CHAPTER I

Introduction

Chapter 1

1.1 Endo-lysosomal system: bringing in and getting there

The endolysosomal network comprises of a series of membrane-enclosed tubulo-vesicular compartments that undergo dynamic interconversions into functionally and structurally distinct compartments including early endosomes, late endosomes, recycling endosomes and lysosomes (Huotari and Helenius, 2011). Arrays of internalised cargo such as transmembrane proteins, receptor-ligands, extracellular proteins enter the endo-lysosomal system and undergo transportation, sorting and/or degradation. Notably, autophagosomes that capture non-functional or damaged intracellular substrates also deliver their contents to lysosomes for their elimination (Lamb et al., 2013). Cargo-laden endosomes and/or autophagosomes ultimately fuse with pre-existing lysosomes that serve to generate an acidic hydrolase rich milieu suitable for proteolysis, breakdown and recycling of complex macromolecules into simpler compounds that can eventually serve as biomolecular building blocks. Macromolecules or foreign particles such as pathogenic microbes ordained for degradation can enter the endolysosomal system via three major routes: endocytosis, autophagy and phagocytosis.

Vesicular trafficking along the endolysosomal pathway, involves the maturation of endosomes which is characterized by four major events: 1) an increase in the number of intraluminal vesicles (ILVs); 2) decrease in luminal pH; 3) spatial shift in the position of these endocytic compartments from the cell periphery to a juxtanuclear location and 4) exchange of Rab proteins (Huotari and Helenius, 2011).

Classical endocytosis begins with the engulfment of extracellular substrates/ materials at the plasma membrane into clathrin-coated invaginations that eventually deepen and bud off from the membrane forming carrier vesicles. These newly formed vesicles instantaneously merge into the pre-existing network of early endosomes which are the first endocytic stations located

in close proximity to the cytosolic surface of the membrane bilayer. The fusion events occurring during the early stages of the endocytic pathway are regulated by a discrete set of proteins of which small GTPase Rab5 is the dominant player (Bucci et al., 1992). Early endosomal lumen offers a slightly acidic environment that allows for the dissociation of the receptor-ligand complexes. These sorting endosomes are highly dynamic and pleiomorphic organelles with a central vacuole and tubular extensions providing a platform that serves as a distribution centre for various types of cargo and recycling of the non-ligand bound receptors and essential membrane components that need to be re-utilised at the cell surface. For this purpose, they undergo extensive fusion and fission and are sometimes referred to as sorting endosomes. The recycling endosomes which transport these membrane receptors and membrane-bound lipids to the plasma membrane are subjected to regulation by small GTPases Rab4 and Rab11 (Hsu and Prekeris, 2010). On the other hand, substrates that need to be degraded move to late endosomes and ultimately reach the lysosomes.

Early endosomes undergo morphological transformation into late endosomes which is accompanied by membrane lipid remodelling, decrease in intraluminal pH, an increase in the number of intraluminal vesicles due to vesicle budding events and "Rab conversion" i.e. transition from Rab5-association to Rab7-association (Poteryaev et al., 2010). Late endosomes act as a second sorting hub of the endolysosomal system. They regulate trafficking of cargo packaged in intraluminal vesicles towards lysosomes for degradation, but prior to that, the cargo-sorting receptors initially bound to luminal hydrolases are sorted into tube-like structures which are recycled to the Golgi network (Cullen, 2008). Late endosomes also act as a sink for ubiquitinated membrane proteins that are eventually degraded into the lysosomes (Raiborg and Stenmark, 2009) (Figure 1.1).

Lysosomes are a reservoir for active hydrolytic enzymes, and their luminal pH is around 4.0 that enable them to carry out numerous proteolytic activities. There are more than 50 different

hydrolases congregated in the lysosomes, the glycosylated precursors or proenzymes for which are delivered upon late endosome-lysosome fusion. The degradative process also requires intra-luminal calcium and calmodulin and prerequisite modification of the lysosomal enzymes with mannose-6-phosphate moieties, the receptors for which are concentrated in the endo-lysosomal system (Luzio et al., 2010).

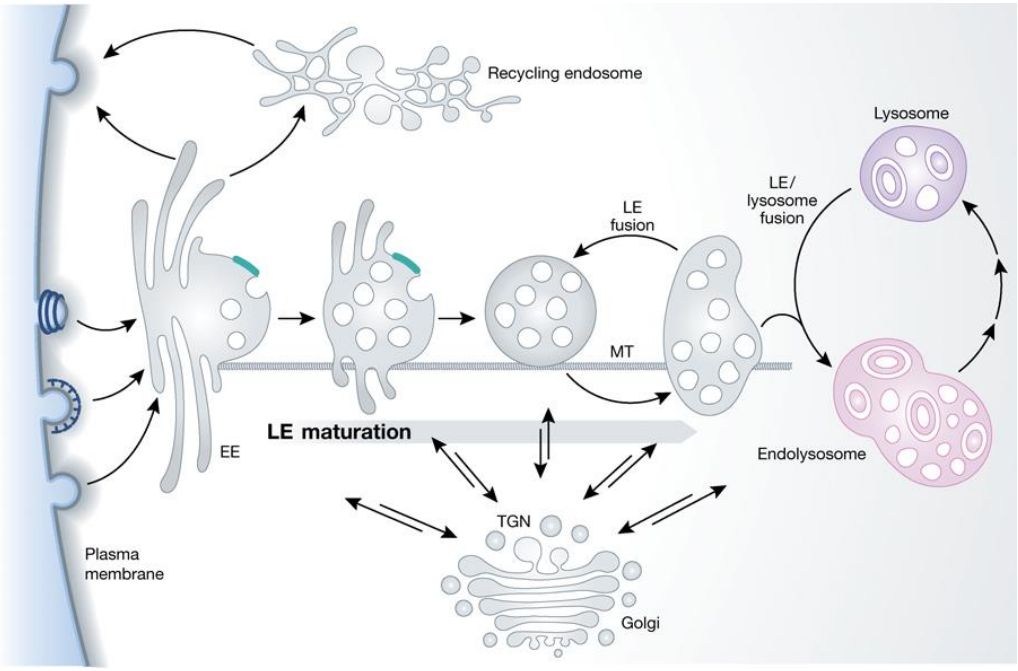


Figure 1.1: Stages of Endosomal maturation. The carrier endocytic vesicles deliver the internalized cargo and membrane to EEs situated near the plasma membrane. This is followed by a brief period during which the endocytosed cargo is sorted into distinct domains of the EEs for recycling to the plasma membrane. While maturation, the endosomes move along the microtubules (MT) towards the perinuclear region and transform into nascent LEs inheriting the vacuolar domains of the EEs. A subset of endocytosed cargo destined for degradation is combined with freshly synthesized lysosomal hydrolases and membrane components from the secretory pathway. The LEs further undergo homotypic fusion, increase in size acquiring more ILVs. Next, the mature late endosomes merge into the lysosomes forming transient hybrid compartments known as endolysosomes where active degradation occurs. These hybrid organelles further differentiate into classical dense lysosomes, acting as storage organelles for a variety of hydrolytic enzymes and membrane components. *Reproduced with permission (Huotari and Helenius, 2011) Copyright © 2011, John Wiley and Sons.*

1.2 Molecular machinery regulating vesicular trafficking along the endolysosomal pathway

Endosomal maturation, fission and fusion processes require a group of conserved proteins which primarily includes Rab GTPases, tethering factors and SNAREs (Figure 1.2). Sorting and fusion involve vectorial transport of vesicles along the cytoskeletal tracts towards the

acceptor organelle membranes, where activated Rab proteins temporally recruit the tethering machinery forming the initial contact or "fusion vertices" (Bonifacino and Glick, 2004; Cullen, 2008; Yu and Hughson, 2010). This interaction is followed by the assembly of membrane-embedded cognate SNARE proteins, present on the apposed membranes leading to the formation of SNARE complexes spanning the membrane bilayers and ultimately effectuating the union of bilayers along with complete luminal amalgamation (Jahn and Scheller, 2006). Each compartment has its own specific set of small GTPases, tethering factors and SNAREs to ensure cargo delivery to the right address essential for the maintenance of cellular homeostasis. Apart from that, these subcellular organelles also have distinct membrane morphologies owing to the presence of specific phosphoinositide lipid-rich domains in their membrane bilayers distinguishing each of these vesicular compartments from the preceding one during the sequential endosome maturation pathway. The detailed molecular mechanism and stages of the endolysosomal trafficking have been described in the subsequent sections.

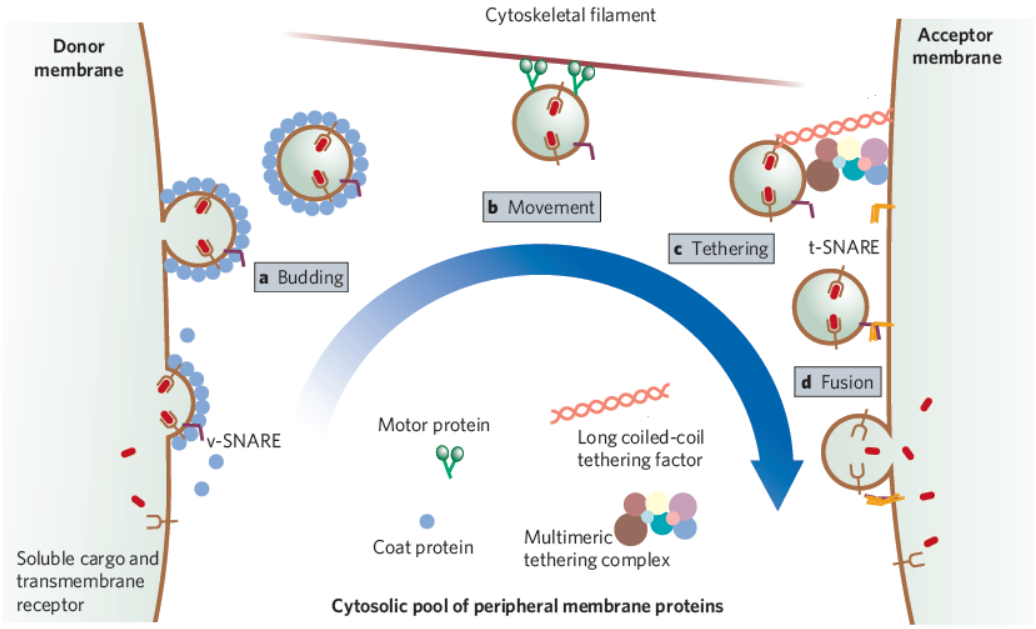


Figure 1.2: Schematic representing major steps of vesicular trafficking and fusion. a. Vesicle formation is aided by coat proteins at the cytoplasmic leaflet of the donor membrane. **b.** Once the vesicle buds off from the membrane the coat proteins are removed and motor proteins are recruited enabling the vesicle transportation along the cytoskeletal tracts. **c.** As the cargo-enriched vesicle reaches close to the target organelle, tethering and docking of the vesicles onto acceptor membranes is initiated by the tethering factors. **d.** Specific SNARE proteins localizing to the vesicular and acceptor membranes are mobilised driving membrane fusion and cargo delivery. *Adapted from: (Behnia and Munro, 2005) Copyright © 2005, Springer Nature.*

1.2.1 Small GTPases play a seminal role in regulating the compartmentalisation of the endomembrane system

The membrane fusion events begin with the recruitment of signalling molecules with a guanine nucleotide switch. Several members of the Ras superfamily are known to steer key functions in the intracellular membrane trafficking. Trafficking and sorting of the materials along the endolysosomal pathway is primarily regulated by a set of guanine nucleotide-bound proteins belonging to Rab, Arf and Arf-like GTPase sub-families that associate with distinct organellar membranes (Figure1.3). The membrane specificity of these GTPases largely bring about the variation in organelles between species and cell types and therefore they are often regarded as the molecular addresses of their cognate endocytic compartments in the cell biology research. (Agola et al., 2011; Barr, 2013; Burguete et al., 2008; de Curtis and Meldolesi, 2012; Hall, 2012; Itzen and Goody, 2011; Mizuno-Yamasaki et al., 2012; Pfeffer, 2013). Rab proteins are the largest subfamily of Ras GTPases encompassing 66 members in humans and are primarily involved in the regulation of sequential steps during endosome maturation process (Mizuno-Yamasaki et al., 2012).

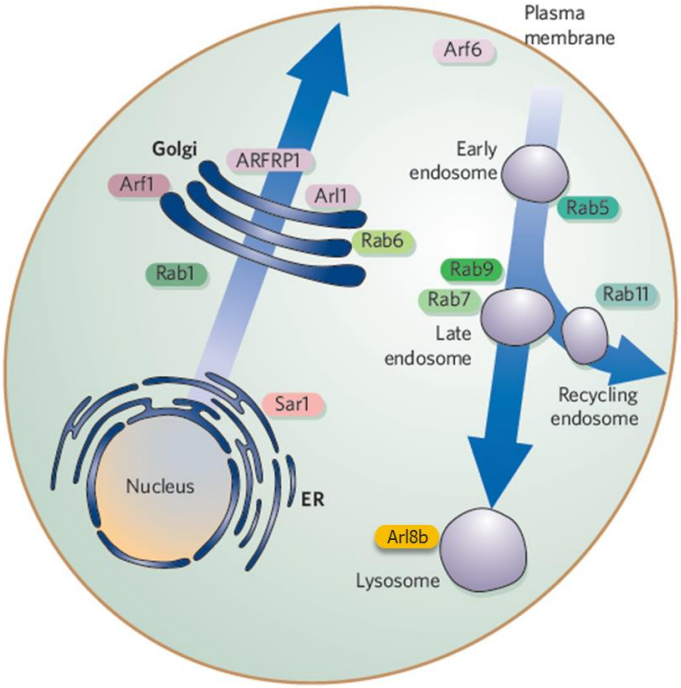


Figure 1.3: GTPases localising to specific organelles of the endolysosomal system. Examples of GTPases that are localised to specific organellar membranes within eukaryotic cells. Arf family GTPases are shown in purples, and Rabs in greens and Arl8b in orange. Some are found in multiple locations for instance, Arf1 is localized throughout the Golgi stack, but most are restricted to only one organelle. The examples shown are the best characterized and ubiquitous cases, but there are many more GTPases that are less well characterized or are found in specialized or polarized cells. *Modified from: (Behnia and Munro, 2005) Copyright © 2005, Springer Nature.*

Rabs are switch-like proteins that exist in association with guanine nucleotides. They usually have poor enzymatic activity (Barr, 2013; Itzen and Goody, 2011) and toggle between inactive GDP-bound and active GTP-bound forms. In the GDP coupled state, these proteins interact with cytosolic proteins known as **GDP dissociation inhibitor** (GDI) through their carboxy-terminal prenyl anchor domain that acts as a chaperone to keep the Rabs soluble in the cytoplasm. The GDIs assist specific **guanine-nucleotide exchange factors** (GEFs) to promote the substitution of the GDP with GTP that allows for the insertion of the prenyl anchor into the organellar and vesicular membranes. The membrane-embedded activated GTPases are recognised by their effectors such as tethering complexes to perform significant downstream cellular functions (Barr and Lambright, 2010; Barr, 2013; Jahn and Scheller, 2006; Lachmann et al., 2011). Rab signalling is turned off by another class of proteins known as GAPs (GTPase

accelerating proteins) which catalyse the hydrolysis of the γ-phosphate of GTP leaving the Rab bound to GDP (inactive) (Barr and Lambright, 2010).

Several Rab GTPases have been reported to perform essential tasks during endosomal trafficking. The ubiquitous GTPases Rab5, Rab4 and Rab11 participate in the early endocytic steps while Rab7 and Rab9 function and regulate the downstream events of this pathway.

Rab5 localises to the early endosomal membranes upon activation by the Rabex5 GEF protein (Cabrera and Ungermann, 2013; Horiuchi et al., 1997; Itzen and Goody, 2011). At this juncture, the activated Rab5 recruits multiple effectors including: the phosphatidylinositol (PI)-3-kinase Vps34, the large coiled-coil tethering factor EEA1 (early endosomal antigen 1), rabaptin-5, rabenosyn-5, and subunits of the CORVET tethering complex (Christoforidis et al., 1999a; Galvez et al., 2012; Rink et al., 2005). Upon recruitment to the early endosomal membranes, the PI-(3)-kinase Vps34 catalyses the phosphorylation of phosphatidylinositol to generate phosphatidylinositol 3-phosphate [PI(3)P] at these sites. Generation of this lipid promotes the recruitment of an effector protein, EEA1 that functions as the core component of vesicle docking and fusion machinery at early endosomes by promoting the homotypic fusion of the early endosomal vesicles with the assistance of specific SNARE proteins.

In addition to Rab5, several other Rab counterparts localise to distinct domains of sorting endosomes. For instance, Rab4 and Rab11 carry out pertinent roles in the recycling pathway and regulate the biogenesis of recycling endosomes with the help of specific effector proteins (Fouraux et al., 2004; Sönnichsen et al., 2000).

As the endosomes mature, they undergo changes in shape and membrane composition that promotes the formation of intraluminal vesicles (ILVs) and gives rise to MVBs. The biogenesis of MVBs involves the consequential replacement of Rab5 by Rab7 which is catalysed by Mon1-Ccz1, a Rab7-specific GEF protein (Gerondopoulos et al., 2012; Nordmann et al., 2010; Poteryaev et al., 2010; Rink et al., 2005; Vonderheit and Helenius, 2005; Zeigerer et al., 2012).

The involvement of a Rab5-GAP (Msb3) and Rab5 effector BLOC1 complex has also been reported to be crucial for this transition and formation of these lysomotrophic organelles (Del Conte-Zerial et al., 2008; Di Pietro et al., 2006; Setty et al., 2007).

Rab7 is a critical component regulating most of the actions at the late endolysosomal step and is also responsible for changes in membrane surface composition rendering them more competent for fusion with lysosomes. Upon recruitment to specific domains in the hybrid endosome, Rab7-GTPase binds to its effector proteins. These include proteins such as RILP (Rab-Interacting lysosomal Protein) and FYCO1 (FYVE And Coiled-Coil Domain Containing 1) that connect the late endocytic compartments to the motor proteins: dynein and kinesin respectively and initiate the microtubule-dependent transport of these organelles. Rab7 also interacts with the components of Retromer complex for regulation of vesicle trafficking towards TGN (Haas et al., 2005; kleine Balderhaar et al., 2010; Liu et al., 2012; Poteryaev et al., 2010; Rojas et al., 2008; Seaman et al., 2009); subunits of HOPS complex that coordinates late-endosomal-lysosomal fusion events (Bröcker et al., 2012; Seals et al., 2000); and Rabring7, a RING-type E3 ligase which is involved in EGFR sorting and degradation (Mizuno et al., 2003; Zhang et al., 2009). Apart from these, proteins such as Rubicon and PLEKHM1 are also recruited by active Rab7 that negatively regulate the endosome maturation process and also promote autophagosome-lysosome fusion (McEwan et al., 2015a; Sun et al., 2010; Tabata et al., 2010).

Rab9 is another essential GTPase that is found to be associated with the late endosomes. The chief function of this protein is to regulate the replenishment of the Golgi mannose-6-phosphate receptor pools by recycling the vacated receptors from late endosomes (Dong et al., 2013; Lombardi et al., 1993; McGourty et al., 2012). This process is primarily executed by the Rab9 effector TIP47 that interacts with the cytoplasmic domains of M6PRs and returning them to the Golgi network for their re-utilisation (Carroll et al., 2001). Besides this, Rab9 also binds

and recruits GCC185, a Golgi tethering factor which promotes the docking of the endosomal vesicles at the Golgi complex facilitating the M6PR-recycling process. GCC185 also catalyses microtubule nucleation at this site through its interaction with CLASP (Cytoplasmic linker–associated) proteins thereby contributing to the stabilisation of the Golgi stack structure (Hayes et al., 2009).

Apart from Rab proteins, GTPases belonging to the ADP-ribosylation factor (ARF) and ARF-like (ARL) families also contribute to endolysosomal trafficking. These proteins either function individually or co-operate with the Rab proteins to perform essential tasks (Agola et al., 2011; Burguete et al., 2008; de Curtis and Meldolesi, 2012; Hall, 2012; Mizuno-Yamasaki et al., 2012; Pfeffer, 2013). For instance, Arf6 localises to the plasma membrane and recycling endosomes. At the plasma membrane, Arf6 generates phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] by activating phosphatidylinositol-4-phosphate-5-kinase, causing alterations in membrane lipid composition. Besides this, it also modulates the cortical actin cytoskeleton to promote the formation of membrane protrusions driving cell migration and assists in clathrin-mediated endocytosis of ligand-activated G-protein-coupled receptors (GPCRs) at the cell membranes. Arf6 is also involved in rapid endosomal membrane recycling back to the plasma membrane through its interaction with a microtubule adaptor protein, JIP4. In conjunction with the Exocyst complex, Arf6 also drives slow recycling of materials from the sorting endosomes (D'Souza-Schorey and Chavrier, 2006; Grant and Donaldson, 2009; Montagnac et al., 2011; Poupart et al., 2007).

Another ARF family member, Arf1 which mainly localises at the trans-Golgi network regulates significant functions at this site. This protein recruits coat proteins such as coatomer complex 1 (COP1), Golgi-localized, γ-ear-containing, ADP-ribosylation factor-binding protein (GGA) and adaptor protein 1 (AP1) and mediates cargo packaging and sorting into COP1-coated vesicles transporting them to the correct destination. Arf1 has also been implicated in clathrin-

independent endocytosis of glycosyl-phosphatidylinositol (GPI)-anchored proteins in some cell types (Kumari and Mayor, 2008; Soni et al., 2009).

Notably, several studies have pointed towards the significant roles played by a GTP-bound protein Arl8b, a member of ARF-like GTPase family during endolysosomal trafficking. This lysosomal GTPase primarily mediates the fusion of multivesicular late endosomal compartments with lysosomes by recruiting its effector HOPS complex (Garg et al., 2011b; Khatter et al., 2015a) and also co-operates with Rab7 for transportation of these organelles along the microtubular tracts in a PLEKHM1-dependent manner during specialized conditions such as autophagy (Marwaha et al., 2017). Arl8b depletion prevents membrane association of the tethering and fusion machinery (HOPS complex) to the lysosomal membranes resulting in the impairment of cargo delivery towards these organelles. Additionally, another Arl8b effector, SKIP (or PLEKHM2), a member of RUN-domain containing protein family interacts with KLC2 (Kinesin Light chain 2) and recruits the motor protein Kinesin1 onto lysosomal membranes thereby, controlling the peripheral displacement of lysosomes along the microtubules (Rosa-Ferreira and Munro, 2011b).

1.2.2 Phosphoinositide (PI) Conversion: A change in the molecular address

Besides, the Rab Switch, the phosphoinositide conversion plays an indispensable role during endosome maturation and cargo trafficking. The early endosomal and late endosomal membranes differ in their lipid content, and their identity is maintained by two specific lipids: PtdIns(3)P and PtdIns(3,5)P_2 respectively. Phosphoinositides bind to proteins containing PI-interaction domains (for example, FYVE, PH, PX and GRAM domains). The generation of specific PI lipids in the organelle membranes is carried out by the action of specific kinases and phosphatases which ensures tight regulation of compartmentalisation during endocytic maturation (Vicinanza et al., 2008). For instance, PtdIns(3)P found on the cytoplasmic surface of early endosomes is mainly synthesised by a class III PI(3) kinase, Vps34 which is recruited

by the activated Rab5 (Schu et al., 1993). Inhibition of PI conversion activity of Vps34 at endosomes results in enlarged late endosomes with fewer ILVs and defects in EGFR trafficking (Futter et al., 2001). The PI conversion machinery acts in concert with the Rab-GTPases specific to the organelle, best exemplified by the interaction of Vps34 with Rab5 and Rab7 during endosomal maturation. Rab5 interacts and recruits Vps34 in the GTP-bound state. However, the binding of Vps34 to Rab7 has been observed with both active and inactive states of this GTPase; although the interaction is more prominent when Rab7 is inactive (GDP-bound) (Christoforidis et al., 1999b; Stein et al., 2003). Therefore, Rab7 activation provides a feedback control to inhibit Vps34-dependent PI(3)P synthesis on the freshly maturing late endosomes.

Later steps of the endosomal maturation involve the exchange of PI(3)P with PI(3,5)P_2 at the limiting membranes of late endosomes. This reaction is catalysed by an enzyme phosphatidylinositol and PI(3)P kinase (PIKFYVE) which was originally characterised in yeast (Fab1). PI (3,5)P_2 is critical for cargo sorting towards MVBs and also mediates the biogenesis of intraluminal vesicles (ILVs) by binding to the ESCRT machinery (Odorizzi et al., 1998; Whitley et al., 2003). Furthermore, PI(3,5)P_2 enables Ca^{2+} efflux at late endosomal membranes and functions in the homotypic and heterotypic fusion of these compartments with lysosomes (Luzio et al., 2007).

1.2.3 Cytoskeletal elements and positioning of the endolysosomal compartments

Endosomes are highly dynamic and motile organelles. Their movements are spatiotemporally regulated and intricately linked to the stage of maturation and functioning of these compartments. The location of endocytic compartments is often a critical parameter that impacts their numbers, dimensions and internal cargo content as they progress through the endosome maturation pathway (Collinet et al., 2010). Owing to the dynamic displacement of these sub-cellular organelles, numerous small-sized endosomal vesicles at the cell periphery are routinely substituted by fewer large-sized endosomes in the perinuclear region. Further, at

the time of biogenesis of early endosomes (EEs) near the plasma membrane, the endosomes undergo slow, short-range back and forth oscillations, but as the maturation progresses; they are subjected to faster and long-range saltatory movements with a net displacement towards the nucleus where the majority of lysosomes are concentrated in majority of cell types such as HeLa. The movement of endocytic compartments along the cytoskeletal tracts is enabled by the action of motor proteins that link the organelles to the microtubules. Endosomal motility is bidirectional and mainly depends on two motor proteins, kinesin and dynein that exert opposing forces and transport them in opposite directions (Murray et al., 2000; Soppina et al., 2009). Disruption of microtubules or defects in motor proteins is detrimental to cellular homeostasis. For instance, there is sufficient evidence that motor protein activity is crucial for the fission of vesicles from early endosomes and is fundamental to maintenance of the balance between cargo sorting into degradative and recycling vesicular compartments (Bananis et al., 2000; Driskell et al., 2007; Hoepfner et al., 2005). Besides this, the position and fusion of endosomes (both homotypic and heterotypic) during different stages of endosomal maturation also requires action of motors (Aniento et al., 1993; Bomsel et al., 1990; Driskell et al., 2007).

Kinesin superfamily comprises of several plus-end directed motor proteins, many of which have been implicated in endosomal motility. For example, KIF16B (retrograde movement of EE); Kinesin2 subunit KIF3A (LE trafficking); Kinesin1 subunit KLC2 (lysosomal distribution) (Brown et al., 2005; Hoepfner et al., 2005; Rosa-Ferreira and Munro, 2011b). On the other hand, the minus-end directed movement of late endosomal and lysosomal compartments depends on a single motor protein; dynein (Aniento et al., 1993; Bananis et al., 2004; Kardon and Vale, 2009). Attachment of organelles to dynein motors can be either direct via dynein intermediate, light intermediate and light chains or indirect through a multiprotein complex, Dynactin. However, the interaction with dynein also requires an additional class of proteins known as adaptors which confer specificity to and increase processivity of dynein-mediated transport of vesicular compartments.

The coordinated interplay of the two opposing microtubular motor proteins has been well characterised for late endosomes and is primarily controlled by the activity of small GTPase Rab7. Activated Rab7 (GTP-bound) on late endosomal membranes recruits its effectors: RILP and ORP1L (oxysterol-binding protein) which further interact with the dynactin subunit $p150^{glued}$ to promote attachment of these compartments to the dynein complex. This interaction mediates the anterograde transport of late endosomes towards the microtubule organising centre (MTOC) (Johansson et al., 2007). A counter function is performed by another Rab7 effector, FYCO1 that links the Rab7-positive late endosomes and autophagosomes to kinesin motor, mediating the plus-end directed movement (Pankiv et al., 2010). An analogous role at the lysosomes and certain LROs (Lysosome-related organelles) has been assigned to another GTPase, Arl8b that regulates the motility of these organelles in conjunction with its effectors PLEKHM1 and SKIP (as described previously) (Figure 1.4).

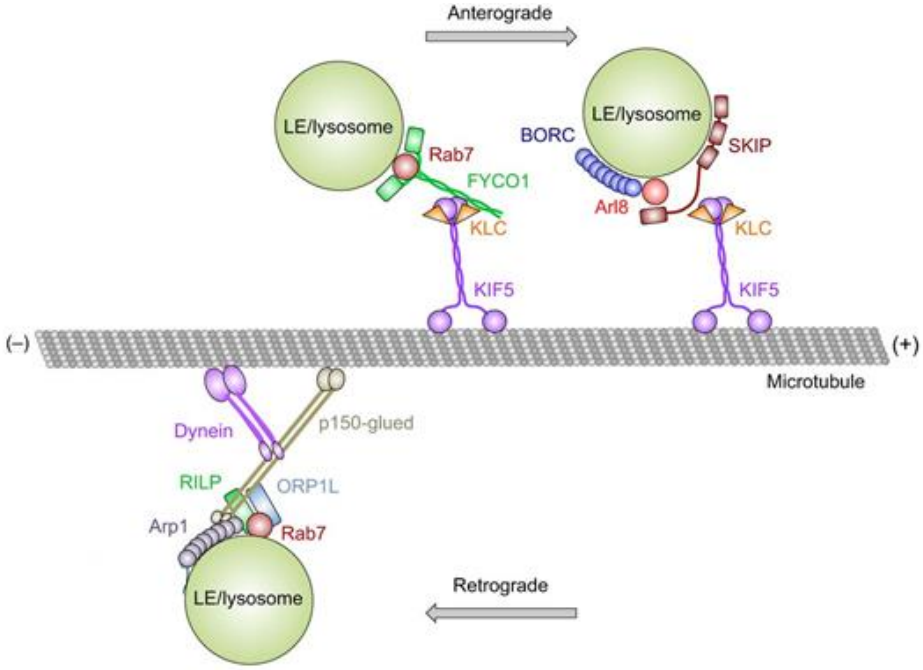


Figure 1.4: Mechanism of late endosome and lysosome movement along microtubules. Plus-end directed movement of late endosome (LE) and lysosomes is jointly driven by BORC, Arl8, SKIP and kinesin-1. An alternative mechanism of anterograde transport is mediated by Rab7 and where its effector FYCO1 acts as an adaptor to kinesin-1. Minus-end directed transport towards the perinuclear space is mediated by Rab7, RILP, ORP1L and dynein–dynactin complex. *Modified from: (Pu et al., 2016).Copyright © 2016. Published by The Company of Biologists Ltd.*

Apart from microtubules, studies have also pointed towards an indirect role played by the actin cytoskeleton in the endolysosomal degradation pathway. It has been reported that actin disruption leads to a blockage in cargo trafficking from early endosomes towards late endosomes (Apodaca, 2001; Durrbach et al., 1996). Recent data from various studies have indicated that Arp2/3-dependent actin nucleation occurring at the endosomal membranes is essential for membrane fission events such as fission of sorting vesicles in the recycling pathway; and cargo delivery towards trans-Golgi network (TGN) and lysosomes (Derivery et al., 2009; Duleh and Welch, 2010; Gomez and Billadeau, 2009; Morel et al., 2009). A clear evidence for the role of actin in regulating fusion events between multivesicular late endosomes and lysosomes/vacuoles, phagosomes and lysosomes, as well as autophagosomes and lysosomes has been provided by studies carried out in both yeast and mammalian cells (Eitzen et al., 2002; Jahraus et al., 2001; Kjeken et al., 2004; Lee et al., 2010).

1.2.4 Tethering factors and SNAREs: mediating intimacy and union

Tethering factors are the molecular bridges mediating interactions between the cargo-loaded intracellular carrier vesicles and the acceptor compartments. On the basis of structural differences, these can be further classified into multisubunit or coiled-coil tethering factors. Tethers are recruited by specific small GTPases in their active conformation to distinct donor or acceptor membranes that are pre-destined for fusion. These proteins bring the membranes projected for fusion into close proximity and mediate docking of the donor compartment onto the target membrane. Subsequently, they assist in the recruitment of cognate SNARE pairs ultimately effectuating the merger of the two vesicular membranes along with luminal mixing.

1.2.4.1 Tethers of the Endo-lysosomal System: CORVET and HOPS

Endolysosomal system comprises of many tethering factors imposing regulation at multiple steps. The endosomal tethering factors are sub-divided into two general classes. The first category includes long coil-coiled tethering molecules that mediate fusion events at early

endosomes. For instance, EEA1 and Rabenosyn5 both of which are recruited by active Rab5 on specific domains of the opposite membranes of sorting endosomes and/or endocytic vesicles form dimers and co-ordinate SNARE assembly eventually resulting in significant fusion events (Christoforidis et al., 1999a; Nielsen et al., 2000; Peterson et al., 1999; Simonsen et al., 1998; Tall et al., 1999). The second category of tethering factors comprises of oligomeric complexes having multiple protein subunits. The organisation of these multimeric protein complexes is also negotiated by specific GTPases on the endosomal membranes. Two such multi-subunit tethering factors that regulate cargo trafficking along the endolysosomal route are: CORVET (Class C core vacuole/endosome tethering) complex and HOPS (Homotypic fusion and Protein Sorting) complex both of which were initially discovered in yeast (Nickerson et al., 2009) (Figure 1.5). Like EEA1, CORVET is also an effector of Rab5 and primarily functions at the membranes of early endosomes, newly formed endocytic carrier vesicles and macropinosomes while HOPS interacts with two distinct GTPases: Rab7 and Arl8b and is associated with late endosomal and lysosomal membranes. The transition of Rab5 to Rab7 during endosomal maturation coordinates the exchange of these tethering proteins on the membranes of transiently formed hybrid endosomes that enables the newly maturing late endocytic compartments either for homotypic fusion with other late endosomes and heterotypic fusion with autophagosomes or lysosomes. According to the studies performed in yeast cells, both CORVET and HOPS are heterohexameric tethering factors that share four subunits nominated as core proteins: Vps11, Vps16, Vps18, and Vps33 and two distinct accessory subunits: Vps3(yeast)/ TGFBRAP1(mammals) and Vps8 in case of CORVET which have been replaced by Vps39 and Vps41 in HOPS complex (kleine Balderhaar and Ungermann, 2013). Notably, all the subunits of these two complexes have been conserved during the course of evolution pointing towards the indispensable functions executed by these two tethers (Kim et al., 2001; Nickerson et al., 2009; Richardson et al., 2004; Seals et al., 2000; Wurmser et al., 2000).

The accessory subunits confer Rab-specificity to these complexes. The yeast CORVET complex tethers and supports homotypic fusion of Vps21/Rab5 positive early-endosomes via Vps3 and Vps8 (Cabrera et al., 2013; kleine Balderhaar et al., 2013; Markgraf et al., 2009; Ohya et al., 2009; Peplowska et al., 2007; Plemel et al., 2011). On the other hand, the HOPS specific subunits Vps41 and Vps39 directly bind to Ypt7 (the yeast homologue of Rab7) positioning this tethering factor on late endocytic compartments (Bröcker et al., 2012; Ostrowicz et al., 2010; Plemel et al., 2011; Seals et al., 2000). Owing to the conservation of these multisubunit tethers across from yeast to humans the localisation and functions of these complexes have been generalised to the mammalian system.

HOPS complex is so far the best characterised endolysosomal tethering factor in terms of structural details, functioning and regulation and appears to be the most active tether in the endolysosomal system (kleine Balderhaar and Ungermann, 2013; Mima and Wickner, 2009; Stroupe et al., 2006; Zick and Wickner, 2012). The structural configuration and mechanism of action of this complex have been discussed in more details in Chapter 3 of the thesis.

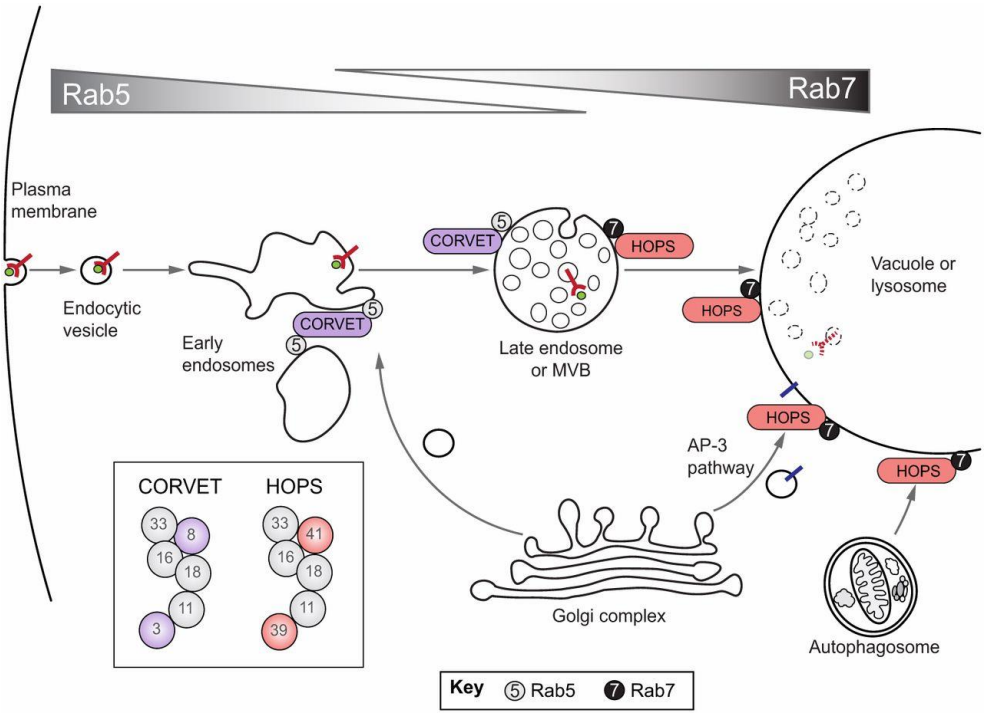


Figure 1.5: Graphical representation of CORVET and HOPS function in yeast. CORVET functions as an effector of the small GTPase Rab5 and regulates homotypic fusion of early endosomal compartments. At the late endosome, Rab5 is substituted with Rab7, which then interacts and recruits HOPS complex to promote tethering and fusion at these compartments. HOPS is also essential for homotypic fusion of vacuoles (or lysosomes) and also mediates heterotypic fusion events such as autophagosome/ late endosome fusion with the vacuole. *Reproduced with permission (kleine Balderhaar and Ungermann, 2013) Copyright © 2013. Published by The Company of Biologists Ltd.*

1.2.4.2 SNAREs and SM proteins

SNAREs (**S**oluble **N**-ethylmaleimide-sensitive factor **A**ttachment protein **RE**ceptors) are highly conserved protein molecules that physically drive multiple intracellular fusion events (Chen and Scheller, 2001; Jahn and Scheller, 2006). Most of the SNARE proteins consist of a cytoplasmic domain which is characterised by amphiphilic heptad repeats alongside a C-terminal transmembrane domain anchoring these proteins at the membrane. The SNAREs form a tetrameric co-complex penetrating the two bilayers intended for fusion. The asymmetric distribution of SNAREs on the donor and acceptor compartments gave rise to the early nomenclature: t-SNAREs concentrated on the target membranes and v-SNAREs on the membranes of donor vesicles. Based on sequence alignments and structural studies the SNAREs nomenclature has been re-defined and is now based on the packing residues in the tetrameric coiled-coil core which is made up of repeats of hydrophobic amino acids, with an exception of a single ionic layer (the **zero layer**) of packing residues at the centre of the helical bundle. In this central layer, the t-SNARE has a conserved glutamine (Q) at the centre of its SNARE domain while v-SNARE has a conserved arginine (R); which combine to form the central packing layer of the tetrameric SNARE complex (Jahn and Scheller, 2006). Accordingly, the t-SNAREs were renamed as Q-SNAREs (with three sub-families with distinct homologies: Qa, Qb and Qc) whereas v-SNAREs were now designated as R-SNAREs. The most commonly found SNARE complexes comprise of three Q-SNAREs in one bilayer and one R-SNARE from the other apposed membrane, form a reversible zipper-like structure (a tetrahelical bundle) at the tethered membranes. The zippering initiates at the distal N-terminal end and proceeds towards the membrane end uniting the two lipid bilayers (Fasshauer and

Margittai, 2004; Pobbati et al., 2006; Schwartz and Merz, 2009; Sørensen et al., 2006). During this process, partially zippered transition state complexes are formed while the Q- and R-SNAREs are still in separate bilayers. These quaternary structures are known as trans-SNARE complexes (or SNAREpin). Post-fusion the Q- and R-SNAREs reside in a single bilayer for a short time-span in a fully zippered conformation and these structures are identified as cis-SNARE complexes which readily undergo dissociation in an ATP-dependent manner carried out by a set of ubiquitous proteins including NSF (N-ethylmaleimide-sensitive factor) and its co-factor α-SNAP (soluble NSF attachment protein) (Ryu et al., 2016).

