rab6 antibody labelling. Once individual objects were identified, the mean fluorescent signal from pixel-to-pixel co-labelled protein was quantified using Volocity software. These values were further partitioned, where appropriate, into those arising from infected and non-infected or transfected and non-transfected cells, determined by GFP or HA labelling. At least 50 TGNs from each condition were quantified for each sample. Unless stated otherwise, all experiments were repeated at least three times. Statistical differences were determined using a two-tailed student t-Test. A probability value of less than 0.05 was used to show a statistically significant difference.

2.13 Plasma membrane-to-TGN transport assays

CI-MPR transport or CTxB transport assays were undertaken as described previously (Ganley *et al.*, 2008; Robinson *et al.*, 2010) with the following modifications. HeLa cells were transfected with Syn6 siRNA as described above or were infected with wild-type or *ssaV* mutant *S.* Typhimurium for 13 h. Then, cells were incubated with culture medium containing either anti-CI-MPR antibody (2G11) or Alexa Fluor 555-CTxB (Invitrogen, U.K.) for 30 min followed by a chase of 30 min with medium only.

Quantification of the number of cells showing CI-MPR or CTxB localisation at the TGN was done by epi-fluorescence microscopy. Quantification was initially conducted in the fluorescent channels associated with CI-MPR or CTxB and TGN46 or Rab6 labelling. The cells were assessed as to whether there was TGN-associated labelling of CI-MPR or CTxB or not. Next, where appropriate, the fluorescent channel was switched to reveal *Salmonella* and the same cells were assessed according to whether they were infected or not. At least 50 cells were analyzed for each condition. Triplicate experiments were conducted for each condition analyzed. Statistical differences were determined using a two-tailed student t-Test. A probability value of less than 0.05 was used to show a statistically significant difference.

2.14 Lysosomal enzyme secretion assays

The activities of secreted β-hexosaminadase and cathepsin D were analyzed as described (Riederer *et al.*, 1994b; Yasuda *et al.*, 1999) with the following modifications: HeLa cells were grown in 6 well plates, seeded at a concentration of 2 x 10^5 cells/well, for 24 h. Cells were infected with wild-type or *ssaV* mutant *S.* Typhimurium. At 1 h after inoculation and for every subsequent media exchange DMSO or amiodarone (Sigma, U.K.) were added to negative and positive control cells, respectively. Amiodarone was used at a concentration of 8 μM. At 8 h after inoculation cells were washed 4 times with pre-warmed collection media (OPTI-MEM, GIBCO BRL) supplemented with 10 mM mannose-6-phosphate (Sigma, U.K.) and 20 μg/ml gentamicin. Collection medium (1.5 ml) was then added to each well and the cells were incubated once more at 37 °C. At 14 h after inoculation extracellular β-hexosaminidase activity was assayed in 480 μl of the collection medium by addition of 120 μl of β-hexosaminidase substrate buffer (0.5 M NaOAc pH 4.4, 0.5% Triton X-100, 5 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-d-glucopyranoside (Sigma, UK)). After 1 h 30 min at 37 °C, reactions were stopped by addition of 600 μl of 0.5 M glycine, 0.5 M Na_2CO_3, pH 10.0. Release of fluorescent substrate was measured by a Cary Eclipse fluorescence spectrophotometer using an excitation wavelength of 360 nm and emission wavelength of 447 nm. Intracellular β-hexosaminidase activity was determined by scraping the cells of one well in 500 μl of cell collection buffer (10 mM phosphate buffer, pH 6.0, 0.15 M NaCl, 0.5% Triton X-100) followed by centrifugation at 60,000 g for 30 min. The supernatant was transferred into a fresh tube and 120 μl of sample and 120 μl of hexosaminidase substrate buffer were diluted with 360 μl of OPTI-MEM. After 1 h 30 min at 37 °C, reactions were stopped by addition of 600 μl of 0.5 M glycine, 0.5 M Na_2CO_3, pH 10.0 and samples were assayed as described for extracellular β-hexosaminidase activity.

At 14 h after inoculation, extracellular cathepsin D activity was assayed in 80 μl of the collection medium by addition of 20 μl of cathepsin D substrate buffer (0.25 M NaOAc, pH 4.2, 0.25% Triton X-100, 100 μM MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-d-Arg-NH2 (Peptide Institute, Japan)). After 1 h at 37 °C, reactions were

stopped by addition of 600 µl of 5% TCA. Release of fluorescent substrate was measured by a Cary Eclipse fluorescence spectrophotometer using an excitation wavelength of 328 nm and emission wavelength of 398 nm. Intracellular cathepsin D activity was determined by scraping the cells from one well in 500 µl of cell collection buffer (10 mM phosphate buffer, pH 6.0, 0.15 M NaCl, 0.5% Triton X-100) followed by centrifugation at 60,000 g for 30 min. The supernatant was transferred into a fresh tube and 20 µl of sample and 20 µl of cathepsin D substrate buffer were diluted with 60 µl of OPTI-MEM. After 1 h at 37 °C, reactions were stopped by addition of 600 µl of 5% TCA and samples were assayed as described for extracellular cathepsin D activity. β-hexosaminidase and cathepsin D activity from intracellular and extracellular samples were expressed as a % ratio of total enzyme activity, which equals the sum of intracellular and extracellular activities. Statistical differences were determined using a two-tailed student t-Test. A probability value of less than 0.05 was used to show a statistically significant difference.

To analyze protein levels of intracellular and secreted cathepsin D, HeLa cells were grown in 10 cm circular plastic dishes and seeded at a concentration of 2.5 x 10^6 cells/dish. Cells were infected with wild-type or *ssaV* mutant *S.* Typhimurium. At 1 h after inoculation and for every subsequent media exchange DMSO, amiodarone (8 µM) or chloramphenicol (34 µg/ml) were added as appropriate. At 8 h after inoculation the cells were washed 4 times with pre-warmed collection media (OPTI-MEM, GIBCO BRL). Collection medium (4 ml) was then added to each dish and the cells were incubated once again at 37 °C. At 14 h after inoculation the collection medium was harvested (extracellular fraction) and concentrated using Amicon Ultra-15 Centrifugal Filter Units according to the manufacturer's protocol (Millipore, U.K.). Cells were collected using a trypsin-EDTA solution (GIBCO BRL), then resuspended in equal volume of lysis buffer (20 mM Tris-Cl pH 8.0, 0.5% NP40, 150 mM NaCl) in the presence of protease inhibitors (Complete EDTA-Free, Roche). Intracellular samples were normalised by dilution to give the same overall protein concentration. The same dilution factor was applied to each of the corresponding extracellular fractions, so that samples reflected an equivalent number of cells. Intracellular and

extracellular proteins were then separated by SDS-PAGE and analyzed by immunoblot using antibodies against cathepsin D and actin (loading control).

2.15 FRAP analysis

HeLa cells, RAW264.7 macrophages or BMM were grown on 35 mm glass bottomed dishes (Matek) seeded at a concentration of 1.0-5.0 x 10^5 cells/dish in appropriate media. Cells were then either transfected or infected with *S.* Typhimurium strains. At 1 h 30 min prior to imaging, growth medium was replaced with imaging medium (OPTI-MEM, GIBCO BRL) and incubated at 37 °C. For imaging, Magic Red cathepsin B peptide (MAGIC RED™ Cathepsin B, ABD Serotec, U.K.) was added to the medium at one tenth the manufacturer's recommended concentration and maintained for the duration of imaging. Live cell imaging and FRAP analysis were done using a LSM510 Zeiss confocal microscope. FRAP bleaching was done on entire cells using 458, 488, 543, and 633 nm lasers with a 40 x objective. Approximately 5 to 8 cells per field of view were bleached for a duration of 100 bleaching cycles according to the bleaching module within the LSM510 software (LSM510 version 3.2). Post-bleaching images were acquired every three seconds for 150, 200 or 300 seconds. Quantification of fluorescence was carried out using Volocity software, with each data set having approximately 25 cells in each condition analyzed. Fluorescence values were converted to relative values by expressing the first post-bleach value as zero. Inter-sample normalisation was done using the fluorescence values of non-infected or non-transfected samples, as appropriate. All experiments were repeated at least three times.

2.16 FACS analysis

For assays to determine intracellular bacterial replication, HeLa cells were seeded at a concentration of 2 x 10^5 cells/well in 6 well plates and treated with mock, scramble, Syn6 or Syn10 siRNA oligos or exposed to a lysosomal protease inhibitor (Cathepsin Inhibitor III, Merck, U.K.) at a concentration of 0.4 μM, 4 h prior to infection. The inhibitor

was maintained through all subsequent media exchanges. Cells were then infected with wild-type *S.* Typhimurium as described above. Cells were collected at 2 h and 8 h after inoculation following exposure to trypsin-EDTA solution and cells from each well were resuspended in 10 ml of 10% FCS DMEM. Cells were then washed twice in PBS, centrifuged and resuspended in 1 ml of PBS. Cells were fixed in 4% paraformaldehyde-PBS for 20 min, washed twice in PBS and then re-suspended in 1 ml PBS for analysis. A total of over 10,000 events were analyzed on a FACS Calibur^{TM} flow cytometer (Becton Dickinson) for fluorescence intensities in channel FL-1. Uninfected cells were used to calibrate the instrument settings. Data were analyzed with FlowJo^{TM} 8.6.3 (Tree Star Inc., U.S.A.) software. Results were expressed as GFP fluorescence per HeLa cell and represent the median of the ratio between 2 h and 8 h after inoculation normalised to mock or DMSO treated cells.

For FACS assays to determine cathepsin B activity, HeLa cells were transfected as described above. At 2 h prior to analysis, cell growth medium was replaced with imaging medium and cells were incubated at 37 °C for 1 h 30 min. Cells were collected and washed twice in PBS, then resuspended in 1 ml of imaging medium. Magic Red cathepsin B peptide was added as described above and maintained for the duration of the analysis. A total of over 10,000 events were analyzed on a LSR Fortessa^{TM} flow cytometer (Becton Dickinson) for fluorescence intensities using a 530 nm emission filter and a 640-670 nm band pass emission filter. Untransfected cells were used to calibrate instrument the settings. Data were analyzed with FlowJo^{TM} 8.6.3 software.

For assays FACS assays to quantify the intracellular levels of Vti1b and CD-MPR, HeLa cells were seeded as appropriate and treated with mock, scramble, Vti1b or CD-MPR siRNA oligos as described above. Mock treated cells were then infected with wild-type or *ssaV* mutant *S.* Typhimurium as described above. Cells were collected at 14 h after inoculation following exposure to trypsin-EDTA solution and cells from each well were resuspended in 10 ml of 10% FCS DMEM. Cells were then washed twice in PBS, centrifuged and resuspended in 4% PFA-PBS and fixation was allowed to proceed for for 20 min. Cells

were then washed twice in PBS and re-suspended in 300 μl of FACS permeablisation PBS solution (0.1% saponin, 1% azide, 10% horse serum). Primary antibodies were added to the cells at concentrations stated in Table 2.1 and Table 2.2. Labelling was allowed to proceed for 1 h at room temperature. Cells were then washed in 4 ml of FACS permeablisation buffer, centrifuged, resuspended in 300 μl of FACS permeablisation buffer and secondary antibodies were added at concentrations iterated above. Labelling was allowed to proceed for 30 min in the absence of light at room temperature. Cells were then washed twice in PBS solution and then resuspended in 1 ml PBS solution for analysis. A total of over 10,000 events were analyzed on a FACS Calibur™ flow cytometer for fluorescence intensities in channel FL-1 (*Salmonella)* and FL-4 (Vti1b or CD-MPR). Unlabelled and uninfected cells were used to calibrate the instrument settings. Data were analyzed with FlowJo™ 8.6.3 (Tree Star Inc., U.S.A.) software.

FACS assays to determine the surface labelling of CI-MPR were conducted as described for the intracellular labelling of Vti1b and CD-MPR with the exception that FACS permeablisation buffer was replaced with FACS non-permeablisation buffer (1% azide, 10% horse serum).

2.17 BFA treatment

Samples were treated with BFA (Sigma) at a working concentration of 5 μg/ml or with DMSO control and left for 2 hrs under the previously specified growth conditions of HeLa cell culture. Cells were then fixed, labelled and analysed by confocal microscopy for effects on Golgi morphology.

2.18 Immunoblot analysis

For immunoblot analysis of the levels of Syntaxin 6, Syntaxin 10, Vti1b, CD-MPR and SKIP cells were seeded in 6 well plates at a concentration of $2x10^5$/well collected using 400 μl trypsin-EDTA solution per well (GIBCO BRL), centifuged at 2000 x g then resuspended

in equal volume of lysis buffer (20 mM Tris-Cl pH 8.0, 0.5% NP40, 150 mM NaCl) in the presence of protease inhibitors (Complete EDTA-Free, Roche). Lysates were centrifuged at 14,000 x g at 4 ^{o}C for 5 min to pellet cell debris. The protein concentration of sample supernatents was determined against a standard curve of BSA concentration using a colourmetric Bradford's-reagent protein concentration assay, according to the manufacturers protocol (BioRad, UK). Supernatants were resuspended in equal volume of 2 x sample buffer and boiled at 100 ^{o}C for 5 min. Samples were used immediately or stored at -20^{o}C.

Proteins in lysates, corresponding to 20 µg of sample, were separated using 12% polyacrylamide gels by SDS-PAGE. Proteins were transferred into polyvinyldene fluoride (PVDF) membranes using a semi-dry transfer cell (BioRad) at 3 mA/cm^2 and 24 V for 1 h. Membranes were blocked with TBS (20 mM Tris-Cl, pH 7.5 150 mM NaCl) containing 5% milk powder, 0.05% Tween 20 for at least 2 h. Membranes were incubated with primary antibodies (Table 2.1; Table 2.2) in TBS 5% milk 0.1% tween 20 overnight at 4 ^{o}C. Following incubation with primary antibodies membranes were washed three times for 15 min in TBS containing 0.2% tween. Membranes were incubated with secondary antibodies diluted in TBS containing 5% milk powder, 0.1% tween for 1 h at room temperature. Following three further washes of 15 min with TBS 0.2% tween, proteins were detected using enhanced chemiluminescence ECL (GE Heallthcare). Tubulin or actin were used as loading controls.

2.19 Statistical Analysis

All results are reported as mean ± standard error of the mean (SEM) unless otherwise stated. Statistical analyses were performed using two-tailed, un-paired Student's t-test on data arising from experimental assays repeated more that two times. P values < 0.05 were considered as statistically significant.

Chapter 3

The interaction of *Salmonella* with host trafficking proteins

3.1 Introduction

As detailed in chapter 1, it is thought that one of the key mechanisms through which *Salmonella* is able survive and replicate in cells is through its interaction with the host vesicular trafficking pathways. By fusing its vacuole with specific endocytic compartments and avoiding fusion with others *Salmonella* could satisfy requirements for nutrients and vacuolar membrane (Ramsden *et al.*, 2007). As discussed earlier, as the SCV matures it recruits cellular proteins such as the lgps (Lamp1, Lamp2, CD63) and v-ATPase and fails to recruit others that are usually present on and within maturing phagosomes and lysosomes, such as MPRs and cathepsins. This suggests that the SCV interacts with LE but has limited interactions with lysosomes (Garcia-del Portillo & Finlay, 1995; Rathman *et al.*, 1997; Steele-Mortimer *et al.*, 1999). To further investigate the selectivity of interactions between the SCV and the late endosomal system I undertook an immunofluorescence screen of infected HeLa cells. A range of proteins were analysed that are involved in trafficking with LE and lysosomes. Initially I analysed the immunofluorescence labelling of proteins according to their recruitment to the SCV and Sifs as well as any obvious changes in distribution compared to non-infected cells. Proteins selected for analysis included cargo proteins transported through endocytic compartments, SNAREs involved in trafficking of

LE compartments and retrograde transport to the TGN, GTPases involved in LE trafficking and/or recycling and other cellular machinery involved in trafficking from endosomes to the TGN.

3.2 Distribution of proteins that traffic in well-defined pathways in infected cells

Within the endomembrane system there are certain proteins that are transported with a well-defined itinerary. Interference with their trafficking often results in changes to their cellular distribution. Therefore, altered distribution can be exploited as indicators of disruption of specific pathways (Ganley *et al.*, 2008; Metcalf *et al.*, 2008; Stechmann *et al.*, 2010). I selected four proteins (TGN46, CD-MPR, CI-MPR and TrfR) that are known to traffic in well-defined pathways. I examined their distributions in HeLa cells infected for 14 h with wild-type GFP-expressing *Salmonella* and compared them to uninfected cells using immunofluorescence analysis.

TGN46 is a glycoprotein of unknown function is commonly used to selectively label the TGN in cells (Prescott *et al.*, 1997; Salcedo & Holden, 2003). The protein is also known to be transported in a retrograde manner from the plasma membrane through endocytic compartment to the TGN (Ganley *et al.*, 2008).

Both CD-and CI-MPRs are known to be recycled back to the TGN after they carry out the function of ferrying newly synthesised lysosomal enzymes to endosomes. Their retrograde transport to the TGN is dependent on Syn10 and occurs via a pathway distinct from that used by TGN46 (Ganley *et al.*, 2008).

TrfR is a carrier protein for transferrin, which is required for import of iron into the cell. It is regulated in response to intracellular iron concentration, internalising the transferrin-iron complex and is transported to the ERC where iron is released. The receptor-transferrin complex is then recycled to the plasma membrane via endocytic recycling pathway dependent on Rab11 (Grant & Donaldson, 2009).

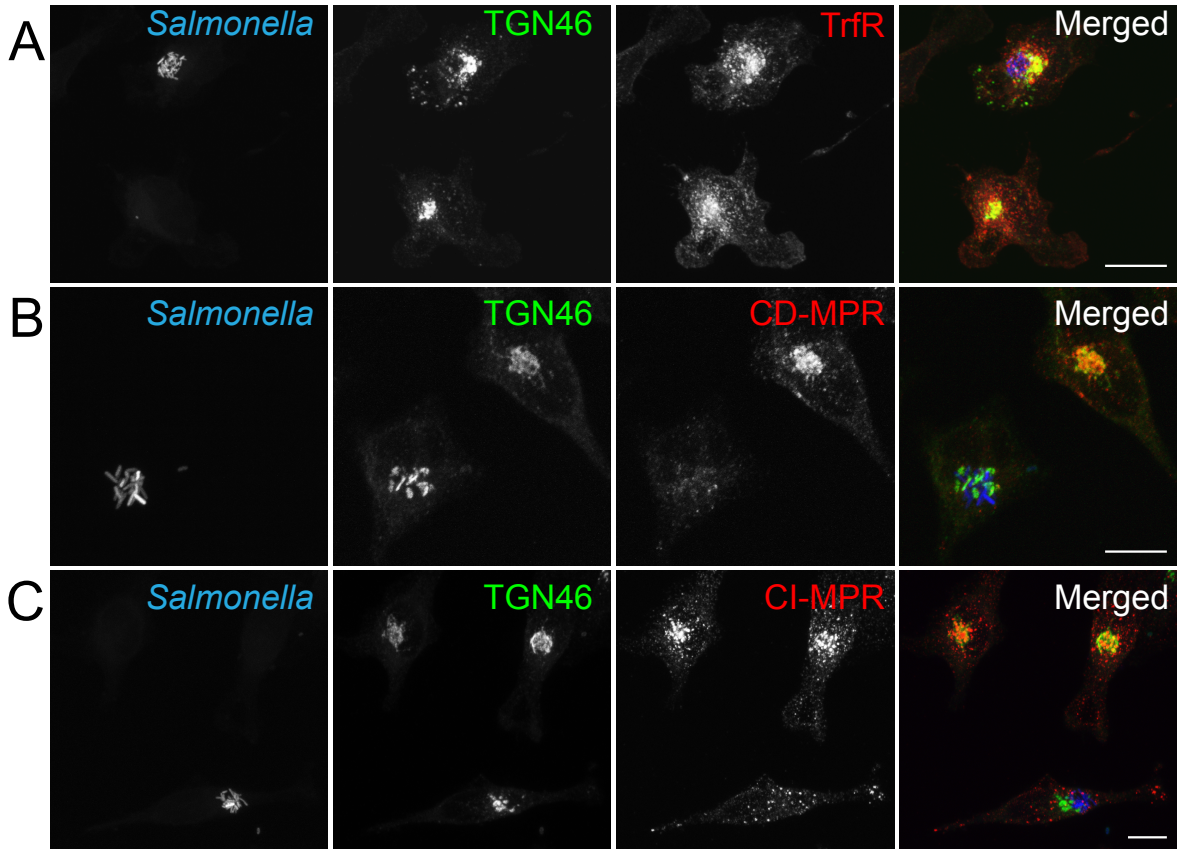


Figure 3.1 : ***Distribution of proteins that traffic in well-defined pathways in infected cells.*** *A) Confocal microscopy images showing the localisation of A) TrfR B) CD-MPR and C) CI-MPR in infected and non-infected cells. Cells were infected with wild-type* Salmonella *constitutively expressing GFP (blue), fixed after 14 h and immunolabelled for the protein of interest (red) and TGN46 (green). Images were false coloured using Zeiss LSM software. All scale bars correspond to 10 µm.*

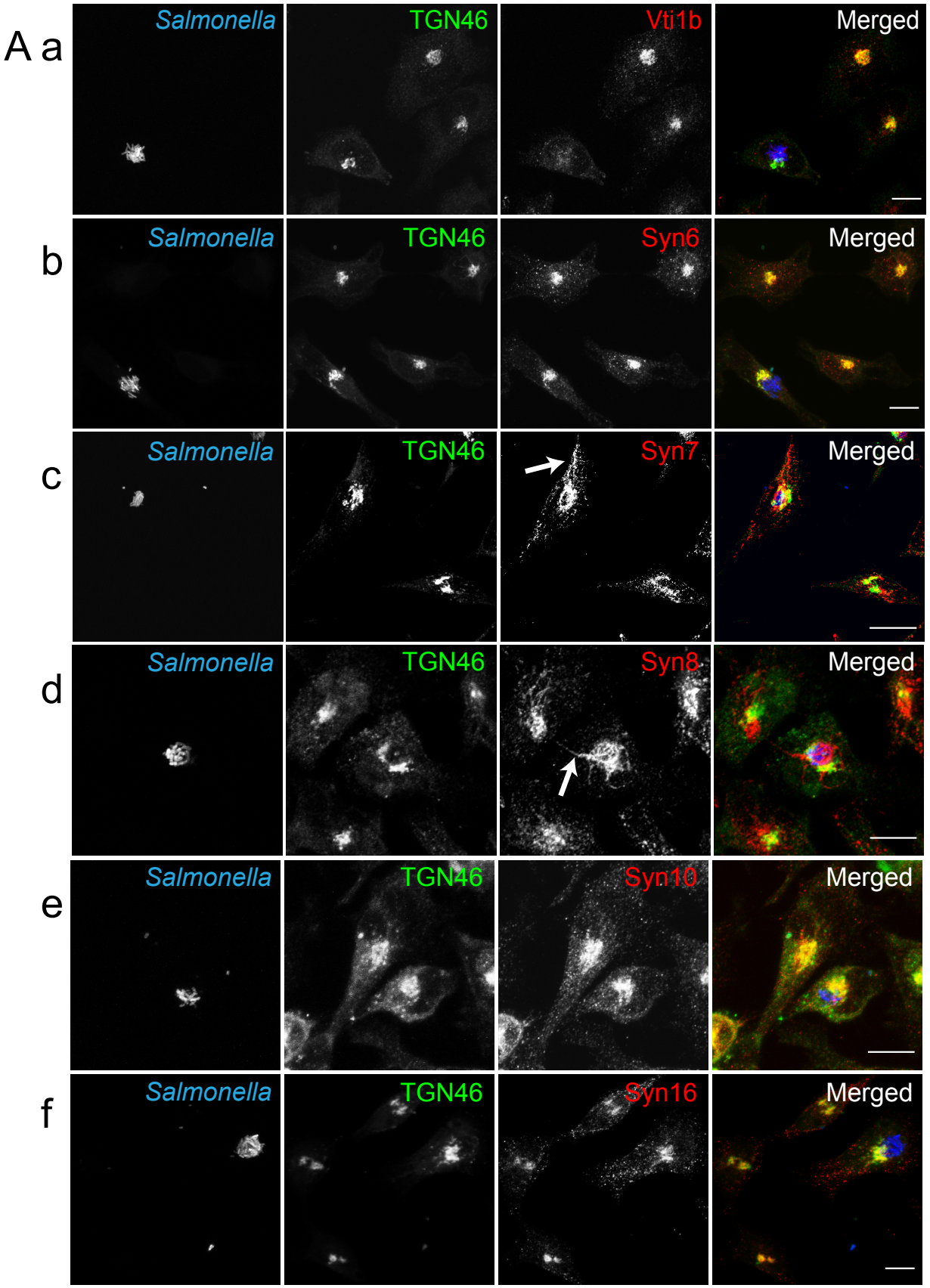
After 14 h of infection there were no obvious changes to overall TGN46 labelling in infected cells when compared to non-infected cells, besides a moderate change to the TGN ribbon morphology associated with the MTOC-associated *Salmonella* microcolony (Ramsden *et al.*, 2007) (Fig. 3.1 A, B, C). Thereafter, I used TGN46 to define the TGN in infected and uninfected cells. After 14 h of infection there was also no obvious changes to TrfR labelling in infected cells when compared with non-infected cells (Fig. 3.1 A). These results indicate that the pathways through which TGN46 and TrfR traffic are likely unaffected by *Salmonella* infection.

In contrast, there was a striking difference observed in the steady state labelling of both CD-MPR and CI-MPR (Fig. 3.1 B, C). In uninfected cells a proportion CD and CI-MPR labelling strongly co-localised with TGN46 at the TGN, however in infected cells there was a substantial reduction in the intensity of labelling of both MPRs co-localising with the TGN46 (Fig. 3.1 B, C), indicative of interference during transport.

3.3 Distribution of endocytic SNAREs in infected cells

Given that the SCV has characteristics of the LE compartment, I analysed the distribution of SNARE proteins known to be involved in LE fusion events or in traffic from endosomes to the TGN that could interact with the LE compartments. The Q-SNAREs, Syn7, Syn8 and Vti1b partner with either of the R-SNAREs VAMP7 or VAMP8 to mediate LE-LE homotypic fusion and LE-lysosome heterotypic fusion events respectively (Pryor *et al.*, 2004). The Q-SNAREs Syn6, Syn16, Syn10 and the R-SNAREs Vamp3 and Vamp4 function in two separate endosome-to-TGN pathways differentiated by the presence Syn10 and Syn6 (Ganley *et al.*, 2008).