Each trafficking pathway utilises its own set of complementary SNAREs, indicating their involvement in determining the specificity of the membrane fusion events. However, the promiscuous nature of these proteins can sometimes lead to the formation of non-cognate SNARE complexes which can drive off-pathway fusion. To prevent these mismatches, the proofreading of cognate-SNARE pairing is performed by tethering proteins resulting in the stabilisation of functional SNARE complexes (Hong and Lev, 2014). The interaction between the tethers and SNAREs is facilitated by a particular class of proteins belonging to the SM (Sec1/Munc18) family. These proteins are highly conserved SNARE master regulators of which four sub-families have been reported until now: Sly1 (ER to Golgi pathway), Sec1/Munc18 (exocytosis), Vps45 (endosomal trafficking) and Vps33 (cargo delivery towards late-endosomes and lysosomes) (Jahn, 2000).

Vps33 is one of the four shared subunits of the two endolysosomal tethering factors: CORVET and HOPS; and coordinates most of the fusion events in multiple trafficking pathways culminating into lysosomes. The lysosomal trafficking involves the interaction of t-SNAREs: Vti1b, Syntaxin7 and Syntaxin8 with v-SNAREs: VAMP7 or VAMP8 at late endosomal or lysosomal membranes (Hong, 2005; Jahn and Scheller, 2006). Nevertheless, autophagosome-lysosome fusion is catalysed by Syntaxin 17 that interacts with a lysosomal SNARE VAMP8

in a HOPS-dependent manner to bring about the merger of these two compartments (Itakura et al., 2012; Itakura and Mizushima, 2013).

1.3 Phagosome maturation and re-wiring of the endocytic pathway by bacterial pathogens

Pathogenic bacteria have evolved diverse strategies to adapt and survive within the host. Invasion of the host cells is beneficial for the intracellular pathogens as they can conceal themselves from the host immune system while still receiving a continuous supply of nutrients from the infected cell. However, the intracellular lifestyle poses a major threat of being delivered to the hydrolytic environment of lysosomes through the formation of phagolysosomes. To thrive intracellularly, pathogens intercept and interfere with the endomembrane trafficking machinery and modulate the host signalling pathways to evade lysosomal degradation. On that account, bacteria secrete specific virulence factors that actively target the host proteins and lipids regulating cargo delivery towards these lytic organelles and create an environment conducive for their survival and proliferation. These bacterial proteins regulate different stages of bacterial infection and function either as activators, inhibitors or mimics of eukaryotic proteins controlling the membrane dynamics in the endocytic pathway.

The mechanism of bacterial uptake (phagocytosis) and various bacterial manipulation strategies to seize the host clearance pathways or commandeer the host cellular processes to generate a favourable environment have been discussed in detail in the subsequent sections.

1.3.1 Maturation of nascent phagosomes into phagolysosomes

Phagosome maturation is an important defence mechanism used by host cells to restrict the growth of vacuolar pathogens. This process involves a sequence of membrane fusion events that massively restructure the protein and lipid composition of the phagosomal membranes accompanied by variations in the phagosomal contents leading to the formation of a degradative compartment.

Phagosomes are specialised, membrane-bound organelles that are mainly generated by professional phagocytic cells in order to entrap and eliminate large particulate materials such as residual apoptotic bodies or microbial pathogens. These compartments are larger in size as compared to endocytic compartments and are central to innate and adaptive immunity. The nascent phagosomes are vacuolar structures that are derived from the invaginations of plasmalemma and are devoid of the microbicidal and degradative capacity. Once formed these nascent vacuoles undergo carefully regulated series of membrane remodelling by multiple fusion and fission events which are accompanied by acquisition and pH-dependent activation of hydrolytic enzymes in a process known as phagosome maturation (Figure 1.6). The phagosome maturation is quite analogous to the endosomal maturation pathway, and likewise, segregated into early, late and lysosome-interacting stages. Similar to early endosomes, Rab5 is present at early phagosomes and is critical for their transition into late phagosomes which are associated with Rab7 (Vieira et al., 2003). Akin to the endolysosomal pathway, Rab5 is activated at the early phagosomal membranes by the GEF protein Rabex5 which catalyses the exchange of GTP for GDP (Horiuchi et al., 1997). Activated Rab5 facilitates the fusion of these compartments with early endosomes through association with a tethering protein EEA1 (Christoforidis et al., 1999a; Scott et al., 2002). Additionally, Rab5 binds and activates Vps34 (the type III phosphatidylinositol 3-kinase) generating phosphatidylinositol 3-phosphate [PtdIns(3)P] on the cytoplasmic leaflet of early phagosomal vacuoles (Kinchen et al., 2008). The synthesis of PtdIns(3)P promotes the docking and activation of several critical proteins like EEA1 and Hrs that are fundamental to the maturation process. Consequently, Rab5 sensibly directs the recruitment and activation of Rab7 in conjunction with Mon1-Ccz1 complex (serve as Rab7 GEF) and the two endolysosomal tethers: CORVET and HOPS, a process best understood in yeast (Nordmann et al., 2010; Peplowska et al., 2007; Plemel et al., 2011). Accordingly, CORVET is replaced by HOPS during the progression of phagosomal maturation by switching two of its accessory components Vps3 and Vps8 by Vps39 and Vps41

respectively forming late phagosomes. Acquisition of Rab7 is central to the biogenesis of mature phagosomal compartments. Upon activation this GTPase binds and recruits the effector molecules RILP (Rab7-interacting lysosomal protein) and a long-splice variant of ORP1 (oxysterol-binding protein related-protein 1; ORP1L) that mutually regulate the dynein-dependent transport of phagosomes towards the microtubule-organizing centre (MTOC) (Harrison et al., 2003; Johansson et al., 2007). Furthermore, these mature phagosomes develop tubular projections rendering them more competent for fusion with perinuclear lysosomes. The merger of the late phagosomes into lysosomes results in the formation of highly acidic and hydrolase-enriched hybrid organelles known as phagolysosomes which possess immense microbicidal capacity. Observations such as the impairment of phagosome acidification in *Drosophila* mutants devoid of one of the core subunits of HOPS complex, Vps16b; and accumulation of apoptotic corpses in Rab7-positive phagosomes upon disruption of HOPS components in *C. elegans* have stipulated a pivotal role of this multi-protein tethering factor in phagolysosomal degradation (Akbar et al., 2011; Kinchen et al., 2008). Once tethered, the phagosomal and lysosomal membranes coalesce together in a reaction catalysed by the SNARE co-complex including syntaxin7, syntaxin8 (Q-SNAREs), VAMP7 and VAMP8 (R-SNAREs) (Becken et al., 2010).

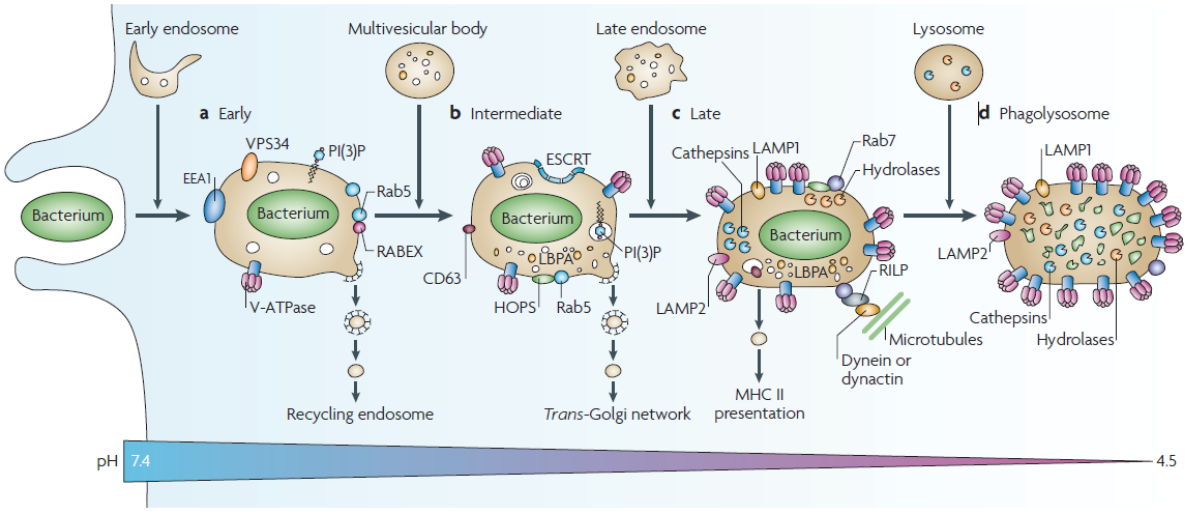


Figure 1.6: Stages of phagosomal maturation. Following ingestion of the pathogen, the nascent phagosome is sequentially modified through interactions with organelles of the endolysosomal system. The phagosome maturation pathway consists of three distinct stages — early (a), intermediate (b) and late (c) phagosomes — concluding with the formation of transient hydrolase enriched hybrid compartments known as phagolysosomes (d). During the maturation process, phagosomes acquire several lytic enzymes and undergo progressive acidification aided by v-ATPases. *Adapted from: (Flannagan et al., 2009) Copyright © 2009, Springer Nature.*

Unlike yeast, the phagosome maturation process in higher organisms also involves the Arf-like GTPase, Arl8b which has recently emerged as a crucial regulator of HOPS function and lysosomal trafficking. Arl8b has been shown to facilitate efficient degradation and removal of phagosomes containing apoptotic cell corpses by recruiting HOPS subunit Vps41 to mediate phagosome-lysosome fusion in somatic-sheath cells (large phagocytes in *C. elegans*) (Sasaki et al., 2013). Several other studies have highlighted the importance of this GTPase in phagosome maturation where knockdown of this protein resulted in a delayed fusion of *E. coli* phagosomes with lysosomes along with affecting microbial clearance in murine macrophages (Garg et al., 2011b). Further, it has been reported that Arl8b (along with its effector SKIP) co-operates with Rab7 to drive lysosomal tubulation in activated macrophages that is essential for the retention of pinocytic content and promoting phagosome maturation and acidification in these cells (Mrakovic et al., 2012). This function of Arl8b can be exploited by certain intracellular pathogens such as *Salmonella* to direct kinesin-dependent endosomal tubulation

of their vacuoles and mobilize the bacteria along the microtubular tracts aiding in cell-to-cell transfer (Kaniuk et al., 2011a).

Additionally, numerous other factors regulating distinct steps of the phagosome maturation pathway have been reported which include: Ca^{2+} ions, components of Retromer complex, and several Rab proteins such as Rab10, Rab11a, Rab22, Rab14, Rab20 and Rab32.

1.3.2 Confiscation of the endo-lysosomal machinery and subversion of phagosome maturation for microbial survival

Vacuolar bacteria induce their own uptake into the non-phagocytic host cells such as intestinal epithelial cells which are often the first cellular targets of many enteric pathogens. To achieve this, various pathogenic bacteria have evolved two distinct mechanisms. Pathogens, for instance, *Yersinia* species and *Listeria monocytogenes* make use of specific proteins expressed on their surfaces, which bind to host receptors and initiate a signalling cascade for actin re-organisation eventually resulting in the internalisation of the organism (zipper mechanism). However, most intracellular bacterial species employ dedicated translocon machineries to inject specific proteins across the host membranes that either interact directly or mimic the cellular proteins regulating actin dynamics to facilitate their entry (trigger mechanism) (Lecuit et al., 1997; Yoshida et al., 2002; Zhou and Galán, 2001). Following the internalisation, these pathogens can either reside in the membrane-bound compartments derived from the plasma membrane of the host or quickly lyse these vacuoles to escape into the cytoplasm.

Both lifestyles impose certain restrictions for bacterial growth that need to be taken care of. The vacuolar bacteria need to evade phagosomal maturation while their cytosolic counterparts face threats like autophagy, induction of inflammatory response and activation of cytokine signalling. Examples of bacterial pathogens using the latter strategy include *Shigella flexneri* and *Listeria monocytogens* which usurp the actin polymerisation machinery for their transport and further dissemination to the neighbouring cells (Ray et al., 2009).

Nonetheless, a majority of intracellular bacterial pathogens manipulate the vesicular trafficking pathways to establish their intracellular niche. They interact with host organelles and proteins to elicit a variety of cellular changes to facilitate the invasion and proliferation while suppressing the immune responses. These pathogens target the phagosomal maturation process at various steps, either by interfering with the fusion events between early endosomes and late endosomes or late endosomes and lysosomes or by modifying the phagolysosomal environment to promote their survival (Duclos and Desjardins, 2000; Haas, 2007; Méresse et al., 1999a). To achieve this, bacteria make use of a sophisticated repertoire of secretory proteins and corresponding injecting apparatus to launch them into the host cell lumen. These virulence factors secreted by the bacteria are known as "effectors" which can either act as mimics, agonists or antagonists of the host proteins to bypass phagosomal maturation and establish a vacuolar niche for their growth and dissemination. Avoidance and inhibition of the trafficking through the canonical endocytic pathway is the most common strategy employed by a variety of intracellular bacterial pathogens such as *Mycobacterium tuberculosis, Legionella pneumophila, and Chlamydia trachomatis.* On the other hand, some of these bacteria, for instance, *Brucella abortus, Coxiella burnetti* and *Salmonella Typhimurium* promote the acidification of their vacuoles and permit interactions of their niches with the lytic organelles (lysosomes) (Figure 1.7). The mechanistic details of the stratagems commonly used by various intracellular bacterial pathogens to hijack host signalling and trafficking pathways to trick the cellular immune defences are discussed below.

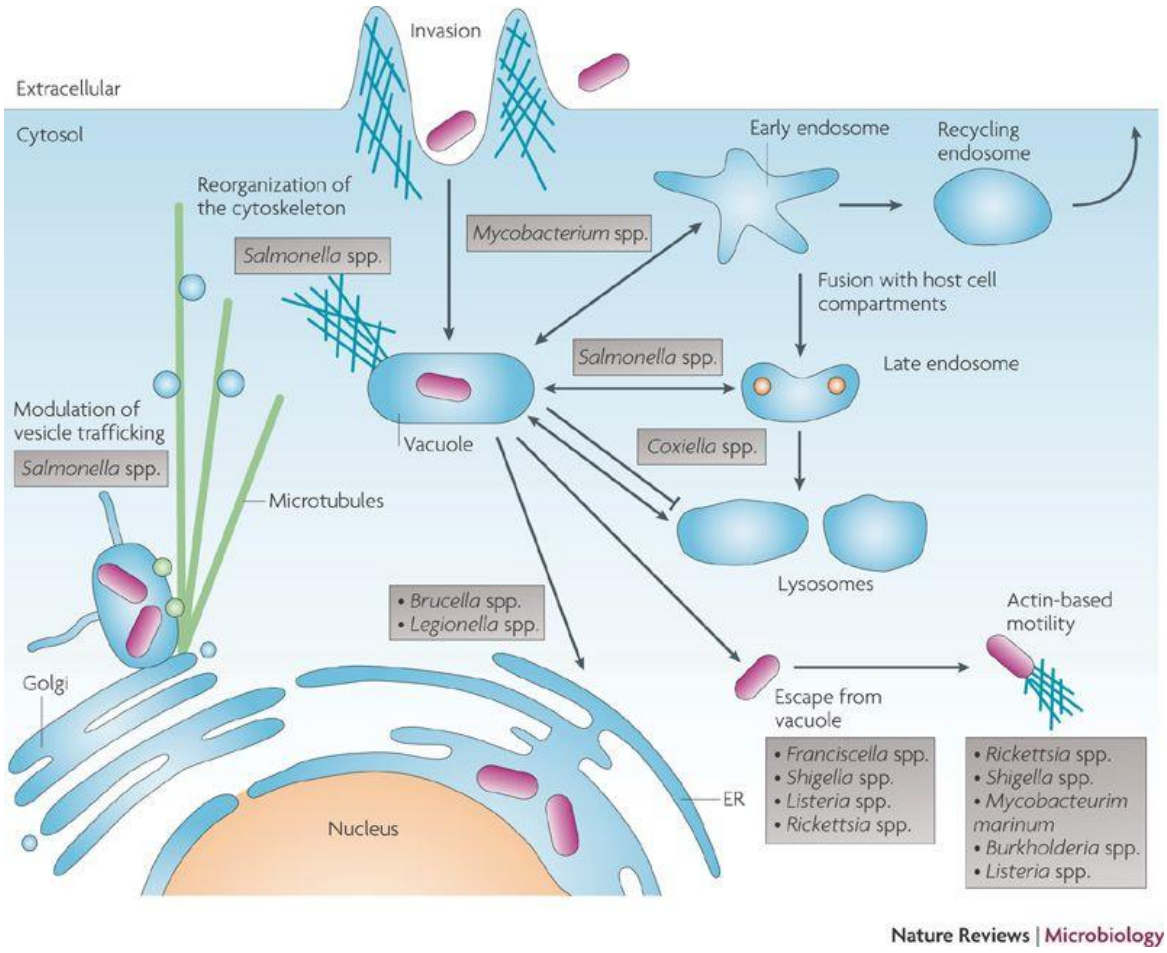


Figure 1.7: Bacterial pathogens manipulate host membrane trafficking to resist immune surveillance and promote their intracellular survival and replication. After invading the host cells, pathogenic bacteria take alternate routes of intracellular trafficking to establish their replication niche. Several such pathogens take residence in specialized membrane bound vacuolar compartments or phagosomes and manipulate host cytoskeletal elements to induce vacuolar and vesicular motility. Some vacuolar bacteria promote their survival through interactions with distinct sub-cellular compartments of the endolysosomal system or replicate in organelles of the secretory pathway such as the endoplasmic reticulum (ER). Other bacteria lyse their vacuoles and escape into the cytosol and promote their actin-dependent motility. Fusion with lysosomes results in bacterial killing by lytic enzymes and acidic pH. In order to successfully establish a replication competent microenvironment, almost all intracellular pathogens either inhibit or delay lysosome fusion. *Adapted from: (Diacovich and Gorvel, 2010)*

1.3.2.1 Inhibition of phagosome maturation by *Mycobacterium tuberculosis*

The *Mycobacterial* species stalls the maturation of their phagosomes to avert the deleterious effects of lysosome fusion. The MCVs or *Mycobacterium tuberculosis* phagosomes are characterised by the presence of early endosomal GTPase Rab5,lysosomal glycoprotein LAMP1 and neutral pH while excluding certain late endocytic markers like Rab7-GTPase and Vacuolar ATPases (Deretic and Fratti, 1999; Li and Xie, 2011; Sturgill-Koszycki et al., 1996). The absence of the Rab5 effector EEA1 from the MCV (*Mycobacterium*-containing vacuoles)

membranes indicates an early block in phagosome maturation pathway. *Mycobacterium tuberculosis* has a dedicated set of proteins to perform this task. For example, mycobacterial lipoamide dehydrogenase (LpdC) interacts with a host protein Coronin1 (or TACO) which has been implicated as a mechanism to prevent the delivery of this pathogen to the lysosomes (Deghmane et al., 2007; Jayachandran et al., 2008). Another important mechanism by which *Mycobacterium* prevents lysosomal fusion is by inhibition of "Rab conversion" on the maturing phagosomes. This process is carried out by two factors: the *Mtb* lipid, mannose-capped lipoarabinomannan (ManLAM) and a phosphatase, SapM which together act to impede the accumulation of PI(3)P on the membranes of mycobacterial phagosomes; ultimately preventing their transition into phagolysosomes (Vergne et al., 2003; Vergne et al., 2005). In addition to this, *Mycobacterium* is also believed to interrupt the interaction of Rab7 to its effector RILP in order to forestall phagosomal maturation (Sun et al., 2007).

1.3.2.2 *Coxiella burnetti* resides in a lysosome-like compartment.

Unlike other vacuolar pathogens, *Coxiella* species survives and replicates in the acidic lysosome-like compartments (Méresse et al., 1999a). The *Coxiella* containing vacuole (CCV) acquires the autophagic marker proteins like LC3, Beclin1 and p62 (Romano et al., 2007; Vazquez and Colombo, 2010; Winchell et al., 2014) within few minutes of infection following which it traffics along the endocytic route for the establishment of the replicative large-cell variant or the mature CCV (Coleman et al., 2004; Howe et al., 2003). The replicative *Coxiella* vacuole shares several features with the lysosomal compartments which include: acidification (pH~4.8), cathepsin D and acid phosphatase (Maurin et al., 1992). The acidification of the mature CCVs promotes bacterial replication and translocation of T4SS (type IV secretion system) effector proteins (Heinzen et al., 1996; Newton et al., 2013). During the later stages of infection, the CCVs undergo a series of homotypic fusion events with other CCVs and heterotypic fusion with endolysosomal and autophagosomal compartments eventually leading

to their expansion into giant vacuoles spanning throughout the cytoplasm of the host cells (Coleman et al., 2004; Larson et al., 2016). Recent studies have unravelled a mechanism by which pathogenic *Coxiella burnetti* inhibits the phosphorylation of HOPS subunit Vps41 to prevent trafficking to microbicidal lysosomes and thrive intracellularly (Barry et al., 2012). However, the mechanistic details of *Coxiella*'s interference with the host vesicular pathways and the roles played by the effector proteins in this process still remain elusive.

1.3.2.3 Hijacking of the secretory pathway by *Legionella, Brucella and Chlamydia*

Both *Legionella pneumophila* and *Brucella abortus* deviate from the endocytic pathway and interact extensively with the endoplasmic reticulum (ER) followed by their retention in specialised compartments known as *Legionella* containing vacuoles (LCVs) and *Brucella* containing vacuoles (BCVs) respectively.

During the early stages of infection, *Legionella* translocates an effector protein SidM (also known as DrrA) through the type IV secretion system encoded within their genome. SidM acts as a GEF for Rab1, a GTPase regulating vesicular trafficking between ER and Golgi and recruits it to the LCV membranes to foster their fusion with ER-derived vesicles (Sherwood and Roy, 2013). In addition to this, another *Legionella* effector RalF recruits and activates the GTPase, Arf1 facilitating the recruitment of ER-derived vesicles to the *Legionella* vacuoles (Nagai et al., 2002).

Similarly, the BCVs recruit a host GTPase, Sar1 to sequester the vesicles originating from the ER and are closely associated with the ER network (Celli et al., 2005). However, the BCVs transiently interact with the late endosomal and lysosomal compartments during early stages of phagosome maturation (Atluri et al., 2011) but later on digress from this pathway for the formation of replication competent vacuoles.

Another paradigm of the intracellular pathogens that drift away from the classical endocytic route to create a specialised intracellular habitat is *Chlamydia trachomatis.* Upon uptake, the

Chlamydia reside in vacuolar structures called "inclusions" which traffic towards the Golgi network. A characteristic feature of a chlamydial infection is the fragmentation of Golgi complex into mini-stacks around the inclusions (Hackstadt et al., 1996; Heuer et al., 2009). The formation of these unique compartments requires a range of bacterial and host proteins that permit selective fusion with sphingomyelin-containing exocytic vesicles while forbidding interaction with late endosomes/lysosomes enabling replication and survival of this pathogen (Capmany and Damiani, 2010; Hackstadt et al., 1996; Van Ooij et al., 2000). Several Rab proteins (Rab1, Rab4, Rab11, Rab6 and Rab14) are recruited by chlamydial effectors to the inclusions where they promote the acquisition of lipids and nutrients (Betts et al., 2009; Capmany and Damiani, 2010; Rzomp et al., 2003). Besides, another effector protein secreted by this pathogen IncA mimics the mammalian SNAREs to facilitate interaction of the inclusions with the incoming vesicular traffic (Delevoye et al., 2008).

1.3.2.4 *Salmonella* maintains a close association with the endocytic pathway

Salmonella trafficking involves extensive interaction with the endocytic and secretory pathways (Jennings et al., 2017; Vogels et al., 2011). Upon ingestion by the host cell, these bacteria reside in a vacuolar habitat that matures through sequential interactions with the endolysosomal pathway and moves along the microtubules to form a replicative niche situated near the Golgi network. The maturation of *Salmonella* phagosomes requires two distinct type III secretion systems whose expression is temporally regulated (Galan and Curtiss, 1989; Shea et al., 1996). The mature *Salmonella* vacuoles (SCVs) continuously exchange materials with late endosomes and lysosomes and are characterised by the presence of proteins such as Rab7, lgps (Lysosomal glycoproteins like LAMP1, LAMP2) and vacuolar-ATPase (Drecktrah et al., 2007b; Garcia-del Portillo and Finlay, 1995; Méresse et al., 1999b). However, late endocytic markers such as M6PRs and cathepsin D are absent from these compartments indicating that

Salmonella ensures a controlled interaction between the SCVs and lysosomes (McGourty et al., 2012; Rathman et al., 1997).

The complex interplay of *Salmonella* effectors and host vesicular trafficking machinery has been discussed in detail in the subsequent sections.

1.4 *Salmonella enterica serovars* and Salmonellosis

Salmonella genus is a group of gram-negative, rod-shaped, peritrichous flagellated, motile, facultative intracellular bacilli; closely related to *Escherichia* genus as both of them are believed to have evolved from a common ancestor. The genus *Salmonella* belongs to the family Enterobactericiae and comprises of two major species namely *Salmonella bongori* (a commensal in cold-blooded organisms) and *Salmonella enterica* that is a leading cause of a multitude of diseases (collectively known as **Salmonellosis**) in warm-blooded organisms. Divergence of *S. enterica* led to host expansion and further division of this group based on sequence variation and biochemical properties into six sub-species: (I) *enterica,* (II) *salamae,* (IIIa) *arizonae,* (IIIb) *diarizonae,* (V) *houtenae,* (VI) *indica (Boyd et al., 1996; McQuiston et al., 2008).* The sub-species *enterica* encompasses more than 2500 serogroups based on differences in O (somatic) antigen due to differences in lipopolysaccharide (LPS) structure and H (flagellar) antigen owing to variations in the expression of protein flagellin a basic component of flagella (Brenner et al., 2000; Lan et al., 2009). The vast biological diversity of serovars categorised under this sub-species are capable of occupying diverse environmental niches due to broad host adaptations and are associated with a variety of diseases (Brenner et al., 2000; Kingsley and Bäumler, 2000).

Based on clinical manifestations in humans, WHO has broadly categorised *Salmonella enterica* into typhoidal and non-typhoidal *Salmonella* (NTS*)*. The former includes human-restricted serovars *Salmonella typhi* and *Salmonella paratyphi* which cause systemic infections (typhoid or paratyphoid fever respectively). The latter category comprises of serotypes such as

Salmonella Typhimurium and *Salmonella enteriditis* that infect a broad spectrum of hosts leading to either localised infections (self-limiting gastroenteritis, enterocolitis or invasive bacteraemia in the immune-compromised host) in humans or systemic infection (typhoid-like disease) in mice.

Typical symptoms of Salmonellosis include: abdominal cramps, diarrhoea, nausea, headache and vomiting often accompanied by acute onset of fever. The emergence of disease symptoms generally occurs 12-36 hours after ingestion of *Salmonella*, and the illness usually lasts 2–7 days. In most cases, the symptoms of the disease are not severe, and the patients mainly recover without any specific treatment. Nonetheless, the bacteria continue to inhabit the intestinal tract of the infected individuals for weeks after the disappearance of symptoms and are routinely shed in the faeces of such patients (on an average, for 4 or 5 weeks in adults and longer in infants or aged individuals). However, in some cases, particularly in children or immune-compromised adult patients, the associated dehydration can occasionally become severe and life-threatening.

Salmonella typhi and *paratyphi* infections generally manifest into bacteraemia (systemic *Salmonella* infection) that usually last for longer durations. This illness can be enteric fever, typhoid (caused by *Salmonella typhi*), or paratyphoid fever (caused by *Salmonella paratyphi*). The disease symptoms appear gradually and typically include fever and chills, headache, malaise, lethargy, bloating, vomiting, diarrhoea and abdominal pain. In infants, it can emerge as a non-specific fever. The incubation period for *S. typhi* is generally 8-14 days, but it can range from 3-60 days depending on the infectious dose and patient compliance. For *S.paratyphi* infections, the latency period is comparable to that of non-typhoidal *Salmonella*, 1 to 10 days (Tarr et al., 2008).

1.4.1 Epidemiology of *Salmonella* infection and Sources of infection

Global outbreaks of *Salmonella* infections (primarily, typhoid fever) are quite common in both developed and developing nations. It has been estimated that typhoid fever affects approximately 22 million people per year worldwide and accounts for almost 200,000 deaths per year. The incidence of *Salmonella* infections is relatively low in developed countries due to improved sanitation and hygiene whereas *S. typhi* infections are still an important public health concern in developing countries with a morbidity rate ranging from 200-1000 incidences per 100,000 inhabitants in these areas.

On the other hand, *Salmonella*-mediated gastroenteritis is one of the most common bacterial food-borne diseases in the developed nations like US, with a prevalence rate of more than one million cases estimated per year. The increasing number of cases of invasive bacteraemia caused by non-typhoidal *Salmonella* is now a cardinal health issue, especially in areas like sub-Saharan Africa where Salmonellosis is considered to be an endemic disease. Opportunistic infections by NTS are regularly reported in HIV-infected adults and immune-compromised children. The outbreaks of *Salmonella* infections are usually not common as 60–80% of cases are not recognised as part of a known epidemic and are classified as sporadic cases, or are not diagnosed as such at all (Tarr et al., 2008).

Higher consumption of raw or partially cooked food, an increase in the population of immune-compromised or chronically ill population, deteriorating infrastructure of public health organizations and emergence of multi-drug resistant strains of *Salmonella*; all have possibly contributed to a consistent increment in the incidences of *Salmonella* infections during the past decades (Darwin and Miller, 1999).

1.4.2 Etiological agents of Salmonellosis

Salmonella infections are usually transmitted by ingestion of contaminated food or water. Sporadic release of the bacteria along with faeces of an asymptomatic chronic carrier or an infected person also serves as a constant reservoir for these infections. Therefore, typhoid fever is often regarded as a disease whose prevalence is inversely proportional to the standards of sanitation and hygiene in a country.

*Salmonella*e are ubiquitously distributed in both domestic and wild animals. The infections are highly prevalent in livestock such as poultry, pigs, and cattle; in domesticated pet animals including cats, dogs, birds, and certain reptiles. *Salmonella* can traverse along the entire food chain from primary production, animal feeds, and all the way to domestic households or food-service and storage organisations or institutions.

In humans, *Salmonella* infections are generally contracted through the consumption of contaminated food of animal origin (mainly eggs, meat and dairy products), although other foods, including green vegetables contaminated by manure polluted by the bacteria, have also been implicated in disease transmission. Human infections also occur where individuals have contact with the infected animals. These infected animals often are asymptomatic carriers with no signs of the disease. Person-to-person dissemination of *Salmonella* can also occur via faecal-oral route (Tarr et al., 2008).

1.4.3 *Salmonella* pathogenesis and disease outcome

Following oral inoculation into its animal host *Salmonella* encounters a series of challenging environmental conditions to colonise the intestinal lumen preceding invasion and/or dissemination to systemic sites. These involve competition with the gut microbes for the available nutrients, space and colonisation resistance along with enduring variations in physiological conditions such as oxygen availability, pH and osmolarity (Erhardt and Dersch,

2015; Patel and McCormick, 2014). At each step, *Salmonella* activates a set of horizontally acquired virulence genes that confer the ability to battle the commensals during colonisation of the intestinal epithelium and breach the aforementioned immunological barriers to invade deeper tissues. After crossing these physiological barriers, *Salmonella* encounters the host innate immune surveillance at the mucosal surface. The antigenic repertoire (**P**athogen-**A**ssociated **M**olecular **P**atterns, **PAMP**s) including lipopolysaccharide and lipoproteins present on the bacterial outer membrane are detected by the host receptors (**P**attern **R**ecognition **R**eceptors, **PRR**s) on the surface of intestinal epithelial cells (Medzhitov and Janeway Jr, 2000). Recognition of these microbe-specific signature molecules initiates cytokine production and massive infiltration of neutrophils leading to inflammation of the intestinal mucosa which is a pathological hallmark of *Salmonella*-induced gastroenteritis (Gewirtz et al., 2001). The rapid extravasation of polymorphonuclear cells into the gut lumen slackens the cell-cell connections in the epithelial monolayer which creates orifices for *Salmonella* to cross the epithelial barrier and allows leakage of the extravascular fluids and electrolytes into the lumen resulting in the characteristic watery diarrhoea observed during *Salmonella* infection (Galyov et al., 1997; Madara et al., 1993; Zhang et al., 2003). *Salmonella* thrives in the inflamed gut as it can better utilise the metabolites produced during this process while the growth of resident microflora is inhibited; following which the bacteria can adhere to the intestinal monolayer using fimbrial appendages (Bäumler et al., 1996).

The pathological investigations of *Salmonella (S. Typhimurium)* infection in mouse models (that closely resembles the pathogenesis of the human-adapted serovar *S. typhi*) have revealed that the apical surface of the microfold cells (M-cells) are the preferential docking sites for *Salmonella* invasion (Figure 1.8). This is because M-cells lack the mucus lining and the apical brush-border found in other cells of the intestinal monolayer which is advantageous for this invader; although entry through non-phagocytic enterocytes and CD18-positive phagocytes has also been reported (Bolton et al., 1999; Frost et al., 1997; Jensen et al., 1998; Jones et al., 1994;

Vazquez-Torres et al., 1999). Uptake of *Salmonella* into M-cells is mediated through a distinct invasion process known as bacteria-mediated endocytosis (a specialised form of macropinocytosis) which is a net result of effector translocation and profound cytoskeletal changes in case of this pathogen (Francis et al., 1992). Once inside, the bacteria manipulate host cell signalling pathways and undergo transcytosis towards the basolateral surface from where they are shuttled to the underlying mucosa-associated lymphoid tissue (MALT) in Peyer's patches. After crossing the epithelial barrier, the sub-mucosal macrophages and dendritic cells predominantly mediate the uptake of this invasive intruder in this layer (Lelouard et al., 2010). Inside these phagocytic cells, *Salmonella* evades the microbicidal activities by virtue of specific effector proteins that modulate the activity of several host proteins to form a replication permissive niche. The bacteria further undergo rapid division and subsequent transmission to other organs of the reticuloendothelial system such as spleen and liver; a characteristic feature of enteric fever (Alpuche-Aranda et al., 1994). Dissemination of *Salmonella* to systemic sites involves massive pyroptosis and necroptosis of macrophages and the unrestricted proliferation of bacteria at these sites causes severe bacteraemia and eventually leads to death of the organism due to lipopolysaccharide (LPS)-induced septic shock at later stages of infection (Khan et al., 1998; Santos et al., 2001).

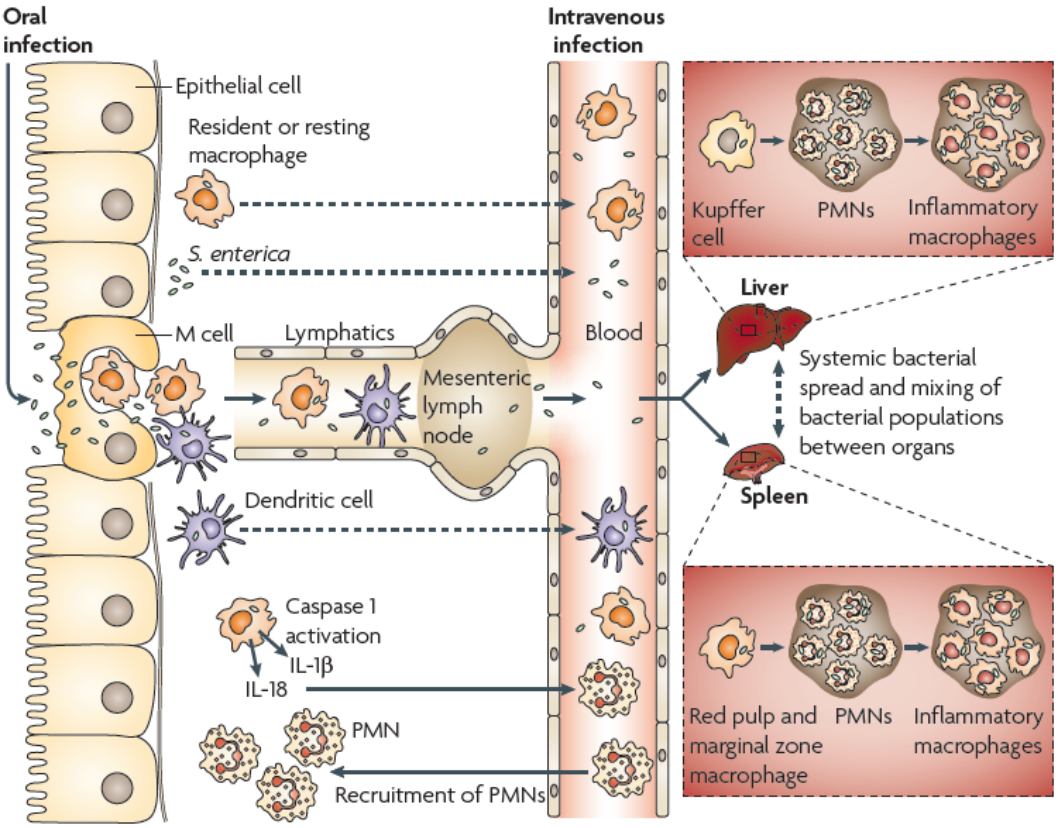


Figure 1.8: Pathology of *Salmonella* infection in mice. *S. enterica* invades M cells (and enterocytes) and is phagocytosed by resident macrophages or dendritic cells. After traversing the epithelial barrier, the bacteria arrive in the submucosal layer, where they activate caspase 1-mediated cell death macrophages, resulting in the production of pro-inflammatory cytokines, that mediate the recruitment of polymorphonuclear phagocytes (PMNs). The pathogenic bacteria then reach the mesenteric lymph nodes and the blood before being captured by resident phagocytes in the spleen and liver. Hepatic and splenic multicellular pathological lesions form at these sites. *Adapted from(Mastroeni et al., 2009)* Copyright © 2009, Springer Nature.

However, during persistent infections *Salmonella* have been shown to preferentially colonise specific sub-types of macrophages in spleen with an anti-inflammatory M2 phenotype also known as hemophagocytic macrophages (as they engulf B- and T- lymphocytes). These cells have a distinct metabolic advantage that promotes the long-term survival of this pathogen inside the host cells (Eisele et al., 2013; Grygiel-Górniak, 2014; McCoy et al., 2012; Nix et al., 2007). While in the liver, *Salmonella* induce immunosuppression by actively inhibiting the proliferation of $CD4^+$ and $CD8^+$ T-lymphocytes to avoid immune surveillance and establishment of a chronic infection at this site (Kullas et al., 2012).

1.4.4 Models to study *Salmonella* infection

The most widely used method to investigate the molecular mechanisms of *Salmonella* pathogenesis is by infecting cells grown *in vitro*. *Salmonella* invades and replicates inside a variety of host cell types, such as intestinal epithelial cells and macrophages. Studying the interaction of these bacteria and the host cell *in vitro* has been invaluable in unravelling mechanisms that are crucial for the establishment of disease. For instance, mechanistic details of the invasion of non-phagocytic cells, intracellular survival and replication of the bacteria in various cell types, biogenesis and maturation of the *Salmonella* containing phagosomes, formation of *Salmonella*-induced tubules and *Salmonella*-mediated cytotoxicity have all been achieved through infections of cell line model systems (Garcia-del Portillo and Finlay, 1995; Garcia-del Portillo et al., 1993a; Knodler and Steele-Mortimer, 2003; Méresse et al., 1999a; Patel and Galán, 2006).