HeLa cells were infected for 14 h with GFP-expressing *Salmonella* and labelled with antibodies to detect TGN46 and the SNARE of interest. Of the SNAREs analysed Syn6, Syn10, Syn16, Vamp3 and Vamp4 had a labelling indistinguishable to that observed in uninfected cells.


A a
Salmonella
TGN46
Vti1b
Merged
b
Salmonella
TGN46
Syn6
Merged
c
Salmonella
TGN46
Syn7
Merged
d
Salmonella
TGN46
Syn8
Merged
e
Salmonella
TGN46
Syn10
Merged
f
Salmonella
TGN46
Syn16
Merged

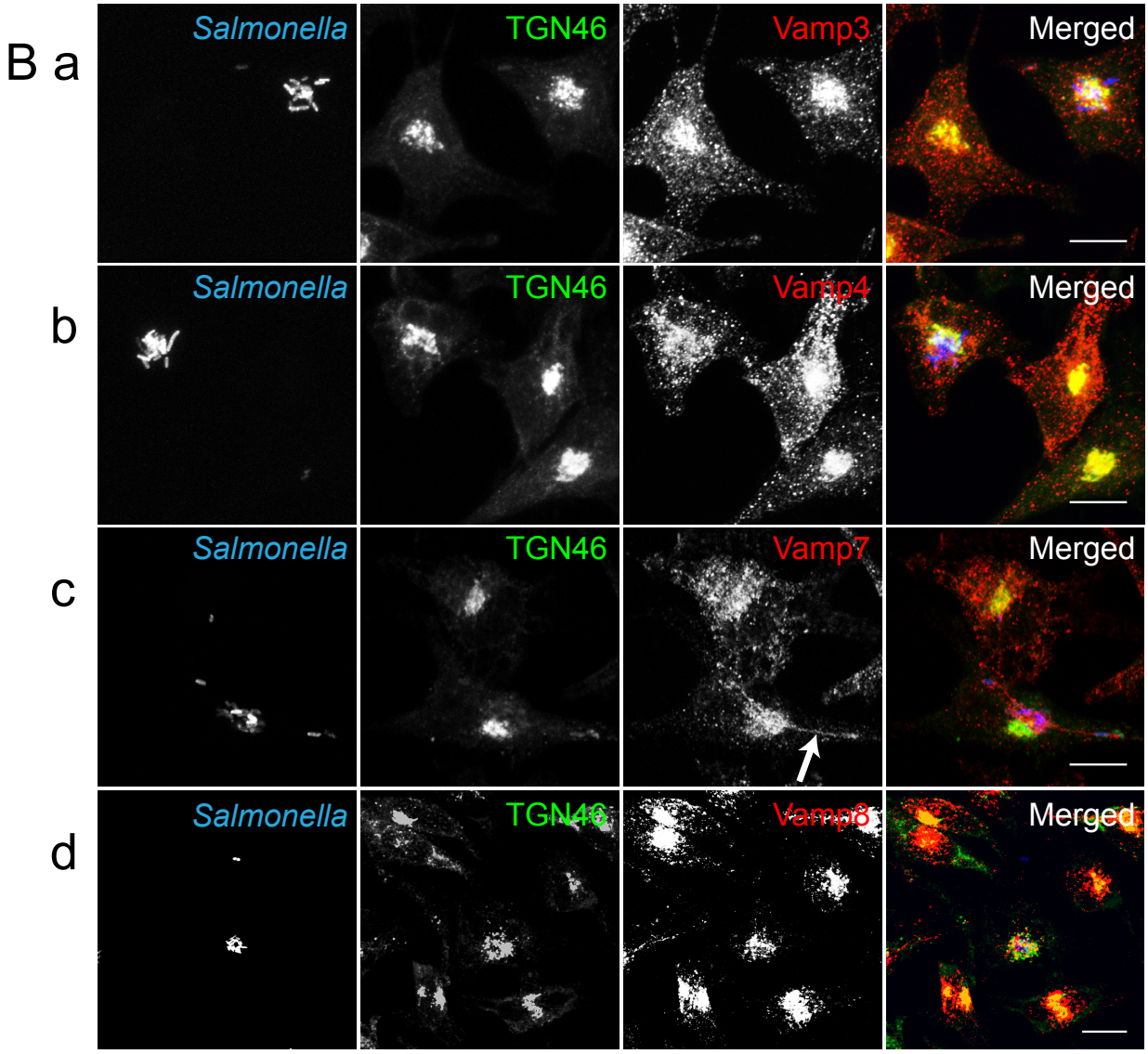


Figure 3.2 : ***Distribution of endocytic SNAREs in infected cells.*** *A) Confocal microscopy images showing the localisation of a) Vti1b b) Syn6 c) Syn7 d) Syn8 e) Syn10 and f) Syn16 in infected and non-infected cells. B) Confocal microscopy images showing the localisation of a) Vamp3 b) Vamp4 c) Vamp7 and d) Vamp8 in infected and non-infected cells. Cells were infected with wild-type* Salmonella *constitutively expressing GFP (blue), fixed after 14 h and immunolabelled for the SNARE of interest (red) and TGN46 (green). Arrows indicate where tubular structures were observed emanating from the region of the microcolony. Images were false coloured using Zeiss LSM software. All scale bars correspond to 10 µm.*

In contrast, the LE SNAREs Syn7, Syn8 and Vamp7 were all recruited to the region of the *Salmonella* SCVs and appeared on tubular structures arising from this region reminiscent of Sifs (Fig. 3.2 Ac, Ad, Bc). The related late endocytic SNAREs, VAMP8 and Vti1b were not recruited to the SCVs and were not detectable on any Sif-like structures (Fig. 3.2 Aa, Bd). In the case of Vti1b in uninfected cells a proportion of its labelling strongly co-localised with TGN46 at the TGN similar to both CD-and CI-MPR. However in infected cells there appeared to be a substantial reduction in Vti1b labelling co-localising with the TGN46 (Fig. 3.2 Aa) similar to the effect seen for both CD-and CI-MPR.

3.4 Syn7, Syn8 and Vamp7 but not Vti1b and Vamp8 are recruited to SCVs and Sifs.

Syn7 has been reported to be recruited to the SCV and Sif membranes (Smith *et al.*, 2007). Given that it functions in combination with Syn8 and Vamp7 and that the SCV and Sif membranes have characteristics of LE compartments it would not be surprising that Syn8 and Vamp7 are also recruited to SCV and Sif membranes. The tubular structures observed during infection suggest that this may be the case (Fig. 3.2 Ac, Ad, Bc).

To further investigate this possibility I infected HeLa cells for 14 h with GFP-expressing wild-type *Salmonella* and then labelled with an antibody to Lamp1, which is known to be recruited to the SCV and Sif membranes and either Vti1b, Syn7, Syn8, Vamp7 or Vamp8. I then analysed by confocal microscopy the co-localisation of these SNAREs with Lamp1 in infected cells. There was a strong co-localisation observed between Lamp1 and Syn7, Syn8 or Vamp7 on tubular structures emanating from SCVs (Fig. 3.3 A, B, C), confirming their recruitment to Sif membranes. This is strong evidence to indicate that the tubular structures seen in infected cells labelled with either Syn7, Syn8 or Vamp7 and TGN46 (Fig. 3.2 Ac, Ad, Bc) were Sifs. In addition there was greatly enhanced labelling in the region of SCVs for all three proteins that also co-localised strongly with Lamp1, indicating recruitment to the SCV membrane.

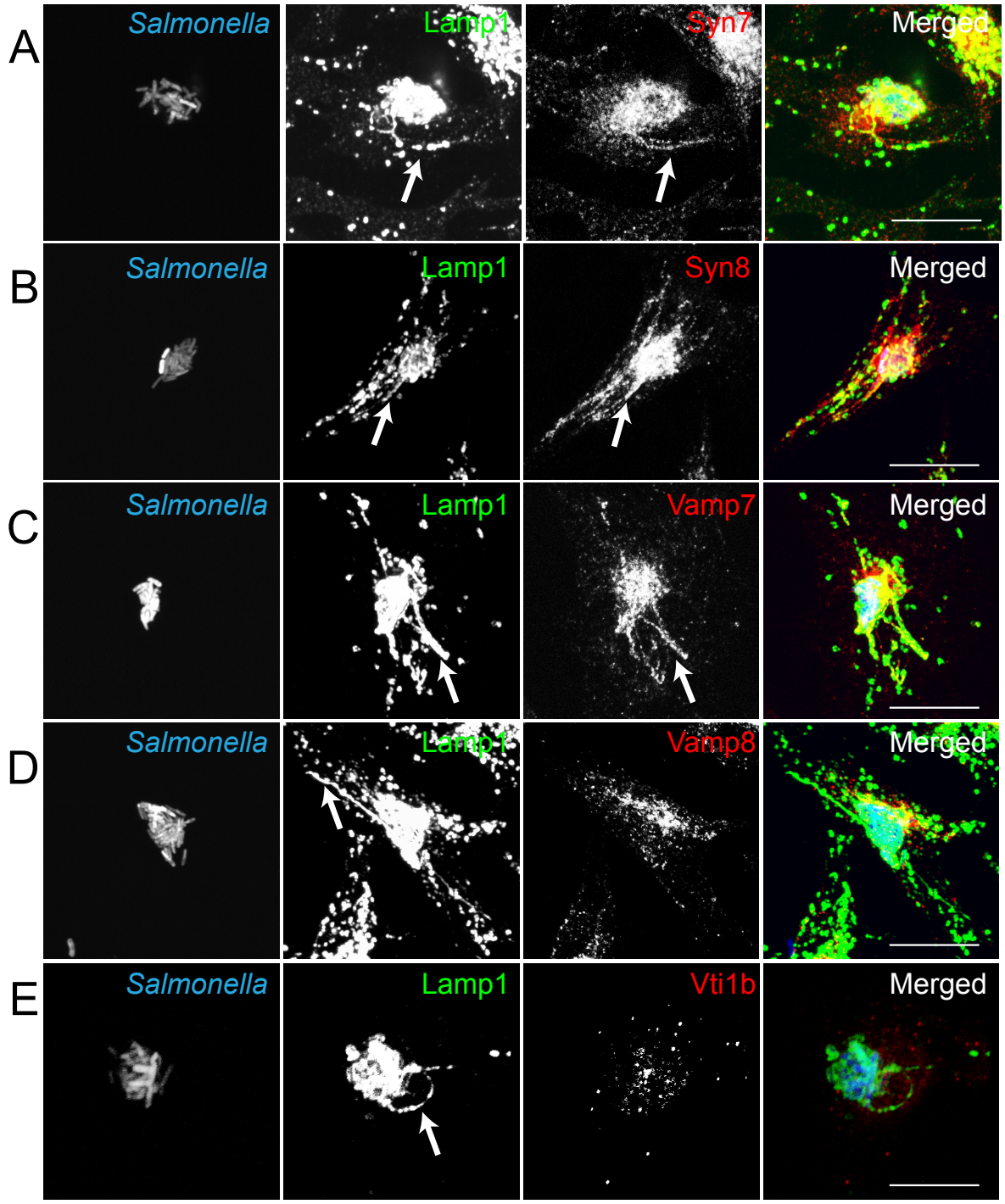


Figure 3.3 : ***Syn7, Syn8 and Vamp7 but not Vti1b and Vamp8 are recruited to SCVs and Sifs.*** *Cells were infected with wild-type* Salmonella *constitutively expressing GFP (blue), fixed after 14 h and immunolabelled for the SNARE of interest (red) and Lamp1 (green). Arrows indicate where tubular structures are observed emanating from the region of the microcolony. Images were false coloured using Zeiss LSM software. All scale bars correspond to 10 µm.*

By contrast when cells were labelled for either Vti1b or Vamp8 and Lamp1 there was no obvious co-localisation with the Lamp1 positive SCV or Sifs (Fig. 3.3 D, E), though this result is subject to the influence of antibody sensitivity and specificity. The absence of Vti1b and Vamp8 from Sifs is conspicuous given that their partner SNAREs in LE/lysosomal compartments (Syn7, Syn8 and Vamp7) are recruited. Depleting the cellular levels of Vti1b and Vamp8 would be required to test the specificity of antibody labelling by immunofluorescence. If they were found to be specific, then it would greatly strengthen the conclusion that they are not recruited to the SCV and Sif membranes.

3.5 Distribution of the retromer components in infected cells

The retromer complex mediates the selection and packaging of endocytic cargo destined for the TGN into vesicular carriers derived from EE/ERC, which then traffic to the TGN (Bonifacino & Hurley, 2008). The multimer including VPS26-VPS35-VPS29 cargo selection complex and tubulating complex of SNX proteins has been shown to be essential for the trafficking both CI-MPR (Arighi *et al.*, 2004; Seaman, 2004; Bonifacino & Hurley, 2008), wntless, Shiga toxin, and polymeric immunoglobulin receptors (Vergés *et al.*, 2004; Bujny *et al.*, 2007; Belenkaya *et al.*, 2008). Furthermore, during early stages of vacuole maturation *Salmonella* has been reported to recruit SNX1 to the SCV and it has been shown to be important for replication (Bujny *et al.*, 2008).

To address whether there was an alteration to the distribution of retromer components during later stages of infection, I labelled HeLa cells infected for 14 h with GFP-expressing *Salmonella* with antibodies to TGN46, SNX1, SNX2, VPS29 and VPS35. Though there was moderate clustering of puncta juxtaposed to *Salmonella* microcolonies, there was no gross change in the labelling of these proteins (Fig. 3.4). In addition they did not display the characteristic labelling of a protein closely associated with the SCV and Sifs as seen with Syn7, Syn8 and Vamp7 (Fig. 3.2 Ac, Ad, Bc; Fig. 3.3 A, B, C).

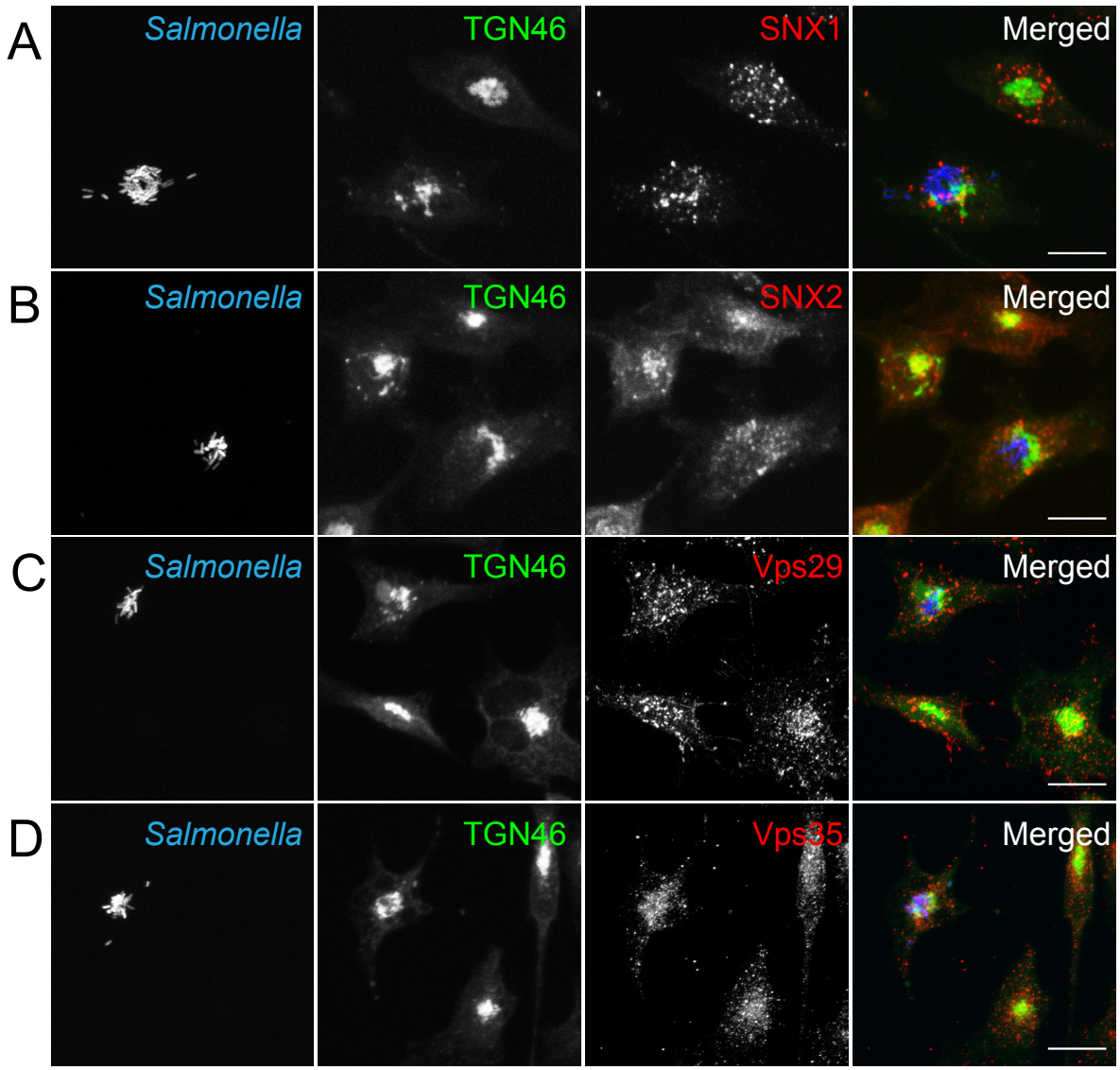


Figure 3.4 : ***Distribution of the retromer components in infected cells.*** *Confocal microscopy images showing the localisation of A) SNX1 B) SNX2 C) Vps29 and D) Vps35 in infected and non-infected cells. Cells were infected with wild-type* Salmonella *constitutively GFP (blue), fixed after 14 h and immunolabelled for the retromer component of interest (red) and TGN46 (green). Images were false coloured using Zeiss LSM software. All scale bars correspond to 10 µm.*

3.6 Distribution of Rab GTPases and the adapter protein EpsinR in infected cells

Rab GTPases function as regulators of trafficking events within the cell (Stenmark, 2009). They are targets of manipulation by several intracellular pathogens, including *Salmonella*, which uses Rab7 (Méresse *et al.*, 1999a; Harrison *et al.*, 2004; Brumell & Scidmore, 2007), to modify its environment. I analysed a selected group of Rab GTPases involved in vesicular trafficking. These included Rab6, a TGN-associated protein that regulates vesicular traffic fusing with and exiting from the TGN (Starr *et al.*, 2010); Rab7, a late endocytic protein that regulates fusion between LEs and lysosomes (Starr *et al.*, 2010); Rab9, a late endocytic protein that regulates retrograde traffic from LE to the TGN (Ganley *et al.*, 2004) and Rab11, an endosomal protein involved in endocytic recycling to the plasma membrane (Jones *et al.*, 2006). In addition, I analysed the distribution of EpsinR, an adapter protein involved in AP-1 and clatherin associated retrograde trafficking to the TGN from the endocytic compartment (Mills *et al.*, 2003).

In agreement with other work, Rab7 was heavily recruited to SCVs (Fig. 3.5 C) (Méresse *et al.*, 1999a). In addition there was moderate clustering of Rab9 puncta juxtaposed to SCVs (Fig. 3.5 D). There was no obvious influence of *Salmonella* on the distributions of Rab6, EpsinR or Rab11 compared to non-infected cells (Fig. 3.5 A, B, E).

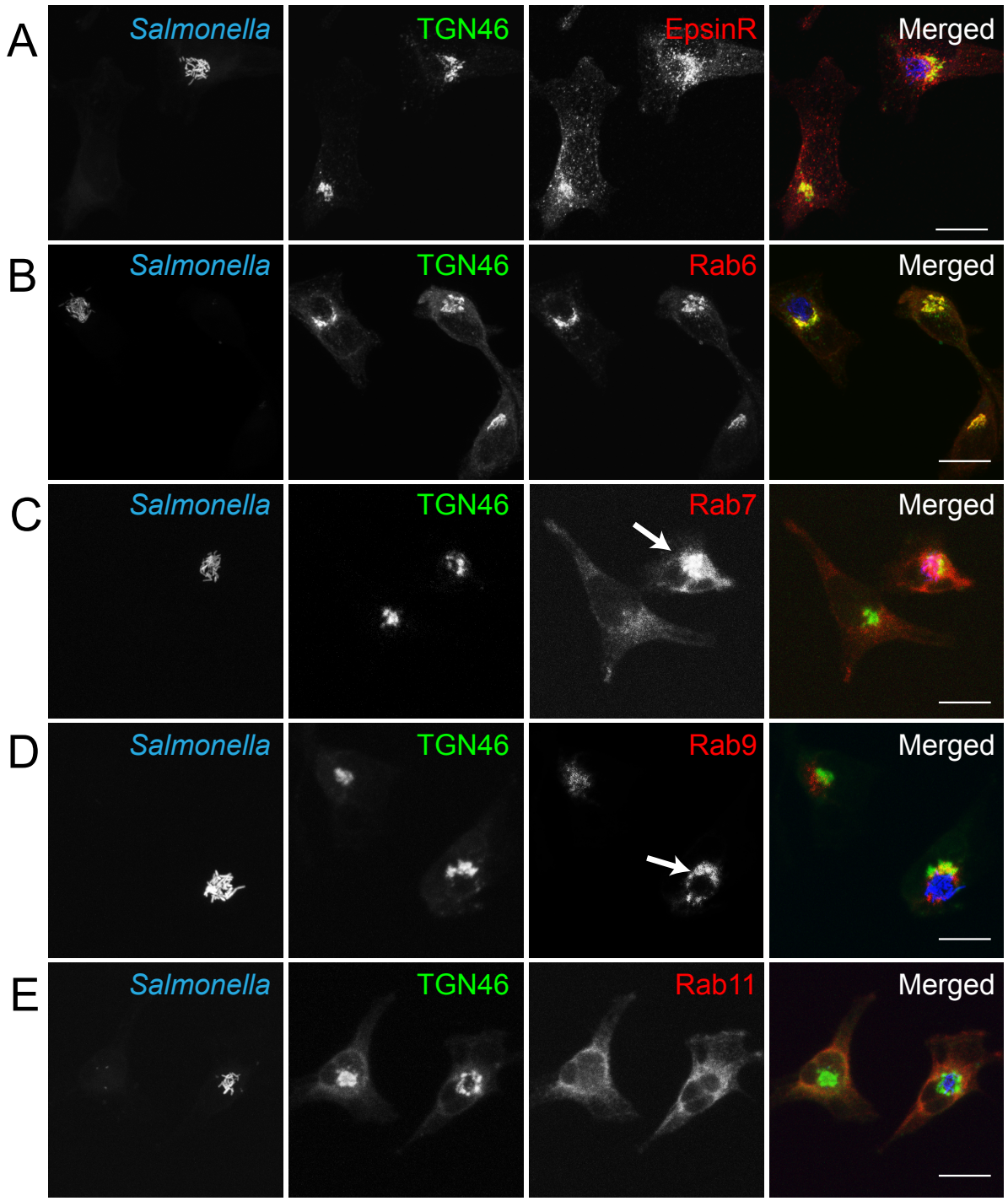


Figure 3.5 : ***Distribution of Rab GTPases and the adapter protein EpsinR in infected cells.*** *Confocal microscopy images showing the localisation of A) EpsinR B) Rab6 C) Rab7 D) Rab9 and E) Rab11 in infected and non-infected cells. Cells were infected with wild-type* Salmonella *constitutively expressing GFP (blue), fixed after 14 h and immunolabelled for EpsinR or Rab protein of interest (red) and TGN46 (green). Arrows indicate where there is recruitment to the SCV (C) or where there is clustering in close proximity to the SCV (D). Images were false coloured using Zeiss LSM software. All scale bars correspond to 10 µm.*

3.7 Characterisation of TGN46-associated labelling of Vti1b in HeLa cells

Given the association between SCVs and the Golgi (Salcedo & Holden, 2003), the difference in Vti1b co-localisation with TGN46 could be relevant in terms of infection. Accordingly, I decided to investigate the TGN46-associated labelling of Vti1b in greater detail.

In work of others, Vti1b was observed in the TGN region of NRK cells but its localisation was only moderately sensitive to brefelden A (BFA) treatment, suggesting a localisation outside the TGN (Kreykenbohm *et al.*, 2002). BFA inhibits nucleotide exchange on the GTPase Arf1 which is involved in the formation of COPI coated vesicles (Chardin & McCormick, 1999). The inhibition of Arf1 results in the redistribution of many Golgi components into the ER. Immunoelectron microscopy has been used to reveal that a considerable amount of Vti1b is found in recycling endosomes of NRK cells (Kreykenbohm *et al.*, 2002).

To determine whether the distribution of Vti1b in HeLa cells is similar to that in NRK cells I investigated its cellular distribution and sensitivity to BFA treatment by immunofluorescence microscopy. Cells were co-labelled for a panel of markers associated with the Golgi/TGN and late endocytic compartments. Immunolabelling of Golgi matrix protein 130 (GM130), a cis-Golgi protein (Nakamura *et al.*, 1995), and TGN46 or Rab6, which are both predominately associated with the TGN (Prescott *et al.*, 1997; Starr *et al.*, 2010), were used as positive controls for BFA-induced Golgi/TGN disruption. Lamp1, which is localised to the late endocytic compartments and therefore should be insensitive to BFA treatment, was used as a control for indirect effects of BFA on late endocytic compartments.

Exposure of HeLa cells to BFA caused a fragmentation of the Golgi/TGN and loss of Golgi/TGN-associated labelling of Vti1b, TGN46, GM130 and Rab6 (Fig. 3.6 A, B, C). The distribution of Lamp1 was not affected (Fig. 3.6 B). These results indicate that the majority of Vti1b observed in HeLa cells is localised to the TGN. Therefore, *Salmonella*

infection appears to reduce TGN localisation of Vti1b.

3.8 Quantification of TGN-associated protein levels in infected cells

To determine in an unbiased manner if there was a reduction of proteins co-localising with the TGN, I employed an automated method for quantitative measurement of the fluorescent signal localised to the TGN. This technique relies on the rendering of images of cells in 3d, which have been acquired by confocal microscopy. The 3d shape of the TGN is automatically differentiated using fluorescence intensity associated with Rab6 or TGN46 labelling (Fig. 3.7 A). The mean fluorescence intensities of the reference and test samples are measured in the computer-defined TGN compartment in infected and non-infected cells (see Materials and Methods for further details). All fluorescence signals are corrected for background fluorescence using a primary isotype antibody controls to determine the signal associated with non-specific labelling (Fig. 3.7 B).

I quantified the TGN associated labelling of CD-MPR, CI-MPR and Vti1b in infected cells in addition to several proteins which did not display any obvious difference during infection: TGN46, Rab6, EpsinR, Syn6 and Syn10. To verify that the juxta-nuclear labelling of TGN46 was indeed TGN-localised after infection with *Salmonella*, HeLa cells were co-labelled with Rab6 (Starr *et al.*, 2010). There was no detectable difference in the levels of TGN46 or Rab6 labelling in TGN compartments of infected cells when compared to non-infected cells (Fig. 3.7 C). This shows that the TGN46 labelling seen after wild-type *Salmonella* infection is indeed localised to TGN compartments and that the extent of this labelling is unaffected by wild-type *Salmonella*. In addition this result confirms the suitability of using TGN46 as a reference protein to identify the TGN in infected cells.

Next I analysed the TGN associated levels of Vti1b, CD-MPR, CI-MPR, EpsinR, Syn6 and Syn10. There was no detectable difference in the levels of TGN-localised fluorescence of EpsinR, Syn6 or Syn10 when comparing infected and non-infected cells (Fig. 3.7 C).

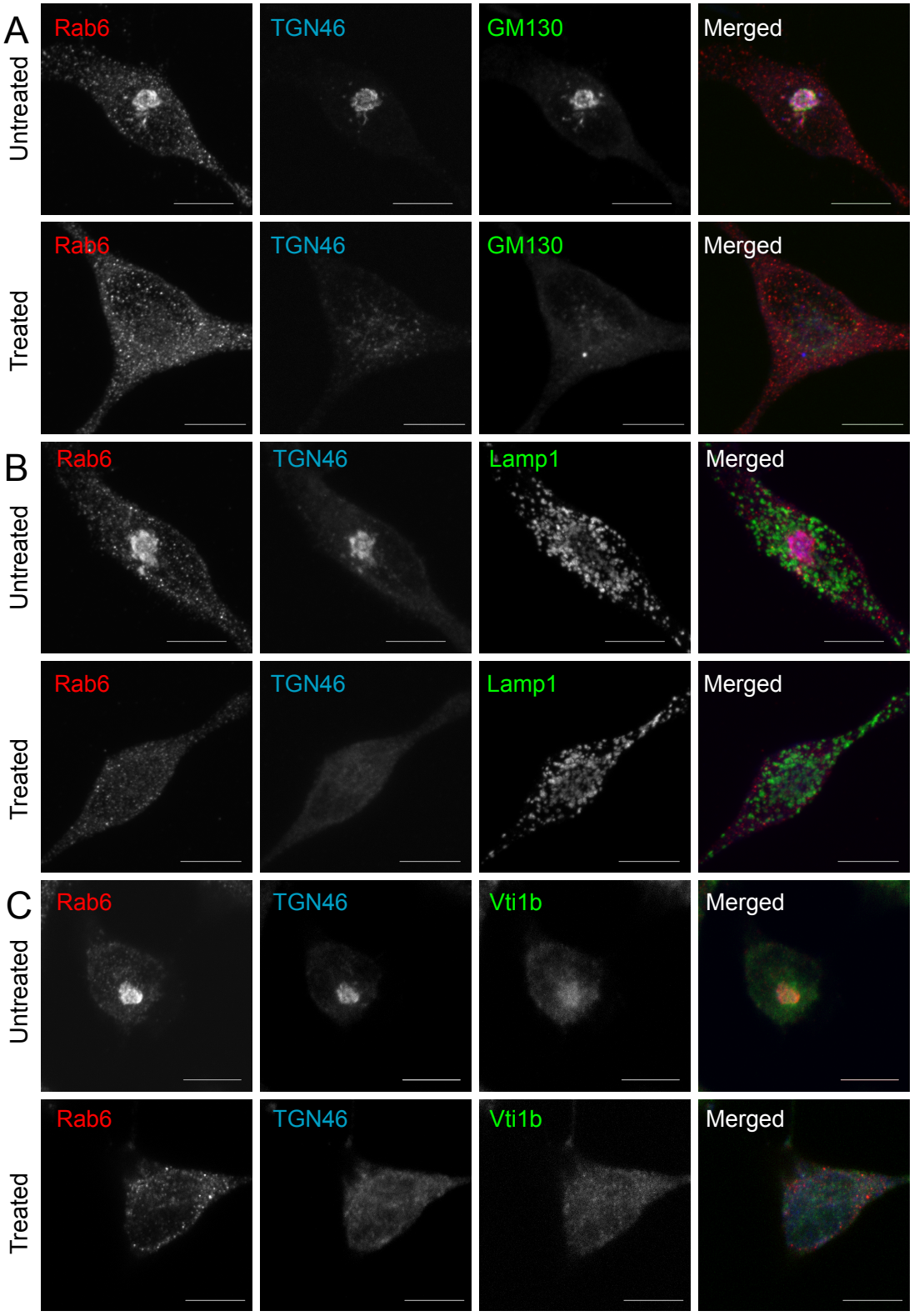


Figure 3.6 : ***Golgi/TGN disruption by BFA in HeLa cells.*** *All panels show either untreated or BFA treated cells labelled with Rab6 (red), TGN46 (blue) and either (A) GM130 (green), (B) Lamp1 (green) or (C) Vti1b (green). Disruption of Golgi/TGN associated labelling of Vti1b, RAB6, TGN46 and GM130 was observed in treated cells whereas Lamp1 labelling was unaffected. All scale bars correspond to 10 µm. Images were false coloured using Zeiss LSM software.*

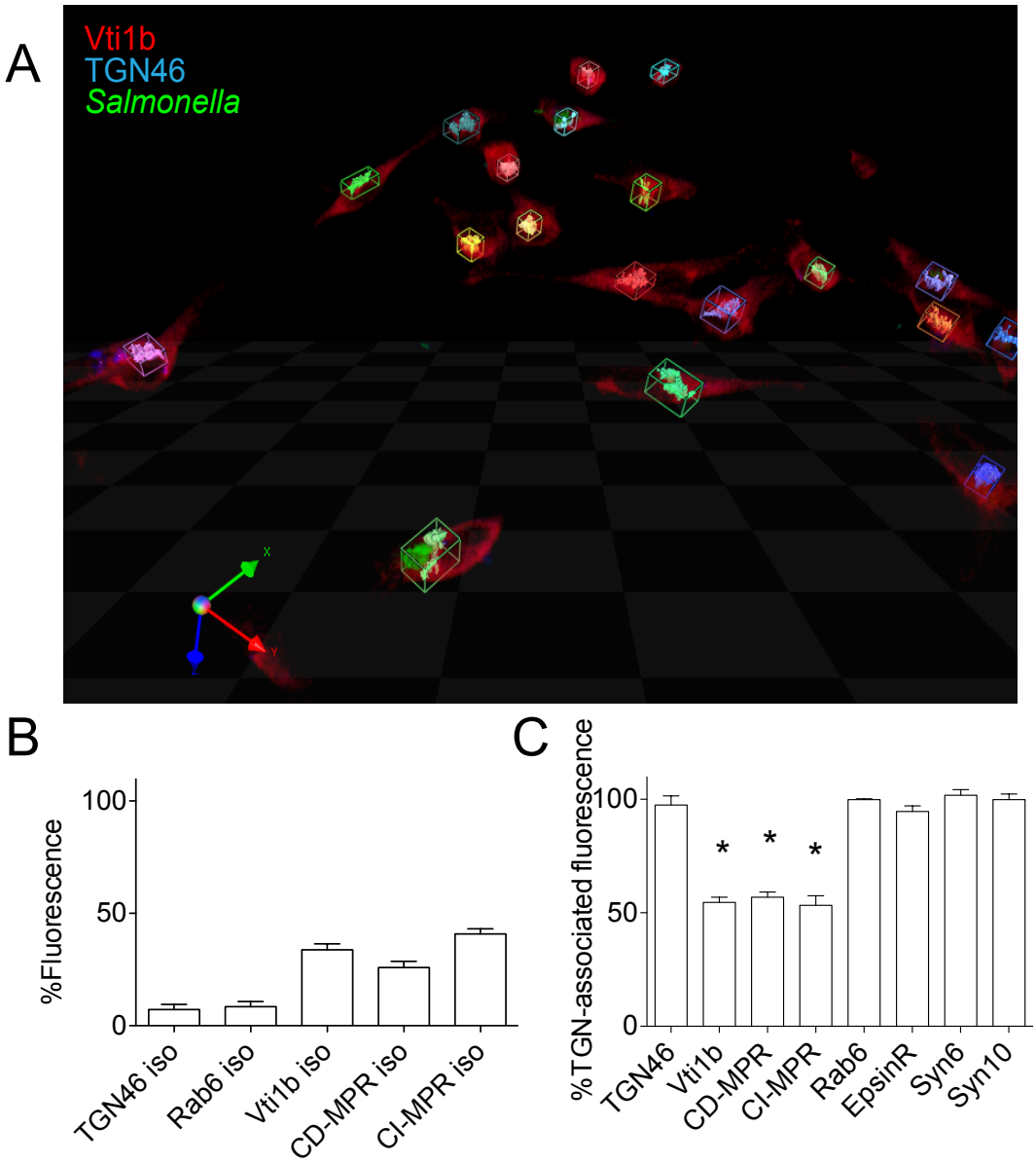


Figure 3.7 : ***Quantification of TGN-associated protein levels in infected cells.*** *(A) Example of a 3 dimensional field of view of infected and non-infected HeLa cells showing the computer-defined TGN regions (boxed) (B) Quantitative analysis of the sum fluorescent signal associated with background labelling determined using relevant isotype controls for TGN46, Rab6, Vti1b, CD-MPR and CI-MPR. Results are expressed as a percentage of the fluorescence signal associated with specific antibody labelling (C) Quantitative analysis of the mean fluorescence of TGN46, Vti1b, CD-MPR, CI-MPR, Rab6,EpsinR, Syn6 and Syn10 in infected cells. Results are expressed as a percentage of the average mean fluorescence signal in TGN compartments of non infected cells corrected for background labelling. Statistically significant relationships in are denoted (* = $P<0.05$).*

However, there was significantly reduced labelling of Vti1b, CD-MPR and CI-MPR co-localising with the TGN of cells infected with *Salmonella* when compared to non-infected cells (Fig. 3.7 C).

These results indicate that intracellular *Salmonella* alters the trafficking to the TGN of CD-MPR, CI-MPR and Vti1b. This was not as a result of a broad reduction of protein levels in the TGN as TGN46, Rab6, EpsinR, Syn6 and Syn10 levels were all unaffected.

3.9 Dynamics of TGN-associated Vti1b in infected HeLa cells

To investigate the dynamics of the effects of *Salmonella* infection on Vti1b protein levels in the TGN, HeLa cells were infected for 8 h and 14 h with GFP-expressing wild-type *Salmonella*. Cells labelled for Vti1b and TGN46 were analysed qualitatively by immunofluorescence microscopy for effects on Vti1b labelling associated with the TGN. Perturbation of Vti1b labelling associated with the TGN in infected cells, compared to non-infected cells was evident for both timepoints after inoculation, but was more pronounced at 14 h (Fig. 3.8 A). This suggests that there is an increasing effect on the TGN localisation of Vti1b as infection proceeds.

To analyse the reduction of Vti1b localised to TGN compartments in a more detailed and quantitative manner samples were analysed over a time-course of infection using quantitative immunofluorescence analysis. HeLa cells were infected with GFP-expressing wild-type *Salmonella*. Cells were fixed every two hours between 2 h to 14 h of infection, then labelled for TGN46 and Vti1b. The decrease in the fluorescent signal localised to the TGN region in infected cells was first detectable 8 h after inoculation (Fig. 3.8 B). This decrease continued beyond 8 h, with the greatest decrease observed at 14 h. There was no significant change in the sum fluorescence signal of TGN46 when comparing infected to non-infected cells for all time points. The average bacterial number per cell between 2 and 14 h was also calculated within the same experiment by counting bacterial cells per infected HeLa

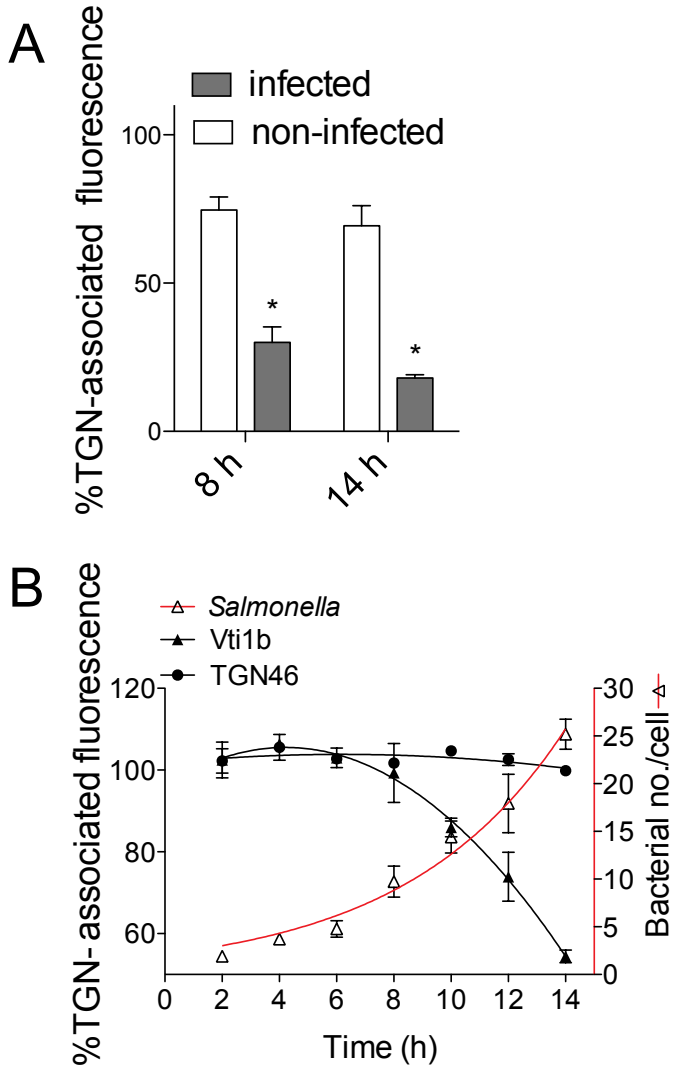


Figure 3.8 : ***Dynamics of TGN-associated Vti1b in infected HeLa cells.*** *HeLa cells were infected with wild-type* Salmonella *expressing GFP and analysed at the specified times pi (A) Quantification of the frequency of TGN associated labelling Vti1b in HeLa cells at 8 and 14 h pi. Scoring of infected and non-infected cells was done by epi-fluorescence microscopy. Statistically significant relationships in are denoted (* = $P<0.05$) (B) Quantitative analysis of the mean fluorescence of Vti1b and TGN46 in infected cells. Results are expressed as a percentage of the average mean fluorescence signal in TGN compartments of non infected cells corrected for background labelling.*

cell by immunofluorescence microscopy (Fig. 3.8 B). The decrease in fluorescence of Vti1b localised to the TGN coincided with increasing bacterial numbers. The greatest difference of Vti1b labelling between infected and non-infected cells was at 14 h. Accordingly, I used this timepoint for further investigation into how *Salmonella* affects proteins in the TGN.

3.10 Reduction in TGN-localised Vti1b in infected cells is dependent on the SPI-2 T3SS

Phenotypes associated with the action of the SPI-2 T3SS effector proteins become apparent 6 h to 8 h after invasion of host cells (Ramsden *et al.*, 2007). Therefore, it was possible that the reduction in TGN associated levels of Vti1b, CD-MPR and CI-MPR was mediated by the activities of the SPI-2 T3SS. To address this possibility HeLa cells were infected with an *ssaV* mutant strain, which has an inactive SPI-2 T3SS (Deiwick *et al.*, 1998) and analysed with respect to TGN-associated Vti1b levels.

After 14 h pi, cells were fixed and analysed by quantitative immunofluorescence microscopy. In contrast to results obtained when cells were infected with wild-type *Salmonella* (Fig. 3.9 B) the TGN-associated levels of Vti1b were unaltered in cells infected with the *ssaV* mutant strain when compared to non-infected cells (Fig. 3.9 A, B).

The *ssaV* mutant strain has a replication defect in HeLa cells (Ochman *et al.*, 1996; Cirillo *et al.*, 1998; Hensel *et al.*, 1998). Therefore it was possible that the differences observed between the wild-type and the *ssaV* mutant strain were a result of the differences in replication rather than a loss of specific SPI-2 T3SS effector(s). To attempt to address possible influences of the differences in the replication rates of wild-type *Salmonella* and those of the *ssaV* mutant strain infected cells were analysed at 20 h and 24 h pi. This was to allow time for greater growth of the *ssaV* mutant strain to occur. When samples were analysed at these timepoints , the levels of Vti1b in the TGN of cells infected with the *ssaV* mutant strain were again similar to those of non-infected cells (Fig. 3.9 B). These data suggest that the reduction in TGN-localised Vti1b in cells infected with wild-type

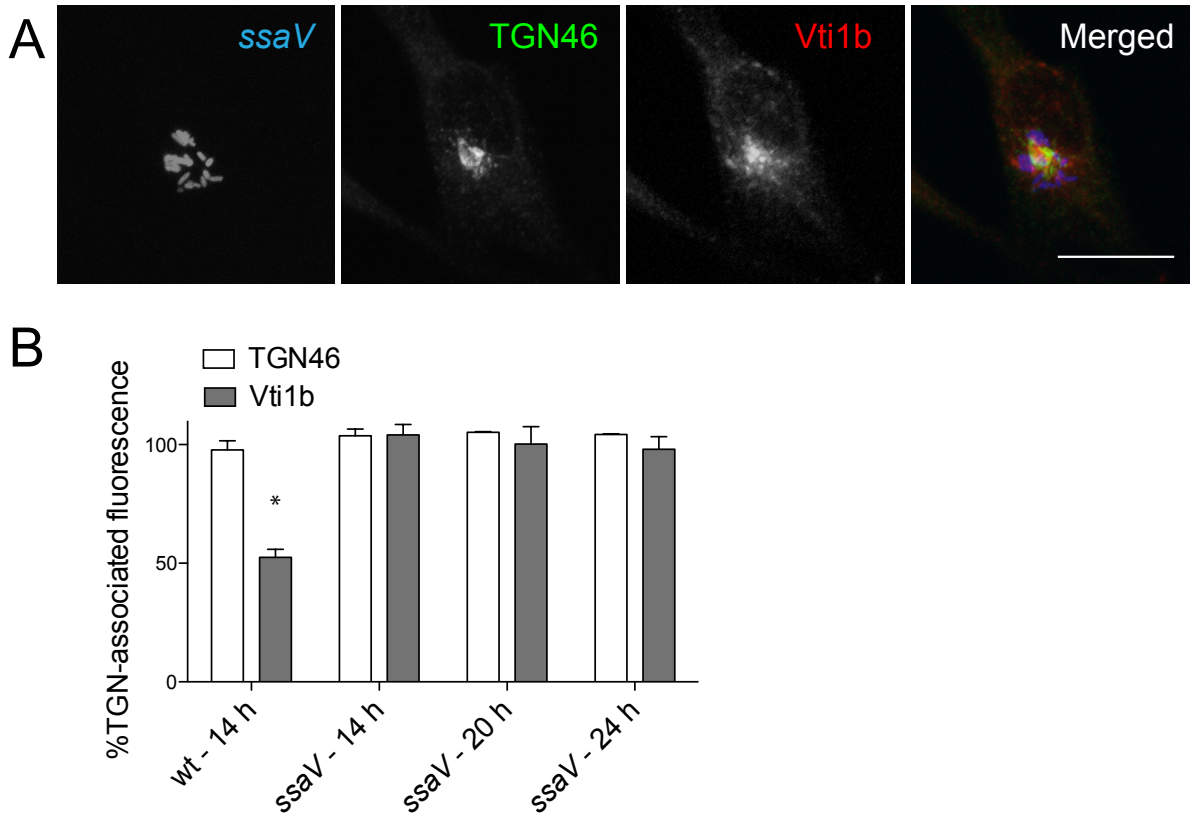


Figure 3.9 : ***Reduction in TGN-localised Vti1b in infected cells is SPI-2-dependent.*** *(A) Confocal microscopy images showing the localisation of Vti1b and TGN46 in an infected HeLa cell. Cells were infected with an* ssaV *mutant strain constitutively expressing GFP (blue), fixed after 14 h and immunolabelled for Vti1b (red) and TGN46 (green). Images were false coloured using Zeiss LSM software. The scale bar corresponds to 10 µm. (B) Quantitative analysis of the mean fluorescence of Vti1b and TGN46 in wild-type and* ssaV *mutant strain infected cells for the indicated timepoint. Results are expressed as a percentage of the average mean fluorescence signal in the TGN of non infected cells corrected for background labelling. Statistically significant relationships in are denoted (* = $P<0.05$).*

bacteria is due to the activities of specific SPI-2 T3SS effector(s).

3.11 Reduction in TGN-associated levels of Vti1b is not due to bacterial replication

To further investigate the possibility of a non-specific effect of replication, I re-analysed, TGN-associated Vti1b in cells containing between 15 to 30 bacteria for both the wild-type and *ssaV* mutant strains and compared them to non-infected cells. Furthermore, I

extended this analysis to include the two MPRs as well as Vti1b.

In cells infected with wild-type *Salmonella* there was a marked reduction in the TGN-associated labelling of Vti1b, CD-MPR and CI-MPR when compared to non-infected cells (Fig. 3.10 Aa, Ac, Ae, C). In contrast, in cells infected with the SPI-2 null mutant strain there was no detectable difference in the levels of protein in the TGN when compared to non-infected cells (Fig. 3.10 Ab, Ad, Ag, C). Therefore the differences observed in labelling of these proteins were not due to indirect effects associated with differential replication.

I also infected cells with an *aroCpurD* mutant strain, which has a severe replication defect (Beuzón *et al.*, 2002) but has a functional SPI-2 T3SS (Fig. 3.10 B). After 14 h of infection, I examined the levels of CD-MPR localised to the TGN. As a positive control for effector translocation I labelled cells for epitope-tagged SseJ (an effector of the SPI-2 T3SS), and compared levels of CD-MPR in cells containing translocated effector with non-infected cells. The levels of CD-MPR localised to the TGN in infected cells were reduced to a similar extent to that observed in cells infected with wild-type *Salmonella* (Fig. 3.10 C), despite greatly reduced bacterial numbers.

Taken together these data indicate that SPI-2 T3SS effector(s), selectively interfere with the TGN localisation of a subset of proteins known to be recycled from endocytic compartments to the TGN.

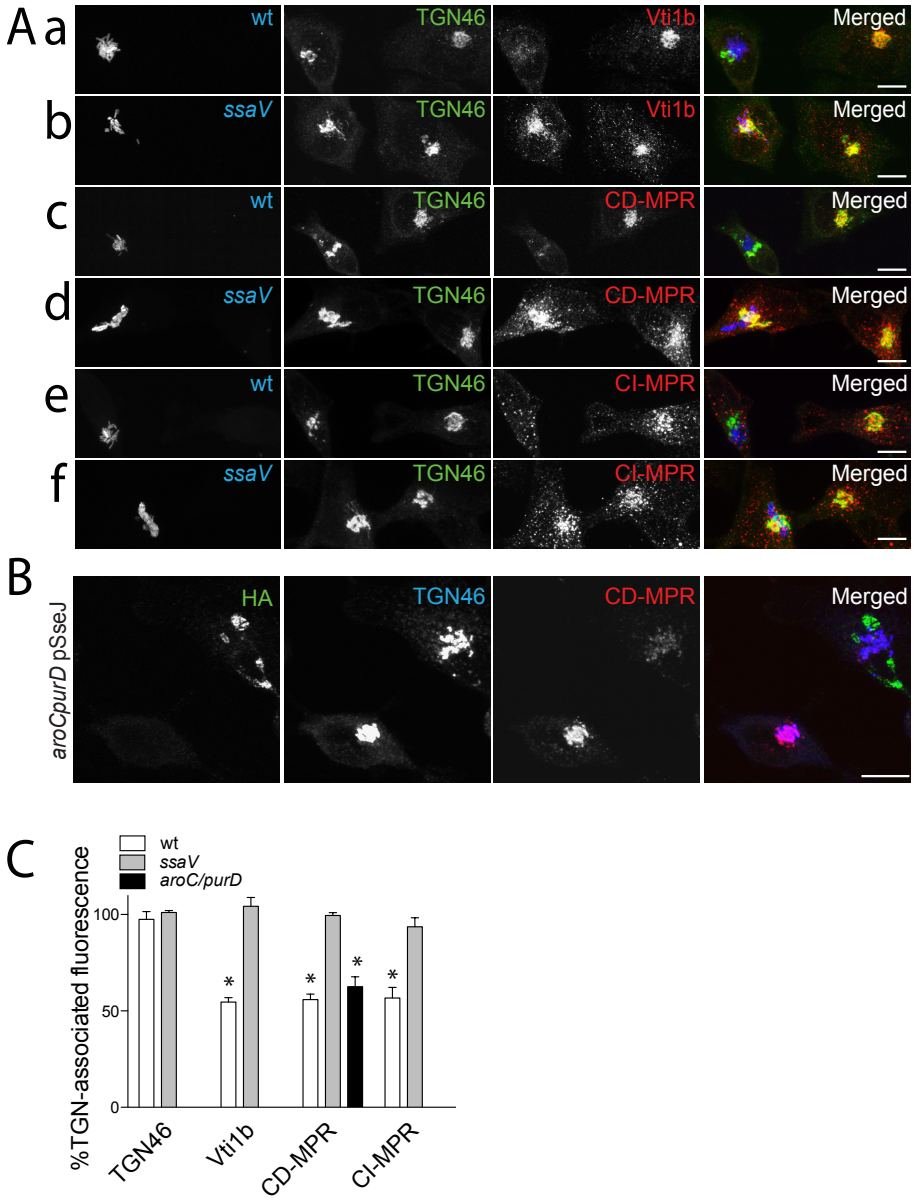


Figure 3.10 : ***Reduction in TGN-localised proteins is SPI-2-dependent and is not associated with replication.*** *(A) HeLa cells were infected for 14 h with either a GFP-expressing wild-type or* ssaV *mutant strains (blue), and were immunolabelled for TGN46 (green) and either Vti1b, CD-MPR or CI-MPR (red). Images were false coloured using Zeiss LSM software. Scale bars represent 10 µm. (B) HeLa cells were infected for 14 h with an* aroCpurD *mutant strain expressing SseJ-2HA and were immunolabelled, without permeabilising the bacterial membrane, for HA (green), TGN46 (blue) and CD-MPR (red). Images were false coloured using Zeiss LSM software. Scale bars represent 10 µm. (C) Quantitative 3d confocal microscopy of mean fluorescence signals of TGN46, Vti1b, CD-MPR and CI-MPR localised to the TGN region in HeLa cells infected for 14 h with wild-type,* ssaV *mutant or* aroCpurD *mutant strains. Only cells containing 15 to 30 bacteria for wild-type and* ssaV *strains were analysed. Statistically significant relationships in are denoted (* = $P<0.05$).*

Chapter 4

Salmonella interferes with retrograde trafficking

4.1 Introduction

In the previous chapter I demonstrated that *Salmonella* alters the cellular distribution of the late-endocytic Qb-SNARE Vti1b, CD-MPR and CI-MPR by reducing their levels in the TGN. All three proteins have a cellular distribution that, at steady state in non-dividing cells, is largely TGN associated and is maintained through cycling between endocytic and TGN compartments. These data led to the hypothesis that *Salmonella* could influence endosome-to-TGN transport of Vti1b, CD-MPR and CI-MPR. Furthermore, the absence of a detectable effect on the levels of TGN46 and Syn6 associated with the TGN suggests that the interference is restricted to a subset of retrograde traffic.