A variety of cell lines (both human and mouse origin) are routinely used to study the intracellular lifestyle of *Salmonella*. Examples include immortalised carcinoma derived mouse-macrophage cell lines, like RAW 264.7 or J774, as well as primary cells, such as mouse bone marrow-derived macrophages (BMDMs) and mouse embryonic fibroblasts (MEFs) are commonly used to study *Salmonella* replication and scavenging inside macrophages which serve as the principal reservoirs of these bacteria during physiological infections. Epithelial cell lines derived from carcinomas, such as HeLa, HepG2 and Caco-2, have also been used extensively to visualise the invasion process and the complex interactions that *Salmonella* establishes with the host vesicular trafficking pathways.

The development of three-dimensional polarised trans-epithelial cell models to study the interaction of *Salmonella* with its host has been a significant advancement as these models imitate the *in vivo* infection more precisely upon *Salmonella* invasion while offering the ability

to control more variables as compared to the whole animal model systems (Nickerson et al., 2001; zu Bentrup et al., 2006).

Apart from the above mentioned *in vitro* model systems, *in vivo* studies can be performed using murine models of *Salmonella Typhimurium* infection which mimic the typhoid fever in humans, caused by the human-restricted serovar *Salmonella typhi*. Infection of genetically susceptible mice strains such as C57/BL6 or BALB/C carrying mutations in the gene encoding for a metal transporter found on SCV membrane: nramp1-/- or slc11a1-/- generate intestinal pathological profiles and inflammatory responses resembling the typhoid disease (Cuellar-Mata et al., 2002; Santos et al., 2001).

More recently, humanised mice strains have been developed that are valuable to study pathological changes occurring in chronic or persistent typhoid infections and the disease profiles of asymptomatic carriers (Libby et al., 2010; Mian et al., 2011).

The amenability of *S. Typhimurium* genome to manipulation using molecular genetics tools alongwith its ability to infect several host cell types and development of robust animal models has allowed for the identification and characterisation of distinct *Salmonella* virulence genes, the host pathways they intercept during infection and various disease phenotypes. Therefore, this serovar is one of the most widely used model systems to study bacterial pathogenesis and host responses during host-pathogen interactions.

1.5 Molecular determinants of *Salmonella* Pathogenesis

The intracellular colonisation of *Salmonella* involves the synchronised release of an arsenal of molecular factors known as “effector” proteins that target the host proteins or have enzymatic activities that interfere with host cell processes in order to establish infection. Some of these effectors are present in all serovars whereas others are unique to specific serovars which determines host specificity and differences in disease outcome. The orchestrated actions of

these proteins mediate bacterial invasion, initiation of inflammatory responses, and assembly of a protective niche conducive for bacterial proliferation and systemic dissemination of this pathogenic organism. Their expression is spatiotemporally regulated and once released they embark a highly coordinated modulation of host signalling and vesicular trafficking pathways in favour of this invader. Effectors are secreted and directionally translocated into the lumen of the host cells with the help of complex supramolecular injecting machines known as type III secretion systems which are fundamental to *Salmonella* pathogenesis. The T3SSs (discussed in more detail below) are structurally similar to the flagella basal body and are almost exclusive to gram-negative bacteria.

1.5.1 *Salmonella* Pathogenicity Islands and Type III Secretion systems: *Salmonella*'s armoury for infection

The *Salmonella* chromosome comprises of discrete genetic loci that encode for a variety of virulence determinants that intercept with the host cell biology at various stages to establish and propagate the *Salmonella* infection. These regions have a low GC content as compared to the rest of the chromosomal segments and are termed as *Salmonella* pathogenicity islands (SPI). SPIs are believed to be acquired via horizontal gene transfer from phage or plasmids of unknown origin during the course of evolution. Many of these pathogenicity islands are highly conserved amongst various *Salmonella* serovars and are distributed throughout genus. Nevertheless, a few of these are present only in specific serovars and therefore are often associated with host specificity (Hansen-Wester and Hensel, 2001; Hensel, 2004; Marcus et al., 2000). *S. Typhimurium* shares at least 11 PAIs (**PA**thogenicity **I**slands) with the typhoid-causing serovar *S. typhi*, although their genetic makeup is quite dissimilar and thus this serovar is extensively used as a model system to mimic and study the pathogenesis of typhoid fever in mice (Sabbagh et al., 2010). Amongst these numerous PAIs encoded in the *Salmonella* genome the major aspects of *Salmonella* pathogenesis can be ascribed to the activities of two distinct

type three secretion systems encoded by SPI1 and SPI2. Type three secretion systems are macromolecular nanosyringe-like organelles that directionally inject bacterial virulence factors into the host cell cytoplasm (Galán and Wolf-Watz, 2006; Mota and Cornelis, 2005). SPI1 encodes for T3SS-1 and effector proteins that are expressed when the bacterium is moving in the gut lumen and even before they encounter the first target cell while SPI2 encodes for T3SS-2 which functions from the *Salmonella*'s intracellular habitat post-invasion.

1.5.2 Type three Secretion systems (T3SS): the Launchpads

Bacterial secretion systems are specialised organelles utilised by these organisms to transport various macromolecules (such as DNA, proteins, ions etc.) across the cell wall. Pathogenic bacteria use them to influence their surroundings and/or hijack the host cell signalling and trafficking machineries. Until now seven different types of such secretory organelles have been described in several bacterial species (Costa et al., 2015). Amongst these, T3SS, T4SS and T6SS are specific to gram-negative bacteria and are used to secrete bacterial compounds into their host interaction partners. These secretion systems vary both in terms of their structure and mechanism of action.

Fundamental to *Salmonella's* successful invasion and virulence strategy is its ability to re-programme the host cell functions to its own benefit; for which it has evolved sophisticated virulence machineries comprising of two distinct type III secretion systems. As mentioned previously, T3SSs are specialised "molecular nano-syringes" that translocate bacterial virulence determinants across the host cell membranes; where they bind or mimic the eukaryotic proteins to bring about relevant phenotypes and promote bacterial invasion, colonisation, survival and eventually systemic dispersion. The expression of the two distinct secretion systems encoded within *Salmonella* genome is spatiotemporally regulated and are responsible for carrying out specific yet sometimes overlapping functions (Deiwick et al., 1998; Hensel et al., 1998). Detailed ultrastructural analyses using electron microscopy have

revealed that these molecular injection complexes comprise of a basal body made up of four membrane-localised rings traversing the bacterial cell wall (both inner and outer membranes) attached to a hollow cylindrical structure that acts as a base for a needle-like appendage externally protruding from the outer layer of the cell wall. A set of translocon proteins function along with these structural elements to form a pore at the target membrane; through which effector proteins are delivered into the cytoplasm of the host cell (Kimbrough and Miller, 2000; Kubori et al., 2000).

The expression of both T3SSs is highly regulated by distinct activating and inhibiting molecules. The first T3SS (SPI1-T3SS) is stimulated by sensing the intestinal environment. Its expression is controlled in a switch on-off pattern by a master regulator HilA and its associated activator proteins. In contrast to SPI1-T3SS, the expression of the second T3SS (SPI2-T3SS) is regulated by two-component regulatory systems such as SsrA/SsrB, PhoP/PhoQ and EnvZ/OmpR of which, SsrA/SsrB is the chief regulator of the majority of SPI2-T3SS functions. The assembly of the second T3SS is initiated at conditions of low pH inside the *Salmonella* vacuoles. However, the effectors are secreted only upon sensing the neutral pH of the host cell cytoplasm. For this purpose, *Salmonella* utilizes a regulatory complex of three *Salmonella* effectors: SsaL, SsaM and SpiC exposed to the cytosol which is dissociated as soon as it encounters the neutral cytosolic pH thereby enabling the release of SPI2-T3SS effectors (Yu et al., 2010a).

The two T3SSs exhibit functional polarisation and function at different stages during *Salmonella* infection. SPI1-T3SS is mainly responsible for secreting effector proteins that mediate docking and invasion by inducing gross modulation of the actin cytoskeleton generating cup-shaped membrane ruffles at the host cell membranes eventually leading to the engulfment of the pathogen into a nascent vacuolar compartment. It also directs the maturation of *Salmonella* vacuoles and contributes to intestinal inflammation by activating the release of

pro-inflammatory cytokines. On the other hand, SPI2-T3SS is essential for the establishment of an intracellular replicative niche for this invasive intruder. SPI2-T3SS effectors are secreted after 3-4 hours post-invasion followed by a lag phase which is required for the maturation of *Salmonella*'s intracellular habitat. Along with its involvement in the intracellular proliferation and survival of *Salmonella* in host macrophages, SPI2-T3SS effectors are also responsible for the systemic spread of *Salmonella* infection to secondary sites such as spleen and liver.

1.6 *Salmonella* opts for an intra-vacuolar lifestyle

Salmonella can penetrate the membrane of a eukaryotic host cell either passively through engulfment by phagocytes (macrophages, neutrophils, dendritic cells) or actively by triggering the invasion of non-phagocytic cells mainly, epithelial cells or fibroblasts. To escape their usual fate during the intracellular lifestyle, *Salmonellae* subvert the bactericidal eukaryotic processes to establish a novel membrane-bound replication-permissive microenvironment known as *Salmonella* containing vacuole (or SCV) as a survival strategy in the otherwise inhospitable host environment. *Salmonella* modifies its vacuolar niche through complex interactions of virulence factors secreted by *Salmonella* and host proteins functioning in the endolysosomal pathway. Recent observations have shown that in order to maintain a stable parasitophorous vacuolar habitat *Salmonella* extensively communicates with the endocytic compartments and re-directs the endomembrane trafficking pathways; that confers protection from the intracellular antimicrobial defence mechanisms while maintaining a constant supply of nutrients coming into the cell via the endocytic route. The mechanism by which *Salmonella* establishes a parasitic relationship with its enemy is described in the subsequent sections.

1.6.1 *Salmonella* invasion and biogenesis of *Salmonella*-containing phagosomes (SCVs)

The complex interplay between *Salmonella* and host begins as soon as it comes into contact with the target cell. Docking of invasion-primed bacteria to the intestinal epithelial cells triggers the release of the pre-formed pool of dozens of SPI1-T3SS effectors through a

multimeric translocon complex (formed by SipB, SipC and SipD) within few seconds. Proficiency of *Salmonella* invasion is governed by the coordinated action of at least five effector proteins including: SipA, SopB, SopD, SopE, SopE2 and SptP; that cooperate to induce actin rearrangement which involves formation of actin fiber protrusions or "ruffles" at plasma membrane to entrap the bacteria followed by internalization into non-phagocytic cells (Patel and Galan, 2005; Zhou and Galán, 2001) (Figure 1.9). SPI1-T3SS effectors, SopE/E2 act as GEFs for activation of Rho-GTPases: Rac1 and Cdc42 to instigate Arp2/3 complex driven F-actin assembly at the docking sites (Patel and Galán, 2006); while SipA and SipC initiate actin nucleation, promote actin bundling and polymerization and stabilize the filaments during ruffle formation thereby facilitating bacterial endocytosis (Friebel et al., 2001; Hayward and Koronakis, 1999; Hayward and Koronakiss, 2002; Lilic et al., 2003; McGhie et al., 2004; Myeni and Zhou, 2010; Unsworth et al., 2004; Wall et al., 2007). Another effector protein, SopB is an inositol phosphatase that performs multiple tasks during *Salmonella* invasion and SCV biogenesis. The ectopic expression of this protein can induce localised actin dependent ruffle-like structures similar to those produced during *Salmonella* invasion (Zhou et al., 2001). The phosphatase activity of this protein has various effects that promote actin re-organization by activating SH3-containing guanine nucleotide exchange factor (SGEF), an exchange factor for the GTPase RhoG, modification of lipid content (through hydrolysis of a range of phosphoinositide and inositol phosphates) and sealing of the nascent bacterial phagosomes during the invasion process (Norris et al., 1998; Patel and Galan, 2005).

Salmonella invasion prompts the release of pro-inflammatory cytokines by the host cells leading to inflammation of the intestinal mucosa. The activation of Rho-GTPases by SPI1-T3SS effectors (SopE, SopE2 and SopB) triggers the downstream signalling of MAPK pathway by activating proteins such as Erk, Jnk and p38 MAPK that further effectuate the AP-1 and NF-κB-dependent transcription of pro-inflammatory cytokines such as IL-8 (Haraga et al., 2008; Hobbie et al., 1997; Patel and Galán, 2008). The release of IL-8 creates a chemical

gradient that directs the diapedesis of neutrophils across the endothelial barrier. *Salmonella* effector, SipA also plays a prominent role in promoting the inflammatory response. SipA triggers a signal transduction cascade resulting in the activation of protein kinase C (PKC)-α that governs the synthesis of another chemoattractant hepoxilin A3 (HXA3) to drive the transepithelial migration of PMNs in concert with IL-8 (Criss et al., 2001; Lee et al., 2000; Srikanth et al., 2010; Wall et al., 2007).

After gaining entry into the host cells; *Salmonella* downregulates the expression of SPI1-T3SS genes and takes up residence into a vacuolar compartment (SCV) that undergoes further maturation as the infection progresses. However, some of the effectors such as SopB, SptP and SipA have comparatively longer half lives and can be detected even at later time-points. Formation of the nascent SCVs triggers activation of SptP, a *Salmonella*-secreted GTPase activating protein (GAP) of activated Rho-GTPases (Cdc42 and Rac1) that restores the plasma membrane integrity by inhibiting ruffle formation (Fu and Galán, 1999; Galán and Zhou, 2000; Humphreys et al., 2009; Patel and Galan, 2005). SptP also negates the host immune responses by inhibiting Erk/MAP kinases to prolong the intracellular survival of *Salmonella* during self-limiting gastroenteritis.

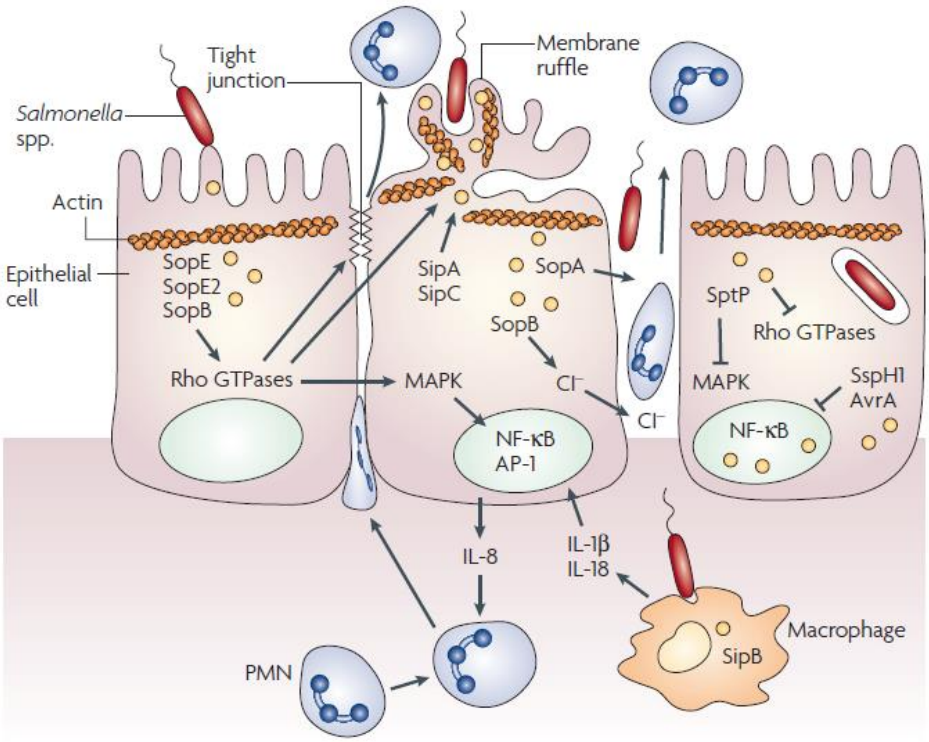


Figure 1.9: SPI1-T3SS mediated *Salmonella* invasion of host non-phagocytic cells. On contact with the epithelial cell, *Salmonella* assembles the SPI1-encoded T3SS and release effector proteins (yellow spheres) into the host cytoplasm. SPI1-T3SS effectors (SopE/E2, SopB) activate Rho GTPases, resulting in the rearrangement of the actin filaments forming membrane protrusions for bacterial uptake, induction of MAPK signalling leading to the production of pro-inflammatory polymorphonuclear leukocyte (PMN) chemokine IL-8 and destabilization of tight junctions for neutrophil infiltration, fluid leakage and bacterial translocation across the epithelium. The changes in the actin cytoskeleton are restored once the bacterium is internalized and MAPK signalling is turned off by the activities of another SPI1-T3SS effector, SptP. *Adapted from: (Haraga et al., 2008) Copyright © 2008, Springer Nature.*

SCV maturation programme comprises of three different stages namely: Early (<30min post infection), Intermediate (30min-5hr post infection) and Late (>5hr post infection) phases; ultimately yielding a mature SCV that is significantly different from the typical phagosomes. These stages are distinguished by the presence of distinct bacterial proteins and host cell markers on the SCV membranes (Figure 1.10).

Early vacuole development begins soon after the bacterium is internalised into the host cells. SPI1-T3SS effector, SopB is the dominant player during initial vacuole development by altering the lipid content of the SCV membrane through hydrolysis of PtdIns(3,5)P2 and PI(3,4,5)P3 to PI(3)P (Malik Kale et al., 2011; Steele-Mortimer et al., 2000; Terebiznik et al., 2002). It also contributes to early maturation of SCVs by recruiting the early endosomal

GTPase, Rab5 and PI(3)Kinase Vps34 to these membranes thereby inducing rapid membrane remodelling through fusion events with the early endosomes mediated by EEA1 within 5min p.i. (Malik Kale et al., 2011; Mallo et al., 2008; McGhie et al., 2009). The nascent SCV at this stage is also characterized by several other endocytic marker proteins such as transferrin receptor (TfnR), Rab4, and Rab11 (Smith et al., 2005; Steele-Mortimer et al., 1999) and acts as a shelter for the internalized bacterium that confers protection against the hostile environment of the host cells. Notably, SopB also interacts with BAR-domain containing proteins: SNX1 and SNX3 which are found to transiently localise onto the SCV membranes during early phases of infection (Braun et al., 2010; Bujny et al., 2008; Dukes et al., 2006). Both SNX1 and SNX3 initiate elongation of the *Salmonella* vacuolar membranes into short tubules and foster the recruitment of late endosomal/lysosomal proteins such as Rab7 and LAMP1 (Braun et al., 2010).

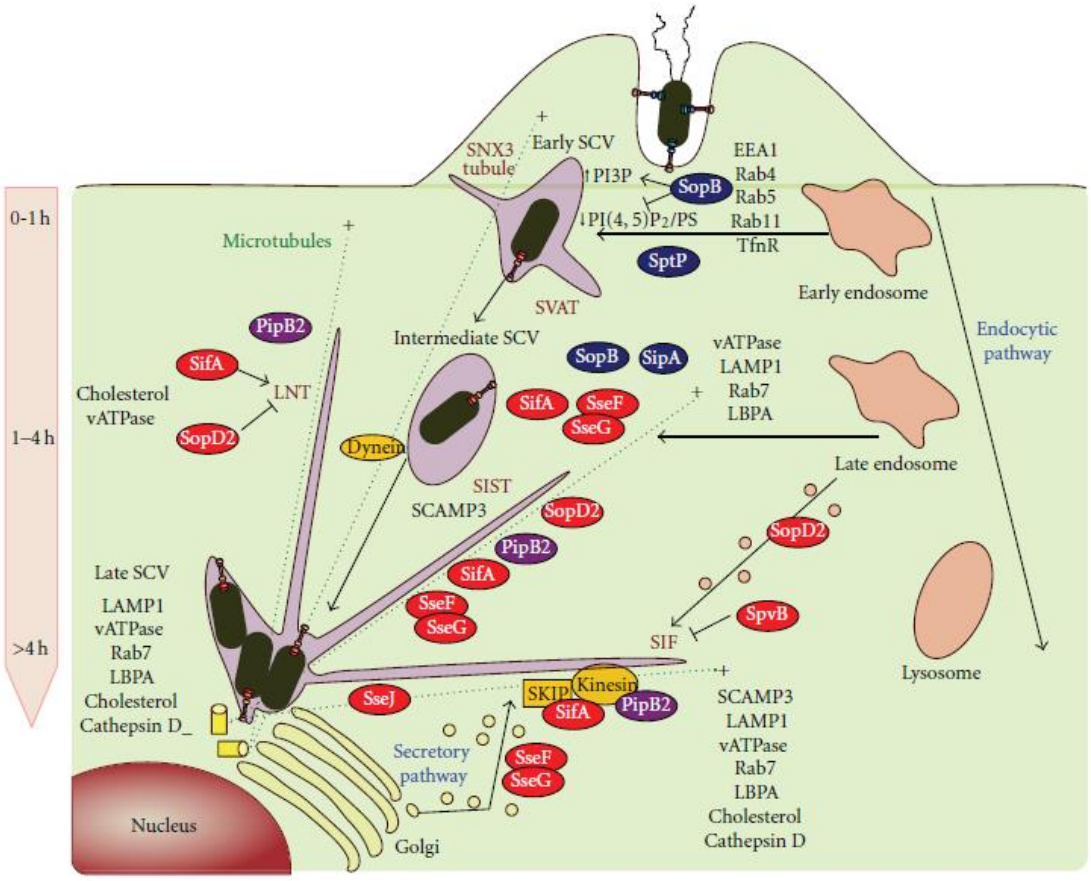


Figure 1.10: SCV maturation and biogenesis of SITs. After internalization, *Salmonella* establishes a replicative niche inside a modified phagosome (SCV). The early steps of SCV biogenesis (0-1 h) are directed by SPI1-T3SS

effectors (in blue) involving the formation of SVATs and SNX3 tubules. During the intermediate stages of development (1-4h), the SCV moves to a juxtanuclear position which is achieved through concerted actions of both SPI1-T3SS effectors (in blue) and SPI2-T3SS effectors (in red). Final stages of maturation and maintenance of a stable vacuole are mainly mediated by SPI2-T3SS effectors. *Salmonella* replication is initiated after a lag of 4–6 h after invasion and are characterized by the formation of three distinct kinds of tubules (SIFs, SISTs, and LNTs) through extensive interactions with the host endocytic and secretory pathways. Effectors involved in the formation of these tubules are depicted in red (SPI2-T3SS effectors) and in purple (effectors of both systems). *Adapted from: (Ramos-Morales, 2012) Copyright © 2012 Francisco Ramos-Morales.*

1.6.2 SCV maturation and formation of *Salmonella*-induced tubular networks (SITs)

As they mature, *Salmonella* phagosomes acquire late endocytic marker proteins such as lysosomal glycoproteins (LAMP1, LAMP2 and LAMP3/CD63), vacuolar-ATPases, Rab7, Rab9 and RILP as soon as 30 mins p.i. (post-invasion) through interactions with late endocytic compartments; while the early, sorting and recycling membrane markers are lost from the SCV membrane during this phase (Beuzón et al., 2000; Drecktrah et al., 2007b; Garcia-del Portillo and Finlay, 1995; Méresse et al., 1999b; Steele-Mortimer et al., 1999). Interestingly, certain late endocytic markers such as lysobisphosphatidic acid (LBPA), lysosomal hydrolases (cathepsins) and Mannose-6-phosphate receptors (M6PRs) are selectively excluded from the maturing SCV membranes (Brumell et al., 2001c; Knodler and Steele-Mortimer, 2005; Méresse et al., 1999a). The recruitment of lysosomal glycoproteins to these maturing SCVs is mainly brought about by Rab7, the principal small GTPase operating at late endocytic vesicles (late endosomes and lysosomes). Rab7 along with its effector RILP can recruit the motor protein dynein to the *Salmonella* vacuolar membranes to impel the centripetal displacement of SCVs and situating them in the vicinity of the Golgi network by ~2 hr p.i.

During the intermediate stages of SCV maturation *Salmonella* shifts gears and upregulates the expression of the second T3SS. The SCV has limited supply of nutrients and as it matures the pH of the vacuole decreases further leading to acidification of the vacuole along with a decrease in Mg^{2+} concentration and accumulation of anti-microbial peptides in the lumen of these compartments; generating stressful conditions. These environmental cues act as signals to stimulate the transcription of SPI2 type III secretion system genes forming pore-like structures

at the SCV membranes (SPI2-T3SS). SPI2-T3SS is induced at approximately 3-4 hr post-infection, and this system operates from the membrane of the maturing *Salmonella* vacuole. The second T3SS galvanizes the maturation of SCVs by secreting another cohort of virulence determinants known as SPI2-T3SS effectors that enhance interactions of the *Salmonella* phagosomes with the late endocytic compartments and facilitates the systemic spread of the infection. Likewise, SPI1-T3SS effectors, the secretion of SPI2-T3SS effectors also requires a functional translocon complex made up of three major proteins: SseB, SseC and SseD; embedded in the SCV membranes (Nikolaus et al., 2001).

The concerted activities of at least eight SPI2-T3SS secreted virulence factors (SifA, SseJ, SopD2, PipB2, SseF, SseG, SpvB, and SteA); modify the lipid and protein repertoire of the SCV membranes and manipulate the cellular transport machinery to establish an interconnected tubular network. The later stages of the intracellular life cycle of *Salmonella* (>5hr post infection) are characterised by the microtubule-dependent elongation of the vacuolar membrane into a dynamic tubular network known as *Salmonella*-induced filaments (or SIFs) (discussed in detail in the subsequent section and Chapter 4 of the thesis).

The two SPI2-T3SS effectors SseF and SseG are integral membrane proteins that play prominent roles during this stage by recruiting dynein motor complex onto the SCV membranes (Abrahams et al., 2006; Ramsden et al., 2007). Recruitment of dynein mediates the centripetal migration of the maturing SCVs along the microtubule tracks placing them in a juxtanuclear position and anchoring them to the Golgi network (Salcedo and Holden, 2003). The placement of the SCVs in Golgi vicinity is followed by the replication of *Salmonella* and formation of "clustered" microcolonies encouraging nutrient and membrane acquisition through interactions with the intense vesicular network in this region (Abrahams et al., 2006; Figueira et al., 2013; Salcedo and Holden, 2003).

Owing to the selective pressures to maintain a stable vacuolar membrane while avoiding fusion with the terminal degradative compartments, *Salmonella* secretes a pleiotropic virulence protein known as SifA that is elementary to the establishment of successful intracellular infection. SifA complexes with a host protein SKIP (**S**ifA and **K**inesin1 **I**nteracting **P**rotein) on the SCV membranes where it functions to detoxify the lysosomes and also co-operates with other SPI2-T3SS effectors, SseJ and SopD2 to stabilise the SCV membrane shielding the bacteria from toxic effects of lysosomes and bactericidal cytosolic environment of macrophages (Beuzón et al., 2000; Ruiz-Albert et al., 2002; Schroeder et al., 2010; Stein et al., 1996).

SKIP (a.k.a PLEKHM2) was first discovered in a yeast-two hybrid screen carried out by Buocrot and co-workers to identify the eukaryotic target proteins of the bacterial effector protein, SifA. Three independent cDNA clones were isolated during the screens which were ultimately found to encode for the C-terminal region of an uncharacterized eukaryotic protein which was named as **S**ifA and **K**inesin1 **I**nteracting **P**rotein (SKIP) owing to its interactions with the above-mentioned binding proteins (Boucrot et al., 2005a). The domain architecture of this protein revealed the presence of at least two functional domains: a RUN (RPIP8, UNC-14 and NESCA) and WD-40 repeats domain at the N-terminus and a pleckstrin homology (PH) domain at the C-terminus. The WD-40 repeat domain of SKIP protein interacts with the TPR domain in the Kinesin1 light chain (Sanger et al., 2017). Under physiological conditions, SKIP (binding through RUN-domain) acts as an effector of the lysosomal small GTPase, Arl8b. Arl8b and SKIP together co-ordinate and interact with Kinesin1 forming a complex that links the lysosomes to the microtubules fuelling the displacement of lysosomes away from the microtubule organising centre (MTOC or the minus-end) towards the cell periphery (Rosa-Ferreira and Munro, 2011b). However, in *Salmonella*-infected cells SKIP has been shown to be a target of SifA and where this protein regulates rapid removal of Kinesin from the

Salmonella vacuolar membranes enabling fission of the kinesin-positive vesicles from the surface of the SCVs. These instant membrane exchange events promote the elongation of the phagosomal membranes along the microtubules and drive plus-end directed motility of the kinesin-positive SCVs mediating cell to cell transfer of the bacteria (Boucrot et al., 2005a; Dumont et al., 2010b).

Lysosomal GTPase, Arl8b has been observed to associate with the SCV membranes during maturation, and is also present on the membrane tubules originating from *Salmonella* endosomes (both LAMP1-positive and LAMP1-negative tubules). On the SCV membranes, this lysosomal GTPase has been shown to be involved in the recruitment of kinesin-1 motor; thereby promoting SIF extension in *Salmonella* infected epithelial cells. Additionally, similar to its function in regulating the anterograde transport of late endocytic vesicles along microtubules, Arl8b also assists in the movement of the SCV compartments towards the cell periphery at later time points (i.e. 20 hours post infection) contributing to *Salmonella* dispersal and invasion of the adjacent uninfected cells (Kaniuk et al., 2011a).

The mature or "late" SCV is, therefore, a replication-competent vacuolar microenvironment primarily enriched with late endosomal/lysosomal proteins (LAMPs, Arl8b) and shows retrograde displacement towards the cell periphery which indeed is essential for bacterial dispersal and propagation of infection later during infection.

The intravacuolar replication of *Salmonella* creates a dire need to partition the existing vacuolar membrane so that each daughter bacterium is enclosed within an individual vacuole. *Salmonella* ensures frequent SCV partitioning through coordinated actions of two SPI-2 effectors, SifA and SteA. The interaction of SifA with a host adaptor protein, PLEKHM1 (having a domain architecture similar to PLEKHM2/SKIP) has been shown to regulate this process as in the absence of this protein the bacteria were found to accumulate in tight clusters in giant "bag-like" vacuoles due to improper partitioning of the SCV membranes (McEwan et

al., 2015c). Likewise, mutant *Salmonella* strains lacking the core virulence factor *steA* also display a phenotype similar to PLEKHM1-depletion thus, contributing to SCV membrane segregation to accommodate the dividing bacterial population (Domingues et al., 2014).

1.6.3 *Salmonella*-Induced tubular (SIT) Networks

A peculiar yet fundamental feature of *Salmonella's* intracellular survival strategy is the generation of an interconnected meshwork of membranous tubules originating from and linking the SCVs; collectively known as *Salmonella*-induced tubules (SITs). SITs are formed by metamorphosis of the host sub-cellular membranes by *Salmonella* into tubular structures often extending across the cytoplasm of the cell and appearing at different stages during infection. These diverse atypical membranous reticulations have been extensively studied in the *in vitro* model systems mainly immortalised epithelial cell lines. But now SITs have also been reported in the interferon-gamma activated macrophage-like cell line RAW264.7, mouse peritoneal macrophages and bone-marrow derived peritoneal macrophages.

Depending on the marker proteins associated with their membrane surfaces and their temporal emergence during *Salmonella* infections, SITs are further sub-divided into four major categories which include: Early or Sorting nexin (SNX) tubules, *Salmonella*-induced SCAMP3 tubules (SISTs), *Salmonella*-induced filaments (SIFs) and LAMP1-negative tubules (LNTs) (Beuzón et al., 2000; Garcia-del Portillo et al., 1993a; Mota et al., 2009; Schroeder et al., 2010; Schroeder et al., 2011).

The sorting nexin (SNX) tubules appear early during infection (15-60 min post-invasion), and their formation is induced by the SPI1-T3SS effector, SopB. SNX1 mediated tubules are formed within the first few minutes after the invasion and are also known as spacious vacuole-associated tubules (SVATs). The formation of these tubules leads to a reduction in the size of nascent *Salmonella* vacuoles and promote removal of M6PRs from these membranes (Braun et al., 2010; Bujny et al., 2008). Subsequently, SNX3 associates with the SCV surface and

initiates formation of short tubules that originate at approximately 30min post infection. Biogenesis of SNX3 tubules is essential for promoting the association of Rab7 and LAMP1 to the surface of the maturing SCVs and mediate the transportation of the *Salmonella* vacuoles towards the bacterial replication site (Braun et al., 2010).

Amongst SITs, the most prominent and extensive ones are the LAMP1-enriched tubules known as *Salmonella*-induced filaments (SIFs) which are often regarded as the hallmark of *Salmonella* infection. SIFs were the first *Salmonella*-induced tubular structures reported by Garcia-del Portillo in the year 1993 (Garcia-del Portillo et al., 1993a). Since their discovery SIFs have gathered enough limelight to the *Salmonella* biology ensuing the interest of many cell biologists working in this field of research and till now SIFs remain the most widely studied tubular networks generated by this invasive intruder. These tubules are much more complex, thicker, longer, dynamic yet stable than the inherent tubules observed in normal cells like lysosomal extensions or early endosomal tubular domains. The formation of SIFs is crucial for the development of *Salmonella*'s replicative niche which enables this foreign invader to thrive in the hostile environment of host cells. Recent studies have now confirmed that SIFs are essential to maintain a constant supply of nutrients to the SCVs and promote intravacuolar bacterial replication (Liss et al., 2017b; Popp et al., 2015a).

SIF biogenesis is concomitant with the end of the lag phase and marks the beginning of *Salmonella* proliferation within the host cells. The composition of SIFs is similar to that of the mature SCV membranes and likewise, these tubules are also decorated with the SPI2-T3SS effector proteins (SifA, PipB2, SseG, SseF) and lysosomal markers (Lgps, Rab7, Arl8b, vATPase, cholesterol and low levels of cathepsin D) of which LAMP1 is the most abundant protein associated with these membranous compartments; and therefore, widely used as a marker protein for these tube-like structures. Maximal SIF formation is observed between 8-9 hr post-invasion. Biogenesis of SIFs involves the concerted and antagonistic actions of many SPI2 effectors such as SifA, pipB2, SseJ, SopD2, SseF, SseG (Knuff and Finlay, 2017;

Schroeder et al., 2011). The particulars of SIF architecture and the coordinated interplay of specific *Salmonella* effectors and host proteins contributing to SIF biogenesis has been described in Chapter 4 of the thesis.

The role of these effectors in SIF formation has been widely studied, and still, there are many continuous revelations regarding them. But still there are many loopholes in the current information available regarding these extensive filamentous networks for instance; the dynamic and complex interplay between *Salmonella* effectors and their host counterparts resulting in the formation of these tubules is still unclear. Although it has been reported that SIF formation is vital for *Salmonella* survival and replication during in vitro infections; the *in vivo* data supporting this evidence is still lacking. SIFs have been observed upon infection of in vitro tissue culture models by *S. Typhimurium*, but the formation of these unique structures have not been reported in the case of human-restricted serovar *S. typhi* until now.

Recent studies have suggested the exploitation of proteins involved in the secretory pathway for the biogenesis of a subset of SITs known as *Salmonella*-induced secretory carrier membrane protein 3 (SCAMP3) tubules (SISTs). The major constituent of SIST membranes is an integral membrane protein found in TGN, sorting and recycling endosomal membranes SCAMP3; indicating that the membrane for SIST formation is derived from these compartments. Likewise, SIFs, the development of SISTs also requires SPI2-T3SS effectors including SifA, PipB2, SopD2, SseF and SseG (Mota et al., 2009).

The infection of host cells with *Salmonella* strains deficient in the two SPI2-T3SS effectors (*sifA*$^{-}$*sopD2*$^{-}$) essential for SIF formation have illuminated the existence of tubules originating from the SCV membranes that are LAMP1-negative but are characterised by the presence of other effector proteins such as PipB2. LNTs (or LAMP1-negative tubules) also undergo kinesin-dependent extension along the microtubules and are believed to be the precursors for SIF formation (Schroeder et al., 2010).

1.7 Rationale and Aim of the thesis

During its intracellular lifecycle, *Salmonella* redirects membranes and nutrients from the host cell's recycling and sorting compartments to the SCV by generating an interconnected network of tubules, also known as *Salmonella*-induced filaments (SIFs) that form a continuum with the SCVs. In order to establish this replication competent microenvironment, *Salmonella* extensively interacts with the components of the endolysosomal system including late endosomes and lysosomes. Although fusion of *Salmonella* phagosomes with lysosomes can be readily observed by live-cell imaging microscopy; little is known about the specific cellular components targeted by the secreted bacterial virulence proteins to instigate the fusogenic activity and recruitment of membrane and nutrients from these compartments for *Salmonella* replication and SIF biogenesis. The extensive recruitment of the components of late endosomal/lysosomal fusion machinery such as small GTPases: Rab7 and Arl8b to *Salmonella* vacuoles has instilled a view that the effector proteins of these GTPases might also be recruited by the bacteria to drive these dynamic intracellular remodelling events. Several studies have previously reported that *Salmonella* does hijack many of such effectors, for instance, RILP, SKIP and PLEKHM1 to promote SCV motility at different stages during infection.

With this in mind, we intended to explore the role of an essential component of fusion machinery, HOPS complex which catalyses most of the fusion events occurring at the lysosomal membranes during *Salmonella* maturation and trafficking. HOPS complex tethers the membranes of distinct cargo-enriched vesicular compartments destined for fusion with the lysosomes bringing them in close proximity to these lytic organelles and expedites the recruitment of SNARE proteins that physically drive membrane amalgamation and luminal mixing. Being an effector of the lysosomal GTPase Arl8b and an interaction partner of Rab7 (through RILP); HOPS is capable of mediating both homotypic and heterotypic fusion processes. Therefore, we hypothesised that HOPS could be a potential target of *Salmonella* effectors to bring about the dynamic and frequent interactions between SCVs and late endosomes/lysosomes which indeed is crucial for *Salmonella* virulence.

To address this, we directed our efforts towards: 1) Analyses and quantification of changes in the subcellular distribution of HOPS subunits in *Salmonella* infected cells. 2) We have also tried to understand the physiological impact of the altered distribution of HOPS complex on SCV maturation and *Salmonella* survival.

The lumen of the maturing SCVs acidifies and signals the transcription and assembly of SPI2-T3SS. Following recognition of the neutral cytosolic pH inside the host cell, the SPI2-T3SS translocon initiates the release of a multitude of effector proteins that intercept with various host signalling pathways. Given the fact that, much of *Salmonella*'s ability to modulate the intracellular environment from within the SCV is dependent on the SPI2-T3SS effectors and some of these proteins are known to target the proteins regulating the endolysosomal trafficking pathways; in the final part of the thesis we also aimed to investigate/assess the influence of these virulence factors on the re-distribution of this lysosomal tethering protein complex to mature SCVs and SIFs.

CHAPTER II

Material and Methods

Chapter 2

2.1 Cell Culture

HeLa, HEK293T, and RAW264.7 cells were obtained from the American Type Culture Collection and maintained in DMEM (Lonza) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Life technologies) at 37ºC in 5% CO_2 humidified incubator. All the cultures were used between passage numbers 5-15.

An Arl8b-KO HeLa cell line was previously described (Marwaha et al., 2017). Arl8b-knockout (KO) HeLa cells were generated using the Arl8b sg/RNA (Target sequence: 5′-GATGGAGCTGACGCTCG-3′) CRISPR/Cas9 All-in-One Lentivector Set (Applied Biological Materials). For stably silencing the expression of Vps41 in RAW264.7 cells, lentivirus mediated shRNA gene silencing approach was used. Briefly, for lentiviral transduction, RAW264.7 cells were seeded in a 35-mm tissue culture dish (Corning) in Polybrene (8 μg/ml; Sigma-Aldrich) and mixed with 500 μl of viral supernatant (day 0). Puromycin (Sigma-Aldrich) was added after 24-48 hr at 5 μg/ml for a minimum of 3 days to select transductants, and experiments were performed on days 5-15 after transduction. shRNA target sequences were as follows: Mission (negative control sequence), CAACAAGATGAAGAGCACCAA and mouse Vps41, GAGTGGCCTGGAGATCTATAT. Development of HeLa-Vps41 shRNA cell line was previously described using Vps41 shRNA, 5′-CCATTGACAAACCACCATTTA-3′ (Khatter et al., 2015b).

Primary mouse embryonic fibroblast (MEF) cells were isolated from the embryos of BALB/c mouse. Briefly, embryos were harvested from female mice 15 days after the appearance of the copulation plug. Embryos were placed in 1 ml of 0.05% trypsin/EDTA solution (Life technologies) and finely minced using a sterile razor blade and repeated pipetting was performed to dissociate cells. The trypsin was inactivated by adding DMEM supplemented with 10% FBS and the culture was centrifuged to pellet MEF cells. The pelleted MEF cells

were resuspended in culture media, and plated at optimal density in tissue culture dishes at 37^{o}C in 5% CO_2 humidified incubator.