Two retrograde trafficking routes from endosomes to the TGN have been described that are distinguishable by the SNARE proteins that function within these pathways. The first pathway, which transports TGN46, Shiga toxin and Cholera toxin, relies on the SNAREpin complex of Syn6-Syn16-Vti1a and Vamp3 (Ganley *et al.*, 2008). The second retrograde trafficking pathway, which transports MPRs, relies on the SNAREpin complex of Syn10-Syn16-Vti1a and Vamp3 or Vamp4 (Ganley *et al.*, 2008) (Fig. 4.1) . It was shown through the use of siRNA experiments that the distinguishing features between these pathways are Syn6 and Syn10, each functioning in separate SNAREpin complexes with Syn16-Vti1a and

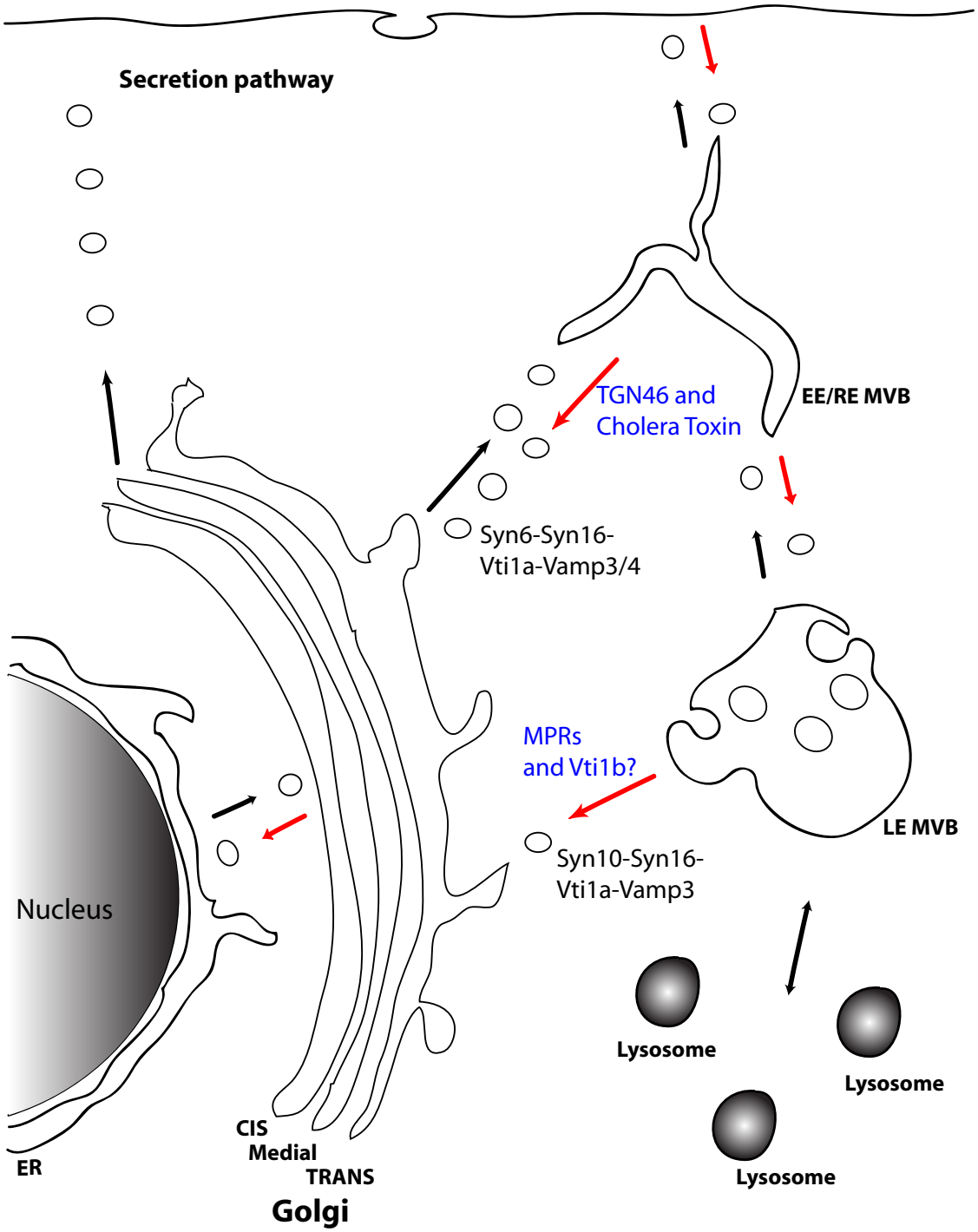


Figure 4.1 : ***Endosome-to-TGN trafficking routes.*** *Retrograde trafficking routes are denoted by red arrows and anterograde trafficking routes are denoted by black arrows. The cargos that are transported by each of the two endosome-to-TGN trafficking routes are shown in blue, adjacent to the route they follow. The SNAREpin complex associated with each of the two transport routes are listed adjacent to relevant route. EE, early endosome; RE, recycling endosome; MVB, multi-vesicular body; ER, endoplasmic reticulum.*

Vamp3 (Ganley *et al.*, 2008). Vti1b has been shown to recycle in a retrograde manner from endosomes to the TGN (Hirst *et al.*, 2004), but it is not known through which of the two routes it travels.

I used siRNA-mediated depletion of Syn6 and Syn10 from host cells to evaluate if the CD-MPR, CI-MPR and Vti1b share a similar endosome-to-TGN trafficking pathway. I also investigated whether the phenotypes observed during infection could be recapitulated in uninfected cells by directly targeting either of the retrograde trafficking pathways through siRNA mediated depletion of Syn6 or Syn10. In addition, to assess the possible interference of endosome-to-TGN transport by *Salmonella* more directly, I undertook assays of molecules that undergo Syn6 or Syn10 dependent transport from the plasma membrane to the TGN, in the context of *Salmonella* infection. Finally I investigated whether there were any physiological ramifications of *Salmonella* infection on the pathways involving MPR trafficking.

4.2 siRNA-mediated depletion of Syn10

To verify the protein level of Syn10 after siRNA treatment, SDS-PAGE and immunoblotting of intracellular protein was undertaken in HeLa cells treated with indicated siRNA oligos (Fig. 4.2 D). The pool of four oligos as well as single oligos 2 and 3 were found to be the most effective and were selected for use in further experimentation. Using 3d confocal microscopy to determine protein levels in TGN compartments, I observed that CD-MPR was reduced after siRNA-mediated depletion of Syn10 using either a pool of 4 oligos or single oligos 2 or 3 targeted against Syn10 (Fig. 4.2 A, C). This agrees with published data showing a dependency on Syn10 for the retrograde traffic of MPR (Ganley *et al.*, 2008). Interestingly, the TGN localisation of Vti1b was similarly reduced indicating that it is also dependent on Syn10 for retrograde recycling (Fig. 4.2 B, C) . Although it is thought to be recycled to the TGN, the pathway through which Vti1b travels has not been described previously. In addition, similar to cells infected with *Salmonella*, there was no detectable

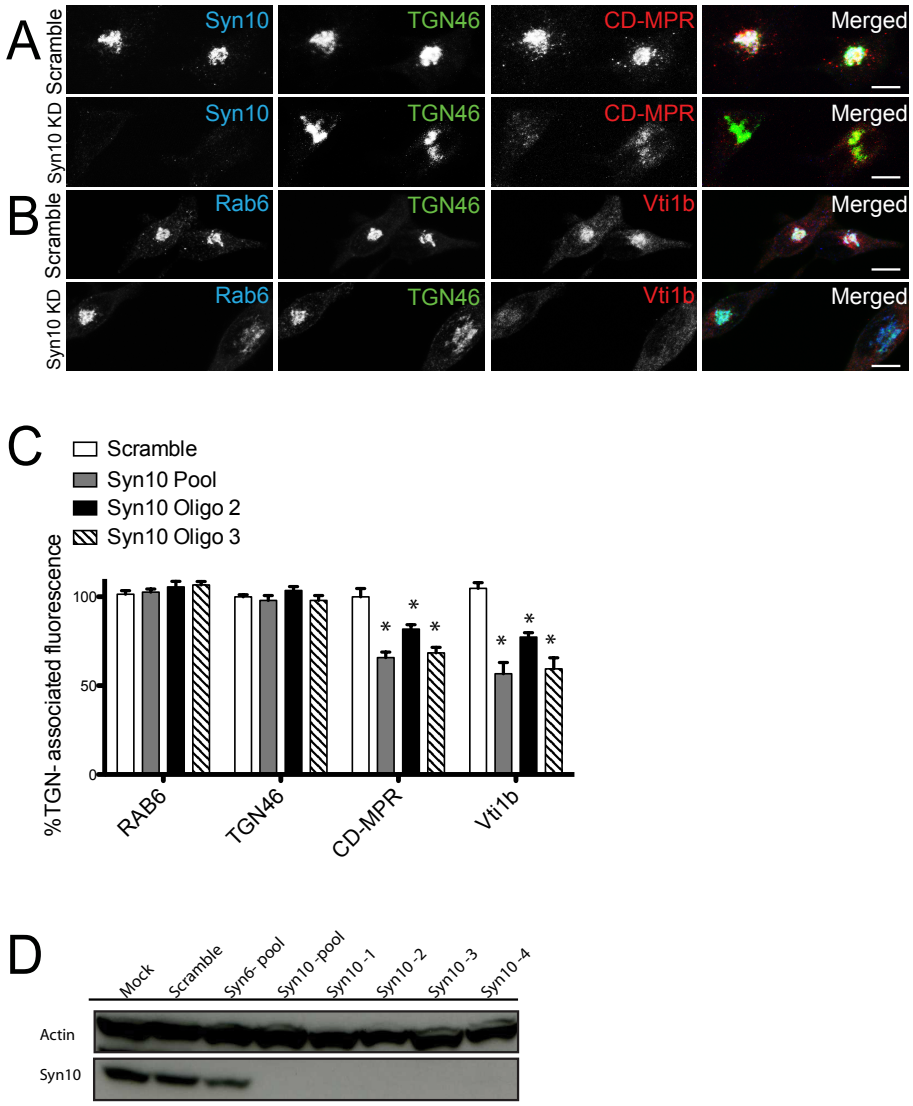


Figure 4.2 : ***siRNA depletion of Syn10.*** *Confocal microscopy images showing the localisation of (A) CD-MPR (red), TGN46 (green), Syn10 (blue), and (B) Vti1b (red), TGN46 (green), and Rab6 (blue) in HeLa cells treated with scramble siRNA or Syn10-3 siRNA targeted against Syn10. Images were false coloured using Zeiss LSM software. The scale bar corresponds to 10 µm. (C) Quantitative analysis of the mean fluorescence of Rab6, TGN46, CD-MPR, and Vti1b after siRNA treatment using scrambled siRNA, a siRNA pool of 4 oligos and 2 single oligos against Syn10. Results are expressed as a percentage of the average mean fluorescence signal in mock treated cells corrected for background. Quantitative fluorescence scoring was done using at least 100 cells in each condition. Experiments were undertaken in triplicate. Statistically significant differences are denoted using a * , as determined through a two tailed student t-test resulting in a $P<0.05$. (D) SDS-PAGE and immunoblotting of levels of Syn10 in HeLa cells treated with indicated siRNA oligos.*

effect of Syn10 depletion on the TGN localisation of TGN46 and Rab6. These observations were confirmed using another non-overlapping, single siRNA oligo against Syn10 (Fig. 4.2 C).

When quantified, the reduction of Vti1b and CD-MPR at the TGN was similar in magnitude to that observed during infection. The reduction of Vti1b and CD-MPR in the TGN compartments, and the lack of an effect on TGN46 localisation (Fig. 4.2 C), recapitulates the effects observed upon infection with *S.* Typhimurium (Fig. 3.7 C). This is consistent with the hypothesis that *Salmonella* interferes with Syn10-dependent retrograde traffic to the TGN.

4.3 siRNA-mediated depletion of Syn6

TGN46 and Syn6 were shown to be unaffected by *Salmonella* infection, suggesting that the retrograde trafficking route defined by Syn6 is not perturbed by *Salmonella*. However, the possibility exists that the technique I employed to assess TGN-localisation of the proteins examined was not sensitive enough to detect disturbances in retrograde trafficking of TGN46. To test this I disrupted the pathway by depleting Syn6 using siRNA. Depletion of Syn6 has been shown previously to reduce the amount of TGN46 in TGN compartments (Ganley *et al.*, 2008).

In agreement with published data, knock-down of Syn6, using a pool of oligos (verified by immunoblot and immunofluorescence analysis (Fig. 4.3 A, C)), resulted in a reduction in the fluorescence of TGN46 in TGN compartments, defined by Rab6 (Fig. 4.3 A, B). Syn6 knock-down did not affect the sum fluorescent signal of Rab6 in TGN compartments, suggesting that the TGN remained intact (Fig. 4.3 A, B). These results indicate that the technique I used is sensitive enough to detect perturbation in the retrograde trafficking route defined by Syn6.

Interestingly, depletion of Syn6 enhanced the levels of CD-MPR in the TGN (Fig. 4.3 B). This is in broad agreement with the reported observation that Syn6 depletion from

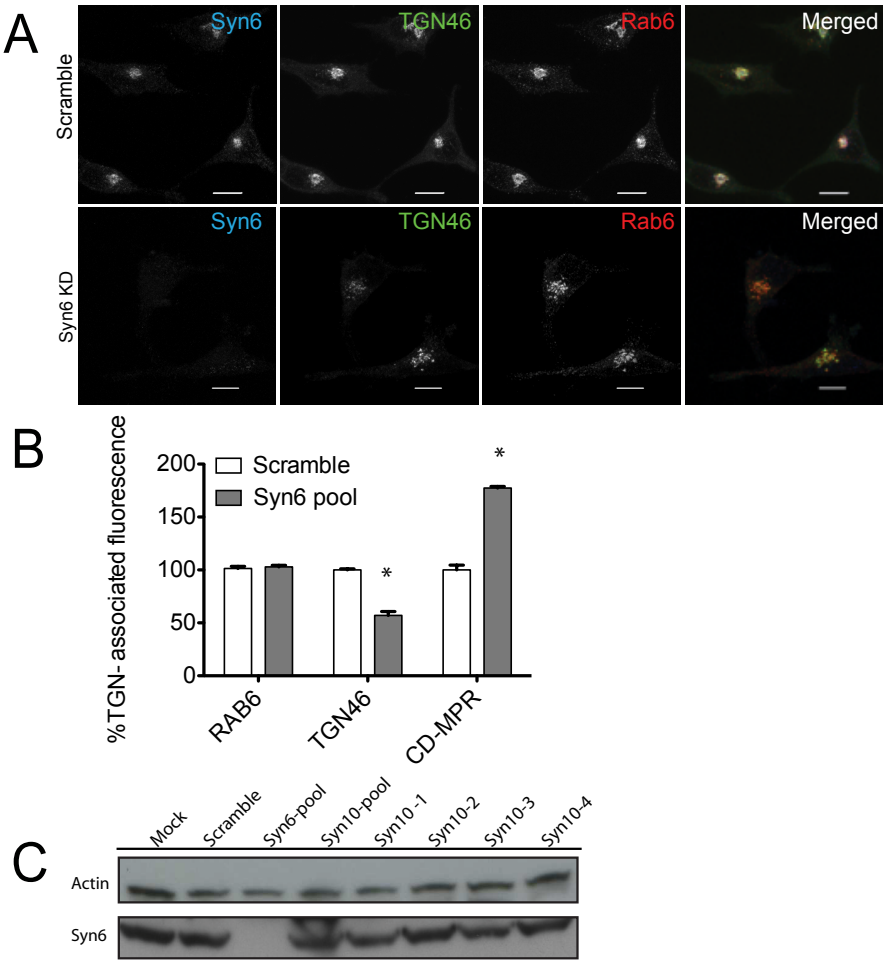


Figure 4.3 : ***siRNA depletion of Syn6.*** *Confocal microscopy images showing the localisation of (A) Rab6 (red) TGN46 (green) and Syn6 (blue) in HeLa cells treated with scramble siRNA or Syn6-oligo pool siRNA consisting of 4 oligos targeted against Syn6. Images were false coloured using Zeiss LSM software. The scale bar corresponds to 10 µm. (B) Quantitative analysis of the mean fluorescence of Rab6, TGN46 and CD-MPR after siRNA treatment using scrambled siRNA and Syn6-pool siRNA consisting of 4 oligos. Results are expressed as a percentage of the average mean fluorescence signal of mock treated cells corrected for background. Quantitative fluorescence scoring was done using at least 100 cells in each condition. Experiments were undertaken in triplicate. Statistically significant differences are denoted using *, as determined through a two tailed student t-test resulting in a $P<0.05$. (C) SDS-PAGE and immunoblotting of levels of Syn6 in HeLa cells treated with indicated siRNA oligos.*

HeLa cells increases the cellular levels of CI-MPR (Ganley *et al.*, 2008). These data also support the hypothesis that *Salmonella* affects retrograde traffic defined by MPR trafficking and does not interfere with retrograde traffic defined by Syn6.

4.4 Effect of *Salmonella* on Syn6 and Syn10 dependent pathways: trafficking assays

Certain molecules undergo retrograde transport from the plasma membrane to the TGN and can be used as probes to detect disruption of this type of transport. These assays exploit the previously described specificity in the retrograde trafficking routes from the plasma membrane through the endocytic compartment to the TGN of cholera toxin B subunit (CTxB) (Ganley *et al.*, 2008), and antibody-labelled CI-MPR (Robinson *et al.*, 2010). A small proportion of CI-MPR undergoes anterograde transport to the plasma membrane, where it can be labelled with antibodies, and from there the labelled receptor traffics to the TGN (Ghosh *et al.*, 2003; Robinson *et al.*, 2010). Transport of CI-MPR to the TGN from endocytic compartments has been shown to be dependent on Syn10 whereas transport of CTxB is dependent on the Syn6 retrograde pathway (Ganley *et al.*, 2008).

HeLa cells were infected for 13 h with wild-type or *ssaV* mutant strains and then incubated in the presence of culture medium containing either anti-CI-MPR antibody or CTxB for 30 min followed by a chase of 30 min with media only. Cells were then fixed and analysed by immunofluorescence microscopy. Strikingly, the trafficking to the TGN of antibody-labelled CI-MPR was strongly perturbed in cells infected with wild-type *Salmonella* (Fig. 4.4 A, B), while there was no apparent effect on CTxB transport (Fig. 4.4 A, C). Furthermore, this interference was dependent on a functional SPI-2 T3SS since there was no effect on CI-MPR traffic to the TGN in HeLa cells infected with the *ssaV* mutant strain (Fig. 4.4 B). To control for the sensitivity of the assay to detect a perturbation in CTxB traffic, siRNA mediated depletion of Syn6 was undertaken. As expected there was a significant reduction in the amount of CTxB associated with the TGN (Fig. 4.4 C).

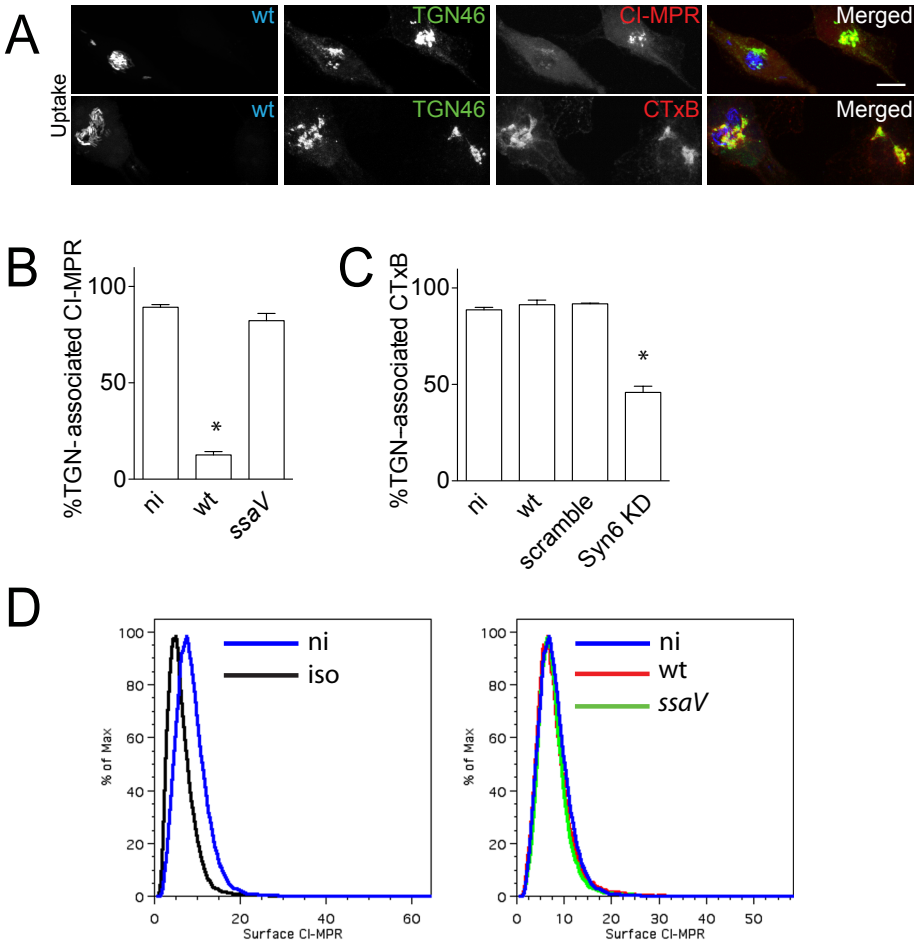


Figure 4.4 : ***Effect of* Salmonella *on CI-MPR and CTxB trafficking.*** *HeLa cells were infected for 1¼ h with wild-type or* ssaV *mutant* Salmonella *strains (blue) or depleted of Syn6 (Syn6 KD). Cells were then allowed to internalise either fluorescent CTxB (red) or an anti-CI-MPR antibody (red) for 30 min followed by a 30 min chase. Cells were fixed and labelled to detect CI-MPR or TGN46 (green). (A) Confocal microscopy images showing the localisation of TGN46 and either internalised CI-MPR or CTxB in cells infected with wild-type* Salmonella *and non-infected cells. Images were false coloured using Zeiss LSM software. The scale bar corresponds to 10 µm. (B) Quantification of the frequency of TGN associated CI-MPR in uninfected cells or cells infected with wild-type or* ssaV *mutant* Salmonella *strains. Experiments were conducted in triplicate. Statistically significant differences are denoted using *, as determined through a two tailed student t-test resulting in a $P<0.05$. (C) Quantification of the frequency of TGN associated CTxB in uninfected cells or in cells infected with wild-type* Salmonella *or in cells depleted of Syn6. Experiments were conducted in triplicate. Statistically significant differences are denoted using *, as determined through a two tailed student t-test resulting in a $P<0.05$. (D) FACS analysis of plasma membrane associated CI-MPR. Cells were infected for 1¼ h with either GFP-expressing wild-type or* ssaV *mutant* Salmonella *then collected and labelled with an antibody against CI-MPR or an isotype (iso) control. Histograms represent the levels of fluorescence labelling associated with CI-MPR or isotype control. Cells were gated for infection by GFP fluorescence. Over 10000 cells were analysed per condition.*

Finally, I analysed the surface expression of CI-MPR by flow cytometry to verify that the differences in antibody labelled CI-MPR transport from the plasma membrane to the TGN in cells infected with wild-type bacteria was not due to differences in the levels of CI-MPR on the plasma membrane. I infected cells with GFP-expressing wild-type or *ssaV* mutant *Salmonella* for 14 h. Cells were collected and labelled for plasma membrane associated CI-MPR. The specificity of the labelling was confirmed by the difference in fluorescence between CI-MPR labelled cells and those labelled with an isotype control (Fig. 4.4 D). There was no detectable difference in the levels of plasma membrane associated CI-MPR between uninfected cells or cells infected with wild-type or *ssaV* mutant *Salmonella* (Fig. 4.4 D). Therefore, differences in CI-MPR transport to the TGN from the plasma membrane seen during infection with wild-type bacteria are not a result of differences in the surface levels of CI-MPR.

These results, together with those showing redistribution of Vti1b and the MPRs from the TGN, provide strong evidence for selective inhibition of Syn10-dependent retrograde trafficking by *Salmonella* SPI-2 T3SS effector(s).

4.5 Levels of Vti1b and CD-MPR in infected cells

As stated in section 1.3.4.5, trafficking of MPRs is very important for generation of functional lysosomes. Mislocalisation of the MPRs away from the TGN would be expected to result in insufficient receptor to bind newly synthesised lysosomal enzymes as they arrive in TGN compartments. The enzymes would then be re-routed via the bulk secretory pathway to the plasma membrane, and secreted outside the cell (Riederer *et al.*, 1994a; Ikeda *et al.*, 2008). If MPR traffic was impaired in cells infected with *Salmonella* then it is possible that there may also be re-routing of lysosomal enzymes in these cells.

Before addressing whether *Salmonella* infection results in rerouting of lysosomal enzymes, I investigated whether the reduced levels of Vti1b, CD-MPR, and CI-MPR at the TGN after 14 h of infection was associated with depletion of these proteins from the cell or

their dispersion into other compartments. Immunoblot analysis and fluorescence-activated cell sorting (FACS) analysis were used to assess whether there was any change in the overall levels of Vti1b and CD-MPR after 14 h of infection with either wild-type or *ssaV* mutant strains of *S.* Typhimurium.

There was no noticeable reduction in the levels of Vti1b or CD-MPR in cells infected with either wild-type or *ssaV* mutant strains when compared to non-infected cells when analysed by immunoblotting (Fig. 4.5 A). However, in this experiment samples contained both infected and uninfected cells. As an alternative approach I analysed protein levels at the single cell level via FACS analysis. In these experiments cells were depleted of Vti1b or CD-MPR by siRNA treatment to act as positive controls for a reduction in protein levels. Prior to FACS analysis, infected cells were gated to ensure that only infected cells were analysed. The results of these experiments showed that, while it was possible to detect reduced protein levels after specific siRNA treatments, there was no detectable reduction in the levels of either Vti1b or CD-MPR after 14 h of wild-type or *ssaV* mutant strain infection (Fig. 4.5 B, C).

These results indicate that the effects on Vti1b, CD-MPR and CI-MPR observed during infection with wild-type bacteria are not a consequence of reduction in overall levels but rather a redistribution of these proteins away from TGN compartments. It is possible that they could be trapped in an early endocytic compartment, as has been described for MPRs localised to SNX1 positive compartments in cells depleted of Syn10 (Ganley *et al.*, 2008).

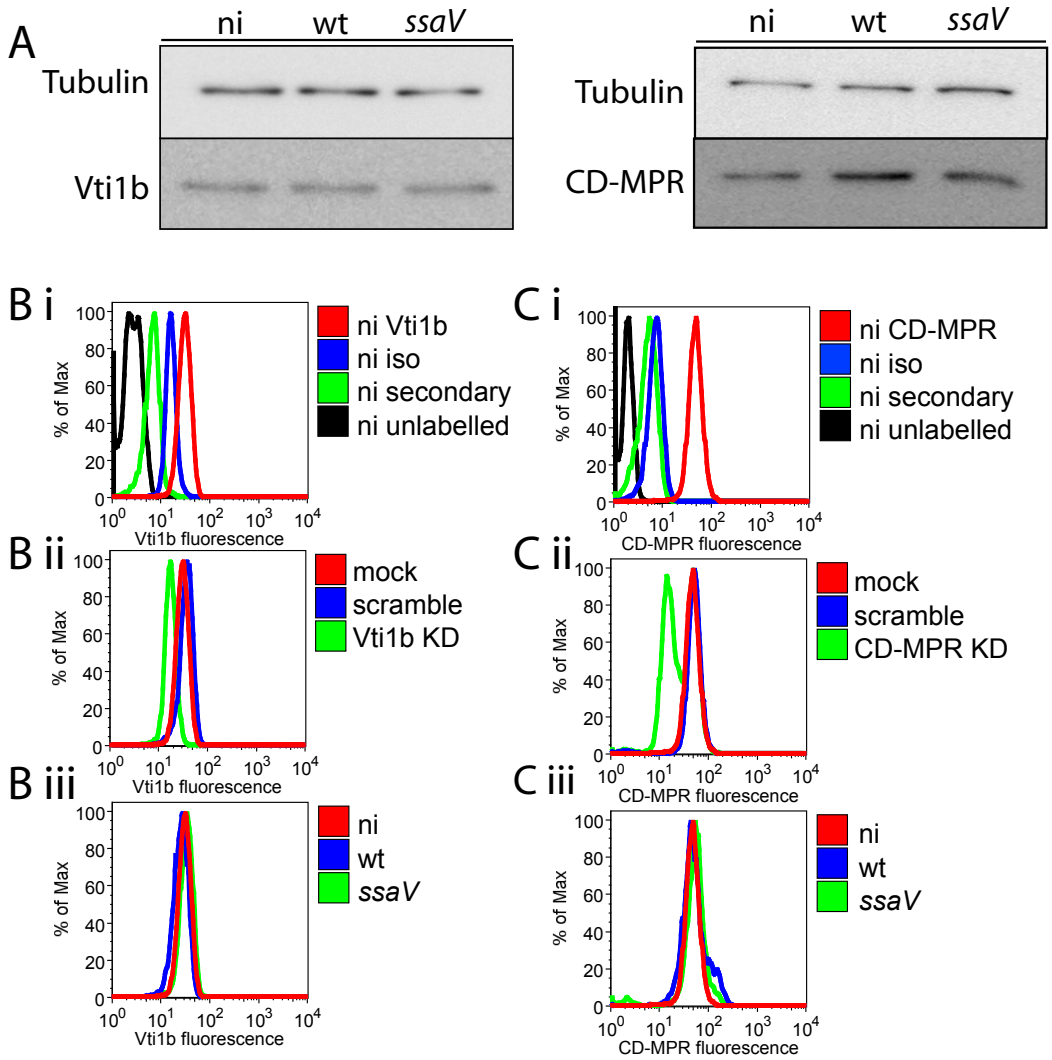


Figure 4.5 : ***Analysis of levels of Vti1b and CD-MPR.*** *(A) HeLa cells were left uninfected or infected for 14 h with either GFP-expressing wild-type or* ssaV *mutant strains. Intracellular proteins were analysed by SDS-PAGE and immunoblotting. (B and C) FACS analysis of levels of Vti1b and CD-MPR in HeLa cells. (Bi, Ci) Uninfected HeLa cells (ni) were prepared according to the following conditions for analysis; left unlabelled, labelled with secondary antibody only (secondary), labelled with IgG isotype control (iso) or labelled with a specific primary antibody to Vti1b or CD-MPR prior to secondary antibody (specific). (Bii, Cii) HeLa cells were treated with mock or scramble controls or indicated siRNA oligos and labelled with antibody to Vti1b or CD-MPR. (Biii, Ciii) HeLa cells left uninfected or infected for 14 h with either GFP-expressing wild-type or* ssaV *mutant strains and labelled with antibody to Vti1b or CD-MPR for analysis.*

4.6 Secreted lysosomal enzyme activity assay

To analyse misrouting of lysosomal enzymes in *Salmonella*-infected cells, the extracellular activities of two lysosomal enzymes, β-hexosaminidase (β-hexo) and cathepsin D (CatD), were measured. The assays involved fluorescent substrates for β-hexo (4-methylumbelliferyl-2-acetamido-2-deoxy-β-d-glucopyranoside) and CatD (MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-d-Arg-NH_2); these substrates have been shown previously to be suitable reagents to assess activities of these enzymes (Ikeda *et al.*, 2008). Both substrates fluoresce upon their cleavage and the sum fluorescent signal from a sample is proportional to the amount of cleavage that occurs. For these assays, the intracellular and extracellular activities of each enzyme were calculated to provide the total enzyme activity and the extracellular activity is presented as a percentage of total enzyme activity (Fig. 4.6 B, C). Exposure of cells to amiodarone causes an increase in secretion of lysosomal enzymes via the secretory pathway due to a blockade of MPR trafficking (Ikeda *et al.*, 2008). Amiodarone was used as a positive control to cause increased secretion of β-hexo and CatD in HeLa cells. The enzymatic activity assays were undertaken in acidified buffer (pH 4.2 or pH 4.4), which allows for autoactivation of any immature inactive enzyme (Turk *et al.*, 2012).

To control for non-specific release of intracellular enzymes due to cytotoxicity, a lactate dehydrogenase (LDH) cytotoxicity assay was used to test all extracellular samples. LDH is a stable cytoplasmic enzyme but when the plasma membrane is damaged, LDH is released into the culture supernatant. The amount of LDH in the supernatant corresponds to the degree of cell lysis within the cell culture (Decker & Lohmann-Matthes, 1988). SPI-1 T3SS mediated invasion by *Salmonella* has been shown to cause cytotoxicity in cells (Monack *et al.*, 1996; Hersh *et al.*, 1999). To remove the effects of SPI-1-associated cytotoxicity within the cell culture, all cells were washed thoroughly 8 h pi (at which time the SPI-1 T3SS is no longer active) and then the secreted lysosomal enzymes were collected at 14 h pi. There was very low cytoxicity detected for all samples and no detectable difference observed between the samples (Fig. 4.6 A).

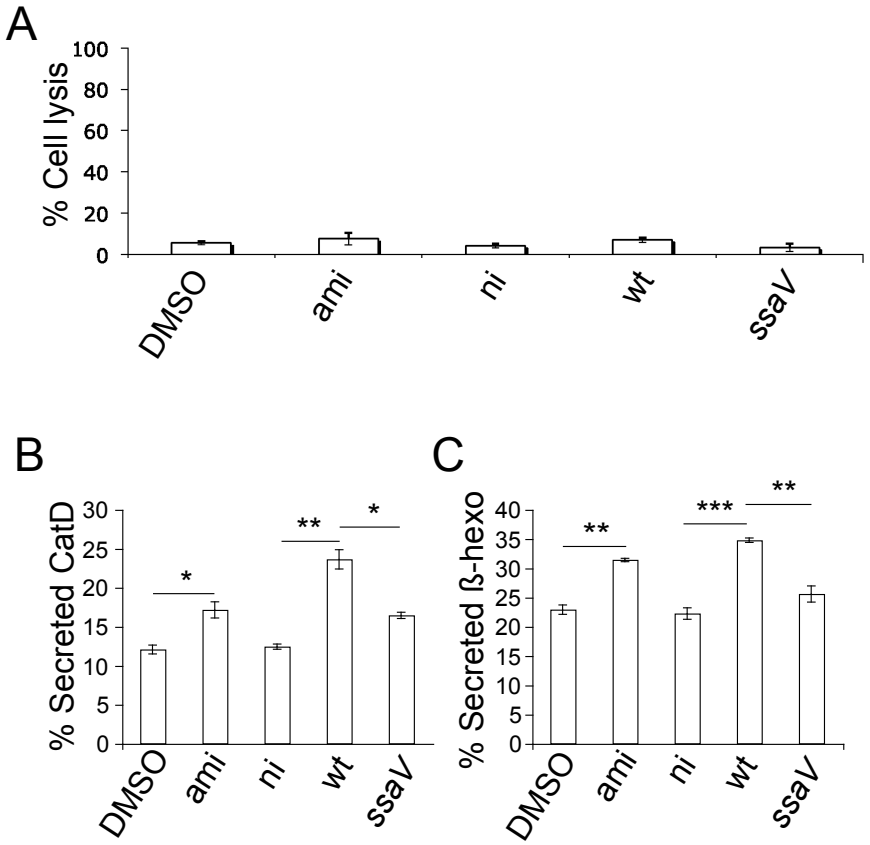


Figure 4.6 : ***Secreted lysosomal enzyme activity assay*** *Cells were either treated with DMSO or amiodarone (ami) or infected with wild-type* Salmonella *or the* ssaV *mutant strain or left non-infected (ni) for 14 h. Cells were washed 8 h pi and samples were analysed 14 h pi. (A) Cytotoxicity was assessed using LDH levels and expressed as a percentage of LDH levels of control samples (uninfected cells completely lysed in 1% Triton X100-PBS). (B and C) Secreted levels of CatD and β-hexo were expressed as a percentage of total CatD or β-hexo, which equals the sum of the intracellular and extracellular levels of the enzyme. Statistically significant relationships in both are denoted using *=$P<0.05$ or **=$P<0.005$ or ***=$P<0.0005$*

As was predicted from published data(Ikeda *et al.*, 2008), a statistically significant increase in the extracellular activity for both CatD and β-hexo was observed in cells treated with amiodarone when compared to cells treated with dimethyl-sulfoxide (DMSO) alone. In addition, a statistically significant increase in the extracellular enzymatic activity of CatD and β-hexo was observed for wild-type *Salmonella*-infected cells compared to non-infected cells (Fig. 4.6 B, C). There was also a significant increase in the extracellular enzymatic activity of CatD and β-hexo in wild-type *Salmonella* infected cells when compared with cells infected with the *ssaV* mutant strain, indicating that the SPI-2 T3SS accounted for a major proportion of the *Salmonella* effect in the levels of extracellular activity of secreted CatD and β-hexo (Fig. 4.6 B, C).

4.7 Levels of secreted lysosomal enzymes

The conditions under which the above assays were conducted were acidic (pH 4.2 or pH 4.4) to ensure conversion of any immature CatD and β-hexo to their active mature forms. If the increased activities of CatD in the extracellular fractions of cells infected with *Salmonella* were due to a misrouted secretion of this enzyme directly from the TGN, then this enzyme would be expected to be present in its inactive, immature form. To test this, intracellular and extracellular fractions were subjected to SDS-PAGE and immunoblotting using an antibody that recognises both immature and mature forms of CatD.

There was a marked increase in the secretion of CatD in cells treated with amiodarone and cells infected with wild-type bacteria when compared with non-infected cells (Fig. 4.7). Furthermore, the vast majority of increased secreted protein was in the immature state indicating that the immature pro-CatD had not encountered an acidic environment (Fig. 4.7). This suggests that it was likely to have been trafficked directly from the TGN to the plasma membrane. In addition, the proportion of the intracellular levels of immature to mature CatD appeared to be higher in cells infected with wild-type bacteria or amiodarone-treated cells when compared to non-infected cells (Fig. 4.7). This is a further indication of an im-

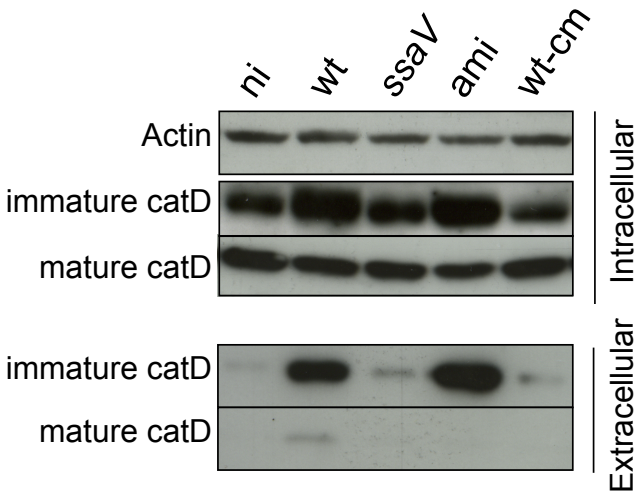


Figure 4.7 : ***Lysosomal enzyme secretion levels.*** *Cells were either treated with amiodarone (ami) or infected with wild-type* Salmonella *or the* ssaV *mutant strain or left non-infected for 14 h. Cells were washed 8 h pi and intracellular and extracellular samples were analysed 14 h pi. In one case cells infected with wild-type bacteria were also exposed to chloramphenicol (cm) 2 h pi to block bacterial protein synthesis. Intracellular and extracellular proteins were analysed by SDS-PAGE and immunoblotting using antibodies against CatD and actin (loading control).*

paired maturation pathway characteristic of a blockade in MPR traffic.

The effect of *Salmonella* on CatD required bacterial protein synthesis as it was not observed in cells infected with wild-type bacteria and then exposed to chloramphenicol 2 h pi (Fig. 4.7). In cells infected with the *ssaV* mutant strain there was a very small increase in the levels of secreted CatD when compared to non-infected cells (Fig. 4.7).

Together the data presented in this chapter indicate that *Salmonella* interferes with a retrograde trafficking route defined by Syn10 in a SPI-2 T3SS-dependent manner, disrupting MPR trafficking. This is accompanied by a re-routing of lysosomal enzymes from the TGN to the extracellular space.

Chapter 5

SifA impairs lysosome function

5.1 Introduction

In this chapter I examine the physiological impact of interference by *Salmonella* on retrograde recycling and investigate the underlying mechanism. Trafficking of MPRs is very important for cellular homeostasis of the lysosomal pathway. Mis-localisation of MPRs from the TGN results in insufficient receptor to bind newly synthesised lysosomal enzymes as they arrive in Golgi compartments from the ER. There is a resultant failure to retain mannosylated lysosomal enzymes as they traffic through the Golgi cisternae, which are then re-routed via the bulk secretory pathway to the plasma membrane, and secreted outside the cell (Riederer *et al.*, 1994b; Ikeda *et al.*, 2008).

From a physiological point of view, it could be beneficial for a vacuolated pathogen to interfere with lysosomal biogenesis, since lysosomes are a major component of the innate immune system. Rerouting of hydrolytic enzymes could be an important mechanism through which *Salmonella* detoxifies lysosomes.

5.2 Lysosome function in infected HeLa cells

If *Salmonella* causes the misrouting of newly synthesised lysosomal enzymes out of the cell, the functionality of lysosomal compartments might be impaired. To test this I assayed

lysosomal cathepsin B (CatB) in living cells infected with *Salmonella* using a fluorescent substrate of cathepsin B (Magic Red cathepsin B (MRcatB)). I used this substrate in combination with fluorescence recovery after photo-bleaching (FRAP) analysis and FACS analysis. FRAP analysis, with this protease detection system, has previously been employed to study lysosome function in the context of MPR trafficking (Metcalf *et al.*, 2008). It has also been used in conjunction with FACS analysis to detect steady state levels of CatB activity in living cells (Creasy *et al.*, 2007) (used during transfection experiments in section 5.9 and 5.10).

Both assays employ the use a membrane-permeant cathepsin B-specific substrate linked to a di-substituted cresyl violet fluorophore with two dipeptide CatB targeting sequences. In this conformation the peptide is non-fluorescent, but upon hydrolysis by CatB the fluorophore is converted to a form that fluoresces strongly in the visible red spectrum. As CatB activity progresses, more of the peptide is converted to the fluorescent state until eventually an equilibrium of red fluorescence intensity is reached within the cell. This steady state equilibrium of fluorescence intensity is the threshold of product formation versus product decay and differences in these levels (which are reflective of overall CatB activity in the cell) can be detected using either confocal microscopy or FACS analysis.

Using FRAP analysis I investigated whether there was any reduction in lysosome activity in infected and non-infected HeLa cells after 14 h of infection. Infected cells containing between 10 and 30 bacteria per cell were selected for analysis together with non-infected cells in the same field of view.

Prior to FRAP it was evident that cells infected with wild-type bacteria had lower steady state levels of fluorescence compared with uninfected cells (Fig. 5.1 A, B). When quantified using a large sample set over 3 separate experiments, these cells also exhibited a marked reduction in rate of recovery after bleaching (Fig. 5.1 A, Movie S1, B) (files for Movies S1 and S2 are included in supplemental material). By contrast, prior to FRAP there was no obvious reduction in levels of fluorescence in cells infected with the *ssaV* mutant strain (Fig. 5.1 A, Movie S2, B), and these cells consistently recovered to higher

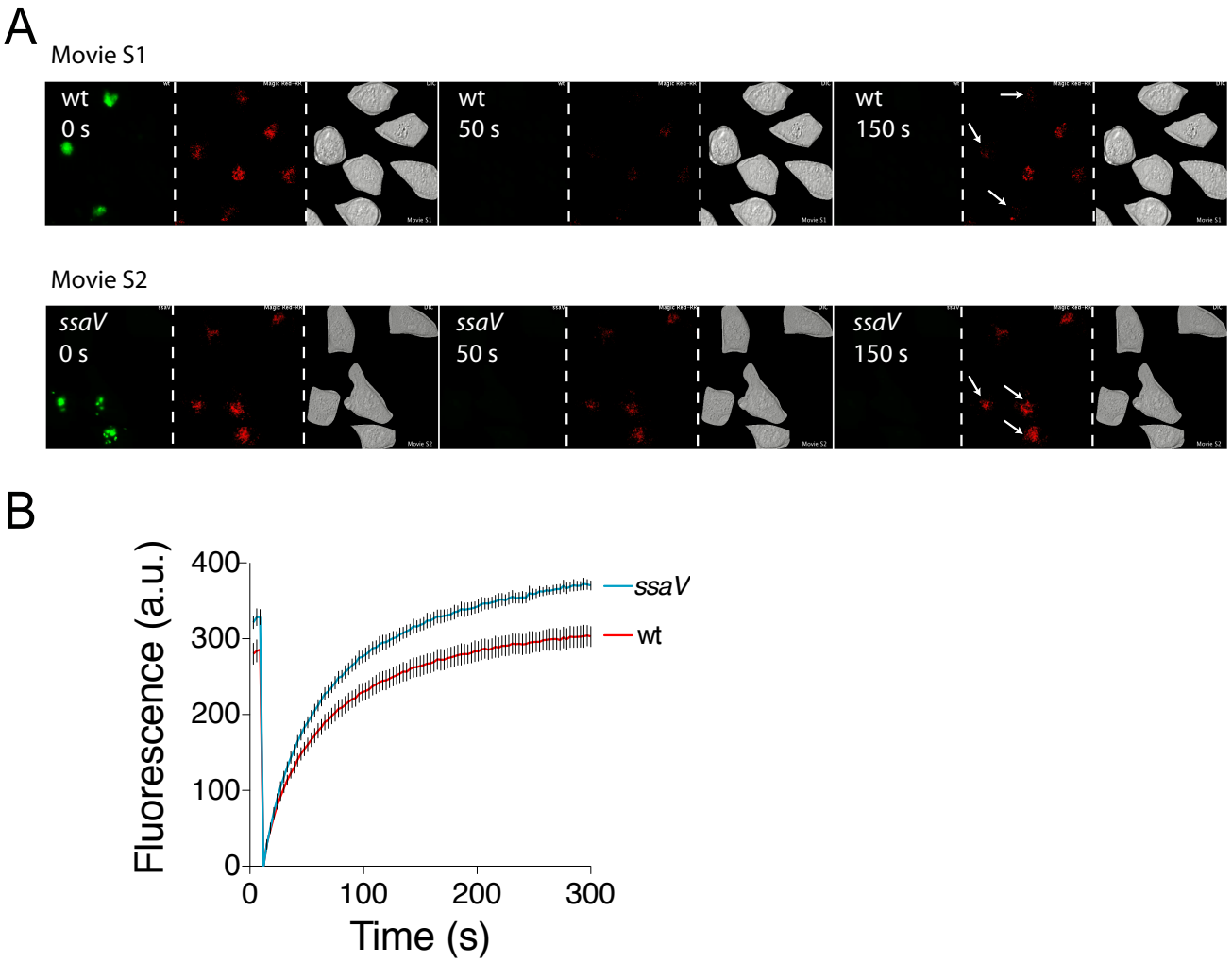


Figure 5.1 : ***CatB activity in infected HeLa cells.*** *HeLa cells were infected for 14 h with wt or* ssaV *mutant* Salmonella *and exposed to a CatB substrate (MRcatB, red). (A) Still frames at indicated time points from Movie S1 (wt, green) and Movie S2 (*ssaV*, green). Entire cells were selected for analysis using differential interference contrast (DIC). Infected and uninfected cells in the same field of view were analysed using fluorescence recovery after photobleaching (FRAP) and were imaged every 3 sec over 150 second (s). Arrows indicate fluorescence recovery in infected cells after 150 s. (B) Infected and uninfected cells in the same fields of view were analysed by FRAP assay over 300 s. Data were normalised to the mean fluorescence of uninfected cells. Error bars represent +/- SEM.*

levels of fluorescence compared to cells infected with the wild-type strain (Fig. 5.1 A, Movie S2, B).

These data suggest that lysosome function is compromised in wild-type infected HeLa cells and that this is dependent on the SPI-2 T3SS. This is consistent with the effect of *Salmonella* on MPR trafficking and the misrouting of lysosomal enzymes reported in chapters 3 and 4.

5.3 Lysosomal function in infected macrophage cells

Much of the virulence of *Salmonella* in systemic disease is due to its ability to survive and replicate in host macrophages. During systemic infection macrophages represent the primary reservoir of bacterial replication (Watson & Holden, 2010). To determine if the SPI-2 T3SS-dependent impairment of lysosome function observed in infected HeLa cells was also evident in infected mouse macrophages, I infected macrophages with wild-type or *ssaV* mutant strain *S.* Typhimurium and analysed them for CatB activity using the FRAP assay.

Two macrophage cell types were used in these experiments. Initially, CatB activity was analysed in RAW264.7 cells, a mouse macrophage-like cell line. In cells infected for 14 h with wild-type *Salmonella*, there was a reduction in both steady state CatB activity, and in recovery of CatB activity following fluorescence bleaching, compared to cells infected with the *ssaV* mutant strain (Fig. 5.2 A).

I also conducted infections in primary C57BL/6 bone-marrow derived mouse macrophages. These cells are much more restrictive to bacterial growth (Helaine *et al.*, 2010) and it can be argued that they are a more physiologically relevant cell type than RAW264.7 mouse macrophages-like cells given that they are derived from an *in vivo* source. Bone-marrow macrophages were infected for 48 h or 72 h with either wild-type or *ssaV* mutant strains. Macrophages containing similar numbers of bacteria were selected for analysis. There was a marked reduction in CatB activity when cells were infected with wild-type

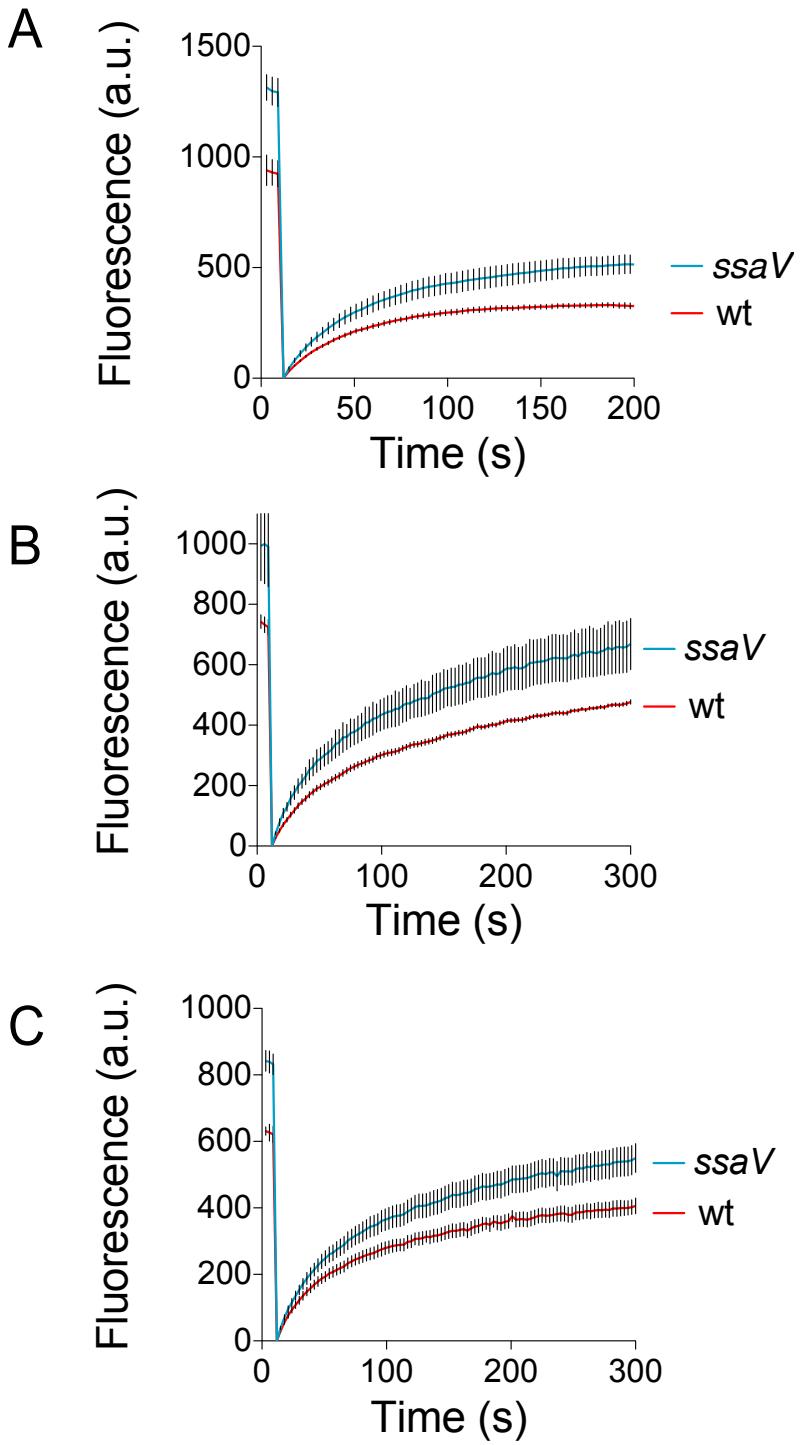


Figure 5.2 : ***CatB activity in infected macrophages.*** *(A) RAW264.7 cells were infected for 14 h with wild-type (wt) or* ssaV *mutant* Salmonella *and were analysed by FRAP assay of CatB activity. (B and C) C57BL/6 mouse bone marrow-derived macrophages were infected for 48 h (B) or 72 h (C) with wt or* ssaV *mutant* Salmonella *and were analysed by FRAP assay of CatB activity. Data were normalised to the mean fluorescence of uninfected cells.*

Salmonella, when compared with cells infected with the *ssaV* mutant strain at both 48 h and 72 h post-uptake. This was evident both at steady state levels and by FRAP (Fig. 5.2 B, C).