2.2 Antibodies and Chemicals

The following antibodies were used in this study: mouse anti-FLAG M2 clone (F1804; Sigma-Aldrich), mouse anti-HA (MMS-101P; Covance), rabbit anti-HA (sc-805; Santa Cruz Biotechnology), rat anti-HA clone 3F10 (11867423001; Roche), mouse anti-Myc 9E10 clone (sc-40; Santa Cruz Biotechnology), mouse anti-α-tubulin (T9026; Sigma-Aldrich), mouse anti-GAPDH (sc-166574; Santa Cruz Biotechnology), mouse anti-EEA1 (610457; BD Biosciences), rabbit anti-EEA1 (ab2900; Abcam), mouse anti-LAMP1 (555798; BD Biosciences), rabbit anti-LAMP1 (ab24170; Abcam), rabbit anti-PLEKHM1 (ab171383; Abcam), rabbit anti-SKIP/PLEKHM2 (HPA032304; Sigma-Aldrich), mouse anti-TGFBRAP1 (sc-13134; Santa Cruz Biotechnology), mouse anti-LBPA (Z-PLBPA; Echelon Biosciences), rabbit anti-Catalase (12980; Cell Signaling Technology), rabbit anti-Rab5 (3547; Cell Signaling Technology), rabbit anti-Rab7 (9367; Cell Signaling Technology), rabbit anti-Cathepsin D (K50161R; Meridian Life Sciences), mouse anti-Cathepsin B clone 4B11 (414800; Thermo Fisher Scientific), rabbit anti-*Salmonella* O-antigen (225341; BD Biosciences), and mouse anti-DnaK (ADI-SPA-880-F; Enzo Life Sciences). Rabbit anti-PLEKHM1 antibody generated against the N-terminal 497 amino acids of human PLEKHM1 protein was a gift from Prof. Paul Odgren (University of Massachusetts Medical School, Worcester, MA) and has been previously used to detect PLEKHM1 by immunofluorescence and Western blotting (Marwaha et al., 2017; Witwicka et al., 2015). Rabbit anti-Arl8 antibody used in this study has been described previously (Garg et al., 2011a).

For detection of HOPS subunits, the following antibodies were used: rabbit anti-Vps11 (ab125083; Abcam), rabbit anti-Vps18 (ab178416; Abcam), rabbit anti-Vps33a (16896-1-AP;

ProteinTech), rabbit anti-Vps41 (ab181078; Abcam), and mouse anti-Vps41 (sc-377271; Santa Cruz Biotechnology).

All the Alexa fluorophore-conjugated secondary antibodies were purchased from Molecular Probes (Thermo Fisher Scientific). HRP-conjugated goat anti-mouse and goat anti-rabbit were purchased from Jackson ImmunoResearch Laboratories. Alexa Fluor 647-conjugated Dextran, LysoTracker Red DND-99 and DAPI were purchased from Molecular Probes (Thermo Fisher Scientific). L-Proline, Cytochalasin D, Bafilomycin A1, Polybrene, Streptomycin, Gentamicin and Puromycin were purchased from Sigma-Aldrich. Yeast drop-out media were purchased from Clontech.

2.3 Bacterial Strains, Plasmids and Infections

All the *Salmonella Typhimurium* strains and plasmids used in this study are described in **Table 1.** For infection of HeLa and MEF cells, late-log *S. Typhimurium* cultures were used and prepared using a method optimized for bacterial invasion (Steele-Mortimer et al., 1999). Briefly, wild-type and mutant bacteria were grown for 16 hr at 37°C with shaking and then subcultured (1:33) in LB (Difco) without antibiotics and grown until late exponential phase (O.D. = 3.0). Bacterial inocula were prepared by pelleting at 10,000 x g for 2 min, diluted 1:100 in PBS (pH 7.2), and added to cells (at the specified MOI) for 10 min at 37°C to allow invasion and synchronized infection. After infection, extracellular bacteria were removed by extensive washing using warm PBS and 50 μg/ml gentamicin was added to the medium at 30 min p.i. for incubation at 37°C. After 2 hr p.i., the concentration of gentamicin in the medium was decreased to 5 μg/ml. Following this infection protocol, cells were processed for microscopy and biochemical experiments as described in the individual figure legends.

For infections of RAW264.7 cells, stationary-phase bacterial cultures incubated at 37°C with shaking were diluted (O.D. = 1) and opsonized in PBS supplemented with 20% FBS for 20 min at 37°C. After three washes in PBS, bacteria were resuspended in growth medium without

antibiotics, and added to the cells (MOI of 50:1) for 20 min to facilitate phagocytosis. The remaining protocol was similar as in case of infection of HeLa cells.

Table 2.1: Descriptions of the strains of *S. Typhimurium* and the plasmids used in this study.

Strains or Plasmids	***Description***	***Source***
Salmonella Typhimurium strains:		
S. Typhimurium SL1344	Wild-type (WT); Streptomycin 50 μg/ml	Kind gift from Dr. John Brumell and described previously (Brumell et al., 2002a).
GFP-*S. Typhimurium* SL1344	*S. Typhimurium* SL1344 transformed with GFP expressing plasmid pFU95; Streptomycin 50 μg/ml Ampicillin 50 μg/ml	This Study
DsRed-*S. Typhimurium* SL1344	*S. Typhimurium* SL1344 transformed with DsRed expressing plasmid pFU96;	This Study

	Streptomycin 50 μg/ml Ampicillin 50 μg/ml	
S. Typhimurium SL1344 Δ*sifA*	*S. Typhimurium* SL1344 with chromosomal deletion of SifA gene; Streptomycin 50 μg/ml	Kind gift from Dr. John Brumell and described previously (Brumell et al., 2002a).
S. Typhimurium SL1344 Δ*sifA*/psifA-2HA	*S. Typhimurium* SL1344 with chromosomal deletion of SifA gene complemented with a plasmid encoding epitope tagged SifA; Chloramphenicol 34 μg/ml	Kind gift from Dr. John Brumell and described previously (Brumell et al., 2002a).
S. Typhimurium SL1344 Δ*sifA*/psifA (L130D)-2HA	*S. Typhimurium* SL1344 with chromosomal deletion of SifA gene complemented with a plasmid encoding epitope tagged SifA having point mutation at amino acid residue position 130 (L to D);	This study

	Streptomycin 50 µg/ml Chloramphenicol 34 µg/ml	
S. Typhimurium NCTC 12023	Wild-type (WT)	Kind gift from Dr. Michael Hensel and described previously (Popp et al., 2015b).
DsRed-*S. Typhimurium* NCTC 12023	*S. Typhimurium* NCTC 12023 transformed with DsRed expressing plasmid pFU96; Ampicillin 50 µg/ml	This Study
S. Typhimurium NCTC 12023 (Δ*sifA*::FRT Δ*sseJ*::FRT)	*S. Typhimurium* NCTC 12023 with a chromosomal deletion of effectors SifA and SseJ	Kind gift from Dr. Michael Hensel and described previously (Popp et al., 2015b).
S. Typhimurium NCTC 12023 (Δ*proC*::FRT)	*S. Typhimurium* NCTC 12023 with a chromosomal deletion of	Kind gift from Dr. Michael Hensel and described

	Pyrroline-5-carboxylate reductase gene	previously (Popp et al., 2015b).
S. Typhimurium SL1344 (Δ*sseF*::aphT)	*S. Typhimurium* SL1344 with a chromosomal deletion of effector SseF; Streptomycin 30 µg/ml Kanamycin 50 µg/ml	Kind gift from Dr. Michael Hensel.
S. Typhimurium SL1344 Δ*sseF*/Pro$_{sseA}$*sscBsseF*::HA	*S. Typhimurium* SL1344 with chromosomal deletion of SseF gene complemented with a plasmid encoding HA-epitope tagged SseF; Streptomycin 30 µg/ml Kanamycin 50 µg/ml Carbenicillin 50 µg/ml	This study
S. Typhimurium SL1344 (Δ*sseG*::aphT)	*S. Typhimurium* SL1344 with a chromosomal deletion of effector SseG; Streptomycin 30 µg/ml Kanamycin 50 µg/ml	Kind gift from Dr. Michael Hensel.

S. Typhimurium SL1344 (Δ*pipB2*::aphT)	*S. Typhimurium* SL1344 with a chromosomal deletion of effector PipB2; Streptomycin 30 μg/ml Kanamycin 50 μg/ml	Kind gift from Dr. Michael Hensel.
Yeast two-hybrid constructs:		
pGADT7 vector	GAL4-activation domain encoding yeast two-hybrid vector	Clontech
pGADT7-Vps39	Human Vps39 cloned into the pGADT7 vector	Described previously (Khatter et al., 2015b).
pGADT7-PLEKHM1	Human PLEKHM1 cloned into the pGADT7 vector	Described previously (Marwaha et al., 2017).
pGADT7-SKIP	Human SKIP cloned into the pGADT7 vector	Described previously (Khatter et al., 2015b).

pGBKT7 vector	GAL4-DNA binding domain encoding yeast two-hybrid vector	Clontech
pGBKT7-SifA	SifA cloned into the pGBKT7 vector	This study
Yeast three-hybrid constructs:		
pBridge vector	Yeast three-hybrid vector	Clontech
pBridge-SifA	SifA (1-330 aa) cloned into the MCS-I of the pBridge vector	This study
pBridge-SifA/SKIP	SifA (1-330 aa) cloned into the MCS-I and full-length SKIP cloned into the MCS-II of the pBridge vector	This study
pBridge-SifA/SKIP (G828D)	SifA (1-330 aa) cloned into the MCS-I and SKIP with point mutation at amino acid position 828 changing G with D, cloned into the MCS-II of the pBridge vector	This study
pBridge-SifA/PLEKHM1	SifA (1-330 aa) cloned into the MCS-I and full-length	This study

	PLEKHM1 cloned into the MCS-II of the pBridge vector	
Mammalian expression constructs:		
pcDNA3.1(-) vector	Mammalian expression vector	Invitrogen
pcDNA3.1(-)- FLAG- TGFBRAP1	N-terminal FLAG-tagged human TGFBRAP1 cloned into the pcDNA3.1(-) vector	This study
pcDNA3.1(-)- FLAG- PLEKHM1	N-terminal FLAG-tagged human PLEKHM1 cloned into the pcDNA3.1(-) vector	Described previously (Marwaha et al., 2017).
pEGFPC1-PLEKHM1	N-terminal GFP-tagged human PLEKHM1 cloned into the pEGFPC1 vector	Described previously (Marwaha et al., 2017).
pcDNA3.1(-)-FLAG-SKIP	N-terminal FLAG-tagged human SKIP cloned into the pcDNA3.1(-) vector	Described previously (Marwaha et al., 2017).
pcDNA3.1(-)-siRNA-resistant-FLAG-SKIP	N-terminal FLAG-tagged full-length rescue construct against	Described previously

	SKIP-siRNA cloned into the pcDNA 3.1(-) vector	(Khatter et al., 2015b).
pEGFPC1-SKIP	N-terminal GFP-tagged human SKIP cloned into the pEGFPC1 vector	Described previously (Marwaha et al., 2017).
pcDNA3.1(+)-Arl8b untagged	Human Arl8b cloned into the pcDNA3.1(+) vector	Described previously (Marwaha et al., 2017).
pcDNA3.1(+)-Mouse Arl8b-GFP	Full-length mouse Arl8b with C-terminal GFP tag cloned into the pcDNA3.1(+) vector	Described previously (Marwaha et al., 2017).
pEGFPC1-Rab7	N-terminal GFP-tagged human Rab7 cloned into the pEGFPC1 vector	Kind gift from Dr. Vesa Olkkonen and described previously (Johansson et al., 2005).

pEBB-HA-Rab7	Full-length human Rab7 with N-terminal HA tag cloned into the pEBB vector	Kind gift from Dr. Jason Kinchen and described previously (Kinchen and Ravichandran, 2010).
pcDNA3.1(-)-HA-Vps41	N-terminal HA tagged human Vps41 cloned into the pcDNA3.1(-) vector	Described previously (Khatter et al., 2015b).
pEGFPC1-Vps41	N-terminal GFP-tagged human Vps41 cloned into the pEGFPC1 vector	This study
pcDNA3.1(-)-HA-Vps39	N-terminal HA tagged human Vps39 cloned into the pcDNA3.1(-) vector	Described previously (Khatter et al., 2015b).
pcDNA3.1(-)-Vps33a-HA	C-terminal HA tagged human Vps33a cloned into the pcDNA3.1(-) vector	Described previously (Khatter et al., 2015b).

GFP-LAMP1	GFP-tagged Lgp120 (rat lamp-1)	Kind gift from Dr. Steve Caplan and described previously (Caplan et al., 2001b).
pEGFPC1-SifA	SifA cloned into the pEGFPC1 vector	Kind gift from Dr. Samuel Miller and described previously (Brown et al., 2006).
Myc-SifA	SifA cloned into the pCMV-myc vector	Kind gift from Dr. Samuel Miller and described previously (Ohlson et al., 2008b).
Myc-SifA (L130D)	Myc-SifA construct with point mutation at amino acid residue position 130 (L to D) of SifA	This Study

Myc-SifA (M131D)	Myc-SifA construct with point mutation at amino acid residue position 131 (M to D) of SifA	This Study
Bacterial expression constructs:		
pGEX-5X-1-SifA	SifA cloned in pGEX-5-X-1 vector	Kind gift from Dr. Kasturi Haldar and described previously (Jackson et al., 2008).
pFU95	Ap^r; *gapA-rbs-gfpmut3.1*; ColE1	Kind gift from Dr. Petra Dersch and described previously (Uliczka et al., 2011).
pFU96	Ap^r; *gapA-rbs-dsred2*; ColE1	Kind gift from Dr. Petra Dersch and described previously

		(Uliczka et al., 2011).
pSifA-2HA	2 HA-tags SifA cloned into the pACYC184 vector	Kind gift from Dr. John Brumell and described previously (Brumell et al., 2002a).
pSifA (L130D)-2HA	psifA-2HA construct with a point mutation at amino acid residue 130 (L to D) of SifA	This Study
Pro$_{sseA}$*sscBsseF*::HA	pWSK29 plasmid containing expression cassette for HA epitope-tagged SseF effector	Kind gift from Dr. Michael Hensel and described previously (Kuhle et al., 2004).

2.4 Transfections and RNAi

Cells grown on glass coverslips (VWR) were transfected with desired constructs using X-tremeGENE-HP DNA transfection reagent (Roche) for 16-18 hr. For gene silencing, siRNA duplexes for non-targeting siRNA pool, control siRNA (5′-TGGTTTACATGTCGACTAA-

3′), human Arl8b (5′-AGGTAACGTCACAATAAAGAT-3′), human Rab7a (5′-CTAGATAGCTGGAGAGATG-3′) human Vps11 (5′-GAGGCTGAGCTGAGCCTCGTATT-3′), human Vps18 (5′-CTAGATAGCTGGAGAGATG-3′), human Vps33a (5′-CATTGCAGTGTTGCCTCGATATG-3′), human Vps39 (ON-TARGET plus SMART pool), mouse Vps39 (ON-TARGET plus SMART pool), human Vps41 (5′-CCATTGACAAACCACCATTTA-3′), mouse Vps41 (ON-TARGET plus SMART pool), human PLEKHM1 (5′-CCGGTCTCTGCAAGAGGTATTGT-3′), human SKIP (5′-CTTCTGAACTGGACCGATT-3′), human Vti1b (ON-TARGET plus SMART pool), human Stx8 (ON-TARGET plus SMART pool), human Stx17 (ON-TARGET plus SMART pool), human Vamp7 (ON-TARGET plus SMART pool) and human TGFBRAP1 (ON-TARGET plus SMART pool) were purchased from GE Healthcare (Dharmacon), and transfection was performed using Dharmafect 1 as per the manufacturer's instructions.

2.5 Immunofluorescence Staining, Confocal Microscopy and Colocalization Analysis

Cells were fixed in 4% p-formaldehyde (PFA) in PHEM buffer (60 mM PIPES, 10 mM EGTA, 25 mM HEPES, and 2 mM $MgCl_2$, final pH 6.8) for 10 min at room temperature. Post fixation, cells were incubated with blocking solution (0.2% saponin + 5% FBS in PHEM buffer) at room temperature for 30 min, followed by three washes with 1X PBS. After this blocking step, cells were incubated with primary antibodies in staining solution (PHEM buffer + 0.2% saponin) for 1 hr at room temperature, washed thrice with 1X PBS, and further incubated for 30 min with Alexa fluorophore-conjugated secondary antibodies made in staining solution. Cells were washed thrice with 1X PBS and mounted in Fluoromount G (Southern Biotech). Single-plane confocal images were acquired using a 710 Confocal Laser Scanning Microscope (ZEISS) equipped with a Plan Apochromat 63×/1.4 NA oil immersion objective and high-resolution

microscopy monochrome cooled camera AxioCamMRm Rev. 3 FireWire (D) (1.4 megapixels, pixel size 6.45 µm × 6.45 µm). For image acquisition, ZEN Pro 2011 (ZEISS) software was used. All images were captured to ensure that little or no pixel saturation is observed. The representative confocal images presented in figures were imported into Adobe Photoshop CS and formatted to 300 dpi resolution. The whole image adjustment of brightness was done using curves function.

For all the colocalization analysis, at least 30 cells for each treatment per experiment were used for three independent experiments. Pearson's Correlation Coefficient (PCC) was determined using the JACoP plugin of ImageJ where the threshold was set using maximum entropy.

2.6 Dextran-647 Uptake and Live-Cell Imaging

In order to trace the endocytic route, HeLa cells were incubated with Alexa-Fluor 647-conjugated dextran (Molecular Probes) for 16-18 hr. The cells were washed once with 1X PBS and infected with GFP-expressing *Salmonella* (MOI 50:1) and further incubated in a dextran-free medium for the rest of the experiment. Live-cell imaging was initiated at indicated time-points.

For live-cell imaging experiments, cells were seeded on glass-bottom tissue culture treated cell imaging dish (Eppendorf) and infected with either DsRed- or GFP- expressing *Salmonella* strains (MOI 50:1) as described above. Post-infection, imaging dish was loaded into a sealed live-cell imaging chamber (37°C and 5% CO_2) for imaging in DMEM. Time-lapse confocal images were acquired at specified time-points using an LSM 710 confocal microscope with an LCI Plan Neofluar objective 63×/1.3 multi-immersion correction and equipped with a high-resolution microscopy monochrome cooled camera AxioCamMRm Rev. 3 FireWire (D). Image acquisition and adjustments to brightness and contrast was performed by using ZEN Pro 2011 software.

2.7 Transmission Electron Microscopy

Sample processing and TEM was performed at the Harvard Medical School EM Facility (Boston, USA). Briefly, control shRNA or Vps41 shRNA transduced HeLa cells were infected with *S. Typhimurium* SL1344 for 10 hr. Post-infection, cells were fixed in routine fixative (2.5% glutaraldehyde/1.25% PFA in 0.1 M sodium cacodylate buffer, pH 7.4) for at least 1 hr at room temperature and washed in 0.1 M sodium cacodylate buffer (pH 7.4). The cells were then post fixed for 30 min in 1% osmium tetroxide/1.5% potassium ferrocyanide, washed in water three times, and incubated in 1% aqueous uranyl acetate for 30 min, followed by two washes in water and subsequent dehydration in grades of alcohol (5 min each: 50, 70, 95, 2× 100%). Cells were removed from the dish in propylene oxide, pelleted at 3000 rpm for 3 min, and infiltrated overnight in a 1:1 mixture of propylene oxide and TAAB Epon (Marivac Canada). The samples subsequently embedded in TAAB Epon and polymerized at 60ºC for 48 hr. Ultrathin sections were cut on a Reichert Ultracut-S microtome, picked up onto copper grids stained with lead citrate, and examined in a JEOL 1200EX transmission electron microscope. Images were recorded with an AMT 2k charge-coupled device camera.

2.8 Double Immunogold EM Labeling

Sample fixation for immunogold EM was carried out as described previously (Marwaha et al., 2017), and double immunogold labeling and imaging was performed at the Harvard Medical School EM Facility (Boston, USA). For preparation of cryosections, control siRNA- and Vps41 siRNA-treated HeLa cells were infected with *S. Typhimurium* as described above. After 2 hr p.i., cells were transfected with HA-Rab7 expressing construct and 10 hr p.i. cells were fixed with 4% PFA + 0.1% glutaraldehyde (Glu) prepared in 0.1 M sodium phosphate buffer, pH 7.4. After 2 hr fixation at room temperature, the cell pellet was washed once with PBS and then placed in PBS containing 0.2 M glycine for 15 min to quench free aldehyde groups. Before freezing in liquid nitrogen, the cell pellets were cryoprotected by incubating in three drops of

2.3 M sucrose in PBS for 15 min. Frozen samples were sectioned at -120°C, and the sections were transferred to formvar/carbon-coated copper grids. Grids were floated on PBS until the immunogold labeling was performed.

The double immunogold labeling was performed at room temperature on a piece of parafilm. All the primary antibodies and Protein A immunogold were diluted in 1% BSA in PBS. In brief, grids were floated on drops of 1% BSA for 10 min to block for unspecific labeling, transferred to 5 µl drops of rat anti-HA, and incubated for 30 min. The grids were then washed in four drops of PBS for a total of 15 min, transferred to 5 µl drops of rabbit anti-rat for 30 min, and washed again in four drops of PBS for 15 min, followed by 15 nm Protein A immunogold for 20 min (5 µl drops). After the 15 nm Protein A immunogold incubation, grids were washed in four drops of PBS, fixed for 2 min with 0.5% Glu followed by four drops of PBS containing 0.2 M glycine for 15 min to quench free aldehyde groups. The labeling process was repeated with rabbit anti-LAMP1 followed by 10 nm Protein A immunogold for 20 min in 5 µl drops. Finally, the grids were washed in four drops of PBS and six drops of double-distilled water. Contrasting/embedding of the labeled grids was performed on ice in 0.3% uranyl acetate in 2% methyl cellulose for 10 min. Grids were picked up with metal loops, and the excess liquids were removed by blotting with a filter paper and were examined in an electron microscope (1200EX; JEOL). Images were recorded with an AMT 2k CCD camera.

2.9 Cell Lysates, Co-immunoprecipitation and Immunoblotting

For lysates, cells were lysed in ice-cold lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitor cocktail). For co-IP experiments, HEK293T cells treated with control or gene-specific siRNA and transfected with indicated plasmids were lysed in ice-cold TAP lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP-40, 1 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, and protease inhibitor cocktail; Sigma-Aldrich). The lysates were incubated with indicated antibody-conjugated agarose beads

at 4°C rotation for 3 hr, followed by four washes in TAP wash buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.1% NP-40, 1 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM NaF, and 1 mM PMSF). The samples were then loaded on SDS-PAGE for further analysis. Protein samples separated on SDS-PAGE were transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories). Membranes were blocked overnight at 4°C in blocking solution (10% skim milk in 0.05% PBS-Tween 20). Indicated primary and secondary antibodies were prepared in 0.05% PBS-Tween 20. The membranes were washed for 10 min thrice with 0.05% PBS-Tween 20 or 0.3% PBS-Tween 20 after 1 hr incubation with primary antibody and 1 hr incubation with secondary antibody, respectively. The blots were developed using a chemiluminescence-based method (Pierce).

2.10 Intracellular Replication Assay

To enumerate intracellular *Salmonella* growth, gentamicin protection assay was performed. Briefly, cells were infected with designated *Salmonella* strain for different time periods using the protocol described above. At the end of every time point p.i. cells were gently washed with PBS followed by lysis using PBS containing 0.1% Triton X-100 and 1% SDS for 5 min at room temperature. The resulting lysates were serially diluted and plated onto LB agar plates containing streptomycin.

2.11 Chloroquine (CHQ) Resistance Assay

To assess cytosolic *Salmonella* replication in HeLa cells upon Vps41 depletion, CHQ resistance assay was performed as described previously (Knodler et al., 2014). Briefly, control siRNA- or Vps41 siRNA-treated HeLa cells were seeded in 24-well plates and infected with *S. Typhimurium* as described above. To evaluate cytosolic replication of *Salmonella*, 1 hr prior to 7 hr p.i. time point, two wells were treated with CHQ (150 µM) and gentamicin (5 µg/ml) for 1 hr (CHQ-resistant bacteria) and another two wells were incubated with gentamicin (5 µg/ml) only (total bacteria). At the end of 7 hr p.i. time point, duplicate gentamicin treated

(total CFU) and duplicate CHQ + gentamicin-treated cells (cytosolic CFU) were solubilized and serial dilutions were plated on LB agar for CFU enumeration.

2.12 *Salmonella* Survival Assay in Morpholino-treated Mice

Six weeks old C57BL/6 male mice were obtained from the CSIR-Institute of Microbial Technology (IMTECH) animal house facility and injected intravenously (i.v.) with 12.5 mg/Kg (of body weight) of either control (CCTCTTACCTCAGTTACAATTTATA) or mouse VPS41-specific (CCATAGCGCAGCCTGAGAGTCAT) vivo-morpholinos (purchased from Gene Tools, LLC) for two consecutive days at an interval of 24 hr, followed by *Salmonella* infection the third day. For *Salmonella* infection, stationary phase culture of *S. Typhimurium* strain SL1344 was diluted to a CFU of ~$1.3X10^3$ in 100 µl of 1X PBS and injected i.v. The infectious dose was quantified by plating plating dilution series on LB agar plates containing streptomycin. Mice were sacrificed after 3 days and dilution series of spleen and liver lysates (prepared in 0.05% sodium deoxycholate in 1X PBS) were plated on LB agar plates containing streptomycin.

2.13 Ethics Statement

This study was carried out in strict accordance to the guidelines issued by the Committee for the Purpose of Supervision of Experiments on Animals (No. 55/1999/CPCSEA) under the Prevention of Cruelty to Animals Act 1960 and amendments introduced in 1982 by Ministry of Environment and Forest, Government of India. All protocols involving mice experiments were approved by the Institutional Animal Ethics Committee (IAEC) of Council of Scientific and Industrial Research-Institute of Microbial Technology (Approval no. IAEC/16/12 and IAEC/17/13).

2.14 Enrichment of *Salmonella*-Containing Vacuoles (SCVs)

Roughly 50 million HeLa cells infected with *S. Typhimurium* SL1344 strain were used for subcellular fractionation of SCVs. At 3 hr and 8 hr p.i., cells were washed thrice with ice-cold PBS and scrapped into a 15 ml centrifuge tube using a rubber cell scrapper. The cells were centrifuged at 1000 rpm for 7 min and the cell pellets were suspended in ice-cold homogenization buffer (250 mM sucrose, 20 mM HEPES (pH 7.2), 0.5 mM EGTA and 5 µg/ml Cytochalasin D) containing protease inhibitor cocktail (Sigma-Aldrich) and transferred to a Dounce Homogenizer with a tight-fitting pestle on ice to break the cells. Approximately 30 strokes were applied until almost 90% of the cells were broken without breaking the nuclei. The intact cells and nuclei were pelleted in microcentrifuge tube at 400 x g for 3 min. The resulting supernatant was collected in a fresh microcentrifuge tube to yield the post nuclear supernatant (PNS). The PNS was brought to a final concentration of 39% sucrose and layered on to 2 ml 55% sucrose which was in turn layered onto 65% sucrose cushion in a 13.2 ml open top Beckman ultracentrifuge tube followed by addition of 2 ml 32.5% and 2 ml 10% sucrose solutions. All sucrose solutions (w/v) were prepared in 20 mM HEPES (pH 7.2) and 0.5 mM EGTA. The PNS layered on sucrose gradient was then subjected for ultracentrifugation in a swinging bucket rotor for 1 hr at 100000 x g at 4°C. The fractions of 1 ml each were collected from top to bottom. Pooled fractions 8-10 were adjusted very slowly to a final sucrose concentration of 11% with homogenization buffer without sucrose and layered on 15% Ficoll cushion (5% sucrose, 0.5 mM EGTA and 20 mM HEPES pH 7.2). The samples in open top Beckman ultracentrifuge tube were spun at 18000 x g for 30 min in a Beckman SW 41 Ti rotor at 4°C. The supernatant was discarded and pellet was resuspended in 11 ml homogenization buffer. The samples were spun again at 18000 x g for 20 min in a Beckman SW 41 Ti rotor at 4°C and the resulting pellet was labeled as "SCV" fraction. The pelleted SCV fractions were resuspended in 20 µl of 4X SDS-sample buffer, boiled for 10 min and analyzed by SDS-PAGE and immunoblotting.

2.15 Enrichment of *Salmonella*-modified Membranes (SMMs) and Affinity Immuno-precipitation

A previously published three-step approach, lysis of infected host cells followed by intracellular compartment enrichment and affinity-IP, was used to enrich and determine the presence of HOPS subunits in SMMs (Vorwerk et al., 2015). Briefly, 16 hr prior to infection, 5 million HeLa cells were seeded in a 10-cm tissue culture dish and four 10-cm dishes were used per IP. For infection, *S. Typhimurium* SL1344 *sseF* harboring a low-copy expression vector with a C-terminal HA-tagged SseF and its cognate chaperone sscB (*sseF*/SseF-HA) was used, and cells infection for a period of 8 hr was carried out as described above. Post-infection, cells were washed thrice with ice-cold PBS and scrapped into a 15 ml centrifuge tube using a rubber cell scrapper, and centrifuged at 1000 x g for 7 min. The resulting cells pellet was suspended in ice-cold homogenization buffer (250 mM sucrose, 20 mM HEPES, 0.5 mM EGTA, pH 7.4), centrifuged at 1000 x g for 10 min, and resuspended in 1 ml of 4°C pre-cooled homogenization buffer containing protease inhibitor cocktail (Sigma-Aldrich). The cells were mechanically disrupted by adding 100 µl of 0.5 mm glass beads (Sigma-Aldrich) using a vortexer (three 1 min strokes) with 5 min of intermediate cooling on ice. The lysate was centrifuged at 100 x g for 10 min at 4°C, and the resulting pellet (labeled as "GEMN pellet") was washed twice with ice-cold homogenization buffer with protease inhibitor mixture. The final GEMN pellet was resuspended in 500 µl of homogenization buffer supplemented with 1.5 mM $MgCl_2$ and treated with DNase I (50 µg/ml) for 30 min at 37°C. The protein concentration in the GEMN protein fraction was determined using Bradford reagent (Bio-rad).

For affinity-IP, 500 µg of GEMN proteins adjusted to a final volume of 500 µl in solubilization buffer (1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40) were added to 20 µl of pre-blocked (in 1% BSA made in PBS for 30 min) anti-HA antibody-conjugated agarose beads or anti-myc antibody-conjugated agarose beads (Sigma-Aldrich) as a control, and were allowed to mix on

rotary shaker at 4°C for 4 hr. At the end of the incubation period, beads were washed five times with 0.1% NP-40 made in PBS to remove non-specific proteins. Finally, the beads were resuspended in 20 µl of 4X SDS-sample buffer, boiled for 10 min and analyzed by SDS-PAGE and immunoblotting.

2.16 GST Pulldown Assay

For protein expression and purification, bacterial expression vectors encoding for GST or GST tagged-SifA were transformed into *E. coli* BL21 strain. Primary cultures of a transformed single colony were set up for 12 hr at 37°C in LB broth containing plasmid vector antibiotic. Secondary cultures were set up using 1% primary inoculums and subjected to incubation at 37°C to an absorbance of 0.6 at 600 nm and then protein production was induced using 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 hr at 30°C. After the incubation period, bacterial cultures were centrifuged at 4,000 rpm for 15 min, washed once with 1X PBS, and resuspended in ice-cold buffer (20 mM HEPES and 150 mM NaCl, pH 7.4) containing protease inhibitor tablet (Roche) and 1 mM PMSF. Cell lysis was performed by sonication, followed by centrifugation at 12,000 rpm for 15 min at 4°C. The supernatants were incubated with glutathione resin (Gbiosciences) on rotation for 2 hr at 4°C to allow binding of GST or GST tagged-SifA, followed by 10 washes with wash buffer (20 mM HEPES, 300 mM NaCl, and 0.5% Triton X-100, pH 7.4). For use in the pulldown assays, protein-bound glutathione resins were blocked with 5% BSA in PBS for 2 hr at 4°C.

For pulldown assays, transfected HEK 293T cells were lysed in ice-cold TAP lysis buffer, and lysates were incubated with protein-bound glutathione resins at 4°C for 3 hr with rotation. Samples were washed four times with TAP wash buffer, and elution was performed by boiling the samples in 1X SDS-PAGE loading buffer and loaded onto SDS-PAGE for analysis.

2.17 Yeast Two-Hybrid and Yeast Three-Hybrid Assay

For the yeast two-hybrid assay, plasmids encoding GAL4-activation domain (AD) and GAL4-DNA binding domain (BD) fusion encoding constructs were co-transformed in *S. cerevisiae* AH109 strain, streaked on SD plates lacking leucine and tryptophan (SD-L/-W) and allowed to grow at 30°C for 3 days. The co-transformants were replated on non-selective medium (SD-L/-W) and selective medium (SD-leucine/-tryptophan/-histidine; SD-L/-W/-H) to assess interaction.

For measuring yeast growth rate, primary cultures were seeded in SD-L/-W broth (Clontech) from single colonies of *S. cerevisiae* AH109 strain co-transformed with indicated plasmids, and grown overnight at 30°C to saturation. The resulting cultures were diluted to approximately 0.1 OD (at 600 nm) in SD-L/-W/-H broth (Clontech) and culture growth was monitored every 4 hr for 48 hr.

For performing the yeast three-hybrid assay, the *S. cerevisiae* Gold strain (Clontech) was made sensitive to methionine (Met) by streaking the yeast on an SD/-Met media at least two times before transforming with the desired plasmids. After co-transformation, yeast cells were re-plated on SD-L/-W (nonselective; selects only for the presence of plasmid) or SD-L/-W/-H/-M (selective; requires interaction of bait and prey proteins through the linker protein for growth).

2.18 LysoTracker Red-uptake Assay

The acidotropic dye LysoTracker Red DND-99 (Thermo Fisher Scientific) was diluted in Opti-MEM without phenol red (Thermo Fisher Scientific). To control siRNA- or Vps41 siRNA-treated HeLa cells cultures 100 nM LysoTracker Red was added and uptake for 1 hr was performed. At the end of the internalization period, cells were washed and then resuspended in fresh pre-warmed medium, and red lysosomal fluorescence of 10,000 cells per sample was determined by flow cytometry (BD Accuri). FlowJo v10 software was used to analyse all of the data from flow cytometric experiments. For visualization of LysoTracker Red-uptake signal

by confocal microscopy, at the end of the dye uptake cells were fixed in 4% PFA in PHEM buffer at room temperature for 10 min. Post-fixation, cells were washed and mounted on glass slides and analysed.

2.19 Statistical Analyses

Statistical analyses were performed with Prism 6 software (GraphPad). Data are presented as mean ± standard deviation (S.D.) unless otherwise indicated. P-values were calculated using two-tailed unpaired Student's *t* test, and differences were considered significant when $P < 0.05$. The sample sizes are specified in the figure legends for all of the quantitative data.

CHAPTER III

Salmonella commandeers the host endolysosomal fusion machinery to promote its intravacuolar replication

The following chapter has been published in PLOS Pathogens, 2017, 13(10): e1006700

Chapter 3

3.1 Introduction

The interaction of *Salmonella* phagosomes with the proteins regulating vesicular trafficking, primarily intersecting at the late endocytic compartments (late endosomes and lysosomes) is necessary for acquisition of membrane for SIF formation, nutrient provision and systemic spread of *Salmonella* infection.

The key cellular candidates that might be involved in the interaction of *Salmonella* phagosomes with the late endocytic compartments and lysosomes include: endolysosomal GTPases Rab7 and Arl8b and their effectors; the late endocytic SNARE proteins (R-SNAREs: VAMP7 and VAMP8 and Q-SNAREs: Syntaxin7, Syntaxin8 and Vti1b) and tethering/docking factors regulating the fusion events in the endolysosomal system: HOPS and CORVET.

Previous studies have indicated the involvement of various endolysosomal proteins such as small GTPases localizing to late endosomes and lysosomes: Rab7, Arl8b, Rab9 and SNAREs (VAMP7, Syntaxin7, and Syntaxin8) during the maturation and trafficking of *Salmonella* phagosomes following infection (Garcia-del Portillo and Finlay, 1995; Garcia-del Portillo et al., 1993a; Méresse et al., 1999a). Although components of the host late endosome-lysosome fusion machinery are known to localize to SCV and SIFs [such as Rab7 (Méresse et al., 1999b) and Arl8b (Kaniuk et al., 2011a)], but their function in *Salmonella* replication and whether *Salmonella* modulates their recruitment for its own survival needs further exploration. Furthermore, none of the studies have shown the direct involvement of tethering factors such as HOPS complex that play critical roles during the fusion process and govern the endolysosomal homeostasis in this scenario.

3.1.1 HOPS Complex

The **HO**motypic fusion and **P**rotein **S**orting **(HOPS)** complex was the first tethering factor identified to be operating in the endolysosomal pathway (Seals et al., 2000; Wurmser et al., 2000). Initial studies were performed in the yeast *S. cerevisiae* where HOPS was established as a critical component of the fusion machinery required for the homotypic fusion of the vacuoles during the inheritance cycle. Later on, these results were extrapolated where HOPS gained appreciation for its role as a tether that regulates the SNARE-driven merger of late endosomes and vacuoles (lysosome-like organelles in yeast) (Bugnicourt et al., 2004; Peterson and Emr, 2001).

The member sub-units of HOPS complex were discovered as part of the genetic screens to identify the yeast mutants that result in aberrant secretion or mislocalization of a vacuolar hydrolase (CPY) into the late secretory pathway (Bankaitis et al., 1986; Rothman and Stevens, 1986). These mutants were classified as *vps (**v**acuole **p**rotein **s**orting)* as they were defective in cargo delivery towards the vacuoles. Based on the biochemical analysis; morphological and phenotypic variations these *vps* mutants were grouped into several classes. Amongst them, the genes that were critical for the assembly of MVBs and vacuoles alongwith regulation of major endolysosomal pathways including autophagy were grouped under VPS-Class C while others with less severe effects on vacuole numbers and morphology as compared to the parent strain were classified into different categories (VPS-Class A, B, D, E or F).

The hetero-hexameric HOPS complex comprises of a Vps-Class C core made up of four proteins: Vps11, Vps16, Vps18 and Vps33. The mutations in the genes encoding for these proteins show severe defects in vacuolar biogenesis and lack coherent vacuoles. This VpsC-core associates with two HOPS-specific accessory proteins namely Vps39 and Vps41. The mutants lacking vps39 or vps41 show a somewhat weaker phenotype with

defects in vacuolar fusion and are characterized by the presence of numerous but smaller vacuole-like compartments which is a classical trait of Vps-class B mutants (Arlt et al., 2011; Wada et al., 1992).