These results show a reduction in lysosome function in a SPI-2 T3SS-dependent manner in three separate cell types of human and mouse origin.

5.4 Lysosomal activity in cells depleted of Syn6 or Syn10

If impairment of lysosome function is due to the effect of *Salmonella* on MPR recycling to the TGN, then blocking MPR traffic in uninfected cells should recapitulate the reduction in CatB activity observed during infection. To investigate whether reduced lysosome function occurs in cells with impaired endosome-to-TGN traffic I depleted HeLa cells of Syn10 or Syn6 by siRNA and examined lysosome function using the MRcatB FRAP assay.

There was a marked reduction in steady state and FRAP levels of CatB activity in cells depleted of Syn10 using either a pool of oligos or a single oligo, compared to cells treated with a scrambled oligo (Fig. 5.3 A). In contrast, Syn6 depletion enhanced levels of CatB activity, both at steady state and during FRAP (Fig. 5.3B). These results are consistent with the data shown in (Fig. 4.2 B; Fig. 4.3 B), of reduced or increased TGN localisation of CD-MPR in cells depleted of Syn10 or Syn6, respectively, and show that Syn10 is required to maintain lysosome enzyme levels.

5.5 Growth of *Salmonella* in cells depleted of Syn10 or Syn6

I next examined the effects of Syn6 and Syn10 depletion, or pharmacological inhibition of cathepsins, on the intracellular growth of GFP-expressing *Salmonella*. Two methods of analysis were employed. Initially the numbers of individual GFP-expressing wild-type bacteria per cell were counted after 8 h of infection using fluorescence microscopy. HeLa

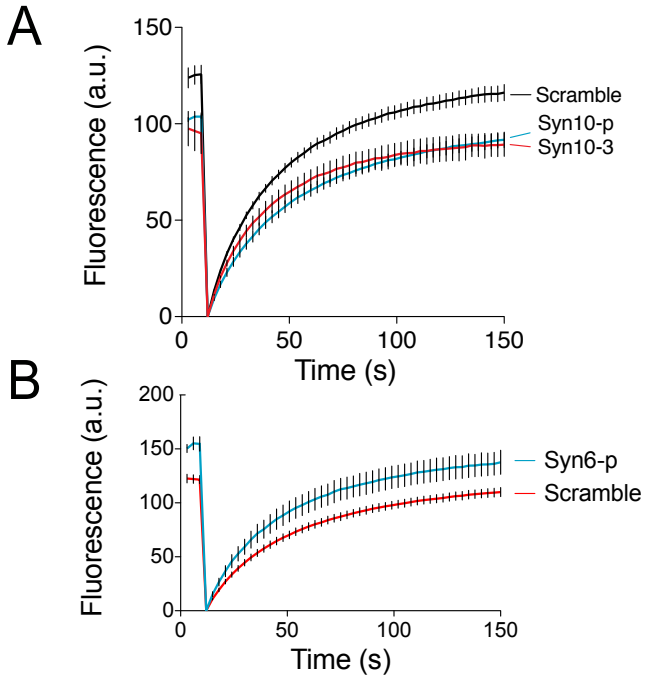


Figure 5.3 : ***Lysosomal activity assay in siRNA treated cells.*** *(A) HeLa cells depleted of Syn10 using either a pool of 4 oligos (-p) or oligo 3 (-3) or exposed to scrambled siRNA were analysed by FRAP assay of CatB activity. Error bars represent +/- SEM. (B) HeLa cells depleted of Syn6 using a pool of 4 oligos (-p) or exposed to scrambled siRNA were analysed by FRAP assay of CatB activity. Error bars represent +/- SEM.*

cells were either treated with the transfection reagent alone (mock), scramble siRNA or siRNA against Syn10. Cells were then infected for 8 h with GFP-expressing *Salmonella*, fixed and labelled for F-actin as a marker for HeLa cells. For each condition, growth of *Salmonella* was assessed by counting individual bacteria per cell. Increased growth was observed when cells were depleted of Syn10 (Fig. 5.4 A).

Bacterial growth in Syn6 or Syn10-depleted cells was also measured by flow cytometry, which provides a sensitive measure of the amount intracellular bacterial growth (Kuijl *et al.*, 2007; Yu *et al.*, 2011). HeLa cells were treated with transfection reagent, scramble siRNA, siRNA against Syn10 or siRNA against Syn6 and were then infected with GFP-expressing wild-type bacteria. At 2 h and 8 h pi cells were collected and analysed by flow cytometry. Results were expressed as GFP fluorescence per HeLa cell and represent the median of the ratio between 2 h and 8 h after inoculation, normalised to mock-treated cells. Cells depleted of Syn6 contained significantly less fluorescence than control cells, while cells

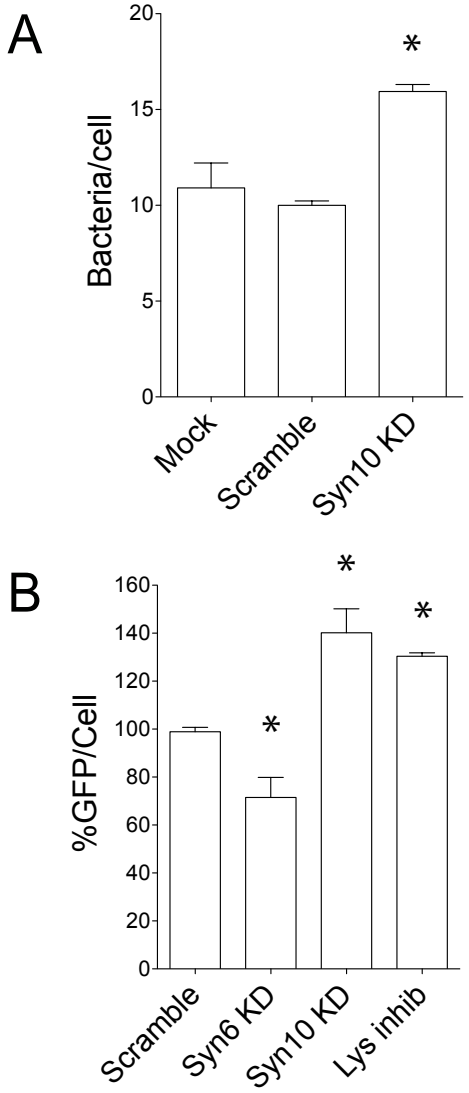


Figure 5.4 : ***Replication of*** **Salmonella** ***in HeLa cells depleted of Syn6 or Syn10*** *(A) HeLa cells treated with mock-transfected, control siRNA or Syn10 siRNA were infected with GFP-expressing wild-type* Salmonella*. The data represent the average bacterial number per cell over 3 experiments with over 200 cells per condition per experiment analysed. (B) HeLa cells treated with control, Syn6 siRNA or Syn10 siRNA, or with lysosome protease inhibitor (Lys) were infected with GFP-expressing wild-type* Salmonella*. Bacterial load, expressed as GFP fluorescence per HeLa cell, was measured by flow cytometry ($n > 10{,}000$). %GFP signal/cell represents the median of the ratio between 2 h and 8 h after infection normalised to mock-transfected or DMSO treated cells.*

depleted of Syn10 contained significantly greater fluorescence, indicating increased bacterial growth (Fig. 5.4 B).

Finally, to investigate more directly the sensitivity of *Salmonella* to lysosomal enzyme activity, cells were exposed to either DMSO or a lysosome cathepsin inhibitor, Cathepsin III. After 4 h of exposure to the inhibitor, cells were infected with GFP-expressing wild-type bacteria, and the inhibitor was maintained in the culture medium for the duration of the infection. After 2 h and 8 h pi cells were collected and analysed by flow cytometry as above. Cells exposed to the inhibitor contained significantly more fluorescence than control cells indicating increased bacterial growth (Fig. 5.4 B). Therefore, the intracellular growth of *Salmonella* was reduced in cells with enhanced lysosomal enzyme activity and enhanced in cells with reduced lysosomal enzyme activity.

5.6 *sifAsseJ* and *sifAsopD2* mutant strains fail to re-distribute CD-MPR

To identify a *Salmonella* effector protein(s) that could impair lysosome function, HeLa cells were infected with various mutant strains for 14 h and examined for effects on CD-MPR localisation. SifA is a SPI-2 T3SS effector that is prenylated by host cell geranyl geranyl transferase, allowing it to insert into the SCV membrane (Reinicke *et al.*, 2005). In this environment it functions in co-operation with the SPI-2 T3SS effectors PipB2, SseJ and SopD2 to mediate Sif formation and reorganisation of the late endosomal network (Stein *et al.*, 1996). SifA is also required to maintain the SCV membrane (Beuzón *et al.*, 2000). Loss of the vacuolar membrane around a *sifA* mutant is inhibited by absence of either SseJ or SopD2 (Ruiz-Albert *et al.*, 2002; Schroeder *et al.*, 2010). Therefore the possible effect of SifA was examined using *sifAsseJ* or *sifAsopD2* double mutant strains along with *sseJ* and *aroCpurD* mutants or wild-type strains. Infection of HeLa cells with the *sifAsseJ* or *sifAsopD2* strains did not induce redistribution of CD-MPR while infection with the *sseJ* or *aroCpurD* mutants or wild-type strains caused its redistribution (Fig. 5.5 A, B).

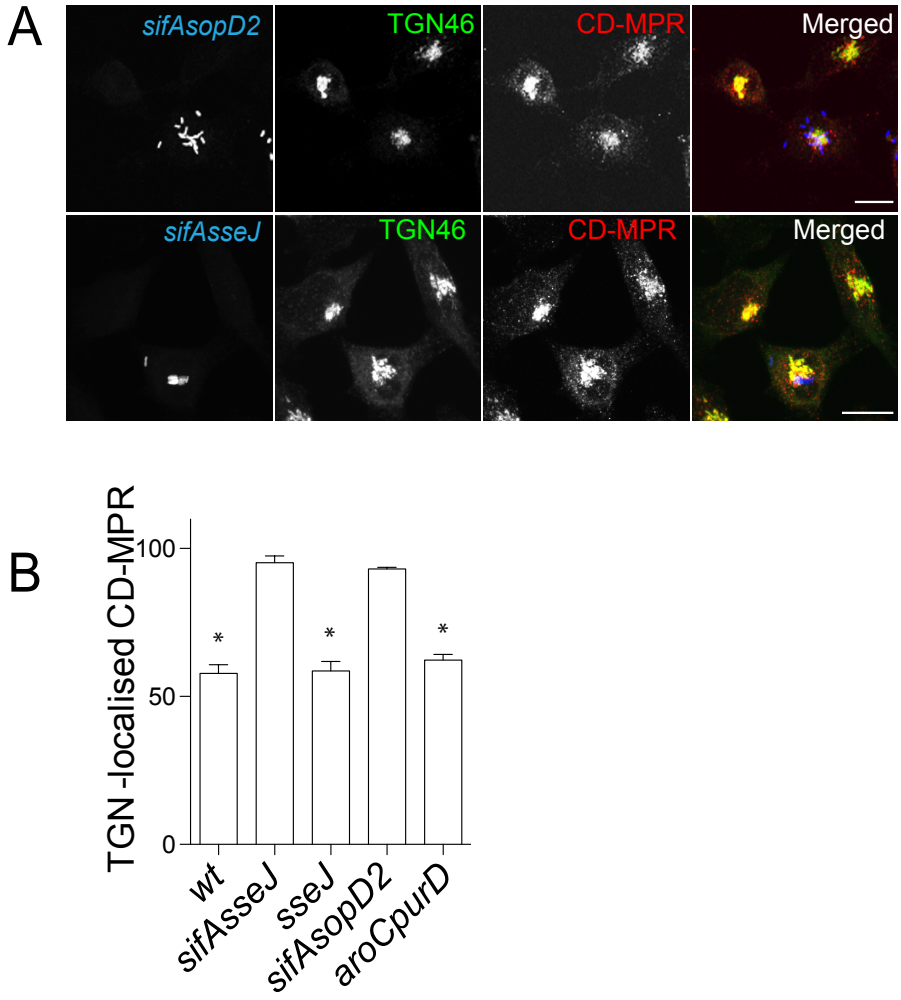


Figure 5.5 : sifAsseJ *and* sifAsopD2 *fail to redistribute CD-MPR*. *(A) HeLa cells were infected for 14 h with either a GFP-expressing* S. *Typhimurium* sifAsseJ or sifAsopD2 *mutant strains (blue) and were immunolabelled for TGN46 (green) and CD-MPR (red). (B) Quantitative 3d confocal microscopy of mean fluorescence signals of CD-MPR localised to the TGN region in HeLa cells infected for 14 h with wild-type,* sifAsseJ, sseJ, sifAsopD2 *or an* aroCpurD *mutant strains. Scale bars represent 10 µm. Statistically significant relationships in are denoted (* = P<0.05).*

These data show that SifA is required by *Salmonella* to interfere with MPR retrograde traffic to the TGN.

5.7 SifA but not SifA$_{(L130D)}$ complements CD-MPR redistribution in a *sifAsseJ* or *sifAsopD2* mutant strain

SifA activity is mediated at least in part through its interaction on the SCV membrane with the host cell protein SKIP (Boucrot *et al.*, 2005). Once bound on the membrane the complex of SifA and SKIP are able to cross-link the SCV with the kinesin-associated microtubule network to promote anterograde movement of membrane, generating Sifs (Dumont *et al.*, 2010; Schroeder *et al.*, 2011). The binding between SifA and SKIP has been characterised at the atomic level and shown to be dependent on a SifA leucine residue at position 130 (L130), which is intimately associated with the PH domain of SKIP (Boucrot *et al.*, 2005; Ohlson *et al.*, 2008; Diacovich *et al.*, 2009).

To test the requirement of a SifA-SKIP interaction in the alteration of MPR trafficking, HeLa cells were infected with the *sifAsseJ* mutant strain harboring a plasmid encoding either an epitope-tagged wild-type SifA (SifA$_{wt}$-HA) or a point mutant (SifA$_{(L130D)}$-HA). Cells were also infected with *sifAsopD2* mutant strain harboring SifA$_{wt}$-HA as well as *ssaV*, *sifAsseJ* mutant or wild-type strains. Complementation of *sifAsseJ* or *sifAsopD2* mutant strains with the wild-type SifA protein, caused redistribution of MPR similar to that caused by wild-type *Salmonella* infection (Fig. 5.6 A, B). However cells infected with *sifAsseJ* mutant bacteria producing the point mutant SifA$_{(L130D)}$-HA, were indistinguishable from cells infected with the *ssaV* or *sifAsseJ* mutants, in terms of accumulation of MPR at the TGN (Fig. 5.6 A, B). This failure to complement was not due to a defect in translocation of effector into the host cell (Fig. 5.6 A) Together these results show that SifA is required to impair MPR trafficking during infection and requirement for L130 strongly implicates SKIP in this process.

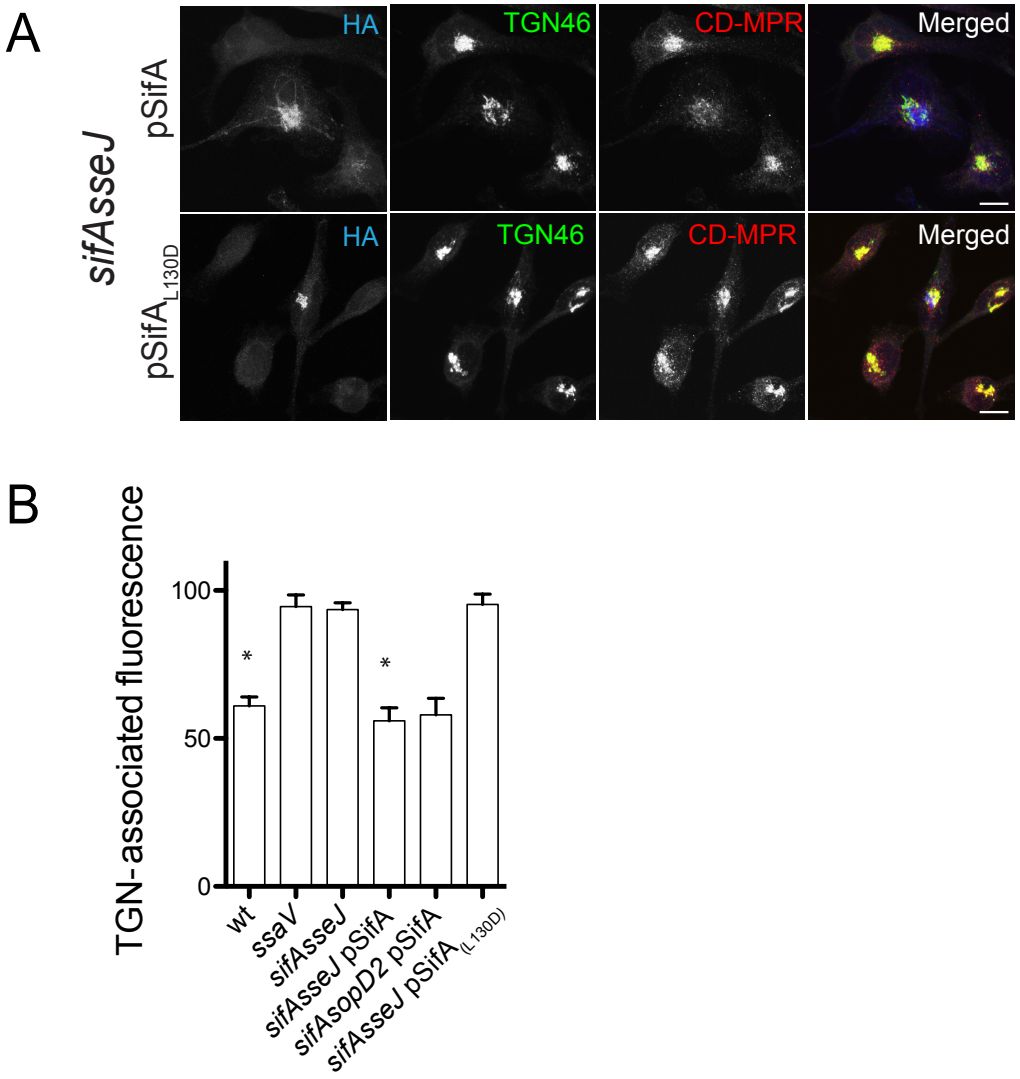


Figure 5.6 : ***SifA but not SifA$_{(L130D)}$ complements CD-MPR redistribution in* sifAsseJ *and* sifAsopD2 *mutant strains*** *(A) HeLa cells were infected for 14 h with a* S. *Typhimurium* sifAsseJ *mutant strain expressing either SifA-HA or pSifA$_{(L130D)}$-HA and were immunolabelled for TGN46 (green) and CD-MPR (red) and HA (blue). Scale bars represent 10 µm. (B) Quantitative 3d confocal microscopy of mean fluorescence signals of CD-MPR localised to the TGN region in HeLa cells infected for 14 h with wild-type,* ssaV, sifAsseJ, sifAsseJ *pSifA-HA,* sifAsopD2 *pSifA-HA or* sifAsseJ *pSifA$_{(L130D)}$-HA mutant strains. Statistically significant relationships in are denoted (* = $P<0.05$). Results are the average of triplicate experiments for all strains except for* sifAsopD2 *pSifA-HA strain which was undertaken 2 times.*

5.8 Leucine 130 of SifA is required for inhibition of lysosome function

If *Salmonella* can alter MPR traffic through the actions of SifA then it is possible that SifA is also responsible for the SPI-2 T3SS-dependent impairment of lysosome function observed during infection. To investigate whether this was the case HeLa cells were infected for 14 h with wild-type, *sifAsseJ* mutant and complemented strains and then subjected to FRAP assay for CatB activity.

Both steady state levels of fluorescence and its rate of recovery during FRAP were higher in HeLa cells infected with the *sifAsseJ* mutant strain, compared to cells infected with wild-type bacteria (Fig. 5.7). Production of SifA in the double mutant strain lowered the fluorescence to that obtained in cells infected with wild-type bacteria, but production of $SifA_{(L130D)}$ in the double mutant strain failed to reduce CatB activity either at steady state or during FRAP (Fig. 5.7). Therefore, SifA accounts for the inhibition of lysosomal function by *Salmonella* and the requirement for leucine 130 implicates SKIP in this process.

5.9 SifA but not $SifA_{(L130D)}$ is sufficient to redistribute CD-MPR and attenuate lysosome function

Next I addressed whether expression of SifA alone was sufficient to redistribute the MPR from the TGN and impair lysosome function. SifA::EGFP or $SifA_{(L130D)}$::EGFP was expressed ectopically in HeLa cells after transient transfection. Levels of MPR localised to the TGN were examined by quantitative 3d immunofluorescence microscopy. Consistent with results of experiments involving infected cells, expression of wild-type SifA was sufficient to reduce CD-MPR localised to the TGN, and this was dependent on leucine 130 (Fig. 5.8 A). Furthermore, expression of SifA was sufficient to reduce both steady state and FRAP-associated CatB activity and again this effect required leucine 130 of SifA (Fig. 5.8 B).

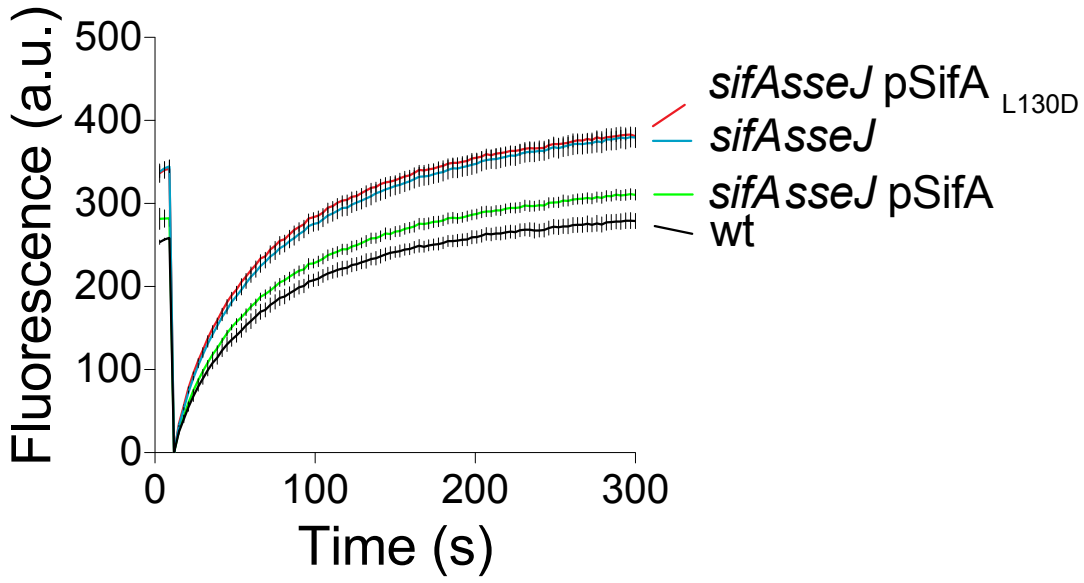


Figure 5.7 : ***Role of SifA in lysosome functions.*** *FRAP assay of CatB activity in HeLa cells infected for 14 h with the indicated strains. Data were normalised to the mean fluorescence of uninfected cells. Error bars represent +/- SEM.*

To examine CatB activity in these cells by an independent method HeLa cells were transiently transfected with a vector expressing SifA::EGFP or $SifA_{(L130D)}$::EGFP. After 24 h of transfection cells were collected and incubated with MRcatB substrate. Cells expressing similar levels of EGFP tagged SifA or $SifA_{(L130D)}$ were selected for analysis of steady state levels of CatB activity using flow cytometry. This method enabled a much larger number of cells to be analysed than the microscopy-based FRAP assay. In agreement with the FRAP assay, we observed a significant reduction in CatB-dependent fluorescence in cells expressing with SifA, compared to cells expressing equivalent levels of $SifA_{(L130D)}$ (Fig. 5.8 B inset).

Together, these data show that SifA is sufficient to inhibit MPR traffic and lysosome function. Since L130 is required for binding of SifA to SKIP, they also provide evidence that SKIP might be involved in lysosome function. These experiments were done together with Dr. Teresa Thurston of our laboratory.

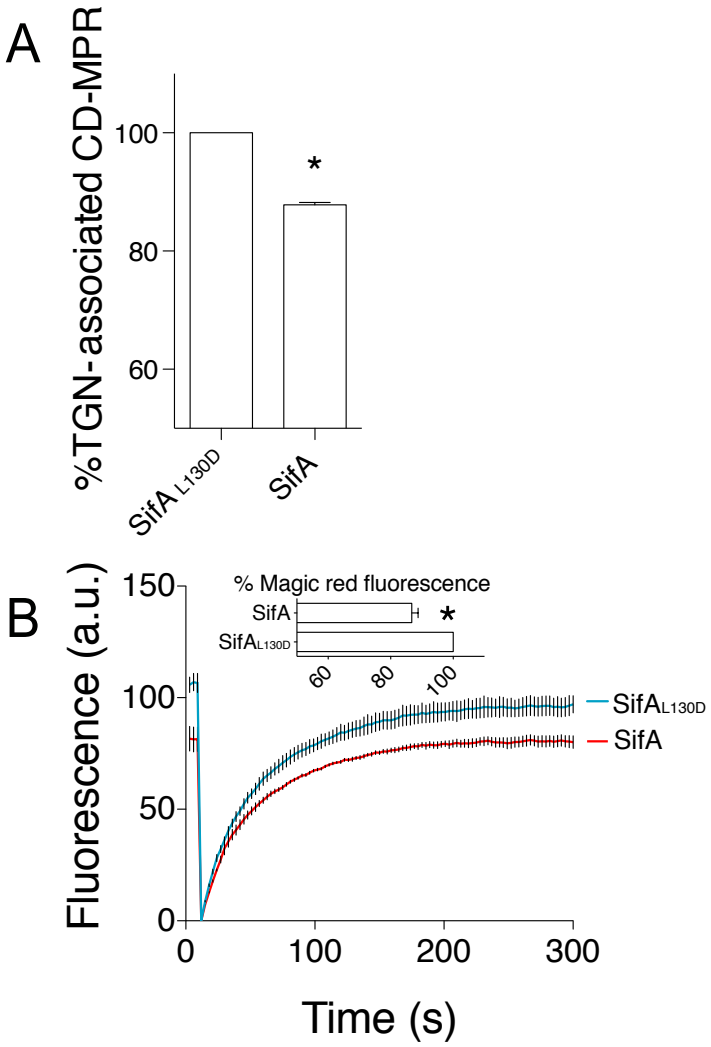


Figure 5.8 : *SifA is sufficient to redistribute MPRs and attenuate lysosome function.* *Cells were transfected with pEGFP-N1 expression vectors expressing GFP C-terminally fused to SifA or SifA$_{(L130D)}$ and then 24 h later were analysed. (A) Quantitative 3d confocal microscopy of mean fluorescence signals of CD-MPR localised to the TGN region in HeLa cells expressing similar levels EGFP tagged indicated proteins normalised to SifA$_{(L130D)}$-transfected cells. (B) FRAP analysis of CatB activity in HeLa cells expressing similar levels of the indicated proteins. (Inset in B) Steady state levels of CatB activity analysed by FACS analysis. Cells were incubated with Optimem medium for 1 h 30 min. MRcatB fluorescent substrate was then added for 10 min and cells were analysed by FACS. Statistically significant differences are denoted using *, as determined through a two tailed student t-test resulting in a $P<0.05$.*

5.10 SKIP functions in MPR trafficking

SKIP is a protein of 1020 amino acids that possesses a kinesin-interacting RUN domain and a PH domain. The N-terminal RUN domain interacts with kinesin-1 and the PH domain interacts with SifA and Rab9 (Boucrot *et al.*, 2005; Jackson *et al.*, 2008a). To date there has only been one function ascribed to SKIP in the absence of SifA. It has been shown that SKIP interacts with the TPR domain of kinesin-1 (Dumont *et al.*, 2010) and affects the distribution of LE and lysosomes. In SKIP-depleted cells Lamp1-positive vesicles cluster in the vicinity of the MTOC, whereas in cells overproducing SKIP, Lamp1-positive vesicles are redistributed to the periphery of the cell (Jackson *et al.*, 2008a; Dumont *et al.*, 2010).

The data presented in this thesis indicate that SKIP might also have a function in MPR trafficking and lysosomal biogenesis. To investigate this, I depleted endogenous levels of SKIP in uninfected cells using an siRNA oligo previously shown to affect SKIP levels (Boucrot *et al.*, 2005) and monitored the impact on CD-MPR TGN localisation and CatB activity. In cells that were depleted of SKIP (Fig. 5.9 A), there was increased MPR localised to the TGN compared to scramble-siRNA treated control cells (Fig. 5.9 B). In addition, depletion of SKIP increased both steady state and FRAP-associated CatB activity (Fig. 5.9 C, C inset).

These results indicate that SKIP acts to negatively regulate MPR trafficking and lysosomal activity. Experiments to analyse the steady state of CatB activity by flow cytometry and the levels of CD-MPR localised to the TGN were done together with Dr. Teresa Thurston of our laboratory.

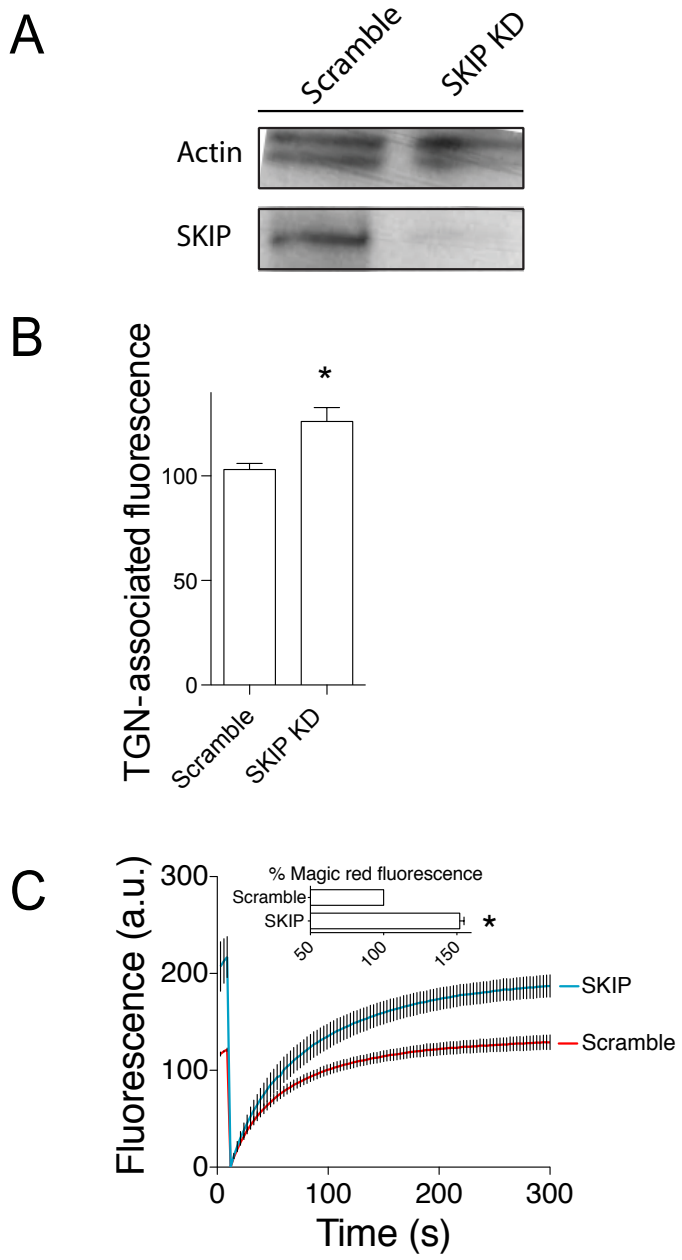


Figure 5.9 : ***SKIP is a negative regulator of MPR trafficking and lysosomal biogenesis.*** *(A) SDS-PAGE and immunoblotting of the intracellular levels of SKIP in HeLa cells treated with siRNA oligo against SKIP or scramble control. (B) Quantitative 3d confocal microscopy of mean fluorescence signals of CD-MPR localised to the TGN region in HeLa cells depleted of SKIP by siRNA or exposed to scramble siRNA control normalised to mock-transfected cells (C) HeLa cells depleted of SKIP by siRNA or exposed to scramble siRNA control were analysed by FRAP assay of CatB activity. Error bars represent +/- SEM. (Inset in C) Steady state levels of CatB activity analysed by FACS analysis. Cells were incubated with Optimem medium for 1 h 30 min. MRcatB fluorescent substrate was then added for 10 min and cells were analysed by FACS. Statistically significant differences are denoted using *, as determined through a two tailed student t-test resulting in a $P<0.05$.*

5.11 Summary

In this chapter I have shown that intracellular *Salmonella* reduces the functionality of lysosomes through the action of SifA, a SPI-2 T3SS effector. Attenuation of lysosome function was shown in human epithelial cells and mouse macrophages. These results are consistent with the effect of *Salmonella* on Syn10-dependent trafficking of MPRs from endosomes to the TGN. I also show that Syn10 is required for full lysosomal enzyme activity. The finding that depletion of Syn6 actually stimulates lysosomal enzyme activity is interesting in relation to the requirement of these two SNARES for shared partner proteins (Ganley *et al.*, 2008) and suggest that one or more of these proteins might be rate-limiting, so that removal of Syn6 or Syn10 could stimulate an equilibrium shift to favour binding to the other partner. Despite the ability of *Salmonella* to interfere with the Syn10 pathway, the growth of wild-type bacteria appears to be inhibited to some extent by the actions of Syn10 and lysosomes, since there was increased bacterial growth in cells depleted of Syn10 or in which lysosome function was further compromised by exposure to a lysosomal protease inhibitor. My experiments with bacterial mutants, SifA and SKIP show clearly that this effect of *Salmonella* is mediated by SifA and is very likely to involve SKIP. Perhaps surprisingly, SKIP appears to be a negative regulator of lysosome biogenesis. Therefore the simplest hypothesis to explain my results is that SifA augments the negative regulatory activity of SKIP (Fig. 5.10). A mechanistic framework for how this could occur will be discussed further in the Discussion.

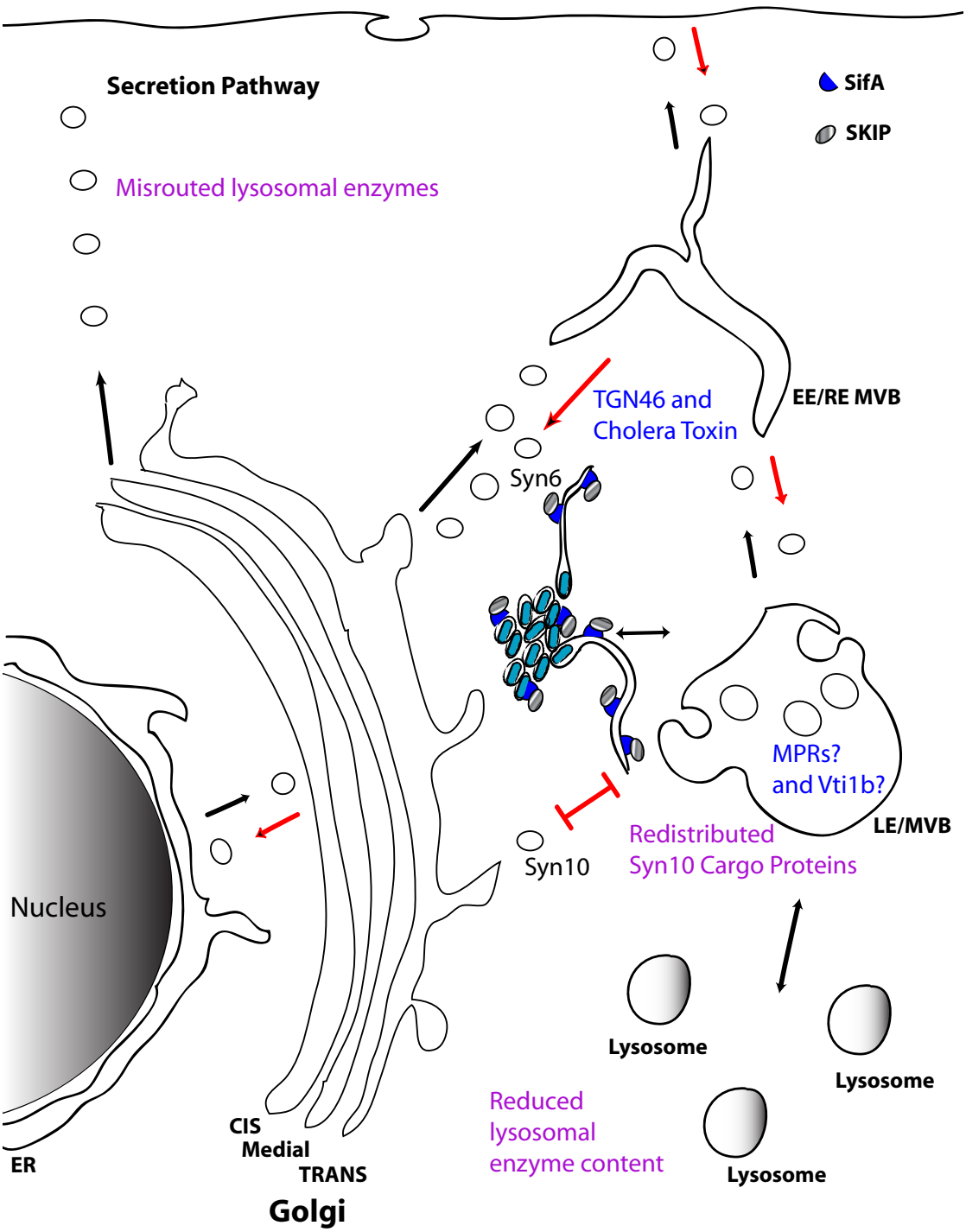


Figure 5.10 : Salmonella *interferes with MPR retrograde traffic through the action of SifA and SKIP to attenuate lysosome function.* Salmonella *(turquoise) in shown in a microcolony associated with the TGN. Retrograde trafficking routes are denoted with the red arrows or the blocked red line to show interference. Anterograde trafficking routes are denoted with black arrows. The cargos that define the two endosome-to-TGN retrograde trafficking routes are shown in blue adjacent to the route they follow or redistributed to LE/MVB. The SNARE defining the route of each of the two endosome-to-TGN transport routes are listed adjacent to relevant route. The physiological impact on the cell are shown in violet adjacent to the region that they are present. SifA and SKIP complexes are shown on the surface of the SCV and Sifs.*

Chapter 6

Discussion

6.1 Introduction

After bacteria enter a cell they are normally enclosed in a vacuole that is rapidly transported in a sequential process through endocytic compartments to lysosomes where hydrolytic enzymes and other bactericidal molecules mediate their killing (Vieira *et al.*, 2002; Haas, 2007; Flannagan *et al.*, 2009). Successful intracellular parasitism by a pathogen is dependent on its ability to manipulate this hostile environment, allowing the establishment of a niche conducive to survival and replication. As discussed in Chapter 1, there are a variety of mechanisms employed by bacterial pathogens to accomplish this, including vaculolar lysis, inhibition of vacuole maturation and deviation of the maturation of the vacuole into one that will not interact with lysosomes.

The intracellular biology of *Salmonella* has been the subject of investigation for over 20 years (Finiay & Falkow, 1989). During this time, the broad consensus of the research community has been that *Salmonella* manipulates the maturation process of the SCV to prevent interaction with lysosomes while maintaining an ability to recruit lgps to the vacuolar membrane. This consensus is based on work from many groups, which has shown the mature SCV to be relatively devoid of MPR-delivered hydrolytic enzymes yet enriched in lgps and the vATPase (Garcia-del Portillo & Finlay, 1995; Rathman *et al.*, 1997; Méresse

et al., 1999b; Hang *et al.*, 2006; Thompson *et al.*, 2011). In spite of intense activity in this area, the underlying mechanisms by which this is achieved are not understood.

Much of the ability of *Salmonella* to modify its environment from within the mature SCV is associated with the SPI-2 T3SS. Acidification of the SCV lumen is used as a signal for *Salmonella* to assemble the SPI-2 T3SS (Yoon *et al.*, 2009; Arpaia *et al.*, 2011) and recognition of the near-neutral cytosolic pH, following formation of the T3SS translocon pore in the vacuolar membrane, results in delivery of many effector proteins into the host-cell cytoplasm, some of which modify endocytic compartments (Yu *et al.*, 2010). To investigate the characteristics of mature SCVs in more detail, I began by investigating the influence that *Salmonella* has on selected proteins of the endomembrane trafficking system in epithelial cells.

6.2 SCVs and Sifs contain a subset of late endocytic SNAREs

SNARE proteins are essential components of the cellular machinery governing the specificity with which intracellular compartments interact. My findings that the late-endocytic SNAREs Syn7, Syn8 and Vamp7 are enriched on SCVs and Sifs are consistent with previously reported findings that SCV and Sif membranes contain proteins normally localised to LEs (Garcia-del Portillo *et al.*, 1993; Garcia-del Portillo & Finlay, 1995; Méresse *et al.*, 1999a; Mota *et al.*, 2009).

Given these observations it was surprising that I failed to detect appreciable levels of Vti1b or Vamp8 on SCV or Sif membranes. Both these SNAREs normally function in conjunction with Syn7 and Syn8 to form SNAREpin complexes (Pryor *et al.*, 2004). Therefore it might be expected that they would be recruited together with their binding partner proteins. These data are preliminary and due to time constraints and the direction that the project has taken these observations have not been investigated as thoroughly as they might otherwise have been. Nonetheless, these results present the intriguing possibility

that *Salmonella* could differentially recruit functionally linked SNARE proteins.

Interestingly, Vti1b and Vamp8 have been identified as critical components for lysosomal fusion events bacterial containing compartments (Furuta *et al.*, 2010). Syn11 has been proposed as a regulator of Vti1b function in macrophages by controlling its incorporation in SNAREpin complexes either with Syn7-Syn8 or with Syn6-Syn7 (Offenhäuser *et al.*, 2011). Binding of Vti1b by Syn11 controls its availability to participate in SNARE complexes that mediate LE to lysosome fusion (Offenhäuser *et al.*, 2011). In addition, Vamp8 and Vti1b have been shown to be essential components enabling lysosomal fusion with the xenophagosome, a bacteria-containing autophagic compartment that normally fuses with lysosomes (Furuta *et al.*, 2010). Of LE SNARE proteins involved in lysosomal fusion only Vamp8 and Vti1b were found to be essential (Furuta *et al.*, 2010). If, as my data suggest, *Salmonella* inhibits the recruitment or retention of Vamp8 and Vti1b on SCV and Sif membranes, then this could indicate a novel mechanism through which fusion with lysosomal compartments is impaired.

6.3 Interactions with post-TGN traffic

The ability of SCVs to localise to the TGN region correlates with the efficiency of intracellular replication of *Salmonella* (Salcedo & Holden, 2003). From this region *Salmonella* is positioned to exploit a site of concentrated transport to and from the TGN. Through interaction with vesicular traffic at this position *Salmonella* could satisfy membrane or nutritional requirements, alter lysosomal enzyme transport and promote the stability of the SCV membrane. My study provides strong evidence to show that *Salmonella* interferes with a subset of cellular traffic that travels from LE to the TGN. However, interactions with vesicular traffic by *Salmonella* are not restricted to this pathway. The most striking example is in the reorganisation of late endosomal vesicles into Sifs that extend from SCVs and the collapse of peripherally distributed LEs towards the MTOC/*Salmonella* microcolony (Garcia-del Portillo *et al.*, 1993; Brumell *et al.*, 2001; Schroeder *et al.*, 2011). Furthermore,

recent work has shown that *Salmonella* specifically recruits SCAMP3, a primarily TGN-localised protein, to the SCV and its associated tubular network (Mota *et al.*, 2009). These data provide evidence that *Salmonella* intercepts and recruits membrane from post-TGN traffic.

There are several intracellular pathogens that interact with the secretory pathway through their association with the ER, including *Legionella pneumophila*, *Brucella* spp., *Toxoplasma gondii* and several viruses (Salcedo & Holden, 2005; Roy *et al.*, 2006). Only *Salmonella*, *Chlamydia trachomatis* and *Chlamydia pneumoniae* are known to interfere with post-TGN exocytic traffic (Hackstadt *et al.*, 1996; van Ooij *et al.*, 2000; Kuhle *et al.*, 2006; Mota *et al.*, 2009; Capmany & Damiani, 2010). For *Chlamydia* a subset of TGN-derived exocytic vesicles rich in sphingomyelin and cholesterol are redirected to the chlamydial inclusion and facilitate replication (Hackstadt *et al.*, 1996; van Ooij *et al.*, 2000; Capmany & Damiani, 2010). Similarly *Salmonella* redirects a subset of post-TGN traffic derived from SCAMP3-containing vesicles to the SCV and tubular membranes (Mota *et al.*, 2009), though experiments using a VSVG secretion assay did not provide evidence of fusion between TGN-derived exocytic vesicles and SCVs (Kuhle *et al.*, 2006). It is plausible that these interactions could help to satisfy membrane or nutrient requirements of replicating bacteria. Similar to post-TGN traffic these data provide evidence that there is selected interference with a subset of retrograde traffic by Salmonella. However, in contrast to SCAMP3-associated traffic I obtained no evidence to suggest that Syn10-dependent traffic is redirected to SCVs or Sifs membranes.

6.4 Counteracting lysosome function at different stages after invasion

It has been reported that at early stages following uptake of *Salmonella* by host-cells, SCVs selectively avoid fusion with lysosomes (Garcia-del Portillo & Finlay, 1995; Bakowski *et al.*, 2010; Thompson *et al.*, 2011). Bacteria pretreated with an inhibitor of protein

synthesis prior to internalisation were unable to prevent being trafficked to lysosomes within the first 2 hours following internalisation, showing that newly synthesised proteins are required for this process (Garvis *et al.*, 2001). Recently it was reported that the SPI-1 T3SS effector, SopB, alters the membrane surface charge of the SCV by reducing the levels of phosphatidylinositol-4,5-bisphosphate and phosphatidylserine on the SCV membrane (Hernandez *et al.*, 2004; Bakowski *et al.*, 2010). This alters the normal recruitment of host-cell proteins involved in endocytic trafficking, such as Rab35, and helps bacteria deviate the maturation of the SCV from trafficking to lysosomes. One of these studies (Bakowski *et al.*, 2010) suggested that SptP also has a role in this function. Early stage avoidance of lysosomes by the SPI-1 T3SS might protect the SCV until the SPI-2 T3SS is assembled and its effectors are translocated into the host-cell, which occurs approximately 2-4 h after bacterial uptake (Yu *et al.*, 2010).

The main finding to come from my work is that *Salmonella* possesses a second mechanism by which the antibacterial activities of lysosomes can be reduced through the activity of the SPI-2 T3SS effector SifA, which somehow interferes with Syn10-dependent retrograde trafficking of MPRs to the TGN, thereby preventing normal delivery of hydrolytic enzymes to endosomes.

6.5 Altered lysosome activity affects the intracellular growth of *Salmonella*

The reduction in lysosome activity in cells infected with *Salmonella* at later stages of infection was also observed when cells were depleted of Syn10 (Fig. 5.3 A). Interestingly, Syn6 depletion had the opposite effect and enhanced lysosome function (Fig. 5.3 B). This was correlated with reduced or increased TGN localisation of CD-MPR in Syn10 or Syn6-depleted cells respectively (Fig. 4.2;Fig. 4.3). Exploiting the differences in MPR localisation and associated lysosomal activity observed as a result of Syn10 or Syn6 depletion I was able to address whether lysosomal function has any bearing on *Salmonella* intracel-

lular growth. I observed increased growth in Syn10-depleted cells and reduced growth in Syn6-depleted cells (Fig. 5.4 A, B). *Salmonella*'s sensitivity to lysosomal activity was also observed when lysosomal enzymes were exposed to a lysosome-specific protease inhibitor (Fig. 5.4 B). Thus, enhanced growth observed during Syn10 depletion or pharmacological treatment is likely to be due to reduced lysosome function and indicates that *Salmonella* is sensitive to lysosomal enzymes. In turn, these results are consistent with the notion that *Salmonella* does not have a robust mechanism for avoidance of SCV-lysosome fusion, but rather depletes MPR-dependent hydrolytic enzymes from lysosomes. This conclusion provides an explanation to resolve the long-standing paradox that SCVs are relatively devoid of hydrolytic lysosomal enzymes (Hang *et al.*, 2006) yet retain lysosomal membrane glycoproteins in the vacuolar membrane (Garcia-del Portillo *et al.*, 1993) and interact dynamically with the endo-lysosomal system (Drecktrah *et al.*, 2007). The actions of SPI-2 T3SS effectors that regulate SCV membrane dynamics (Knodler & Steele-Mortimer, 2005; Drecktrah *et al.*, 2008; Dumont *et al.*, 2010; Schroeder *et al.*, 2010) could promote interactions between SCVs and enzyme-depleted lysosomes to provide a source of vacuolar membrane and possibly nutrients to enable bacterial growth. If such a process occurs, it seems unlikely that it would involve the activity of Vti1b or Vamp8, which were not recruited to the SCV or Sif membrane (Fig. 3.3 D, E).

6.6 MPR recycling to the TGN

MPR trafficking in the cell is one of the most extensively studied vesicular trafficking pathways (Hille-Rehfeld, 1995; Ghosh *et al.*, 2003; Pfeffer, 2009). One critical aspect of its activity is how, after it delivers its cargo of lysosomal enzymes to endosomes, it is recycled back to the TGN. Some reports have suggested that MPRs are not recycled in a retrograde fashion from LE compartments (Meyer *et al.*, 2000; Arighi *et al.*, 2004). Depletion of AP1 or retromer component proteins (Meyer *et al.*, 2000; Arighi *et al.*, 2004) prevented MPRs recycling to the TGN and resulted in their retention in EEs. These results led to the

proposal that MPRs are trafficked directly from EEs to the TGN. However, MPRs bind their ligands in a pH-dependent manner. At a pH <6.0 the ligands dissociate from the MPRs (Tong & Kornfeld, 1989; Olson *et al.*, 2008). The pH of EE is between pH 6.2-6.3, while the pH of LEs is between pH 5.2-5.8 (Mukherjee *et al.*, 1997; Mellman & Warren, 2000). Therefore, MPRs would need to traffic to LE to be exposed to an environment with a pH less that 6.0 to enable the efficient dissociation of their enzyme cargo. Furthermore, components of LEs that are involved in retrograde traffic to the TGN, such as Rab9, have been shown to be essential for MPR retrieval (Riederer *et al.*, 1994a; Seaman *et al.*, 2009). Similar to what was observed on interference of retromer function (Arighi *et al.*, 2004; Seaman, 2004), when dominant negative Rab7 was expressed in cells, MPRs were retained in EEs (Rojas *et al.*, 2008), implicating a role for Rab7 in MPR traffic. Interestingly Rab7 was reported to function in the correct localisation of the retromer complex (Rojas *et al.*, 2008; Seaman *et al.*, 2009). Therefore MPRs probably pass through EEs on their way to LEs. This EE to LE step is probably dependent on retromer, Rab7 and perhaps AP1. Subsequently, upon arrival in acidic LEs ligands dissociate and the receptor is recycled to the TGN in a Rab9-dependent fashion.