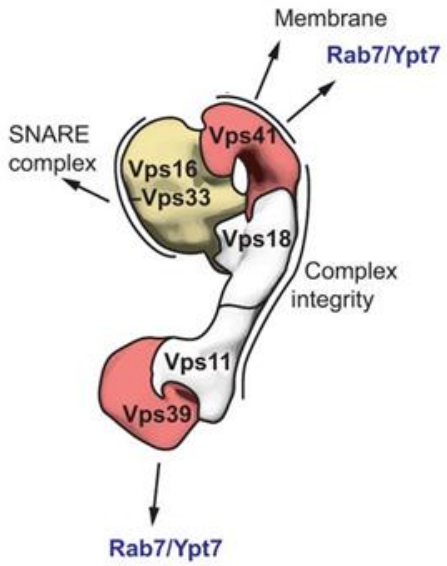


Figure 3.1: The architecture of yeast HOPS complex. Secondary-structure arrangement of yeast HOPS complex comprising of four class C subunits (Vps11, Vps16, Vps18, Vps33) that form the core and two membrane binding Rab-specific subunits. The two Ypt7/Rab7-binding sites are situated at opposite ends and the complex binds to SNAREs through the SM-protein, Vps33. *Modified from: (kleine Balderhaar and Ungermann, 2013) Copyright © 2013. Published by The Company of Biologists Ltd.*

Recent studies using a combination of cryo-electron microscopy and single particle analysis have unveiled the three-dimensional architecture of yeast HOPS complex. The molecular arrangement was found to be in complete agreement with the biochemical analysis of partially purified HOPS complex and gives insights into the tethering mechanism. Accordingly, the Rab-binding subunits (Vps41 and Vps39) are situated at opposite ends of this hexameric complex forming an elongated structure with a large head and a petite tail giving it a sea horse-like appearance. The molecular mass of the multimeric complex is approximately 633kDa. The bulgy head of the complex consists of Vps41 in close association with the Vps16-Vps33 sub-complex whereas Vps39 is positioned in the tail section. The remaining two subunits Vps18 and Vps11 lie in the middle connecting the head and tail. The subunits are arranged in a ~30nm extended

configuration such that this multimeric tether is flexible enough to undergo contraction, expansion and adopt various confirmations without altering overall structure and organisation of the complex (Bröcker et al., 2012). These structural revelations alongwith the analysis of fusion reactions carried out using isolated yeast vacuoles have provided insights into the directionality and order of events during the Rab-dependent tethering mediated by HOPS complex. Accordingly, the small GTPase Ypt7/Rab7 located on the opposing membranes interacts with the membrane-binding subunits Vps39 and Vps41 (Ostrowicz et al., 2010). The recruitment of one subunit expedites the assembly of other protein components through subunit-subunit interactions. The assembly of HOPS at these fusion vertices on the late endocytic membranes is followed by reorientation of the complex on the endosomal membrane leaving the other Rab-binding end available for interaction with Ypt7/Rab7 present on the vacuolar membranes. This results in formation of a bridge-like structure linking the two organelles intended for fusion (late endosomes and vacuoles) which was also observed during ultrastructural cryo-EM tomography. The docking of the two membranes allows the SM (Sec1/Munc18-like SNARE-master) subunit Vps33 to interact with the trans-SNARE complexes and direct the fusogenic activity (Baker et al., 2015; Collins et al., 2005; Krämer and Ungermann, 2011; Lobingier and Merz, 2012; Lobingier et al., 2014; Subramanian et al., 2004). HOPS now acts as a chaperone for SNARE selectivity at the tethered membranes and performs the "proofreading" function by permitting the correct SNARE pairing while forbidding the errors in SNARE complex formation. The vacuolar SNARE proteins are organized in a canonical 3Q:1R conformation which includes: Nyv1 (R-SNARE), Vam3, Vti1 and Vam7 (Q-SNAREs). The four complementary SNARE proteins associate to form a fusion proficient tetrameric helical bundle; that triggers the amalgamation of the membrane lipids and luminal content mixing. Post-fusion the cis-SNARE complex disassembles which requires another set of proteins including: Sec18 (the yeast ortholog

of mammalian NSF), Sec17 (SNAP, soluble NSF attachment protein) and ATP; making them available for another fusion event (Bröcker et al., 2012).

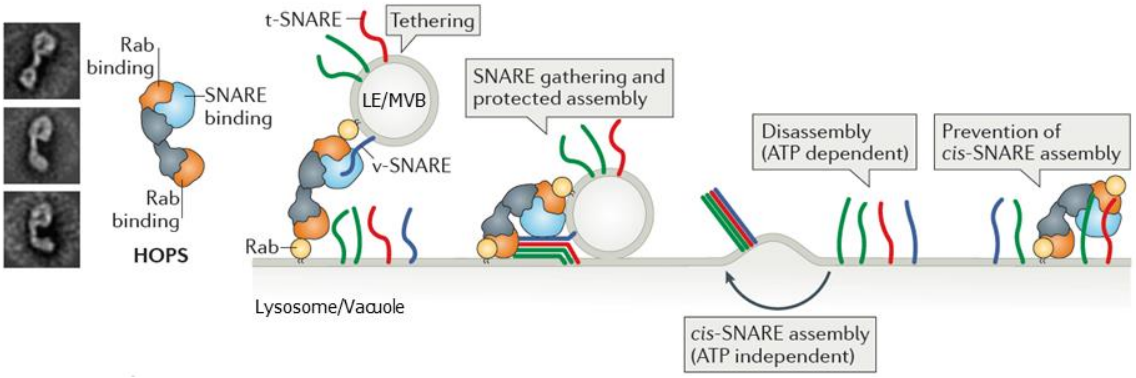


Figure 3.2: Model for HOPS complex-dependent membrane tethering and fusion in yeast. HOPS functions first as a tether, by binding to Rab proteins (Ypt7/Rab7) on two different membranes, and then acts as a chaperone for the assembly of SNAREpin complex. HOPS may also block the premature disassembly of trans-SNARE complexes and the reassembly of cis-SNARE complexes post fusion. *Modified from: (Baker and Hughson, 2016) Copyright © 2016, Springer Nature.*

Although most of the information pertaining HOPS has been derived from studies performed in yeast, there is sufficient evidence for the conservation of HOPS functions in higher eukaryotes such as *C. elegans*, *Drosophila*, zebrafish, and mammals (Jiang et al., 2014b; Schonthaler et al., 2008; Sevrioukov et al., 2005; Solinger and Spang, 2014; van der Kant et al., 2015; Wartosch et al., 2015).

The metazoan HOPS complex has a similar organisation and comprises of all the six subunits described in yeast. The arrangement of the subunits is largely conserved with some modifications. Studies have indicated the involvement of the metazoan HOPS in the biogenesis of late endocytic compartments and cargo trafficking to these organelles apart from their fusogenic and SNARE-chaperoning functions at lysosomal, late endosomal and autophagosomal membranes. Unlike yeast, that does not depend on endocytosis for its survival and is able to survive upon depletion of HOPS subunits; HOPS knockouts in eukaryotes are lethal. Similar to its yeast counterpart, the metazoan

HOPS complex also includes six subunits: hVps11, hVps16, hVps18, hVps33a, hVps39 and hVps41 (kleine Balderhaar and Ungermann, 2013; Solinger and Spang, 2013a).

Interestingly, in higher eukaryotes, there exist two isoforms A and B of both Vps16 and Vps33. Vps33A and Vps16A (a.k.a. Vps16) have been shown to be a part of mammalian HOPS complex functioning at late endosomes and lysosomes (Gautreau et al., 2014; Khatter et al., 2015a; Tornieri et al., 2013) whilst Vps16b (or VIPAR/SPE-39/fob) has been shown to be crucial for phagocytosis and therefore may be operating at early endosomes (Akbar et al., 2011; Cullinane et al., 2010; Pulipparacharuvil et al., 2005). Vps33b combines with a protein VIPAS39 (Vps33b-Interacting Protein, Apical-Basolateral Polarity Regulator, a SPE-39 homolog) (van der Kant et al., 2015) to form a complex that has been implicated to function in the biogenesis of lysosome-related organelles (LROs), establishment of cell polarity and has been linked to ARC (**A**rthrogryposis **R**enal dysfunction and **C**holestasis) syndrome. However, the exact function of this complex is being thoroughly examined and remains elusive.

The mammalian homologues of HOPS subunits have been implicated in endosomal maturation, cargo delivery and fusion of lysosomes with late endosomes, phagosomes or autophagosomes (Jiang et al., 2014b; McEwan et al., 2015a; Pols et al., 2013). Recent advancement in this regard has suggested a similar organization and assembly of the metazoan HOPS through subunit-subunit interactions. Interestingly, the recruitment of mammalian HOPS complex to the lysosomal membranes is catalysed by a separate GTPase, Arl8b which is absent in yeast. Arl8b directly interacts with HOPS subunit Vps41 and expedites the assembly of HOPS complex onto the organellar membranes (Garg et al., 2011b; Khatter et al., 2015a).

Numerous reports have evidenced that dysfunction of HOPS components is associated with a variety of animal diseases, strong deficiencies, embryonic lethality and

developmental defects due to impaired biogenesis of endosomal, phagosomal and lysosomal compartments (Caplan et al., 2001a; Kinchen et al., 2008; Pols et al., 2013; Poupon et al., 2003; Sriram et al., 2003; Swetha et al., 2011). Deficiency of VPS11, VPS16, VPS18 and VPS39 may result in aberrant pigmentation and defective melanosome maturation resulting in oculocutaneous albinism in zebrafish mutants (Maldonado et al., 2006; Pulipparacharuvil et al., 2005; Schonthaler et al., 2008; Thomas et al., 2011). Further, a missense point mutation in murine VPS33a gene results in abnormal melanosomes and gives rise to buff mouse characterized by loss of platelet activity and motor deficiencies (Zhen and Li, 2015). Besides, several other diseases such as Parkinson's disease, Hermansky-Pudlak syndrome (HPS), neurodegenerative disorders (Peng et al., 2012) and cancer (Roy et al., 2011) have been attributed to mutations in HOPS complex subunits.

Besides, HOPS complex being the major regulator of fusion events at lysosomes is also a potential target of pathogenic surveillance as a defence strategy to prevent fusion of their vacuoles with the degradative lysosomes. Few studies have explored the role of mammalian HOPS subunits in maturation of pathogen-containing vacuoles. Previous work has shown that HOPS complex plays an inhibitory function in regulating intracellular survival of the pathogen *Coxiella burnetti* by mediating fusion of bacterial phagosomes with lysosomes (Barry et al., 2012). Consequently, *C. burnetti* mediated phosphorylation of Vps41 subunit of the HOPS complex prevents its membrane localization, and thereby, its function in phagolysosome fusion. HOPS complex has also been shown to be essential for Ebola virus replication with loss of HOPS subunit expression preventing viral escape to the cytosol from host's late endosomes/lysosomes (Carette et al., 2011).

Although *Salmonella* phagosomes readily fuse and acquire contents from the late endocytic compartments of the host cell (Drecktrah et al., 2007b; Rajashekar et al., 2008b), little is known if *Salmonella* employs HOPS complex to mediate fusion with host endolysosomes. Previous studies have shown that HOPS subunit Vps39 interacts with SKIP/PLEKHM2 and PLEKHM1, both of which bind to the *Salmonella* effector SifA (Khatter et al., 2015a; McEwan et al., 2015c). Moreover, HOPS complex is an effector of the small GTPase Arl8b that localizes to SCVs and SIFs in *Salmonella*-infected HeLa cells (Kaniuk et al., 2011; Khatter et al., 2015). A more direct evidence of HOPS function during *Salmonella* infection was shown where depletion of HOPS subunits (similar to PLEKHM1 depletion) altered SCV morphology with multiple bacteria present within a single enlarged vacuole (McEwan et al., 2015). However, *Salmonella* infection in these experiments was visualized after 20 hr p.i. while SCV interaction with host late endosomes/lysosomes and SIF formation is observed as early as 6 hr p.i. (Drecktrah et al., 2007b). Thus, with regard to the role of HOPS complex in *Salmonella* infection, several important questions remain unanswered, for instance, whether HOPS complex regulates *Salmonella* replication, does it regulate SCV maturation and SIF formation and what are the bacterial and host factors required for recruitment of HOPS complex to SCVs and SIFs.

In this chapter of the thesis, I have described the experiments performed for characterization of HOPS function during different stages of *Salmonella* infection in the light of previous reports.

3.2 Results

3.2.1 HOPS complex subunits are recruited to *Salmonella* phagosomes (SCVs) and SIFs at different stages of *Salmonella* infection

To determine the role of HOPS complex in SCV maturation and SIF formation, we first looked at the localization of individual subunits of this hetero-hexameric protein complex in infected cells using confocal microscopy at different time points starting at 10 min post infection until 10 hr post infection. Vps41, one of the membrane binding subunits exclusive to HOPS complex was observed in close apposition to the maturing SCVs at 1h post infection (53±4% for Vps41); although a weak association with early SCVs (EEA1-positive) was spotted as soon as 10 mins after the infection of human epithelial cell line (HeLa). The recruitment of endogenous Vps41 became more evident on the LAMP1-positive *Salmonella* vacuoles by 3 hr post infection (74±1% SCVs were positive for Vps41) while maximum accumulation was observed at 6 hr post infection. At much later time-points (10 hr post infection) endogenous Vps41 was clearly decorating both SCV (>93±4% SCVs) and SIF membranes while co-localizing with the lysosomal membrane protein, LAMP1 which is often used as a marker for these tubular extensions (**Figs 3.3d** and **3.3e**).

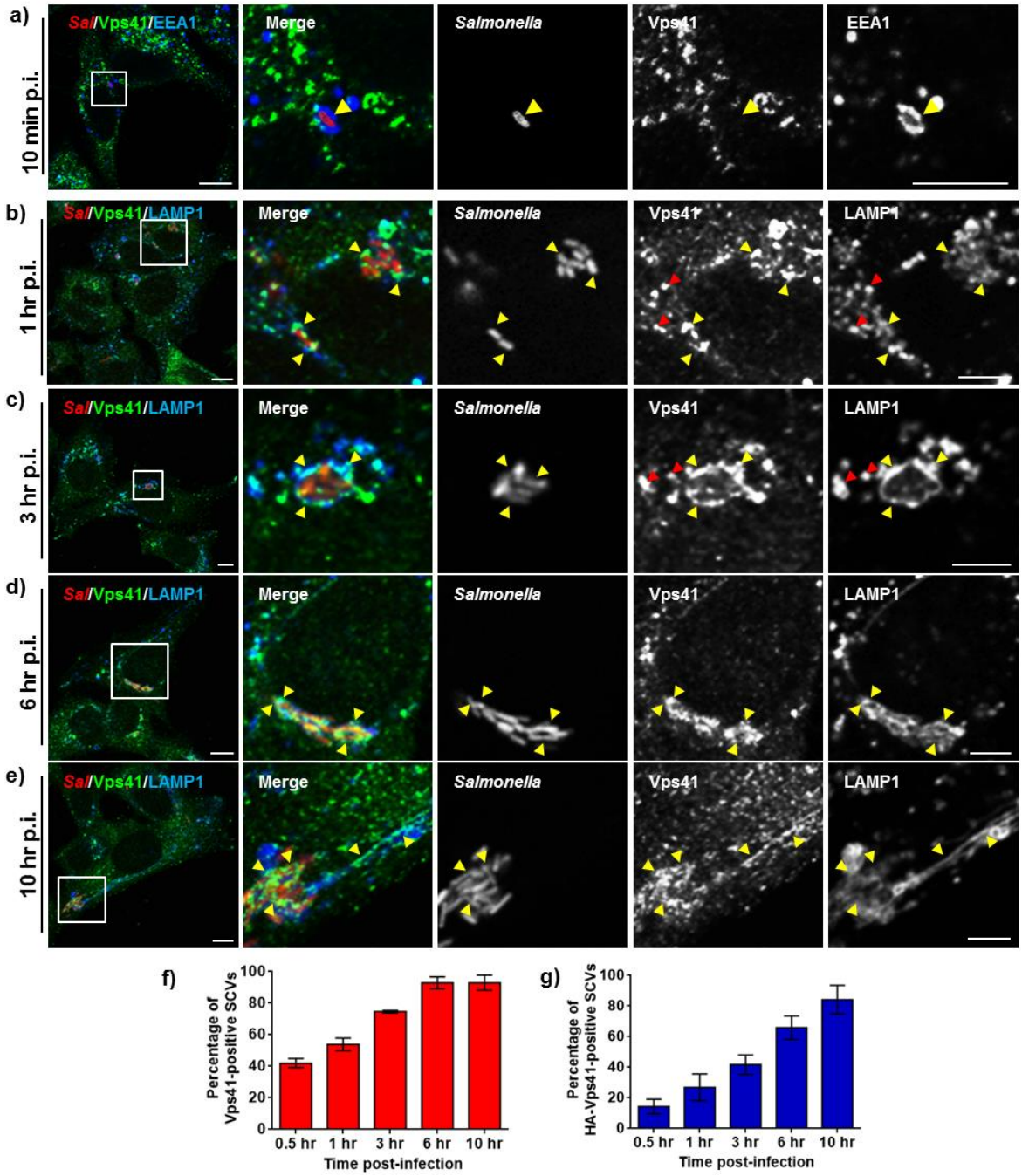


Fig 3.3: HOPS subunit, Vps41 is recruited to LAMP1-positive SCVs and SIFs during *Salmonella* infection. a) Representative confocal micrographs of HeLa cells infected with DsRed-expressing *Salmonella* (red). At 10 min p.i., cells were fixed and stained for endogenous Vps41 (green) and EEA1 (blue). Different panels represent a higher magnification of the boxed areas, showing absence of Vps41 but presence of EEA1 around SCVs (marked by arrowheads) at this time point of infection. **b-e)** Representative confocal micrographs of HeLa cells infected with DsRed-expressing *Salmonella* (red). At different times after infection, cells were fixed and stained for endogenous Vps41 (green) and LAMP1 (blue). Different panels represent a higher magnification of the boxed areas, showing recruitment of Vps41 on SCVs and SIFs (marked by yellow arrowheads). Bars: (main) 10 µm; (insets) 5 µm. **f and g)** Quantification of endogenous (**f**) or HA-tagged Vps41 (**g**)-positive SCVs at different time points p.i. Data represent percentage of Vps41-postive SCVs scored for ~100 SCVs for each time point. The mean ± S.D. is shown for three independent experiments.

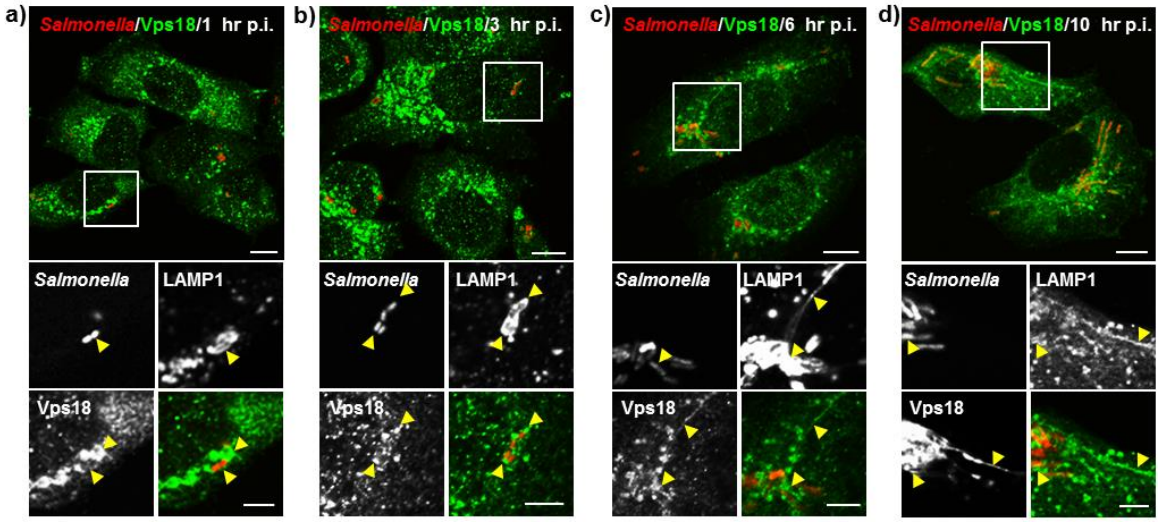


Fig 3.4: Localization of HOPS subunit, Vps18 to SCVs and SIFs at different time points of *Salmonella* infection. a-d) Representative confocal micrographs of HeLa cells infected with DsRed-expressing *Salmonella* (red). At different times after infection, cells were fixed and stained for endogenous Vps18 and LAMP1. Insets depict recruitment of Vps18 on SCVs and SIFs as marked by yellow arrowheads.

A similar trend was observed in case of endogenous Vps18 which is one of the core subunits of HOPS complex. Likewise, Vps18 also showed a striking accumulation on SCVs and SIFs beginning at 6hr but more prominent at 10hr post infection (**Figs3.4a-d**).

Parallel experiments were performed using epitope-tagged Vps41 and Vps33a HOPS subunits; which were also found to accumulate on the maturing SCVs and SIF membranes in a temporal manner. However, in these experiments, HeLa cells were briefly treated with a mild detergent (0.05% Saponin in PBS for 30 seconds) prior to fixation to get rid of the cytosolic background noise due to overexpression of the proteins (**Figs 3.5a-h);** quantification of HA-Vps41 SCVs shown in **Fig 3.3g**). Notably, both endogenous and over-expressed HOPS subunits also localized to the LAMP1-positive vesicles, supporting their normal sub-cellular distribution to late endocytic compartments late endosomes and lysosomes (see red arrowheads in insets of **Figs 3.3b and 3.3c**). The localization of the remaining two HOPS subunits (Vps39 and Vps16) during *Salmonella* infection could not be observed microscopically due to lack of antibodies against these proteins.

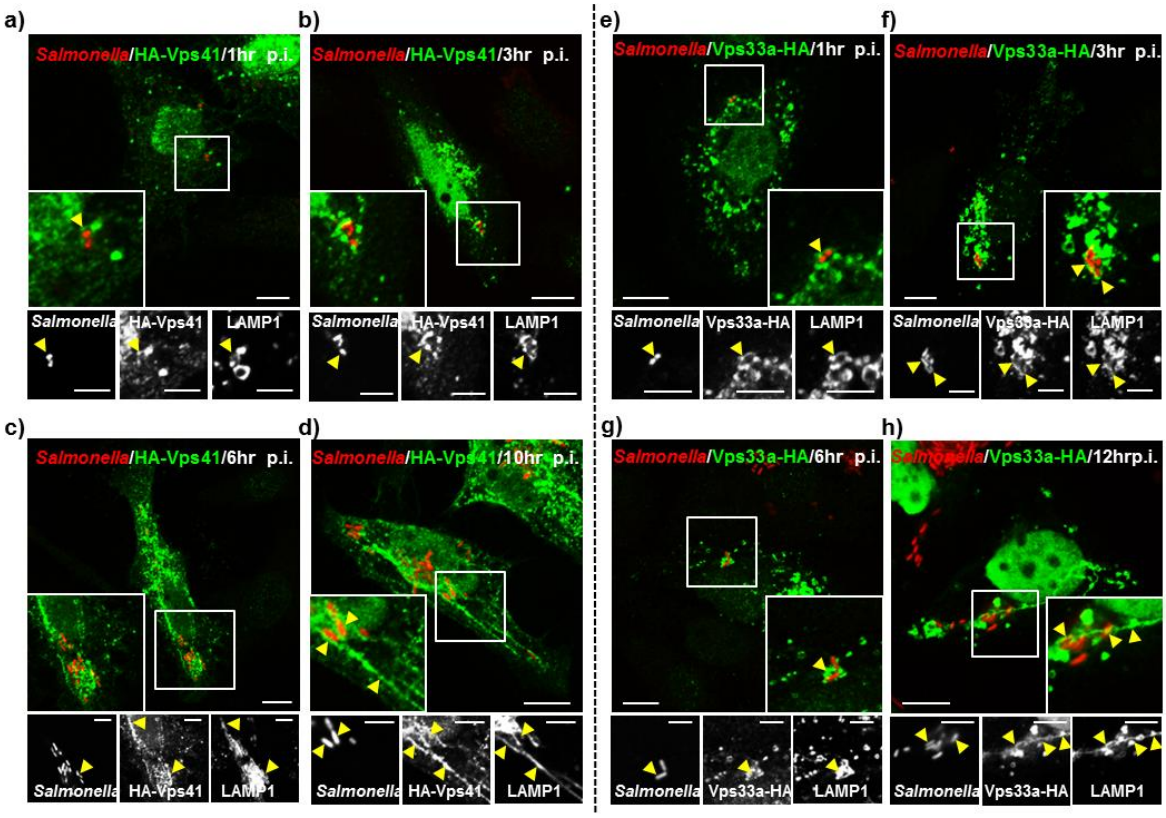


Figure 3.5 Epitope-tagged HOPS subunits localize to SCVs and SIFs during infection. a-h) Representative confocal micrographs of HA-Vps41 or Vps33a-HA transfected HeLa cells infected with DsRed-expressing *Salmonella* (red). At different times after infection, cells were fixed and stained using anti-HA (green) and anti-LAMP1 (blue, shown only in inset) antibodies. Insets depict recruitment of epitope-tagged HOPS subunits on SCVs and SIFs as marked by arrowheads. Bars: (main) 10 µm; (insets) 5 µm.

3.2.2 CORVET-specific subunit, TGFBRAP1 fails to localize at SCVs and SIFs

As mentioned before, the four out of six subunits of this multimeric tethering factor (Vps11, Vps16, Vps18 and Vps33a) are shared with an early endosomal tether, CORVET. To determine the localization of CORVET complex in *Salmonella* infected cells, we looked at the distribution of the epitope-tagged CORVET-specific subunit, TGFBRAP1 in these cells at different time-points: 10 min, 30 min, 1 hr, 3 hr and 6 hr p.i. (**Figs 3.6a-e**). Although, TGFBRAP1 could be clearly seen localizing to the EEA1-positive endosomes as reported previously (Perini et al., 2014); we did not find the recruitment of this protein subunit on SCVs and SIFs at any time-point (**Figs 3.6a-e).**

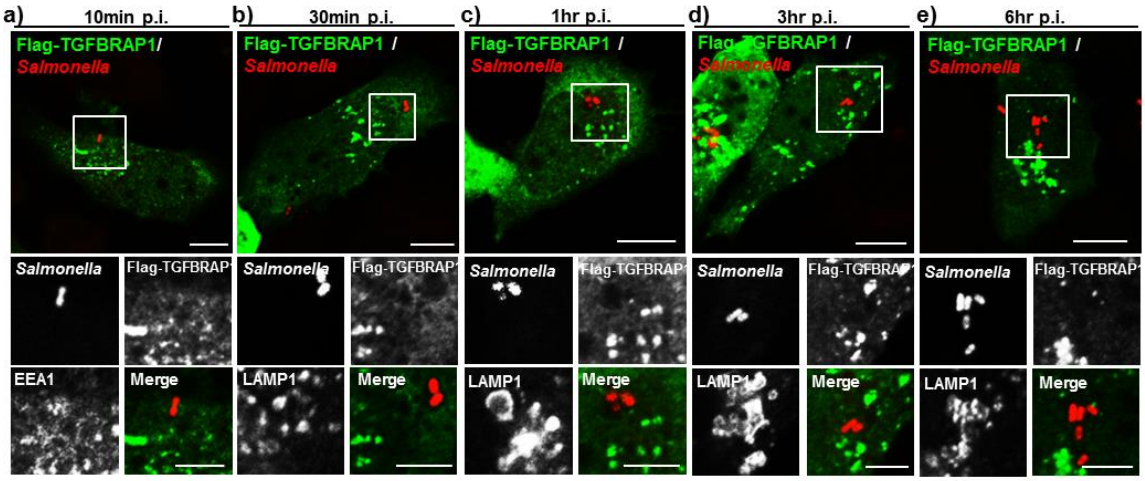


Figure 3.6: CORVET-specific subunit, TGFBRAP1 is not recruited to the SCVs. a-e) Representative confocal micrographs of FLAG-TGFBRAP1 transfected HeLa cells infected with DsRed-expressing *Salmonella* (red). At different times after infection (as indicated), cells were fixed and stained using anti-FLAG (green) and anti-EEA1 (a, blue) or anti-LAMP1 (b-e, blue, shown only in inset) antibodies. Arrowheads in inset from panel (a) depict colocalization of TGFBRAP1 with EEA1.

3.2.3 Live imaging reveals the extensive recruitment of HOPS subunit Vps41 to the SCVs and tubules emanating from and inter-connecting these compartments

Owing to the poor preservation of tubular morphology in fixed cells, we observed a discontinuous and punctate distribution of HOPS subunits along the length of SIFs in the above-mentioned steady state experiments. Previous studies have reported a similar discrete and patchy distribution of LAMP1 on these *Salmonella* induced tubules due to fixation (Drecktrah et al., 2008; Rajashekar et al., 2014). To rule out the possibility that fixation could be the reason for this disconnected distribution of HOPS subunits on SIF membranes we performed live imaging experiments. For this we infected HeLa cells with *Salmonella* constitutively expressing fluorescent monomeric DsRed (DsRed-*Salmonella*) followed by transfection of GFP-tagged Vps41 at 2hr p.i. The live imaging was initiated at 9 hr p.i. In live cells, Vps41 was continuously distributed at both SCV and SIF membranes unlike the previously observed discrete distribution seen in steady state imaging.

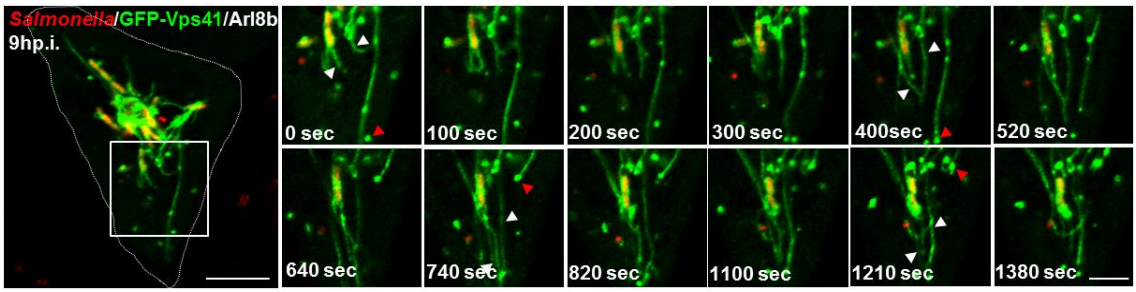


Figure 3.7: Vps41 extensively associates with SCVs and SIFs in *Salmonella*-infected live cells. Time-lapse microscopy of HeLa cells co-transfected with plasmids encoding GFP-Vps41 and untagged-Arl8b, and infected with DsRed-expressing *Salmonella* (red). Time-lapse series were recorded 9 hr p.i., and still images shown here correspond to S2 Movie. Different panels represent a higher magnification of the boxed area indicating Vps41-positive SIFs emanating from the SCVs showing extension, retraction and bifurcation (white arrowheads). Red arrowheads indicate fusion of Vps41-positive vesicles with SIFs. Bars: (main) 10 µm; (insets) 5 µm.

We also observed rapid contraction, expansion and bifurcation of the Vps41-positive tubules during live imaging depicting a dynamic distribution similar to LAMP1 in *Salmonella* infected cells (**Fig 3.7** and **S2 Movie**). Further, the Vps41-positive tubules undergoing continuous fusion and fission with the existing filaments were also seen in these experiments (see red arrowheads in **Fig 3.7**).

3.2.4 Arl8b is essential for the recruitment of HOPS subunit, Vps41 to SCV membranes

In order to reduce the cytosolic background signal that interfered with the visualization of membrane-bound overexpressed Vps41 protein, we co-transfected the cells with a plasmid encoding for the small GTPase Arl8b, which has been previously shown to regulate the membrane localization of HOPS onto lysosomal membranes via this subunit (**Fig 3.7** and **S2 Movie**) (Khatter et al., 2015a). Previously, Brumell and co-workers have demonstrated the localization of Arl8b to both SCVs and SIFs where this GTPase directs the kinesin-dependent motility of SCVs and tubulation of *Salmonella* phagosomes (Kaniuk et al., 2011a). Accordingly, we also find the similar distribution of Arl8b at various time-points during *Salmonella* infection that can be appreciated in **Figs 3.8a-e.** Interestingly, the localization of GFP-Vps41 to the SCVs was completely abrogated in *Salmonella*-infected CRISPR/Cas9 Arl8b-knockout cells, and the protein was entirely

cytosolic (**Figs 3.8f** and **3.8g; S3 Movie**). Furthermore, the quantification data for Vps41 localization to the SCVs in infected WT- and Arl8b knockout cells revealed a dramatic decrease in the percentage of Vps41-positive SCVs (**Fig 3.8i;** mean percentage Vps41-positive SCVs in WT: 91±2% and Arl8b KO: 6±1%) in absence of this GTPase pointing towards the crucial role of Arl8b in the recruitment of this tethering complex to the SCV membranes that needs to be explored further.

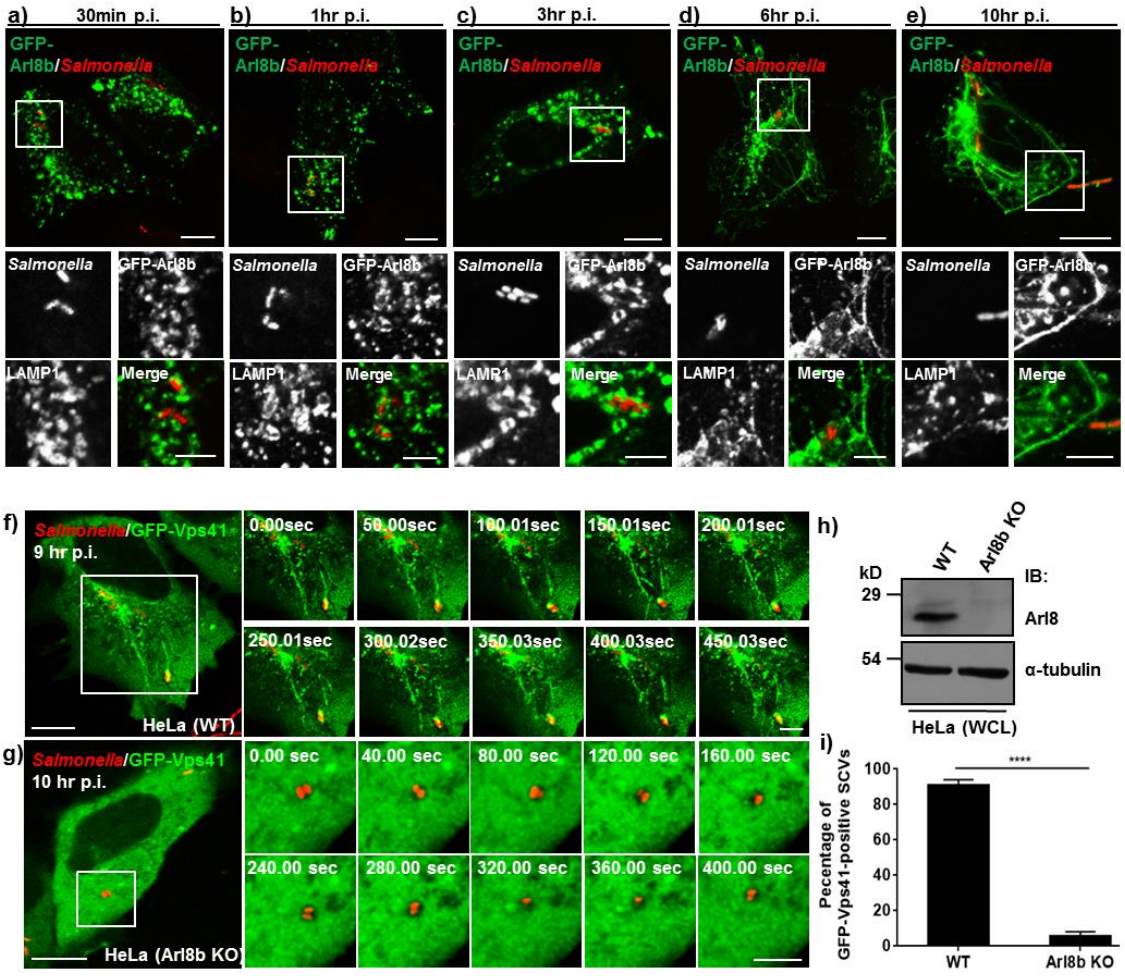


Figure 3.8 The recruitment of HOPS-specific subunit, Vps41 to the SCV is dependent upon expression of lysosomal small GTPase Arl8b. a-e) Representative confocal micrographs of Arl8b-GFP transfected HeLa cells infected with DsRed-expressing *Salmonella* (red). At different times after infection (as indicated), cells were fixed and stained using anti-LAMP1 (blue, shown only in inset) antibody. Insets depict higher magnification of boxed areas. Bars: (main) 10 µm; (insets) 5 µm. **f and g)** Time-lapse microscopy of WT or CRISPR/Cas9 Arl8b KO HeLa cells transfected with plasmid encoding GFP-Vps41, and infected with *Salmonella* expressing DsRed (red). Time-lapse series were recorded at the indicated times p.i., and still images correspond to movies shown as S1 and S3 Movies. Bars: (main) 10 µm; (insets) 5 µm. **h)** WT- and CRISPR/Cas9 Arl8b KO-HeLa cell lysates were immunoblotted with anti-Arl8 antibody for assessing the knockdown efficiency and with anti-α-tubulin antibody as a loading control. **i)** Quantification of GFP-Vps41-positive SCVs in WT- and Arl8b KO-HeLa cells. Data represent mean ± S.D. over three independent experiments at 10 hr p.i. where 100 SCVs were counted in each experiment (****, $P < 0.0001$; Student's *t* test).

3.2.5 HOPS subunits are enriched at *Salmonella* vacuoles during later stages of *Salmonella* infection

To corroborate our confocal data, we biochemically analysed the temporal recruitment of HOPS subunits to the *Salmonella* membranes. For this purpose, we isolated the *Salmonella* phagosomes from infected HeLa at 3 hr p.i. (early SCVs) and 8 hr p.i. (late SCVs) using a two-step density gradient ultracentrifugation followed by immunoblotting with antibodies against individual HOPS subunits (Vps41 and Vps18) and a prokaryotic chaperone DnaK to confirm the presence and replication of *Salmonella* at the two time-points (**Fig 3.9a**, fractions 8-10 (labelled as SCV fraction). The comparison of *Salmonella* infected homogenates processed at the early and later time-points demonstrated the enhanced recruitment of endogenous HOPS and LAMP1 (SCV marker) in the SCV fraction from 3 hr to 8 hr p.i. (**Fig 3.9a**). Accordingly, the early endosomal marker EEA1 was weakly associated with the SCVs at 3hr p.i. but not at 8 hr p.i. We also checked for the presence of CORVET-specific subunit TGFBRAP1 in the SCV fractions at both time-points and found only negligible association of this protein at 3 hr p.i. while no such association was observed at 8 hr p.i. supporting our confocal microscopic results. Additionally, to verify HOPS enrichment on late SCVs and SIFs, we also employed a recently described method of SCV isolation by immunoprecipitation (IP) of SseF-an integral membrane SPI2-T3SS effector protein (Vorwerk et al., 2014). As shown in **Fig 3.9b,** HOPS subunits were specifically enriched in the SseF-IP eluate but not control IP with levels comparable to the known SCV markers, such as LAMP1 and Rab7. In contrast, little or no co-IP of GAPDH or Catalase with SseF was observed, substantiating the specificity of this approach for SCV isolation (**Fig 3.9b**).

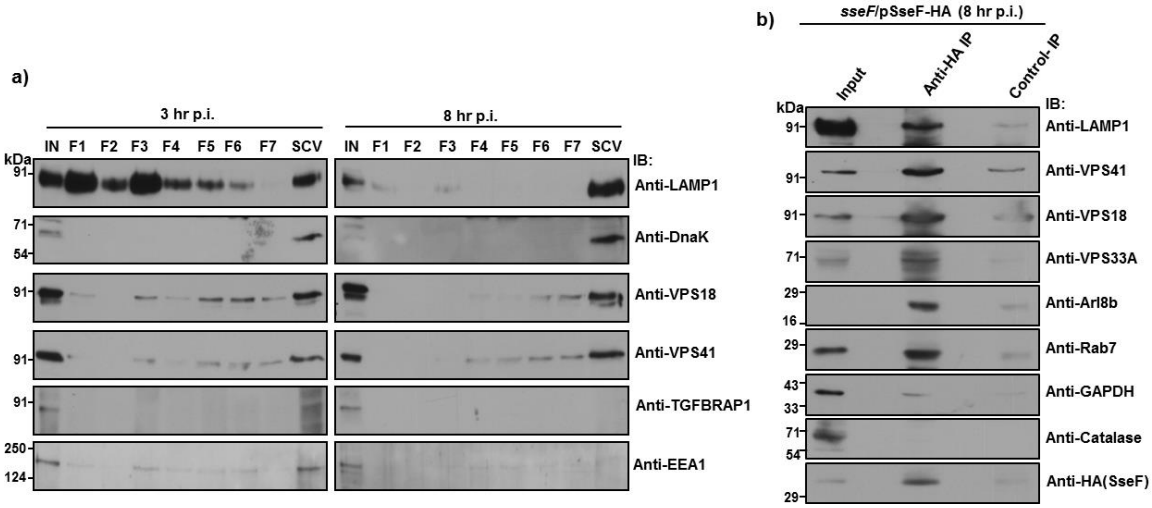


Figure 3.9: Enrichment of HOPS complex subunits at SCVs at later time-points during infection. a) SCVs were isolated from *Salmonella*-infected HeLa cells at 3 hr and 8 hr p.i. using sucrose density ultracentrifugation, followed by second round of ultracentrifugation of fractions 8-10 on a ficoll cushion (labeled as SCV). Different fractions were resolved on SDS-PAGE gel and immunoblotted using indicated antibodies. **b)** *Salmonella*-modified membranes were isolated from HeLa cells infected with *sseF*-deficient strain of *Salmonella* harboring an expression vector with a C-terminal epitope-tagged *sseF* and its cognate chaperone *sscB* (*sseF*/pSseF-HA) at 8 hr p.i. by differential centrifugation. The enriched fraction was further subjected to affinity immunoprecipitation (IP) using anti-HA antibody-conjugated agarose beads or anti-Myc antibody-conjugated agarose beads as a control. The eluted samples were analysed for presence of effector protein (SseF) and host proteins by Western blotting as indicated.