The data presented in this thesis support the view that MPR is recycled to the TGN from LEs and that there are no effects on EE to TGN traffic. I show that Syn10-dependent retrograde traffic to the TGN is impaired during infection by way of MPR and Vti1b depletion in the TGN (Fig. 3.7) and impaired CI-MPR traffic from the plasma membrane to the TGN (Fig. 4.4). In addition, results indicate that Syn6-dependent traffic is not influenced by *Salmonella* infection as TGN46 levels in the TGN remained unchanged and there was no detectable impairment in the retrograde traffic of CTxB (Fig. 3.7; Fig. 4.4).

6.7 Rab9 and Rab9 effectors function in MPR traffic and LE distribution

MPR trafficking from LEs to the TGN has been shown to be dependent on Rab9 and it effectors Tip47, P40, RhoBTB3 GTPase and GCC185 (Pfeffer, 2009). Though the evidence is still somewhat contradictory (Bulankina *et al.*, 2009) it is thought that a Rab9:Tip47 complex functions in the initial stages of MPR recycling from LE to the TGN (Díaz & Pfeffer, 1998; Carroll *et al.*, 2001; Aivazian *et al.*, 2006). Tip47 is primarily a cytosolic protein that is recruited by membrane-associated Rab9 on LEs (Díaz & Pfeffer, 1998; Carroll *et al.*, 2001; Ganley *et al.*, 2004; Aivazian *et al.*, 2006). Here the Rab9:Tip47 complex recognises the cytoplasmic tails of the MPRs by complex-enhanced direct binding of Tip47 (Carroll *et al.*, 2001; Hanna *et al.*, 2002; Burguete *et al.*, 2004). MPRs are then packaged into nascent vesicles for transport to the TGN (Barbero *et al.*, 2002). At the TGN interface, incoming MPR vesicles are guided towards fusion by their interaction with the vesicle-associated Rab9 and TGN-associated GCC185, a coiled-coil tethering protein localised to the TGN by Rab6 and Arl1 (Reddy *et al.*, 2006; Burguete *et al.*, 2008; Hayes *et al.*, 2009). Upon arrival at the TGN, RhoBTB3 GTPase interacts with Rab9 to mediate the uncoating of transport vesicles at which point Syn10-Syn16-Vti1a and Vamp3 enable membrane fusion (Espinosa *et al.*, 2009) (Fig. 6.1). In addition to having a role in MPR recycling, Rab9 has been shown to function in the maintenance of the peripheral distribution of late endosomal compartments (Ganley *et al.*, 2004). This function has been ascribed to the interaction between Rab9 and its putative effector SKIP (Jackson *et al.*, 2008a). SKIP interaction with Rab9 has been localised to the PH domain of SKIP (Jackson *et al.*, 2008a), which presumably allows for SKIP's recruitment to the membrane of LEs. This enables SKIP's RUN domain to cross-link the membrane-associated complex with the TPR domain of kinesin-1 to maintain peripheral distribution of LEs by movement on microtubules (Dumont *et al.*, 2010). In cells depleted of either Rab9 or SKIP there is an abrogation of this function and LEs concentrate at the MTOC (Ganley *et al.*, 2004;

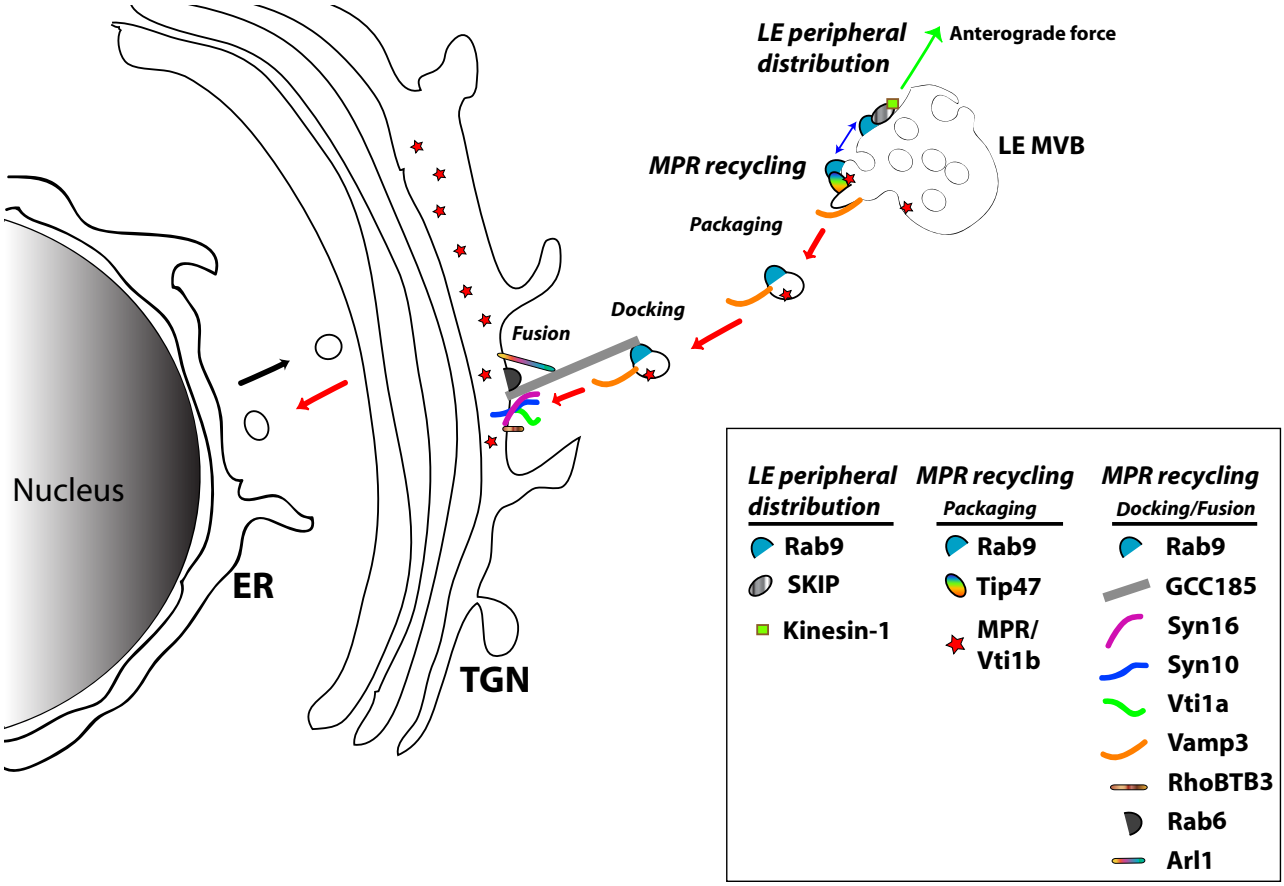


Figure 6.1 : ***Rab9-mediated retrograde transport from LE/MVBs to the TGN.*** *Schematic showing proteins known to function in Rab9-mediated transport from LE/MVBs to the TGN. Retrograde trafficking routes are denoted with the red arrows and anterograde trafficking routes are denoted with black arrows. Equilibrium of Rab9 binding to its effectors, Tip47 and SKIP, is indicated by the double headed blue arrow. Kinesin-mediated microtubule-associated anterograde force is indicated with the green arrow.*

Jackson *et al.*, 2008a; Dumont *et al.*, 2010).

Two lines of evidence implicate SKIP as having a second function in the lysosomal biogenesis pathway. Firstly, the N-terminal region of SifA interacts with the PH domain of SKIP and this is dependent on L130 of SifA (Diacovich *et al.*, 2009). I showed that the effect of bacterially-translocated SifA on lysosome function was dependent on L130 (Fig. 5.7), supporting the idea that SKIP is involved. However, it is also possible that SifA interacts with other protein(s) in an L130-dependent manner. Secondly, and more directly, in cells depleted of SKIP, there was an increase in the TGN localisation of MPRs and an overall increase in CatB activity (Fig. 5.9). These data support the notion that SKIP

acts as a negative regulator of lysosomal biogenesis and that SifA augments this activity. Interestingly, PLEKHM1, a SKIP-related protein, has recently been reported to have a negative regulatory role on Rab7-driven processes to lysosomes (Tabata *et al.*, 2010). This function requires its direct interaction with Rab7 (Tabata *et al.*, 2010). Given that SKIP binds GTP-bound Rab9 (and is likely to be a Rab9 effector) (Jackson *et al.*, 2008a) and Rab9's role in MPR trafficking and lysosomal biogenesis, I hypothesise that SKIP functions as a modulator of Rab9-driven processes in MPR traffic in an analogous manner to which PLEKHM1 modulates Rab7.

The dramatic phenotype of collapse of LEs in infected cells also suggests that Rab9-driven processes in the cell are antagonised (Stein *et al.*, 1996). SifA binds SKIP with an even greater affinity than Rab9 (Jackson *et al.*, 2008a). These authors suggest that SifA displaces Rab9 from SKIP and therefore antagonises Rab9:SKIP function, giving rise to LE clustering at the MTOC. This higher affinity interaction might enhance the negative regulatory activity of SKIP, thereby inhibiting MPR traffic and lysosome function.

6.8 Putative tri-molecular interaction of SifA, SKIP and Rab9

As an alternative to the model of SifA competing with Rab9 for binding to SKIP (Jackson *et al.*, 2008a), I would like to propose a second model which agrees with my data and data that have been published previously (Riederer *et al.*, 1994a; Aivazian *et al.*, 2006; Seaman *et al.*, 2009; Dumont *et al.*, 2010). As mentioned there are two functions described for Rab9 and Rab9-binding proteins with respect to LE trafficking. The first is Rab9:Tip47-mediated retrograde recycling of the MPRs and maintainance of lysosomal homeostasis (Díaz & Pfeffer, 1998). Secondly, Rab9:SKIP interactions maintain the peripheral distribution of LEs (Ganley *et al.*, 2004; Jackson *et al.*, 2008a). Results of my experiments suggest that Rab9:SKIP can also influence MPR recycling and lysosomal homeostasis (Fig. 5.9). One explanation that addresses the interrelatedness of these phenomena would be that mem-

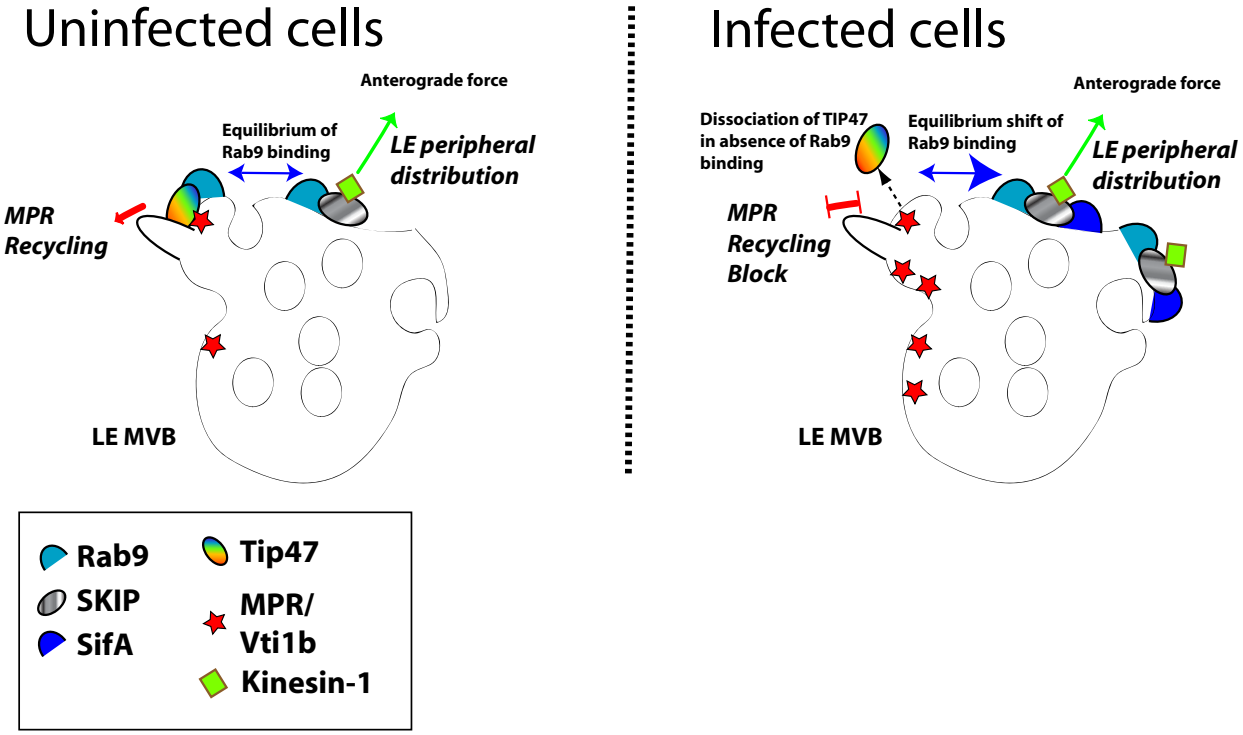


Figure 6.2 : ***Rab9 equilibrium model on LE/MVB membranes in the presence or absence of SifA.*** *Schematic showing Rab9 on LE/MVBs interacting with SKIP or Tip47 in the absence of SifA and the putative Rab9, SKIP and SifA tri-molecular complex in the presence of SifA. Equilibrium of Rab9 binding to its effectors, Tip47 and SKIP, is indicated by the double headed blue arrow which is pushed towards Rab9, SKIP interaction in the presence of SifA. Retrograde trafficking routes are denoted by a red arrow or a blocked red line which indicates interference. Dissociation of Tip47 from the membrane in the absence of Rab9 binding is denoted by a dashed black arrow. Kinesin-mediated microtubule-associated anterograde force is indicated with the green arrow.*

brane associated Rab9 available for binding is limited. In this model (Fig. 6.2) GTP-bound Rab9 on the LE membranes is in a state of equilibrium between binding its effectors, Tip47 and SKIP. If the availability of either of these partners increases then binding to the other decreases. Thus, the ratio of Rab9:Tip47 and Rab9:SKIP binding regulates the steady state level of MPR recycling and LE distribution in cells.

Under normal steady state conditions, Rab9 effector interactions would allow Tip47:Rab9 complex formation, leading to sufficient rates of MPR recycling to the TGN. It has been shown that in the absence of Rab9 both MPR recycling to the TGN and LE positioning are perturbed: MPR concentration at the TGN is reduced (Seaman *et al.*, 2009) and Lamp1-positive vesicles cluster at the MTOC (Ganley *et al.*, 2004; Jackson *et al.*, 2008a). In the

absence of SKIP there would be an increase in the amount of Rab9 available to recruit and bind Tip47. This could give rise to an increased rate of MPR recycling to the TGN (Fig. 5.9 B), increased lysosomal activity (Fig. 5.9 C) and, as a result of the inability to engage kinesin, the clustering of LEs and lysosomes at the MTOC (Dumont *et al.*, 2010). Finally, it has been shown that Tip47 function is required for MPR recycling to the TGN (Díaz & Pfeffer, 1998).

In the context of infection it is possible that membrane-associated SifA acts as a "sink" for SKIP due to its higher affinity for SKIP and permanent membrane association (Reinicke *et al.*, 2005). If the membrane-recruited SKIP were free to interact with GTP-bound Rab9 this might reduce the proportion of Rab9 available to interact with Tip47. The Tip47:Rab9 complex co-stabilises the membrane association of each component (Díaz & Pfeffer, 1998; Ganley *et al.*, 2004; Aivazian *et al.*, 2006). As a result of reduced Rab9 interaction Tip47 would be released from the membrane thereby impairing MPR recycling to the TGN. However, for this hypothesis to be valid, during infection SifA would need to be able to bind to SKIP without preventing the binding of Rab9 to SKIP, which is not compatible with published results (Jackson *et al.*, 2008a). Both SifA and Rab9 have been reported to bind to SKIP through its PH domain (Jackson *et al.*, 2008a) and this binding was found to be competitive, though this was only shown in vitro using a truncated recombinant form of SKIP composed solely of the PH domain. The structure of SifA in complex with the PH domain of SKIP has been solved (Diacovich *et al.*, 2009) revealing that the binding interface between these two molecules is formed by the interaction of two β-sheets from both proteins. Rab9 binding to human Tip47 has been shown to be dependent on the residues ^{161}GVD**KTKSVV**TGG^{172} of Tip47 (Hanna *et al.*, 2002). The greatest influence on binding was associated with the residues **KTKSVV** (Hanna *et al.*, 2002). Interestingly, the N-terminal region of the SKIP PH domain also contains a similar sequence: ^{791}HW**KTCFVV**L^{799}. This sequence maps to a surface exposed β-sheet on an opposing face of the PH domain to the SifA:SKIP interaction site (Fig. 6.3). If this motif binds Rab9 then SKIP might be able to simultaneously bind SifA and Rab9. If it were

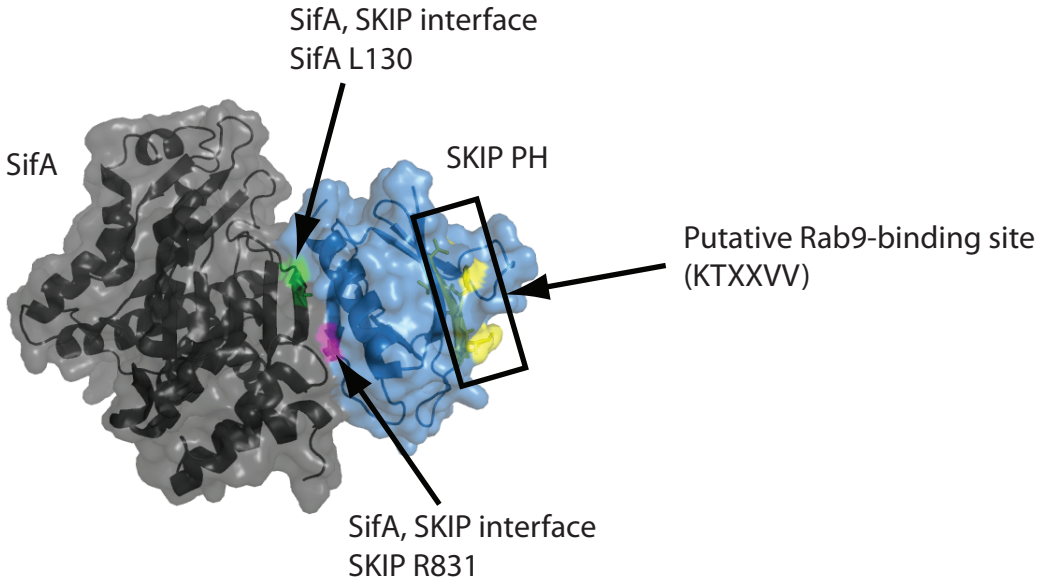


Figure 6.3 : *SifA:SKIP PH-domain crystal structure.* *SifA:SKIP PH-domain co-crystal cartoon and surface topology model showing highlighted residues critical for binding of SifA to SKIP and highlighted putative Rab9 binding site: SifA (L130, green), SKIP (R831, pink) and the putative Rab9 binding site in SKIP (KTXXVV, yellow).*

found to be the case, then it is conceivable that the increased proportion of membrane-associated SKIP localised to the membrane could titrate the available Rab9 free to interact with Tip47, thereby inhibiting its function in MPR traffic (Fig. 6.2).

Salmonella is not unique in its ability to interfere with Rab9 function. Rab9 and Rab9 effectors are important during the infection cycles of Marburg virus, Ebola virus, HIV and measles for viral particle production (Pfeffer, 2009). Notably both HIV and Vaccinia virus require Rab9 and Tip47 during their biogenesis (Murray *et al.*, 2005; Chen *et al.*, 2009; Bauby *et al.*, 2010). In addition the Pseudomonas exotoxin uses a Rab9-dependent pathway to traffic through the cells, in contrast to cholera and Shiga toxins (Smith *et al.*, 2006; Deng *et al.*, 2007), providing further evidence of a segregated retrograde trafficking route dependent on Rab9.

6.9 Future areas of investigation

The observation that Salmonella attenuates lysosomal function has opened many avenues of research to explore this phenomenon from both a mechanistic and physiological point of

view. Here I describe in broad terms some of the areas that could be pursued in further investigations.

6.9.1 Do SifA, SKIP and Rab9 form a tri-molecular complex?

To address this question immunoprecipitation experiments could be carried out with SKIP, Rab9 and SifA. As proposed above, if SifA enhances SKIP recruitment to the membrane to increase its activity then in the presence of SifA there should be increased SKIP and SKIP-bound Rab9 associated with membrane fractions. By carrying out membrane fractionation and co-immunoprecipitation experiments in presence or absence of SifA it would be possible to assess SifA, SKIP and Rab9 do form a trimolecular complex and would provide an explanation as to how SKIP could have enhanced negative regulation of lysosome function during infection.

If evidence of a tri-molecular complex is attained, then the mechanism of interaction could be investigated by mutation of the putative Rab9 binding motif in SKIP **(KTXXVV)** to determine if SKIP binding to Rab9 is abrogated. In addition R831 of SKIP, which has been reported to be essential for SifA binding, could be mutated to evaluate if it still interacts either Rab9 and SifA. The protein-protein interactions could then be further characterised through a range of biochemical techniques including chromatography, surface plasmon resonance and structural x-ray crystallography.

6.9.2 What is SKIP's function in the cell in presence and absence of infection?

To continue to investigate the function of SKIP , SKIP knock-out mouse embryonic fibroblasts cells could be used. SKIP knock-out mice have already been generated by the Sanger Research Institute in Cambridge as part of a genome-wide mutation study in mice and are available as a resource for the research community. Cells with this "clean" genetic background could be transfected to express wild-type SKIP and mutant versions to investigate

their effects on lysosome function in the presence or absence of Salmonella.

SKIP has other reported binding partners including both Arl8, an Arf-like GTPase involved in endocytic trafficking, and the kinesin-1 motor protein (Dumont *et al.*, 2010; Kaniuk *et al.*, 2011). Both of these interactions have been localised to the RUN domain of SKIP (Dumont *et al.*, 2010; Kaniuk *et al.*, 2011). Interestingly, depletion of Arl8 from *C. elegans* has been reported to induce a pleolysosomal phenotype (Nakae *et al.*, 2010). These interactions and phenotypes in our systems could be examined to assess if they contribute to the phenotypes I observed during infection.

6.9.3 What is the fate of MPRs and Vti1b?

An unanswered question raised by my results concerns the fate of both MPRs and Vti1b in infected cells. In cells that are depleted of Syn10, MPRs are primarily re-localised to compartments that are retromer (SNX1)-positive (Ganley *et al.*, 2008). When I examined SNX1 distribution in cells infected for 14 h with Salmonella I found that this protein, like MPRs, was not recruited to the membranes of the SCV and Sifs. It would be interesting to examine more closely by confocal microscopy the distribution of SNX1, MPRs, Vti1b and other markers of EEs, LEs and REs in infected cells. This might help to define at which point *Salmonella* blocks transport of MPRs and Vti1b.

6.9.4 What is the role of Rab9 in lysosome biogenesis?

Though interference in Rab9 function has been shown to impair MPR recycling and induce increased secretion of lysosomal enzymes (Riederer *et al.*, 1994b; Seaman *et al.*, 2009), it has not been established if its loss could impair lysosome activity. The MRcatB assays could be used to assess if in cells depleted of Rab9 there is impaired CatB activity. If found to be the case it would be interesting to assess if the phenotypes of disrupted MPR traffic to the TGN and reduced lysosome function observed during infection could be reversed by ectopically expressing Rab9 in infected cells. If SifA titrates available Rab9, preventing it from functioning in MPR recycling, then the over-expression of Rab9 could rescue the

phenotype and prevent the effects on lysosome function.

GCC185 tethering is reported to be dependent on the clathrin adaptor AP1 and Rab9 (Brown *et al.*, 2011). GCC185 depletion gives rise to MPR localised to AP-1-positive vesicles (Brown *et al.*, 2011). GCC185 also binds Rab9 to guide fusion of incoming traffic at the TGN (Hayes *et al.*, 2009). My results show that SifA inhibits MPR trafficking by specific interference with the Syn10-dependent endosome to TGN pathway. This is accompanied by re-routing of lysosomal enzymes and impairment of lysosome function. It is unlikely that SifA mediates its effect directly through Syn10, as SPI-2 T3SS-dependent inhibition of lysosome function was also observed in mouse macrophages that lack Syn10 (Ganley *et al.*, 2008) and Syn10 distribution was not altered at the TGN in infected HeLa cells (Fig. 3.7; Fig. 5.2). Syn10 is a component of a SNARE complex that also interacts with GCC185 (Ganley *et al.*, 2008). It is possible that SifA inhibits Rab9 function by preventing or limiting its interaction with GCC185. If so MPRs could re-localise to AP1-positive compartments. It is also possible that Rab9 function is being interfered with at an earlier stage where the Rab9:Tip47 complex packages MPRs into nascent recycling vesicles. Defining the endocytic compartment that MPRs re-localise to during infection will help to address where the block in MPR traffic is occurring.

6.9.5 Is lysosomal function compromised in infected mice?

An important question remaining is whether lysosomal function is attentuated in the context of an in vivo infection. Isolation of macrophages containing bacteria from spleens of infected mice has been undertaken in our laboratory previously (Helaine *et al.*, 2010). This procedure could be used in conjunction with the MRcatB assays to address whether lysosome activity is reduced in these cells. If found to be the case further experiments to explore the phenotype in vivo could involve doing infections with a combination of mutant and wild-type bacteria in conjunction with wild-type or SKIP knock-out mice. These experiments would involve examining lysosomal function in uninfected and infected cells and would help to establish whether the phenotypes observed during infection of cells in vitro

are also present in cells from infected tissues . They could also address whether lysosome function affects bacterial growth in vivo as was observed in vitro (Fig. 5.4).

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