Our results indicate correlation between recruitment of HOPS complex with time points wherein SCV is known to acquire content from late endosomes and lysosomes (Drecktrah et al., 2007b). Indeed, in a recent study by Santos et al., where proteomes of early SCV and late SCV were compared, enrichment of HOPS subunits Vps11, Vps16, and Vps18 was observed in the late SCV fractions (Santos et al., 2015). *This experiment was carried out in collaboration with Subhash Babu Arya at IMTECH, Chandigarh.*

3.2.6 HOPS complex is critical for intracellular *Salmonella* replication during infection of both *in vitro* and *in vivo* model systems

To comprehend the significance of extensive HOPS recruitment to *Salmonella* vacuoles and *Salmonella*-modified membranes we assessed the intracellular replication rate of *Salmonella* in cells depleted of individual HOPS subunits. The siRNA-mediated silencing of different HOPS subunits in HeLa cells was confirmed using western blotting and qRT-PCR analysis (**Figs 3.10a-f**). The intracellular bacteria were enumerated post-

fixation using immunofluorescence microscopy at two different time-points (2 hr p.i. and 10 hr p.i.) in both Control- and HOPS specific-siRNA treated HeLa cells infected with *Salmonella* (labelled with anti-*Salmonella* antibody). At 2hr p.i there was not much difference in the bacterial load in Control- and HOPS-siRNA treated cells (with ~35% cells containing 6-10 bacteria/cell, ~17-30% of cells containing 11-20 bacteria/cell, and ~2-7% of cells were containing >20 bacteria/cell) suggesting that HOPS complex is not required for *Salmonella* invasion into the host cells (**Fig 3.10g**). On the contrary, at the later time-point (10hr p.i.) around ~73% of the control-siRNA treated cells had >20 bacteria indicating a marked increase in bacterial load in these cells while only 10-30% of HOPS-siRNA treated cells showed a similar bacterial load with almost equal distribution of cells containing either 6-10 bacteria/cell (20-30%) or 11-20 bacteria/cell (30-35%) demonstrating a significant defect in bacterial proliferation upon HOPS depletion (**Fig 3.10g**).

To substantiate the microscopic observations, we further estimated the number of Colony Forming Units (CFU) in *Salmonella* infected Control- and HOPS-depleted HeLa cell lysates at two different time points (2h p.i. and 10 hp.i.) using a standard Gentamicin protection CFU assay. In the control-siRNA treated cells we observed significant bacterial replication (~3-4 fold increase from 2hr p.i. to 10 hr p.i.) while in case of HOPS-depleted cells the fold increase in the CFU was negligible (~1.09-1.4 fold). As expected and consistent with our colocalization and biochemical data showing little or no association of the CORVET subunit TGFBRAP1 with SCVs and SIFs, we observed a ~2.8 fold increase in bacterial load upon the depletion of this subunit (**Fig 3.10h** and **Fig 3.10f** showing knockdown efficiency >70%), that was not significantly different from the control cells. These results suggest that HOPS, but not CORVET is critical for replication and intracellular survival of *Salmonella*.

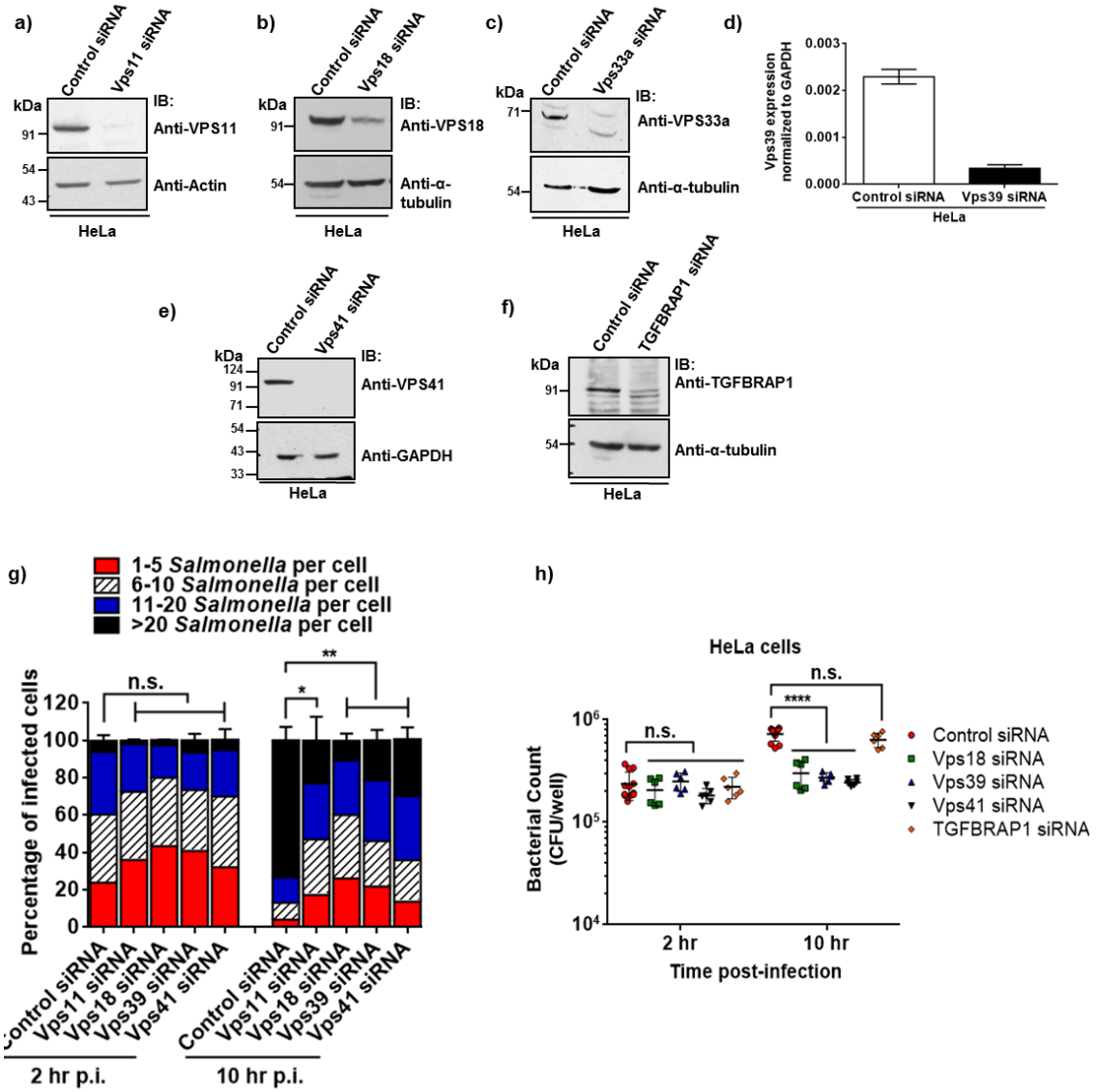


Fig 3.10: Depletion of HOPS subunits impairs *Salmonella* replication. a-f) Western blotting or qRT-PCR analysis of different cell types transfected with indicated siRNA was performed to measure the gene silencing efficiency. **g)** HeLa cells transfected with indicated siRNA were infected with *Salmonella*, fixed at the indicated time points, and immunostained with antibodies to *Salmonella* and LAMP1. Using confocal microscopy, the number of intracellular bacteria was enumerated in ~300 cells per experiment. These numbers were grouped according to the legend, and expressed as a percentage of the total infected cell population. The mean ± S.D. is shown for three independent experiments (n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; Student's t test**). h) Intracellular replication assay.** HeLa cells treated with indicated siRNA and infected with *Salmonella* were harvested at indicated times p.i. The number of colony forming units (CFU) per well are shown as dot plot and data represent mean ± S.D. (n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; Student's t test).

Since HOPS complex is an essential component of the late endocytic fusion machinery, we compared the bacterial burden in the HOPS-depleted cells with those depleted of small GTPases that regulate HOPS assembly on these compartments and the SNARE proteins that physically drive the dynamic fusion events between late endosomes and lysosomes. To this end, we evaluated the bacterial load in cells treated with siRNAs directed against Rab7, Arl8b and late endosomal/lysosomal SNAREs: Vti1b, Syntaxin 8, Syntaxin 17, and Vamp7. The knockdown efficiency was determined using Western blotting and qRT-PCR analysis **Figs 3.11 a-f)**. Comparable to HOPS depletion, the bacterial replication in Rab7 and Arl8b-depleted cells was also defective with only ~1.1-1.3 fold increase in the bacterial load from 2 hr p.i. to 10 hr p.i. (**Fig 3.11g)**. Amongst SNAREs, Syntaxin8 showed the most significant decrease in bacterial replication (~1.15 fold; **Fig 3.11h**) followed by Vti1b and Syntaxin 17 (~1.75 and ~1.8 fold, respectively; **Fig 3.11h**) whereas bacterial replication was modestly (but significantly) decreased in Vamp7-depleted cells (~2.3 fold; **Fig 3.11h)**. In addition to HeLa cells, we also verified that HOPS subunit-Vps41 is required for *Salmonella* replication in primary mouse embryonic fibroblasts, MEFs (**Figs 3.12d** and **3.12h**; knockdown efficiency >70%; control siRNA: ~3.3 fold, Vps41 siRNA: ~2.5fold increase in bacterial burden from 2 hr p.i. to 10 hr p.i.).

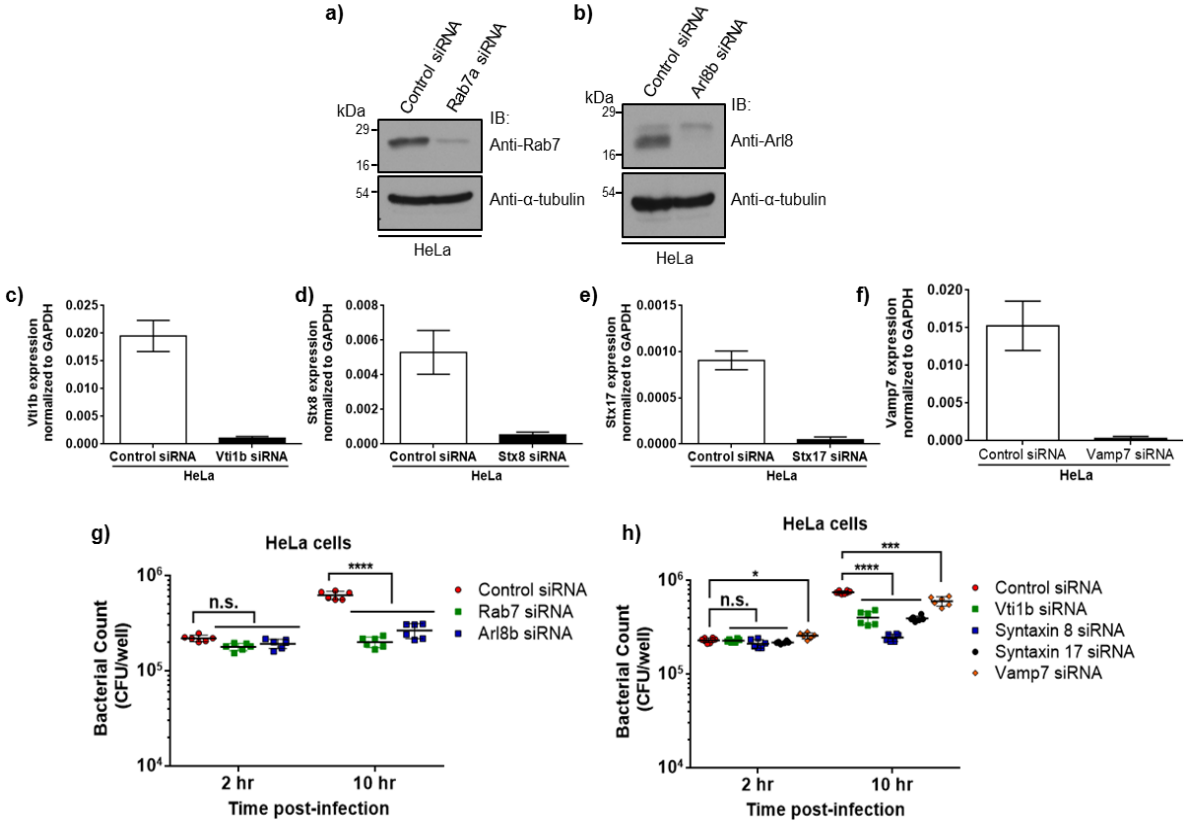


Figure 3.11 a-f) Characterization of the functions of other components of late endosomal fusion machinery during *Salmonella* replication. Western blotting of different cell types transfected with indicated siRNA was performed to measure the gene silencing efficiency. **g and h) Gentamicin-protection assay.** HeLa cells treated with indicated siRNA and infected with *Salmonella* were harvested at indicated times p.i. The number of colony forming units (CFU) per well are shown as dot plot and data represent mean ± S.D. (n.s., not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; Student's *t* test).

It has been well established that host macrophages serve as host cells during both acute and persistent *Salmonella* infections and systemic dissemination of this pathogen (Monack et al., 2004). The dynamics of *Salmonella* invasion, survival and replication in macrophages; eventually leading to cell death and escape of bacteria are central to the pathogenesis of *Salmonella* infection. In view of that, we next analysed whether HOPS complex is necessary for *Salmonella* replication in a macrophage-like cell line RAW264.7, a widely used cell line model for *in vitro* studies of *Salmonella* infection. We performed CFU assays in control-, Vps39- and Vps41-siRNA treated mouse-RAW 264.7 macrophages (**3.12a** and **3.12b Figs**; knockdown efficiency >80%). A significant decrease in bacterial burden was observed in case of HOPS-depleted cells (~2.8 fold and

~1.3 fold increase in *Salmonella* burden upon Vps39 and Vps41 depletion, respectively) as compared to the cells treated with control-siRNA (~4 fold increase in bacterial replication); reinforcing that HOPS complex is indeed a crucial host factor for intracellular *Salmonella* replication in both epithelial and macrophage cells (**Figs 3.12e** and **3.12f**). A similar trend (but overall less replication) was observed in the control and Vps41 lentiviral-mediated shRNA transduced cells (**Figs 3.12c and 3.12g**; knockdown efficiency >70%; control shRNA: ~2 fold and Vps41 shRNA: ~ 0.9-fold change in bacterial burden from 2 hr to 10 hr p.i.).

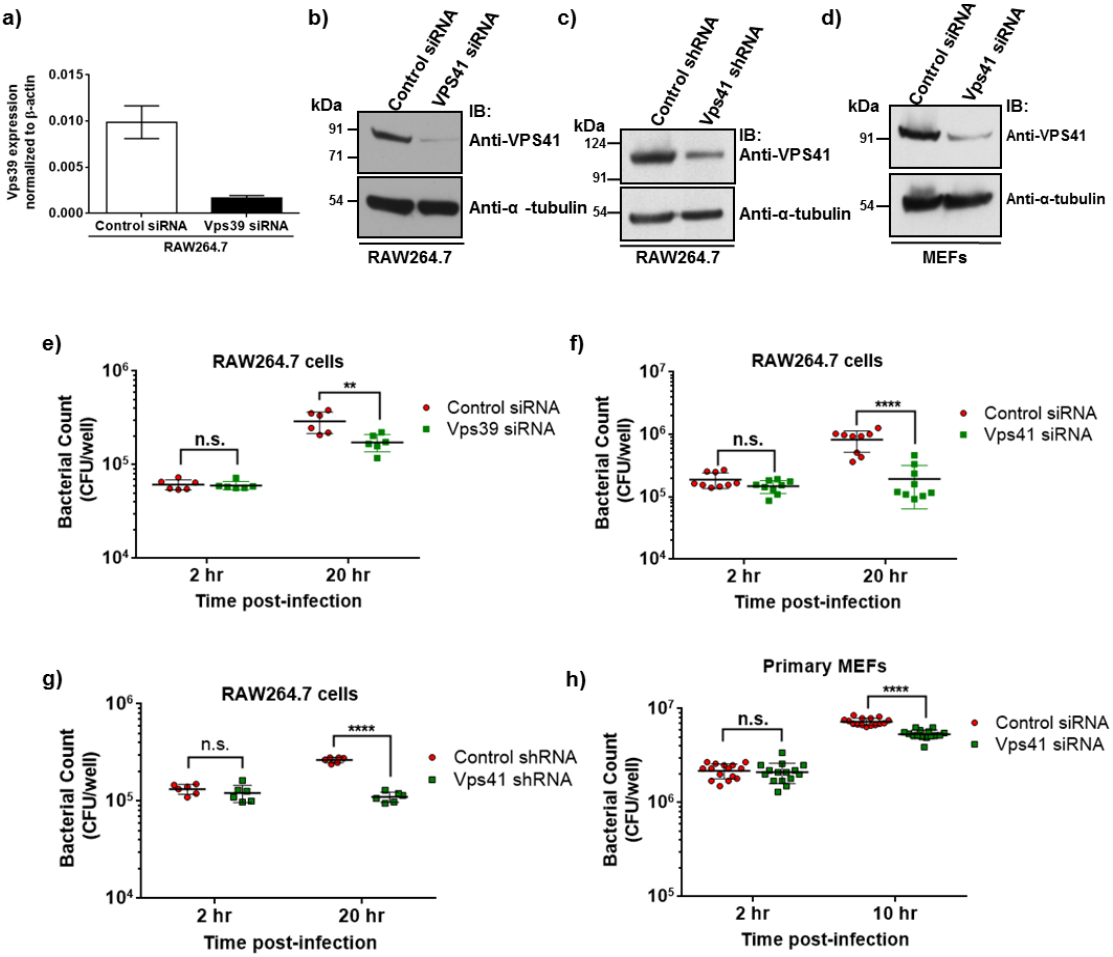


Figure 3.12: HOPS subunits are required for intracellular replication of *Salmonella* in different cell types. a-d) Western blotting or qRT-PCR analysis of different cell types transfected with indicated siRNA or shRNA was performed to measure the gene silencing efficiency. **Intracellular replication assay.** RAW264.7 **(e-g)** cells treated with indicated siRNA or shRNA and infected with *Salmonella* were harvested at indicated times p.i. The number of colony forming units (CFU) per well are shown as dot plot and data represent mean ± S.D. (n.s., not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; Student's *t* test). **h) Gentamicin-protection assay.** primary MEF cells treated with indicated siRNA, and infected with *Salmonella* were harvested at indicated times p.i. The number of CFU per well were determined and shown as dot plot. Data represent mean ± S.D. (n.s., not significant; ****, P < 0.0001; Student's t test).

To corroborate the bacterial infection experiments performed under *in vitro* cell culture conditions, we next assessed whether HOPS subunits are required for *in vivo* replication of *Salmonella* in a mouse model. To determine this, we used morpholino-based approach to downregulate Vps41 expression in mice that were further infected with *Salmonella* by intravenous injection. As a control, standard negative control morpholino was injected in age-matched mice. At day 3 p.i., CFU counts were analyzed from the liver and spleen homogenates of control- and Vps41-morpholino treated mice. The efficiency of Vps41 depletion in both liver and spleen was found to be >80% and >70%, respectively, while no change in the levels of Vps18 (that directly binds to Vps41) was observed (**Fig 3.13a**). Similar to our previous findings in cultured cells, striking decrease in *in vivo* replication of *Salmonella* was observed upon Vps41 depletion (**Fig 3.13b**). Consistent with this, DnaK signal was also strikingly reduced in tissue homogenates from Vps41 morpholino-injected mice (**Fig 3.13a**, *third panel*). Overall, our findings reveal HOPS complex as an essential host factor required for *Salmonella* proliferation in multiple cell types and in a murine infection model. *This experiment was carried out in collaboration with Subhash Babu Arya at IMTECH, Chandigarh.*

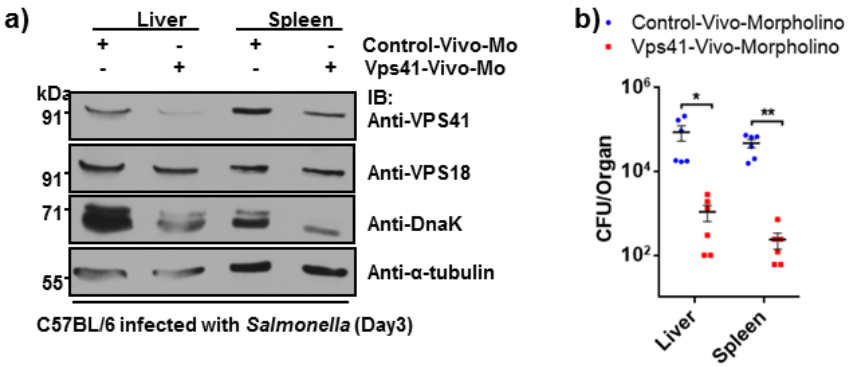


Figure 3.13: Depletion of HOPS subunit, Vps41 impairs *Salmonella* replication in mice. a and b) Mice were injected intravenously (i.v.) with control- or Vps41 specific-vivo-morpholinos (sample size of six for each treatment) for 2 days at an interval of 24 hr, followed by an i.v. challenge with 1.3×10^3 CFU *Salmonella*. On day 3 p.i., mice were sacrificed and bacterial loads in spleen and liver were determined by plating serial dilutions of tissue homogenates on agar plates containing streptomycin (*, $P < 0.05$; **, $P < 0.01$; Student's *t* test). The tissue homogenates were resolved on SDS-PAGE and immunoblotted with indicated antibodies by Western blotting.

3.2.7 Depletion of HOPS subunits delays but does not block the maturation of *Salmonella* vacuoles

The establishment of a replicative intracellular habitat is the key to successful *Salmonella* infection, for which these bacteria dynamically interact with the components of endolysosomal trafficking pathway and procure both membrane and luminal contents from these compartments during SCV maturation (Liss and Hensel, 2015a). As the SCVs mature, they acquire markers of late endocytic compartments such as LAMP1, Rab7 (within 30 min p.i.) while the early endosomal markers like EEA1 are rapidly lost from their membranes.

Since HOPS complex plays a crucial role in regulating the fusion of cargo-laden vesicles with lysosomes across multiple pathways we speculated that this multi-subunit tether might also regulate the fusion of *Salmonella* phagosomes with late endosomes and lysosomes during SCV trafficking. To test this assumption, we followed the recruitment of EEA1, a marker for early SCVs and LAMP1, a late SCV marker protein at different time-points post invasion (10min, 1h and 6h) microscopically. We also quantified the recruitment of these proteins by calculating the percentages of SCVs positive for EEA1 and LAMP1 in both control- and HOPS specific-siRNA treated HeLa cells at these time-points. At the early time point i.e. 10 min p.i. there was no significant difference in the percentages of EEA1-positive SCVs in control cells and HOPS-depleted cells indicating that HOPS is not necessary for the invasion process (**Figs 3.14a-c** and quantification shown in **Figs 3.14j** and **3.14k**; control siRNA: ~78-84%, Vps41 siRNA: ~75%, and Vps39 siRNA: ~71%). %). LAMP1 acquisition was not observed in either control or HOPS depleted cells at this early time point of infection (**3.14a-c Figs**; see intensity profile). Interestingly, we found that at the 1 hr p.i. time-point; while in control cells the majority of SCVs (~70%) had now acquired LAMP1 and were EEA1-negative (as reported previously and **Fig 3.14d,** see intensity profile; quantification shown in **Figs**

3.14j and **3.14k**), only ~14-25% of the SCVs could acquire LAMP1 in case of HOPS-depleted cells and around 40% of the SCVs still retained the early marker protein, EEA1 (**Figs 3.14e** and **3.14f**; quantification shown in **Figs 3.14j** and **3.14k**). These observations implicated that there might be a slight delay in SCV maturation upon depletion of this multi-subunit tethering factor. However, at a much later time-point (6hr p.i.) we observed that ~62-70% of SCVs were now positive for LAMP1 in the HOPS-siRNA treated cells and the early endosomal protein, EEA1 was completely absent from the SCV membranes (**Figs 3.14g-i**; quantification shown in **Figs 3.14j** and **3.14k**), suggesting a delay but not a complete block in the maturation of SCVs in case of HOPS-depletion.

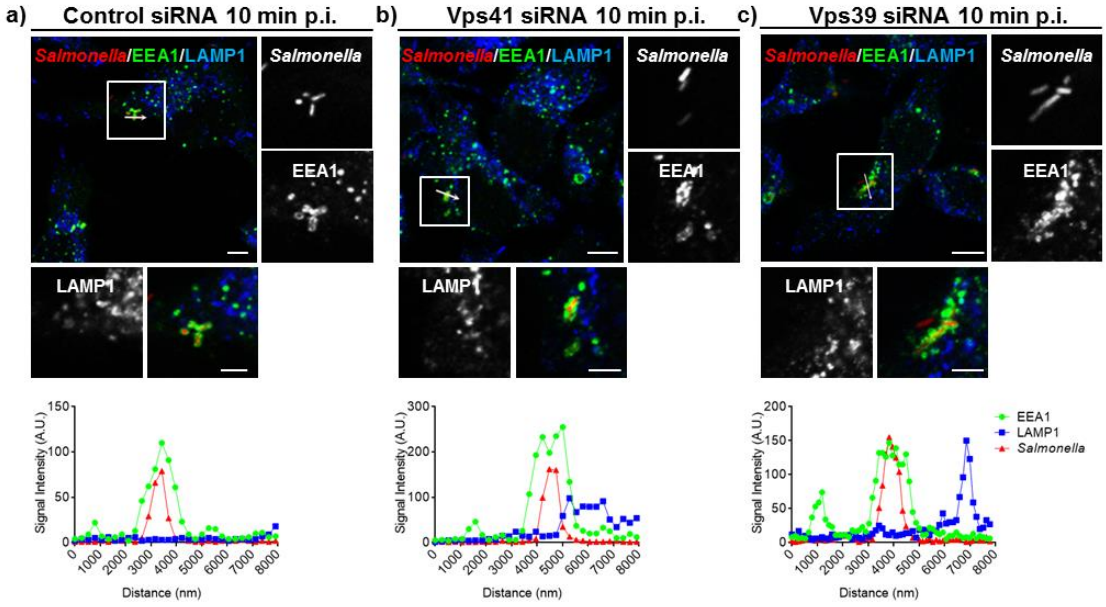


Figure 3.14: Depletion of HOPS subunits does not affect *Salmonella* invasion a-c) Representative confocal micrographs of control siRNA-, Vps41 siRNA- or Vps39 siRNA-treated HeLa cells infected with DsRed-expressing *Salmonella* (red). At 10 min p.i., cells were fixed and stained for early endosomes marker, EEA1 (green) and LAMP1 (blue). Insets depict higher magnification of the boxed areas showing localization of different markers on the SCVs. Shown below the image is the intensity scan profile to visualize colocalization of *Salmonella* (red) with EEA1 (green) and LAMP1 (blue).

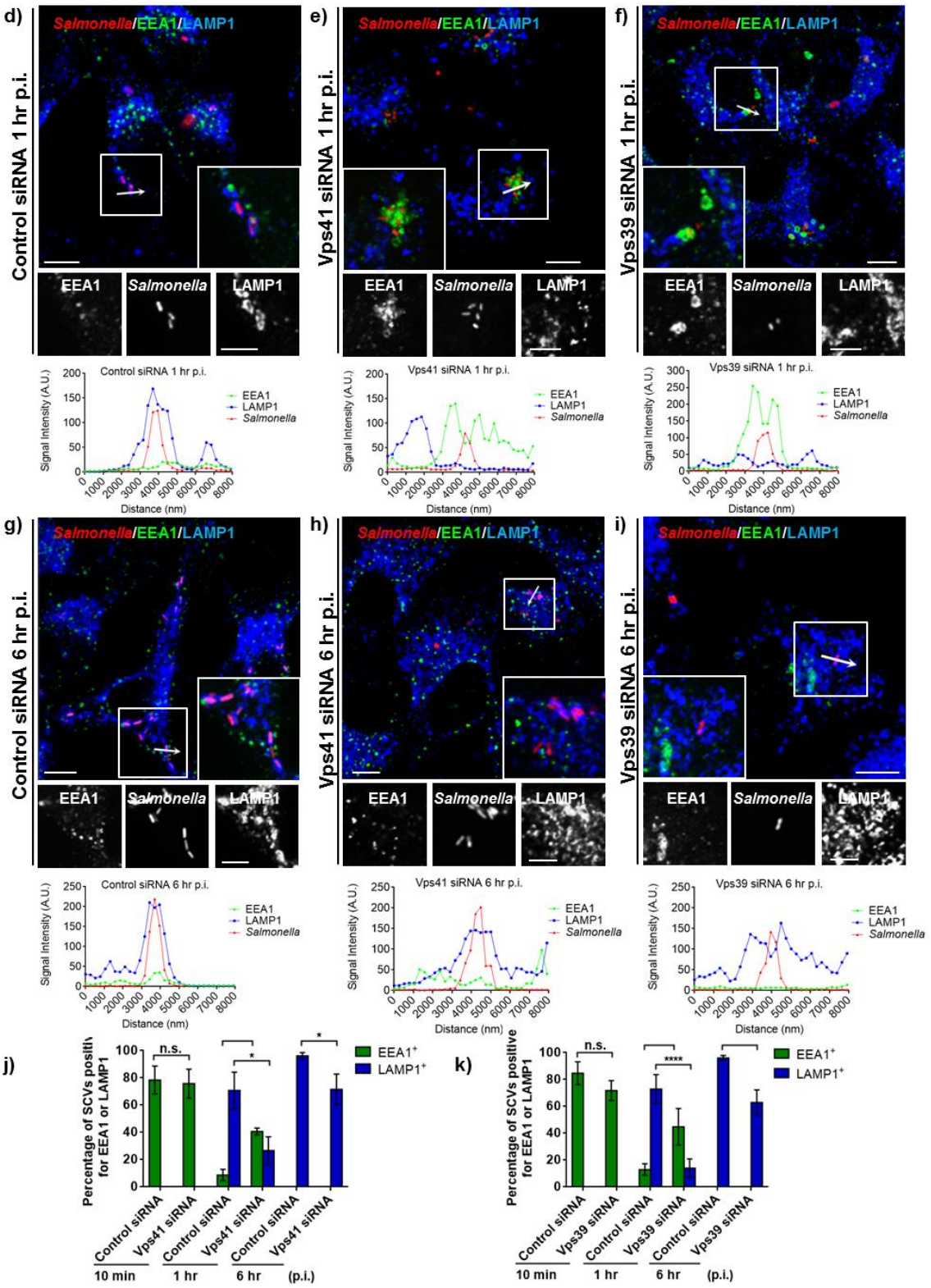


Figure 3.14: Depletion of HOPS subunits delays but does not block SCV maturation. d-i) Representative confocal micrographs of control siRNA-, Vps41 siRNA- or Vps39 siRNA-treated HeLa cells infected with DsRed-expressing *Salmonella* (red). At different time points p.i., cells were fixed and stained for early endosomes marker, EEA1 (green), and LAMP1 (blue). Insets depict higher magnification of the boxed areas showing localization of different markers on the SCVs. Intensity line scan profile of EEA1/LAMP1 across the width of a single SCV (indicated by an arrow in the boxed region) is shown below the individual image. Bars: (main) 10 µm; (insets) 5 µm. **j and k)** Quantification of percentage of infected cells displaying EEA1/LAMP1-accumulation around SCVs at the indicated time point p.i. Data represent mean ± S.D. for ~50 SCVs from three independent experiments (n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; Student's t test).

To validate the above findings, we also analysed the localization of other late endocytic marker proteins such as Rab7 and LBPA (lysobisphosphatidic acid) in control and HOPS-depleted cells at 1hr and 6 hr p.i. As previously reported (Garcia-del Portillo and Finlay, 1995), we did not observe acquisition of the late endosomal lipid-lysobisphosphatidic acid (LBPA) to SCV membranes either in control or HOPS depleted cells at 1 hr and 6 hr p.i. (**Fig 3.15 a-f**).

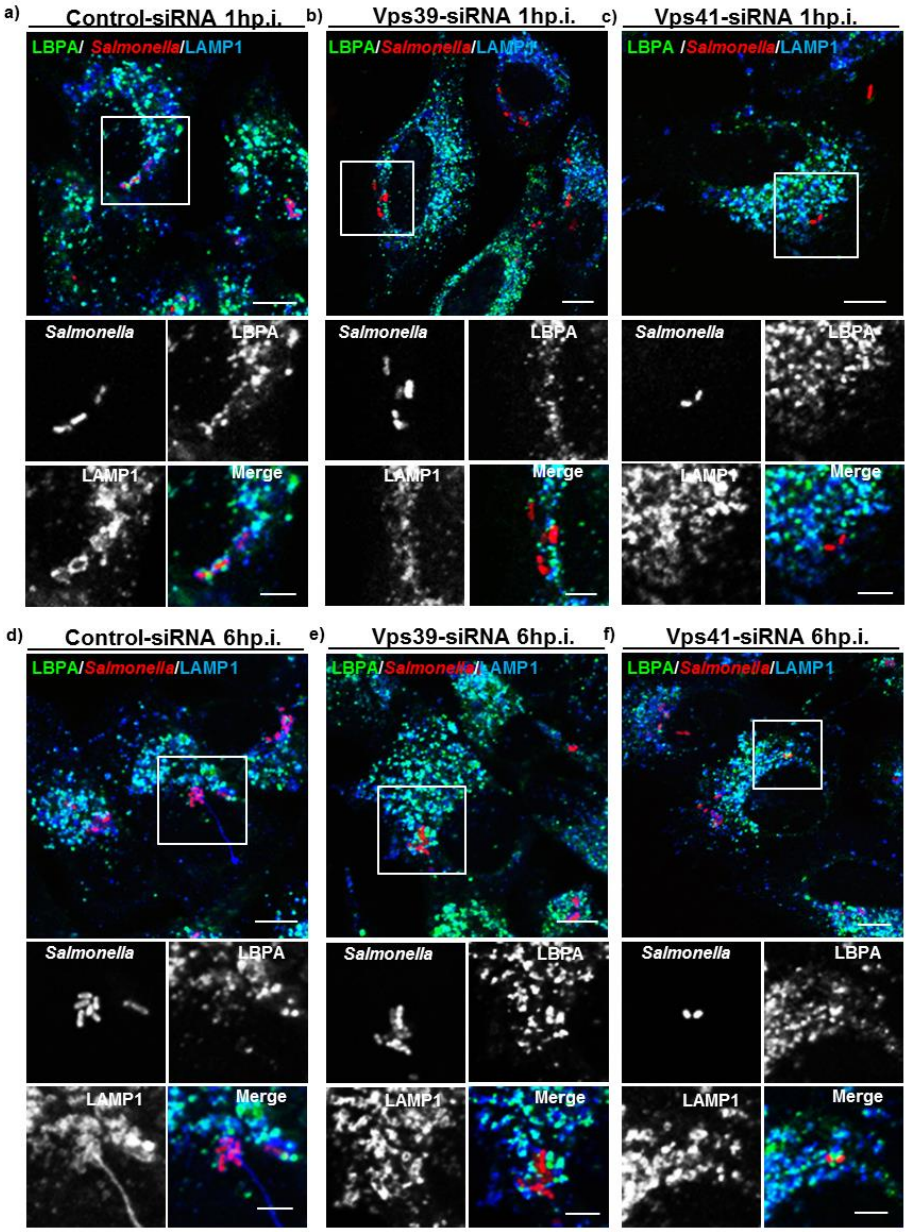


Figure 3.15: LBPA is not acquired around the SCVs in control and HOPS depleted cells. a-f) Representative confocal micrographs of control siRNA-, Vps39 siRNA- or Vps41 siRNA-treated HeLa cells infected with DsRed-expressing *Salmonella* (red). At 1 hr (a-c) and 6 hr (d-f) p.i., cells were fixed and stained for LBPA (green) and LAMP1 (blue). Insets depict higher magnification of the boxed areas showing localization of different markers on the SCVs. Bars: (main) 10 µm; (insets) 5 µm.

However, in the case of Rab7 although we did not find any difference in the localization of this protein to the SCVs in both control- and HOPS-siRNA treated cells (wherein >80-90% SCVs were positive for Rab7 at 1 hr, 3 hr, and 6 hr p.i in both control and HOPS depleted cells, **Figs 3.16a-f;** quantification shown in **Fig 3.16g)**; we did notice a modest yet significant decrease in the Rab7 intensity around the SCVs in HOPS-depleted cells at 1hr p.i. as compared to the control-siRNA treated cells infected with *Salmonella*. Likewise, LAMP1, the Rab7 intensity around the SCVs was recovered at the later time-point (3hr p.i.; **Figs 3.16h and 3.16i**). Our findings suggest that especially at 1 hr p.i., several SCVs in HOPS-depleted cells retain characteristics of both early endosomes and late endosomes (see quantification shown in **Figs 3.14j, 3.14k** and **3.16i**) and that the acquisition of Rab7 to these compartments might be independent of HOPS complex. Collectively, these results signify that depletion of HOPS complex might play a role in SCV maturation but is not solely responsible for this process.

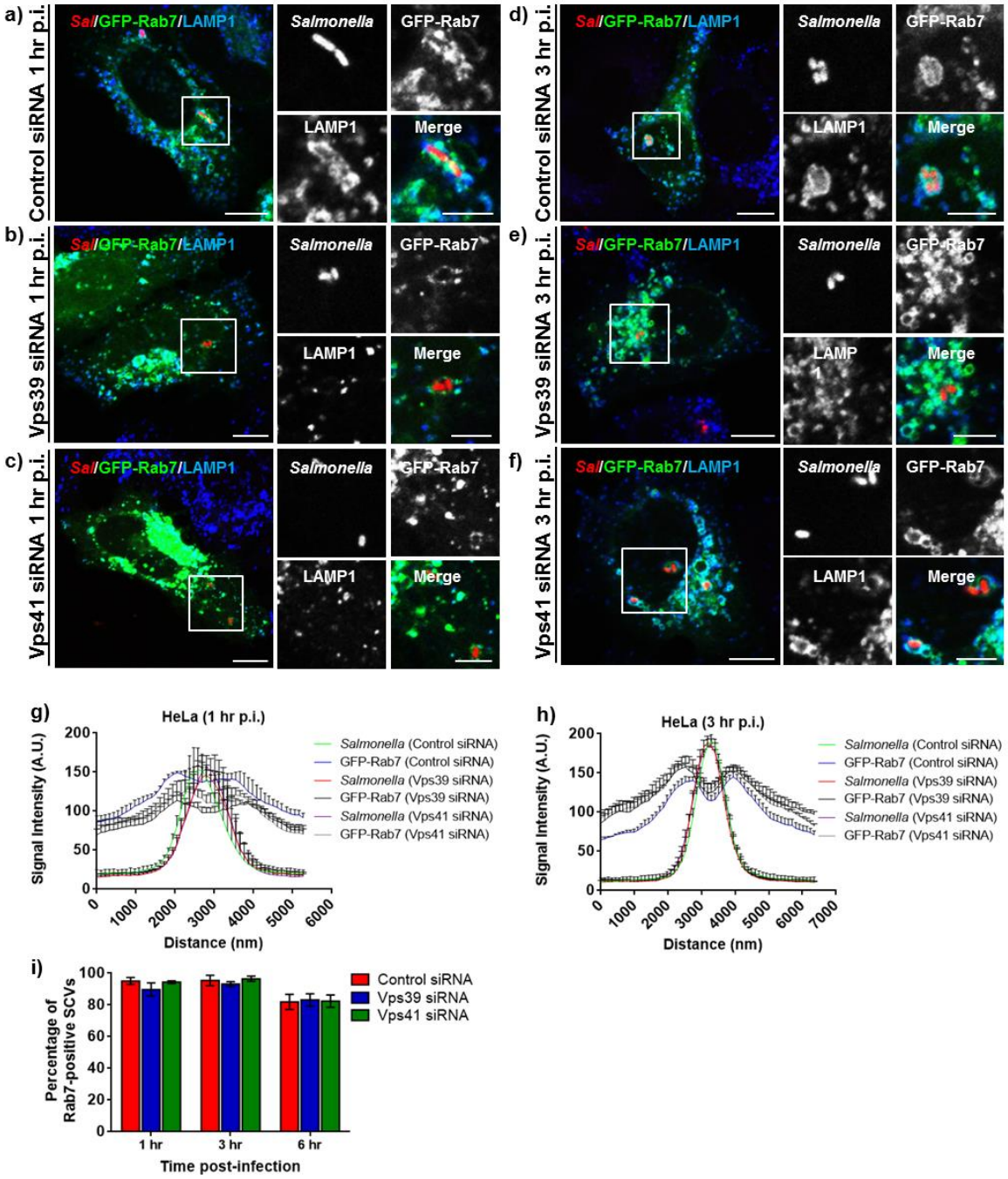


Figure 3.16: Depletion of HOPS subunits does not alter Rab7 recruitment to SCV. a-f) Representative confocal images of control-, Vps41-, or Vps39-siRNA treated HeLa cells, and subsequently transfected with GFP-Rab7 and infected with DsRed-expressing *Salmonella* (red). At different times p.i., cells were fixed and stained for LAMP1 (blue). Insets depict higher magnification of the boxed areas showing localization of Rab7 and LAMP1 around the SCVs. Bars: (main) 10 µm; (insets) 5 µm. **g)** Quantification of Rab7-positive SCVs in control-, Vps41- or Vps39-siRNA treated HeLa cells. Data represent mean ± S.D. over three independent experiments at the indicated time points where ~100 SCVs were counted in each experiment. **h and i)** Quantification of GFP-Rab7 intensity around the SCVs in control-, Vps41- or Vps39-siRNA treated cells over three independent experiments at the indicated time points p.i. where intensity profile of ~50 SCVs were quantified in each experiment. Data represent mean ± S.E.M.

Our findings indicate that SCV maturation follows a scheme similar to maturation of early endosomes to multi-vesicular bodies/late endosomes upstream of HOPS-mediated fusion of late endosomes and lysosomes (Poteryaev et al., 2010; Rink et al., 2005). In agreement with this, previous studies have shown that endocytic machinery required for early to late endosome maturation such as Vps34 and Rab7 is also required for SCV maturation (Brumell et al., 2001c; Brumell et al., 2002b; Mallo et al., 2008; Méresse et al., 1999b). To confirm that LAMP1 acquisition by SCVs is not inhibited upon fusion with lysosomes, we treated cells with Bafilomycin A1 (Baf A1), a routinely used chemical inhibitor of vesicle fusion with lysosomes (Yoshimori et al., 1991). Baf A1 inhibits fusion of lysosomes with other compartments by inactivating the ER Ca^{2+}-ATPase (SERCA) whose activity is required to maintain the lysosomal Ca^{2+} stores (Garrity et al., 2016; Mauvezin et al., 2015). As shown in **3.17a** and **3.17b Figs**, LAMP1 acquisition around SCVs was not impaired in cells pre-treated with Baf A1 (see intensity profile graphs in **3.17c** and **3.17d Figs**) although SIF formation was abrogated in the presence of this drug. These findings support our conclusion that LAMP1 acquisition by SCV does not require heterotypic fusion with lysosomes, which in turn is mediated by HOPS complex. An alternative explanation for this could be that in case of Baf A1 treatment *Salmonella* phagosomes fail to acidify. The acidification of SCVs is necessary for activation of OmpR-dependent transcription of SPI-2 genes which and secretion of SPI2-T3SS effectors from the SCV membranes. In the next chapter of this thesis we have shown that the secretion of SPI2-effector SifA is essential for recruiting HOPS complex to the SCV membranes thereby facilitating fusion with the lysosomes leading to defective bacterial replication and SIF formation in BafA1 treated cells. In concordance with these studies, immunogold-EM of ultrathin sections of *Salmonella*-infected Vps41 depleted cells at 10 hr p.i. showed presence of several vacuolar bacteria surrounded by limiting

membrane positive for late endosomal and lysosomal markers-Rab7 and LAMP1 (**Figs 3.17f and 3.17g**).

Previous studies have shown that *Salmonella* colonizes and hyper-replicates within the cytosol of epithelial cell (Knodler et al., 2014; Malik-Kale et al., 2012). To address whether the cytosolic hyper-replicating *Salmonella* population is increased upon HOPS depletion, we determined bacterial burdens in control and Vps41 depleted cells using the previously described modified gentamicin protection assay where cells are treated with chloroquine (CHQ) before the end of infection time point. CHQ is a lysosomotrophic agent that accumulates within endosomes/lysosomes and has been shown to preferentially target vacuolar but not cytosolic bacteria. We observed a modest but not a statistically significant increase in the number of cytosolic bacteria at 7 hr p.i (peak time point of cytosolic replication (Malik-Kale et al., 2012) in Vps41 siRNA treated cells (**3.17e Fig**: control siRNA: 28±3% and Vps41 siRNA: ~36±4%), suggesting that majority of bacteria (~70%) continue to harbor their vacuolar niche upon HOPS depletion. *This experiment was carried out in collaboration with Subhash Babu Arya at IMTECH, Chandigarh.*

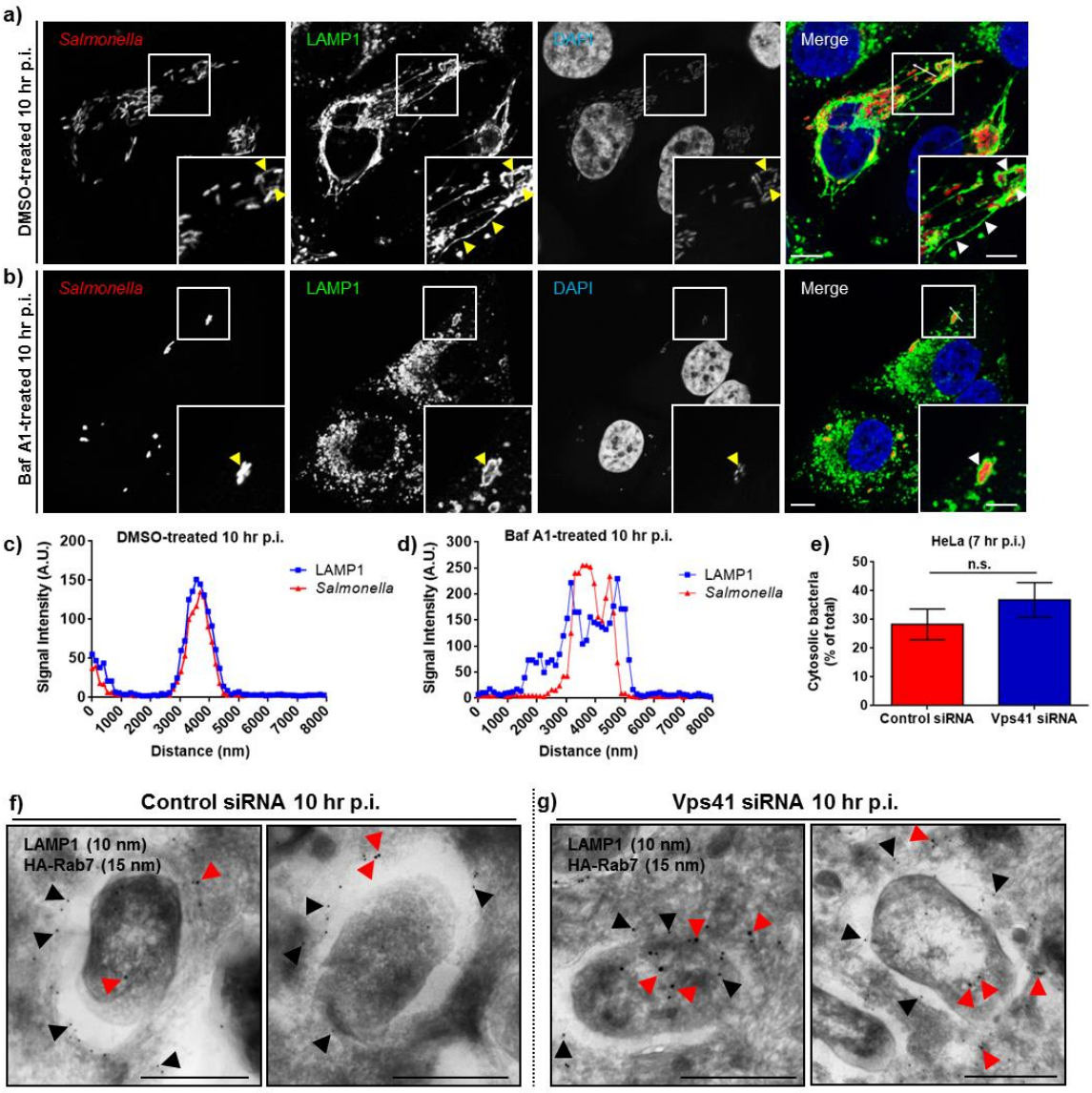


Figure 3.17 LAMP1 acquisition around SCVs does not require fusion with lysosomes. a-d) HeLa cells pre-treated with either DMSO (vehicle control) or Bafilomycin A1 (Baf A1) (50 nM) overnight were infected with DsRed-expressing *Salmonella* (red). At 10 hr p.i., cells were fixed and immunostaining for LAMP1 (green) was performed. The nuclei were stained using DAPI (blue). Insets depict higher magnification of the boxed areas showing localization of different markers on the SCVs. Bars: (main) 10 µm; (insets) 5 µm. f and g) The intensity scan profile to visualize colocalization of *Salmonella* (red) with LAMP1 (blue) in DMSO or Baf A1 treated HeLa cells is shown. **e)** Chloroquine (CHQ) resistance assay was performed to quantify the percentage of cytosolic bacteria in total population upon Vps41 silencing. HeLa cells seeded in a 24-well plate were transfected with control- or Vps41-siRNA, and infected with *Salmonella*. After 6 hr p.i., two wells were incubated with CHQ and gentamicin (CHQ-resistant bacteria, cytosolic bacteria) and two wells were incubated with gentamicin only (total bacteria) for 1 hr. At the end of 7 hr p.i., cells were harvested and the number of CFU per well were determined and the percentage of cytosolic bacteria proliferation was calculated as the ratio of CFU obtained at 7 hr p.i. in CHQ + gentamicin treated wells/CFU obtained at 7 hr p.i. in gentamicin alone treated wells. Data represent mean ± S.D. from three independent experiments (n.s., not significant; Student's *t* test). **f and g)** Representative immunogold EM images of control siRNA (j and k)- or Vps41 siRNA (l and m)-treated HeLa cells infected with *Salmonella* for 2 hr and transfected with HA-tagged Rab7 and fixed at 10 hr p.i. Cells were processed for immunogold labeling with anti-LAMP1 (10 nm) and anti-HA (15 nm) antibodies. Arrowheads indicate localization of Rab7 (red) and LAMP1 (black) around the SCVs. Bar: 500 nm.

3.2.8 *Salmonella* exploits HOPS complex to mediate interaction and acquire contents from late endosomal and lysosomal compartments

A number of live cell imaging studies along with recent ultrastructural analysis using correlative light and electron microscopy (CLEM) have revealed that the SCVs are accessible to fluid phase cargo from lysosomes and incoming endocytic vesicles through direct interactions with these compartments as they undergo maturation inside the host cells (Drecktrah et al., 2007b; Krieger et al., 2014). Furthermore, these studies have pointed out that the SCVs significantly associate with late endosomes/lysosomes as soon as 30 min p.i. and maintain a prolonged interaction with them until several hours post invasion which is concomitant with SIF formation (Drecktrah et al., 2008). However, the fusion machinery regulating the extensive and dynamic interactions at this host-pathogen interface between remains elusive. Our results demonstrating that HOPS complex localizes to SCV and SIFs suggest that similar to its role in mediating late endosome-lysosome fusion, this tethering factor could facilitate SCV fusion with lysosomes. To test this, prior to infection we pre-loaded control siRNA- and Vps41 siRNA-treated HeLa cells or control and Vps41 shRNA stably transduced RAW264.7 macrophages with Alexa 647-conjugated dextran (dextran-647) to specifically label lysosomes, as shown schematically in **Fig 3.18a**. Live-cell imaging performed at 10 hr p.i. in control HeLa and RAW264.7 cells showed several dextran-positive endosomes undergoing fusion with the SCVs, resulting in acquisition of this fluid phase cargo by the SCVs (**Figs 3.18b** and **3.18d**; **S7** and **S9 Movies**). SIF formation was also observed in both control siRNA/shRNA-treated cells (**S7** and **S9 Movies**). In contrast, little or no interaction of SCVs with the dextran compartment was observed in Vps41 depleted HeLa and RAW264.7 cells (**Figs 3.18c** and **3.18e**; **S8** and **S10 Movies**). Quantification of SCVs positive for dextran-647 and its signal intensity, revealed significantly lower dextran acquisition in Vps41 depleted cells compared to control (**Figs 3.18f-i;** percentage of

dextran-positive SCVs in HeLa and RAW264.7 cells-control: ~65-70%, Vps41 depletion: 10-15%). These results suggest that HOPS complex mediates the acquisition of fluid-phase content by the SCVs from late endosomes and lysosomes.

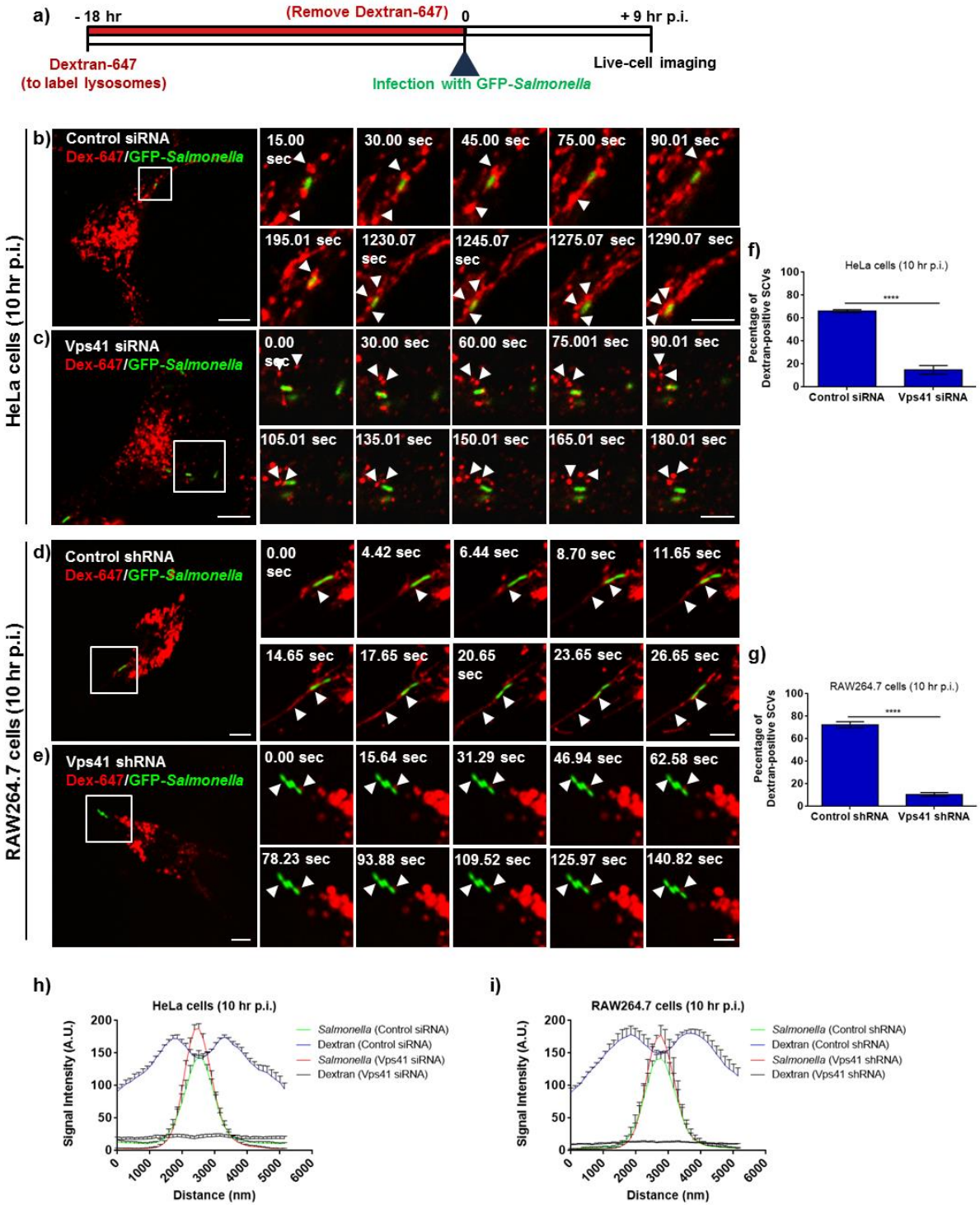


Figure 3.18: Interaction of dextran-loaded lysosomes with SCVs is impaired upon Vps41 depletion. a) Schematic illustrating the protocol used for loading of lysosomes with Alexa-Fluor 647-conjugated dextran in HeLa and RAW264.7 cells, prior to infection with GFP-expressing *Salmonella*. **b-e)** HeLa cells treated with control siRNA **(b)** or Vps41 siRNA **(c)** or RAW264.7 cells transduced with control shRNA **(d)** or Vps41 shRNA **(e)** were pre-incubated with Alexa-Fluor 647-conjugated dextran (red) to label lysosomes, followed by infection with GFP-expressing *Salmonella* (green). Time-lapse series for Alexa-Fluor 647-conjugated dextran loaded and infected cells were recorded at 10 hr p.i., and still images from representative time lapse series are shown **(S7-S10 Movies).** Different panels represent a higher magnification of the boxed area and the white arrowheads indicate the SCVs. Bars: (main) 10 µm; (insets) 5 µm. **f and g)** Quantification of Alexa-Fluor 647-conjugated dextran-positive SCVs in control and Vps41 depleted HeLa and RAW264.7 cells fixed at 10 hr p.i. Data represent mean ± S.D. over three independent experiments where ~100 SCVs were counted in each experiment (****, $P < 0.0001$; Student's t test). **h and i)** Quantification of Alexa-Fluor 647-conjugated dextran signal intensity around the SCVs in control and Vps41 depleted HeLa and RAW264.7 cells. Data represent mean ± S.E.M. of signal intensity from three independent experiments at 10 hr p.i. where ~50 SCVs were counted in each experiment.

In agreement with these findings, imaging of ultrathin sections of *Salmonella*-infected control cells by TEM demonstrated several late endosomes (containing numerous MVBs) and lysosomes (containing lamellar membrane sheets) docked at or in close apposition to the SCVs (**Figs 3.19a** and **3.19b**; **3.20b** and **3.20c Figs**; see magnified insets). In contrast, late endosomes/lysosomes docking at the SCVs were highly reduced in Vps41 depleted cells (**Figs 3.19c** and **3.19d**; **3.20a, 3.20d** and **3.20e Figs**; see magnified insets). Further, as previously noted in another study (McEwan et al., 2015c), we also observed several abnormal "bag-like" SCVs upon Vps41 depletion (**Fig 3.19d**; see magnified inset).

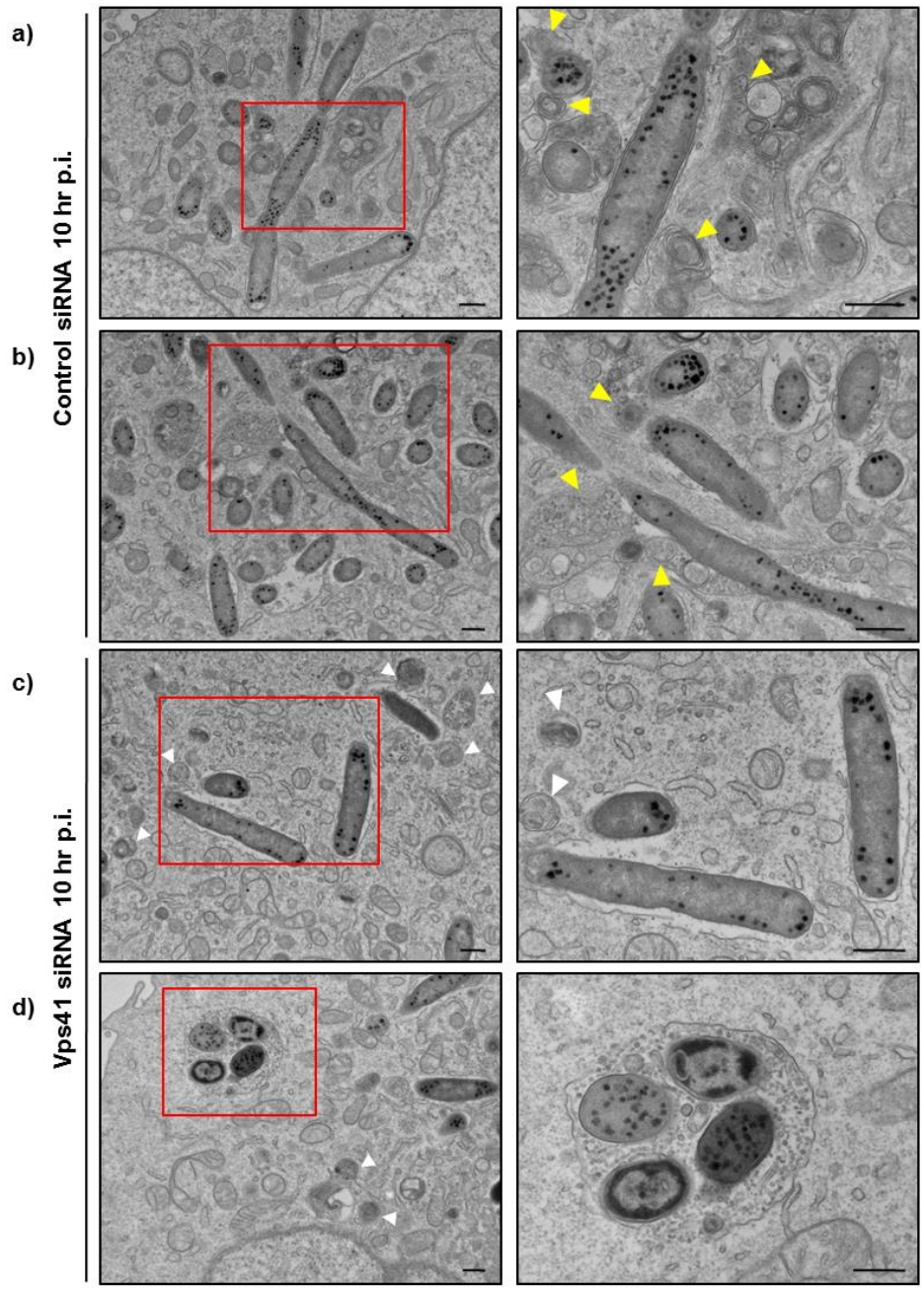


Figure 3.19: Silencing of Vps41 abrogates docking of late endosomes and lysosomes at SCVs. a-d) Representative TEM images of control **(a and b)** and Vps41 **(c and d)** siRNA treated HeLa cells infected with *Salmonella* for 10 hr. Higher magnification of multiple SCVs (marked by yellow arrowheads) interacting with late endosomes (containing MVBs) and lysosomes (containing lamellar membrane sheets) in control siRNA treated cells are shown in the panels on the right. In Vps41 siRNA treated cells, white arrowheads depict the MVBs containing late endosomal compartments. Bar: 500 nm.

Additionally, in few TEM sections, SIF-like tubular extensions were also observed in control but not in Vps41 depleted cells (**3.20 Fig**, *middle panel*). Analysis of several TEM images in control cells revealed that of the ~100 SCVs imaged, ~40 SCVs had closely apposed late endosomes, whereas only ~10 of the 100 SCVs in Vps41-siRNA treated

cells and none of the ~60 SCVs imaged in Vps41-shRNA transduced cells showed docked late endosomes.

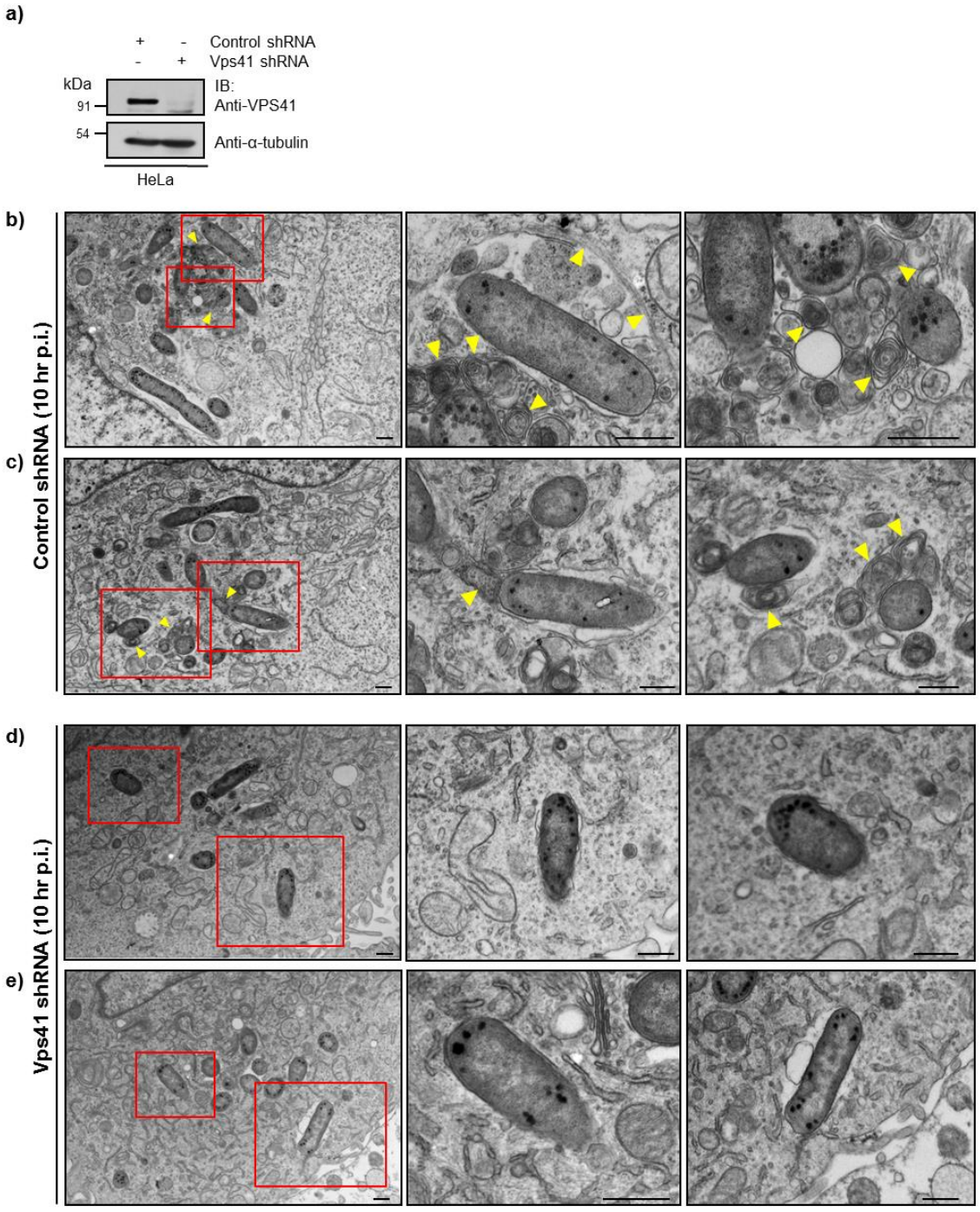


Figure 3.20: Silencing of Vps41 abrogates docking of late endosomes and lysosomes at SCVs. a) Lysates of control shRNA or Vps41 shRNA transduced HeLa cells were immunoblotted with anti-Vps41 antibody for assessing the knockdown efficiency and with anti-α-tubulin antibody as the loading control. b-**e)** Representative TEM images of control shRNA **(b and c)** and Vps41 shRNA **(d and e)** transduced HeLa cells infected with *Salmonella* for 10 hr. Higher magnification of multiple SCVs interacting with late endosomes and lysosomes in control shRNA transduced HeLa cells are shown (marked by arrowheads). Arrowheads indicate SIF formation in inset of panel **(b).** Bar: 500 nm.

As previously reported (Pols et al., 2013), we also noted that lysosomes (containing lamellar membrane sheets) were reduced in Vps41 siRNA treated cells while several large MVB-containing compartments were observed (**Figs 3.19c** and **3.19d**; indicated by white arrowheads). Although docking of late endocytic compartments at the SCVs was reduced upon Vps41 depletion, this did not indicate a general defect in the formation of late endocytic compartments. This was confirmed by LysoTracker Red uptake in control and Vps41 depleted cells, which is a selective probe that labels acidic organelles and routinely used as a specific marker to label late endosomes and endolysosomes. Immunofluorescence analysis and quantification of LysoTracker Red signal intensity by flow cytometry revealed no significant difference in control and Vps41 depleted cells (**3.21a-e Figs)**. The specificity of this probe was confirmed by treating cells with Baf A1 that neutralizes the pH of late endocytic compartments, and hence the signal intensity was reduced to background fluorescence levels (**3.21e Fig**). We also confirmed that functional endo-lysosomes are formed upon Vps41 depletion by comparing levels of mature cathepsin B and D in control and Vps41 siRNA treated cells (**3.21f Fig**). *This experiment was carried out in collaboration with Subhash Babu Arya at IMTECH, Chandigarh.* Taken together, our findings suggest that HOPS complex is a crucial host factor required for SCV fusion with the late endocytic compartments that provide membranes for the formation of a replicative vacuolar niche for this pathogen.

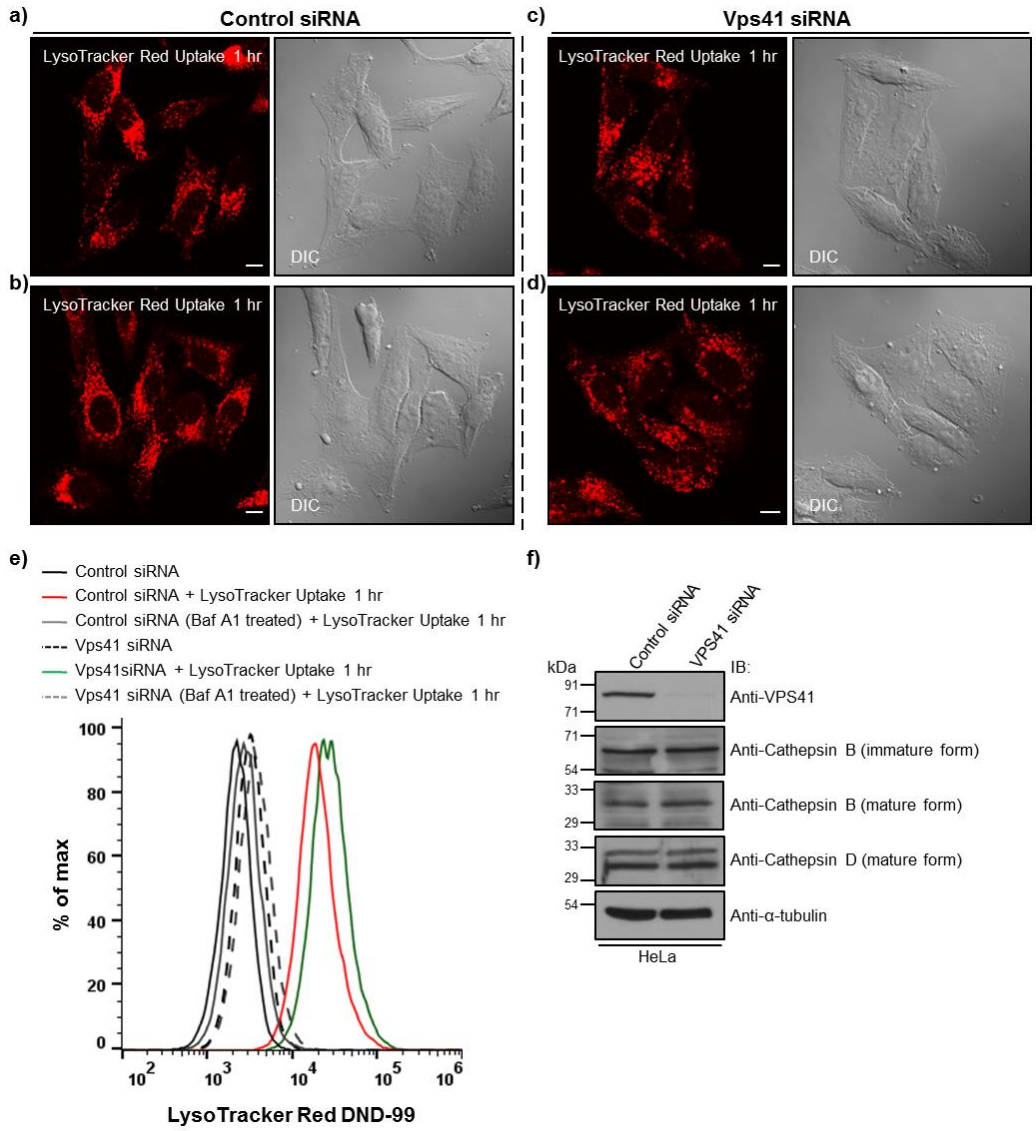


Figure 3.21 Late endocytic compartments are acidic and functional in Vps41 depleted cells. a-d) Representative confocal micrographs of control- and Vps41-siRNA treated HeLa cells incubated with LysoTracker Red (100 nM) for 1 hr. Bar: 10 µm. **e)** Control siRNA- and Vps41 siRNA-transfected HeLa cells were incubated with DMSO (vehicle control) or with Baf A1 incubated for 1 hr. After 1 hr, LysoTracker Red (100 nM) uptake was performed for 1 hr. At the end of the internalization period, cells were washed and fluorescence was determined by flow cytometry. **f)** Lysates from control- and Vps41-siRNA treated HeLa cells were resolved by SDS-PAGE and immunoblotted with indicated antibodies by Western blotting.

3.2.9 Depletion of HOPS complex abrogates SIF formation

The intracellular *Salmonella* uses the endocytic system to expand its vacuolar niche into an extensive tubular network (SIFs) that extends throughout the cytoplasm during later stages of *Salmonella* infection (8-10 hr p.i.). Multiple studies have evidenced that SIFs link the SCVs to the exocytic and endocytic pathways and allows *Salmonella* to obtain nutrients and luminal contents endocytosed by the host cell to promote its intravacuolar

replication (D'Costa et al., 2015; Drecktrah et al., 2008; Kuhle et al., 2006; Perrett and Zhou, 2013; Popp et al., 2015a; Rajashekar et al., 2008b; Zhang and Hensel, 2013). Recently, a seminal study by Hensel and co-workers demonstrated that the ability of *Salmonella* to re-direct the host vesicular pathways to form the SIF network is essential to its metabolism inside the vacuole; further mounting to the importance of this unique phenotype exclusive to *Salmonella* infection (Liss et al., 2017b). SIF biogenesis begins at 5-6 hr p.i. and through dynamic interactions with the late endocytic compartments these tubules form a highly stable network by 8-10 hr p.i. which is best visualized by immunostaining for lysosomal glycoproteins (especially LAMP1) in *Salmonella*-infected cells (Drecktrah et al., 2008). Since, SIF biogenesis is so intricately linked to the intracellular survival and replication of *Salmonella* we next examined its ability to form these tubular networks at two different time-points (6 hr and 10 hr p.i.) in HOPS depleted cells. Strikingly, we observed that as compared to control cells where we could see an extensive network of SIFs; these tubules were completely absent in the cells depleted of either of the six HOPS subunits (**3.22a-i Figs**). Notably, we did find SIFs in TGFBRAP1-depleted cells infected with *Salmonella*, although they appeared to be "beaded" and somewhat thinner as compared to the control cells, which might explain the modest defect in *Salmonella* replication upon depletion of this CORVET-specific subunit as shown in **Fig 3.10h**.

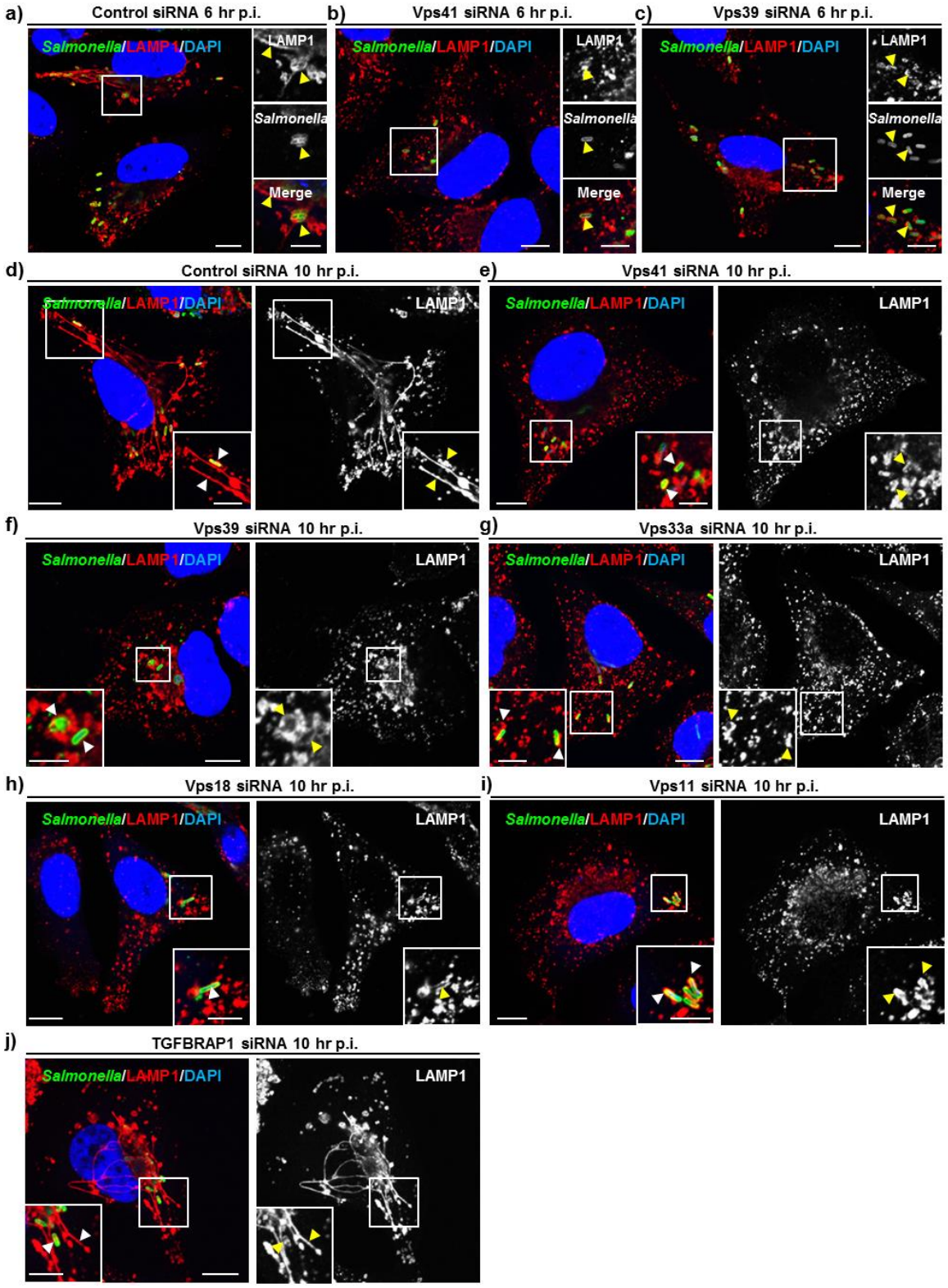


Figure 3.22: Depletion of HOPS complex subunits leads to absence of SIF formation. a-j) Representative confocal micrographs of control siRNA **(a and d)**-, HOPS subunits specific siRNA **(b, c, and e-i)-** or TGFBRAP1 siRNA **(j)-**transfected HeLa cells and infected with *Salmonella*. At different times after infection (as indicated), cells were fixed and immunostained with antibodies to *Salmonella* (green) and LAMP1 (red). The nuclei were stained using DAPI (blue). Insets represent a higher magnification of the boxed areas with arrowheads depicting LAMP1 localization on individual SCVs. Bars: (main) 10 µm; (insets) 5 µm.

Lately, several studies have underlined the importance of live-imaging analyses for the study of intracellular phenotypes induced upon *Salmonella* infection due to the appearance of severe artefacts owing to fixation of the infected cells (Rajashekar et al., 2014). Accordingly, to reinforce and further establish whether formation or stability of SIFs was reduced upon HOPS depletion, we performed live-cell imaging to visualize GFP-LAMP1 (marker for SIFs) dynamics in control-, Vps39- and Vps41-siRNA treated cells that were infected with DsRed-expressing *Salmonella*. At 9 hr p.i., time-lapse imaging revealed extensive SIF formation in control cells that was completely absent in Vps41- and Vps39-depleted cells (**S4-S6 Movies**). Moreover, as compared to the control cells, significantly fewer LAMP1-positive vesicles were found to interact with SCVs in Vps41- and Vps39-depleted cells (**S5** and **S6 Movies**). Collectively, our live imaging and steady state data suggest that *Salmonella* utilizes this multimeric tethering factor to fuse with the late endosomes and lysosomes to initiate SIF biogenesis.

3.2.10 Depletion of HOPS subunits blocks the access of vacuolar *Salmonella* to the extracellular source of nutrition

There have been numerous reports indicating that the SCV is a nutritionally-deprived environment. However, despite this seclusion, *Salmonella* efficiently replicates within the SCVs pointing towards the successful adaptation of this pathogen to thrive intracellularly. Recently, in a pioneering work Hensel and co-workers have highlighted the physiological relevance for a continuous exchange of contents of SCVs with late endosome/lysosomes and SIF formation in providing direct access to specific C-sources from the extracellular medium (Liss et al., 2017b). This could be a possible explanation for the mechanism by which the nesting *Salmonella* obtain nutrients essential for replication. This was partially established by using the auxotrophic strains of *Salmonella* that could not initiate *de novo* synthesis of particular amino acids like asparagine and

proline. However, these mutant bacteria were able to obtain the supplemented amino acids from the external growth medium and replicate only if they were proficient in SIF formation. Furthermore, the results obtained by using auxotrophic strains of *Salmonella* have elucidated that the *Salmonella* population residing inside the SCVs utilize extracellular or medium-supplemented amino acids rather than host cell-derived amino acids indicating that SCVs not only have direct access to the incoming endocytic cargo but also that the amount of host cell nutrients are not sufficient for meeting the demands of the growing *Salmonella* population (Popp et al., 2015a). Based on our findings that HOPS complex plays an indispensable role during SIF formation, we further investigated the role of this multisubunit tethering factor in procuring nutrients for the intravacuolar *Salmonella*. To this end, we infected control- and Vps41-siRNA treated cells with proline auxotrophic strain of *Salmonella* (*proC*). This strain lacks the enzyme catalysing the last step in the proline biosynthetic pathway and is strongly attenuated for intracellular replication unless proline is supplemented in the growth medium in both epithelial and macrophage-like cell line models. As described in a previous study by *Popp et al. 2015*; the *proC* strain showed a severe replication defect as compared to the wild-type (WT) *Salmonella* strain in HeLa cells. Accordingly, this growth defect was completely restored by addition of proline to the culture media in the case of control-siRNA treated cells (**Fig 3.23b**). On the contrary, upon depletion of the HOPS subunit Vps41, only a modest increase in the replication of *proC* strain was observed in the presence of extracellular proline, which was significantly lower as compared to the control cells under the same experimental conditions (**Fig 3.23b**). These results suggest that recruitment of HOPS complex is the strategy used by vacuolar *Salmonella* to continuously access nutrients from the host late endosomes and lysosomes to promote intracellular replication; highlighting the physiological importance of HOPS engagement at SCV and SIF membranes.

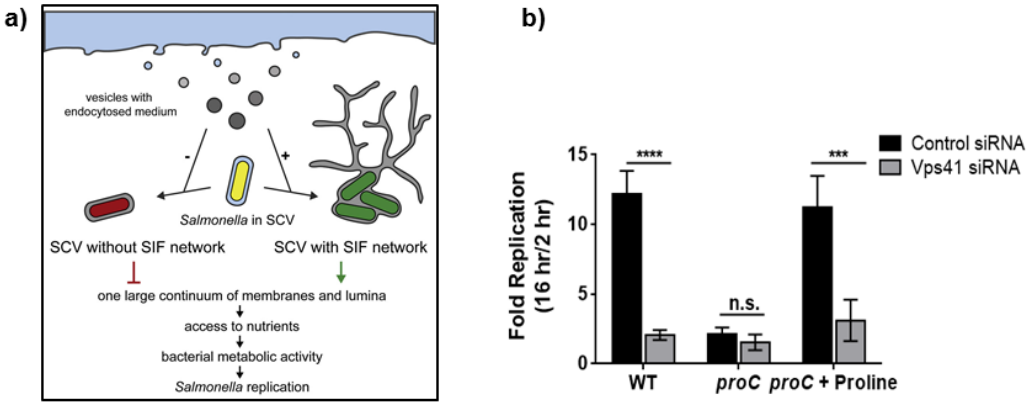


Figure 3.23: Silencing of Vps41 impairs nutrient acquisition by auxotrophic strain of *Salmonella*. a) A schematic demonstrating that formation of the SCV-SIF continuum allows *Salmonella* to bypass nutritional restriction in the intracellular environment by acquiring nutrients from the host cell endosomal system *(Adapted from: (Liss et al., 2017b)*. **b)** Intracellular replication of wild-type (WT) *Salmonella* and a mutant strain auxotrophic for proline *(proC)* was determined at indicated times p.i. in control siRNA- or Vps41 siRNA-transfected HeLa cells. The fold change in intracellular proliferation was calculated as the ratio of CFU at 16 hr p.i./CFU at 2 hr p.i. To complement the intracellular proliferation of *proC Salmonella* strain, cell culture medium was supplemented with proline (0.8 mM). Shown are the means ± S.D. from three independent experiments (n.s., not significant; ***, P < 0.001; ****, P < 0.0001; Student's t test).

CHAPTER IV

Salmonella effector SifA regulates the assembly of HOPS complex onto SCV and SIF membranes

The following chapter has been published in PLOS Pathogens, 2017, 13(10): e1006700

Chapter 4

4.1 Introduction

Salmonella's intracellular lifestyle is regulated by the concerted activity of a group of virulence determinants secreted by the second T3SS encoded within *Salmonella* pathogenicity island, SPI2. These effectors are translocated from the vacuolar membrane into the host cell lumen in response to the intracellular stress inside the SCVs.

Numerous phenotypic changes have been attributed to this cohort of virulence determinants that includes: modulation of host cytoskeletal elements (both actin filaments and microtubules) to mediate juxtanuclear positioning of the mature *Salmonella* vacuoles, redirection of the membranes from endocytic organelles of the host for membrane growth (SIF biogenesis); acquisition of nutrients for vacuolar proliferation; interception and hijacking of endosomal trafficking pathways to confer protection against the lysosomal damage in macrophages; propagation of infection for establishment of a systemic disease by modulating the host cell motility; and poly-ubiquitination of major histocompatibility complex (MHC) class II molecules to inhibit antigen presentation (Figueira and Holden, 2012; Kuhle and Hensel, 2004) . Although the functions regulated by a few of these effectors have been widely studied; the contributions of several other SPI2-T3SS effectors in *Salmonella* virulence still remain elusive.

The collaborative actions of the SPI2-T3SS-secreted effectors convert the "early SCV" into a unique replication-permissive abode known as "late SCV". Several SPI2-T3SS effectors including: SifA, SseJ, SopD2, PipB2, SseF, and SseG perform vital functions during SCV maturation pathway and SIF biogenesis.

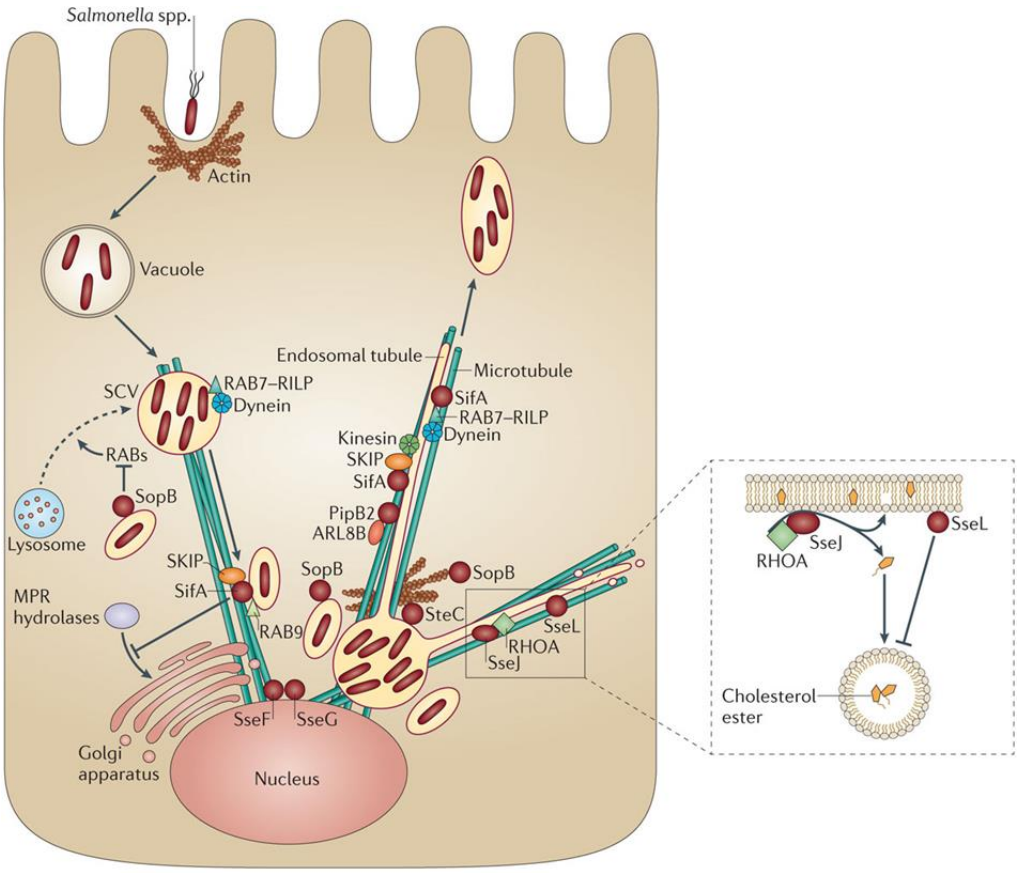


Figure 4.3: *Salmonella*-induced manipulation of host membranes by the action of SPI2-T3SS effectors. *Salmonellae* redirect the host membranes for establishing their intracellular niche (SCV). SPI2-T3SS effectors (shown in dark red) are translocated across the SCV membranes to facilitate microtubule-based SCV transport towards the bacterial replication site (near Golgi) and later on drive the movement of the SCV towards the cell membrane, to release the bacteria for infection of neighbouring cells and dissemination to systemic sites. These effectors also promote the formation of dynamic endosomal tubules during later stages of *Salmonella* infection. *Adapted from: (LaRock et al., 2015) Copyright © 2015, Springer Nature.*

The SPI2-T3SS effector, SifA is the main driver of the majority of intracellular changes during the process of SCV maturation. SifA co-operates with other SPI2 effector proteins to regulate multiple SPI2-induced phenotypes such as the recruitment of LAMP1 while excluding other lysosomal proteins (Mannose-6-phosphate receptors and Cathepsins) from the SCV membranes, elongation of the SCV membranes into tubular structures (SIF biogenesis), microtubule-dependent motility of SCVs , maintenance of vacuolar integrity and intraorganellar proliferation of *Salmonella* (Figueira and Holden, 2012; LaRock et al., 2015; Van Der Heijden and Finlay, 2012).

The *sifA* gene encodes for a 36kDa protein with a bi-modular architecture comprising of two distinct domains. The N-terminal domain (1-136 amino acid residues) is involved in interaction with a eukaryotic host protein SKIP whilst the larger C-terminal domain has a structure similar to bacterial guanine exchange factors and also consists of a potential isoprenylation motif (CAAX motif) similar to those found in Rab-GTPases that facilitates the localization of this protein to the membranes. A *S. Typhimurium* strain with a deletion of the codons encoding for the last six residues of SifA including the CAAX motif is attenuated for systemic virulence in mice and shows a prominent replication defect. The membrane localization of this protein is essential for LAMP1-recruitment, and almost 30% SCVs fail to maintain their vacuolar membrane integrity when infected with a *sifAΔ6 Salmonella Typhimurium* strain (Boucrot et al., 2003; Reinicke et al., 2005).

Several studies have demonstrated an indispensable requirement of SifA for intracellular replication of *Salmonella* in macrophages. Notably, a *ΔsifA* mutant is also strongly attenuated for systemic virulence in mice (Beuzón et al., 2000; Brumell et al., 2001a; Ruiz-Albert et al., 2002; Shea et al., 1996; Stein et al., 1996). In a seminal work, Holden and co-workers showed that a *ΔsifA* deletion mutant is unable to maintain the integrity of its SCV membrane and rapidly escapes into the cytosol of the infected host cells which is detrimental for its survival especially in macrophages (Beuzón et al., 2000). Interestingly, SPI2-null mutants (*ΔssaV, ΔssrA* or *ΔssaVΔsifA*) that fail to translocate any of the SPI2-T3SS effectors maintained an intact vacuolar membrane even after several hours of infection, indicating the involvement of other SPI2-T3SS effectors that might act to destabilize the SCV membranes in absence of this protein. Lately, several other SPI2-T3SS secreted proteins like PipB2, SseJ and SopD2 have been shown to contribute to the instability of SCV membranes in *ΔsifA* mutant strains (Ruiz-Albert et al., 2002;

Schroeder et al., 2010). A closer analysis of this phenotype revealed that the *ΔsifA* deletion mutants accumulate PipB2 (a SPI2 effector that recruits Kinesin1 to SCV and SIF membranes) and Kinesin motor on their vacuolar membranes. The loss of SifA-mediated fission of kinesin1-positive vesicles at the SCV membranes leads to destabilization and eventually lysis of the *Salmonella* vacuoles releasing the bacteria into the cytoplasm; where they succumb to immune surveillance in case of macrophages (Leone and Méresse, 2011).

The prime function of SifA is the elongation of *Salmonella* vacuolar membranes into an interconnected meshwork of tubules (SIFs) as mutant *Salmonella* strains lacking *sifA* are unable to generate SIFs (Beuzón et al., 2000; Stein et al., 1996). Expression of this protein in the uninfected cells results in extensive vacuolation of the LAMP1-positive vesicles leading to the formation of globular lysosomes indicating that SifA alone is sufficient enough to induce tubulation of endosomal compartments by initiating fusion of these vesicles (Brumell et al., 2001a; Ruiz-Albert et al., 2002).

As far as the *Salmonella*-induced phagosomal tubulation is concerned, it is believed that this phenomenon is a result of combined but antagonistic actions of atleast two *Salmonella* effectors: SseJ and SifA. A double mutant lacking both genes, *ΔsifAΔssej* maintains a stable vacuole unlike the *ΔsifA* strain indicating that the loss of membrane integrity in the absence of SifA is dependent on SseJ. Both SifA and SseJ interact with RhoA GTPase although in different states. It has been proposed that upon activation by RhoA, SseJ modifies the SCV membrane via its deacylase and glycerophospholipid: cholesterol acyltransferase (GCAT) activity (Christen et al., 2009; LaRock et al., 2015; Lossi et al., 2008; Ohlson et al., 2005) and interacts indirectly with SifA-SKIP complex to regulate dynamics of SIF formation (Ohlson et al., 2005; Ohlson et al., 2008a; Ruiz-Albert et al., 2002).

Detailed ultrastructural analysis of the SIF network using a combination of EM tomography and live cell correlative light and electron microscopy (CLEM) has provided new insights into the process of SIF biogenesis and the architecture of these atypical tubular networks. It has been shown that although SifA is the key protein involved in the formation of nascent SIFs emerging from the SCVs, there are other SPI2-T3SS effectors that mediate the conversion of a subset of these single-membrane SIFs into double membrane SIFs during later stages of *Salmonella* infection stabilizing the SIF network. The formation of these double membrane SIFs was found to be dependent on SseF and SseG as the *Salmonella* mutants unable to synthesize these proteins failed to show this phenotype and could only induce formation of single membrane SIFs. A more intricate dissection of the membrane logistics of these double membrane *Salmonella*-induced tubular networks has revealed that these bacteria reside in a space (outer lumen) between the inner and outer SIF membranes that is accessible to the endocytic cargo and forms a continuum with the SCVs maintaining a constant supply of the internalized nutrients to the growing *Salmonella* population within the vacuoles. Whereas, the inner lumen is mainly composed of cytoplasmic elements such as ribosomes and cytoskeletal filaments to confer stability to the extensive SIF network. Recent studies have now confirmed that *Salmonella* instigates these unique membrane arrangements to gain access to the endocytic nutrients to survive in the otherwise nutrient-poor environment within SCVs. Besides, there are strong speculations that the enormous volume of the extensive SIF network might contribute towards diluting the concentrations of lethal antimicrobial and proteolytic compounds and mitigating the acidification profile of the SCV lumen while concealing the bacteria (Krieger et al., 2014; Liss and Hensel, 2015).

As mentioned previously, SifA mediates most of its functions through its interaction with a eukaryotic host protein SKIP (**S**ifA and **K**inesin1 **I**nteracting **P**rotein or PLEKHM2)

upon translocation into the host cells. SKIP was originally discovered as a binding partner of this effector protein yielding a functional complex. Later on, this protein was ascribed a role in LE and lysosomal dispersion through its interaction with the TPR domain of Kinesin1 (Dumont et al., 2010b). Almost all the phenotypes observed in the absence of *sifA* can also be observed in case of SKIP-deleted cells infected with wild-type *Salmonella* strains or upon infection with strains carrying a mutated version of the *sifA* gene that is unable to interact with SKIP; highlighting the fact that this interaction is central to *Salmonella*'s intracellular pathobiology. SifA recruits SKIP to the SCV membranes which in turn activates the auto-inhibited Kinesin molecules on the cytosolic leaflet of the SCV membranes (Boucrot et al., 2005a). Although SKIP interacts with Kinesin but it is another effector PipB2 which acts as an adaptor for the recruitment of this motor protein on SCV membranes (Henry et al., 2006). The interaction of SKIP and Kinesin1 is rather thought to mediate the vesicular removal of Kinesin from the SCVs and centrifugal transport of the Kinesin-positive vesicles derived from the SCVs along the microtubular tracts. This process is important for cell to cell transfer eventually leading to systemic spread of the infection and colonization of new infection sites such as spleen and liver (Dumont et al., 2010b; Leone and Méresse, 2011). Another important study by McGourty and co-workers, highlighted the physiological relevance of SifA-SKIP interaction. In this study, they demonstrated that SifA-SKIP complex secures Rab9, a small GTPase involved in the recycling pathway such that this GTPase is now unavailable for transporting the mannose-6-phosphate receptors from the late endosomes to Golgi network. This impedes the delivery of premature hydrolytic enzymes to the lysosomes consequently leading to their secretion, hence compromising the potency of these lytic compartments. Therefore, *Salmonella* vacuoles actually fuse with lysosomes that are detoxified by the action of this protein rendering them incompetent and devoid of their degradative capacity (McGourty et al., 2012).

Salmonella's interference with lysosomal trafficking through HOPS recruitment and the essential requirement of this host factor for bacterial replication and SIF formation has been described in much detail in the previous chapter of this thesis. To gain more coherent understanding of the molecular mechanism by which this tethering factor is recruited to the *Salmonella* vacuoles and SIFs we screened for bacterial virulence proteins regulating the later stages of *Salmonella* maturation programme and SIF biogenesis; since HOPS complex was not found to direct the early maturation events during *Salmonella* infection.

4.2 Results

4.2.1 HOPS recruitment to the SCV and SIF membranes is regulated by the SPI2-T3SS effector, SifA

Previous studies have revealed that in *Salmonella* infected cells SifA co-operates with several other SPI2-T3SS effectors (SseJ, SopD2, PipB2, SseF, and SseG) to mediate the extension of the phagosomal membranes into an extensive tubular network a.k.a SIFs (Figueira and Holden, 2012; LaRock et al., 2015; Rajashekar et al., 2008b; Van Der Heijden and Finlay, 2012). The *Salmonella* mutant strains with deletions in any of these effectors are associated with either altered SIF morphology or variable frequency of these tube-like structures (Birmingham et al., 2005; Rajashekar et al., 2014; Stein et al., 1996). Amongst these, the most severe phenotype has been observed in *Salmonella* strain lacking *ΔsifA* where SIF formation is completely abrogated and vacuolar integrity is disrupted; leading to bacterial release in the host cytosol (Stein et al., 1996). In view of our previous observations regarding the paramount importance of HOPS complex in regulating fusion events at *Salmonella*-modified membranes to obtain a continuous supply of membrane and nutrients for SIF formation and vacuolar replication; we next tried to elucidate if the *Salmonella* effectors involved in SIF biogenesis promote

recruitment of this tethering factor to mediate these effects. To this end, we visualized and quantified the association of HOPS subunits Vps41 (both epitope-tagged and endogenous) and Vps18 (endogenous) to the LAMP1-positive SCVs in mutant strains deficient in either *ΔsifAΔssej*, *ΔpipB2*, *ΔsseF* or *ΔsseG* effectors (**Fig 4.2 and Fig 4.3)**. Surprisingly, as compared to the WT strain, the recruitment of both HOPS subunits (Vps41 and Vps18) to the LAMP1-positive SCVs was abrogated in case of the *ΔsifAΔssej* mutant (**Figs 4.2a** and **4.2b**; quantification shown in **Fig 4.2g**; **4.3a, 4.3b, 4.3g** and **4.3h Figs)**. We used the *ΔsifAΔsseJ* double-mutant strain in these experiments instead of *ΔsifA* single mutant strain as the latter loses its vacuolar integrity over time and becomes cytosolic (Beuzón et al., 2000). Notably, both HOPS subunits continued to localize to at the SCVs in cells infected with other SPI2-T3SS effector deficient strains *pipB2*, *sseF* and *sseG* (**Figs 4.2c-f**; quantification shown in **Fig 4.2h**; **4.3c-f** and **4.3i-l Figs**). These findings indicate that SifA, but not other *Salmonella* effectors involved in SIF formation are crucial for the recruitment of HOPS complex to SCV and SIF membranes.

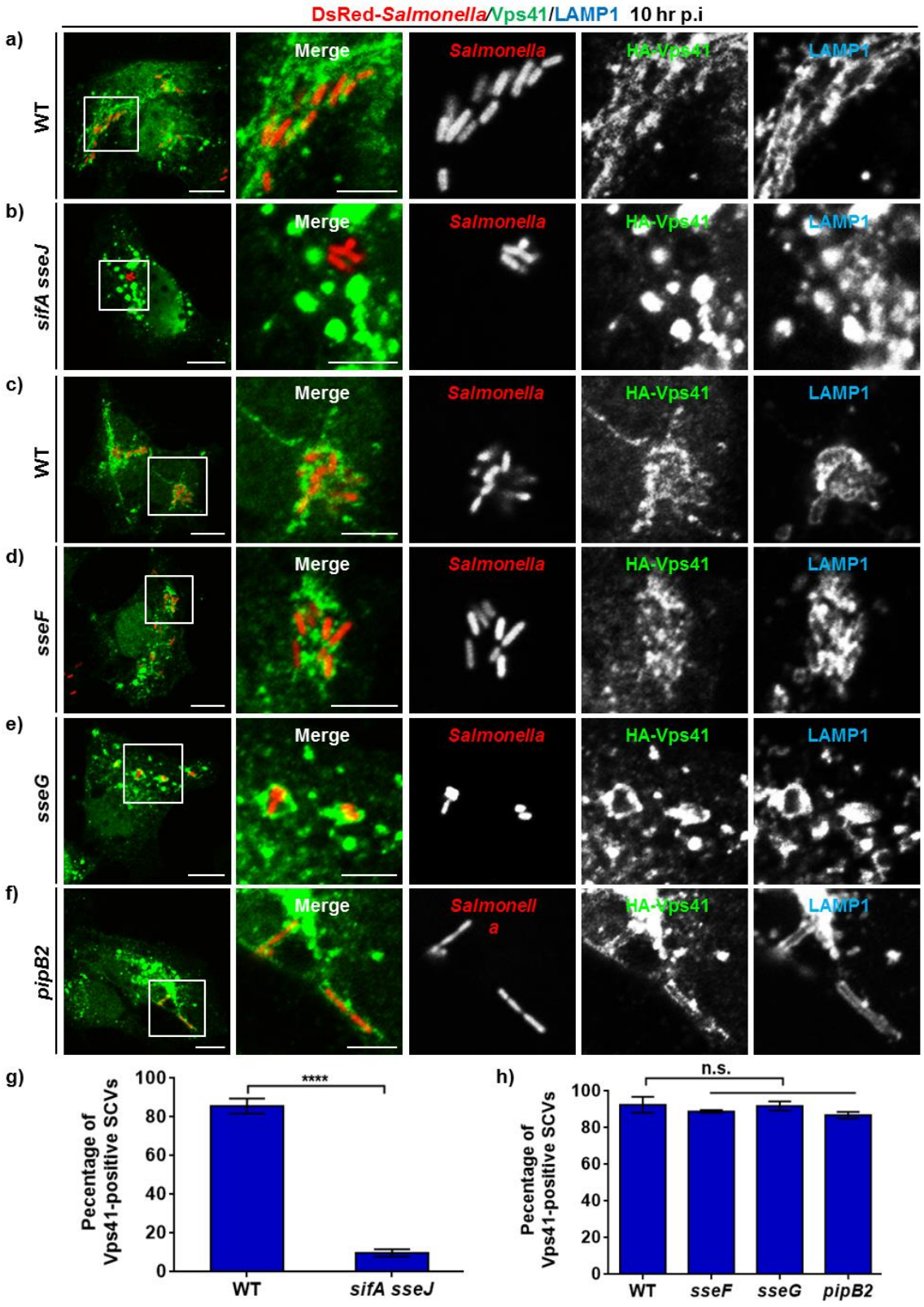


Figure 4.2: Deletion of *Salmonella* effector SifA abrogates Vps41 recruitment to SCV membranes. a-f) HeLa cells were infected with either DsRed expressing-wild-type (WT) strain of *Salmonella* (NCTC 12023 in **(a)** and SL1344 in **(c)** or *sifA sseJ*, *sseF*, *sseG*, and *pipB2* strains, followed by transfection with HA tagged-Vps41. Cells were fixed at 10 hr p.i., and co-stained with anti-HA (green) and anti-LAMP1 (blue) antibodies. Different panels represent a higher magnification of the boxed areas. Bars: (main) 10 µm; (insets) 5 µm. **g and h)** Quantification of Vps41-positive SCVs in HeLa cells infected with different *Salmonella* strains (as labeled) and fixed at 10 hr p.i. Data represent mean ± S.D. from three independent experiments where ~100 SCVs were counted in each experiment (n.s., not significant; ****, $P < 0.0001$; Student's *t* test).

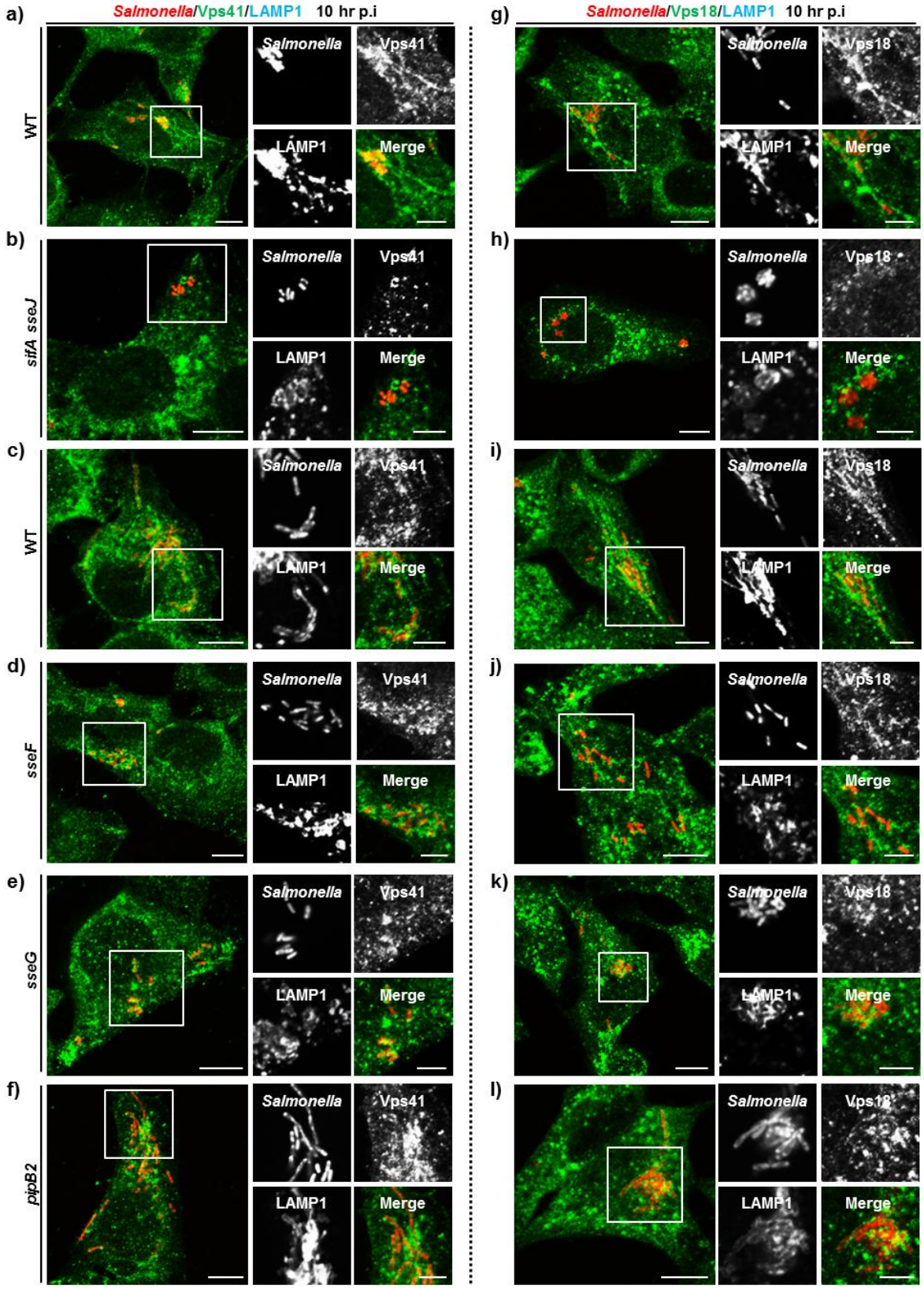


Figure 4.3: Deletion of *Salmonella* effector SifA abrogates acquisition of endogenous HOPS subunits to SCV membranes. a-l) HeLa cells were infected with DsRed expressing-wild-type (WT) strain of *Salmonella* (NCTC 12023 **(a and g)** and SL1344 **(c and i)** or sifA sseJ **(b and h)**, sseF **(d and j)**, sseG **(e and k)**, and pipB2 **(f and l)** strains. Cells were fixed at 10 hr p.i., and co-stained with anti-Vps41 (green, a-f) or anti-Vps18 (green, g-l) and anti-LAMP1 (blue, shown only in inset) antibodies. Different panels represent a higher magnification of the boxed areas. Bars: (main) 10 µm; (insets) 5 µm.

To gain a deeper understanding of SifA mediated HOPS recruitment we checked the localization of HOPS subunits Vps41 and Vps18 in cells expressing epitope-tagged SifA. A previous study has shown that the expression of a vector encoding SifA in mammalian cells leads to extensive vacuolation of the LAMP1- and vacuolar ATPase-positive compartments (Brumell et al., 2001a; Brumell et al., 2002b). We also observed a similar phenotype upon co-expression of epitope-tagged SifA and LAMP1 in HeLa cells. Interestingly we could observe endogenous HOPS subunits-Vps41 and Vps18 were enriched in vertices of the clustered LAMP1-positive vacuoles induced by ectopic expression of SifA (**Figs 4.4a** and **4.4b**).

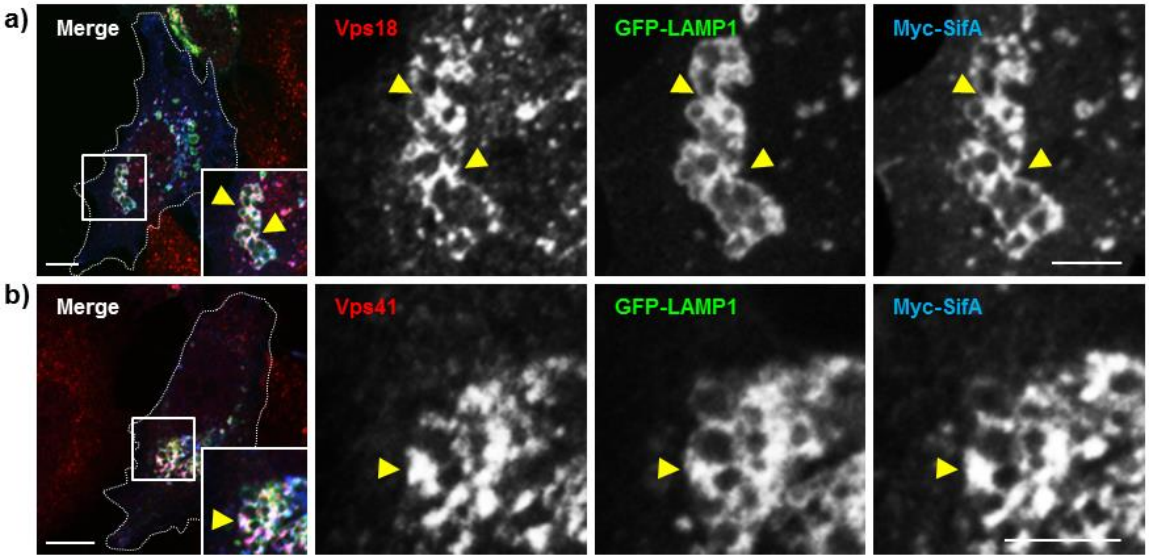


Figure 4.4: HOPS subunits colocalize on SifA-induced globular lysosome clusters. Representative confocal micrographs of HeLa cells co-transfected with Myc-SifA and GFP-LAMP1 plasmids. The cells were fixed and co-stained using anti-Myc (blue) and anti-Vps18 (a, red) or anti-Vps41 (b, red) antibodies. Different panels represent a higher magnification of the boxed areas and HOPS subunits enrichment on clustered lysosomes is indicated by the arrowheads.

Next, to validate our microscopic observations we did a biochemical assay using purified SifA protein (with an N-terminal GST tag) as bait to pulldown HOPS subunits from cell lysates expressing epitope tagged Vps41 or Vps18. As expected, SifA was able to interact with both HOPS subunits and SKIP which is a well characterized binding partner of this protein. Taken together, these results suggest that SifA indeed could promote the fusion of SCVs and SIFs with late endosomes and lysosomes by virtue of its interaction with this multi-subunit lysosomal tethering factor. Further, we performed a co-immunoprecipitation assay using Vps41-transfected *Salmonella*-infected cells where SifA was expressed through a plasmid encoding for an epitope-tagged SifA protein to corroborate the GST-pulldown results (**Figs 4.5a and 4.5b**).

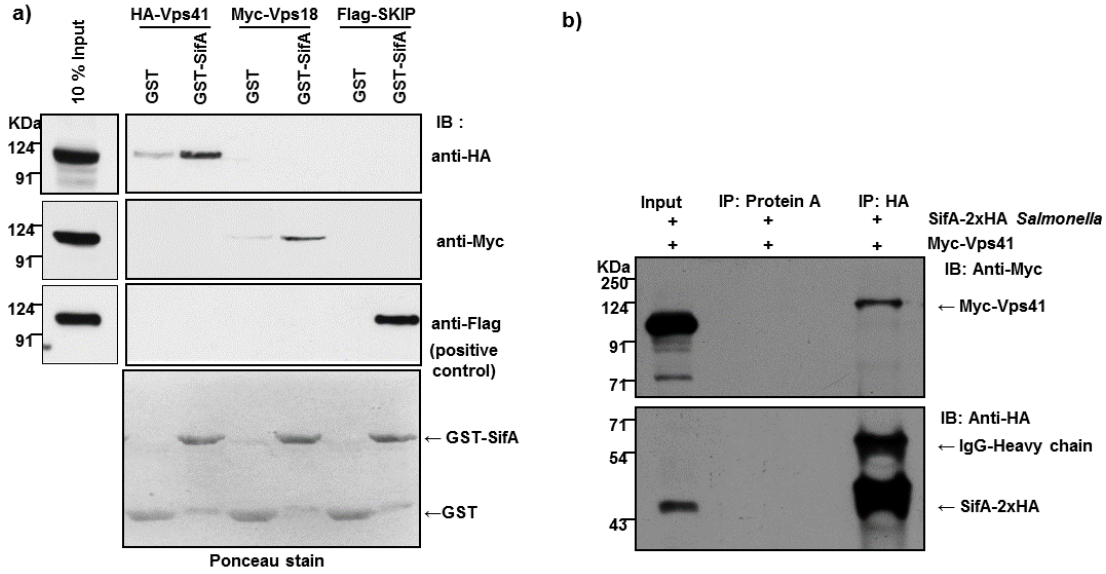


Figure 4.5: SifA interacts with and recruits HOPS complex subunits. a) GST or GST tagged-SifA were immobilized on resin and incubated with lysates prepared from HEK293T cells transfected with either HA-Vps41, Myc-Vps18 or Flag-SKIP. The precipitates were resolved by SDS-PAGE and immunoblotted with indicated antibodies. Ponceau S stain was done to visualize purified proteins. **b)** Lysates from HeLa cells infected with *Salmonella* strain expressing epitope-tagged SifA (*S. Typhimurium* SL1344 *sifA*/pSifA (WT)-2HA) and transfected with Myc-Vps41 were co-immunoprecipitated using either Protein-A conjugated- or anti-HA antibodies-conjugated resins. The precipitates were resolved on SDS-PAGE and immunoblotted with indicated antibodies.

4.2.2 SifA but not SifA (L130D) could promote recruitment of HOPS complex to *Salmonella* phagosomes and SIFs

Translocated SifA protein interacts with a host protein SKIP on the SCV membranes. The SifA-SKIP complex links the SCVs with the microtubule network in a kinesin-dependent manner, to promote the anterograde movement of the membrane, generating SIFs (Dumont et al., 2010b; Schroeder et al., 2011). The structure of SifA-SKIP complex has been resolved at the atomic level and shown to be dependent on a SifA leucine residue at position 130 (L130) which is a part of the binding interface linking the N-terminal domain of SifA and PH domain of SKIP **(Fig 4.6a)** (Boucrot et al., 2005a; Diacovich et al., 2009; Ohlson et al., 2008a).

Intriguingly, in our previous study we had characterized that this interaction partner of SifA (SKIP) co-localizes and interacts with Vps39, the other membrane-binding subunit of HOPS complex (Khatter et al., 2015a). Based on these observations we hypothesized that SifA in complex with SKIP might target HOPS complex to SCVs and SIF membranes.

To test the requirement of the SifA-SKIP interaction in recruitment of HOPS to *Salmonella* modified membranes, HeLa cells were infected with a *ΔsifA* mutant strain transformed with a plasmid encoding either an epitope-tagged wild-type SifA (pSifA-2xHA) or a point mutant of SifA (pSifA(L130D)-2xHA) that is defective in binding to SKIP (Ohlson et al., 2008a; Zhao et al., 2015a). Using co-IP approaches, we confirmed that SKIP does not interact with the SKIP-binding interface mutants of SifA as reported previously (**Fig 4.6b**). It has been shown previously that this mutant retains an intact vacuole unlike the *ΔsifA* strain (Zhao et al., 2015a), which allowed us to analyse whether HOPS is recruited to the SCVs surrounded by a LAMP1-positive vacuolar membrane. Interestingly, we found that as compared to the cells infected with the *sifA* strain

expressing SifA (WT)-2xHA plasmid, in cells infected with *sifA* strain expressing point mutant SifA (L130D)-2xHA, little or no association of HOPS subunit-Vps41 with SCVs was observed at 10 hr p.i. (**Figs 4.6c** and **4.6d**). Quantification of Vps41-positive SCVs infected with either strain demonstrated that the association of HOPS subunit Vps41 to SCV membranes was abrogated in the presence of SKIP-binding defective mutant of SifA in infected cells **(Fig 4.6e)**.

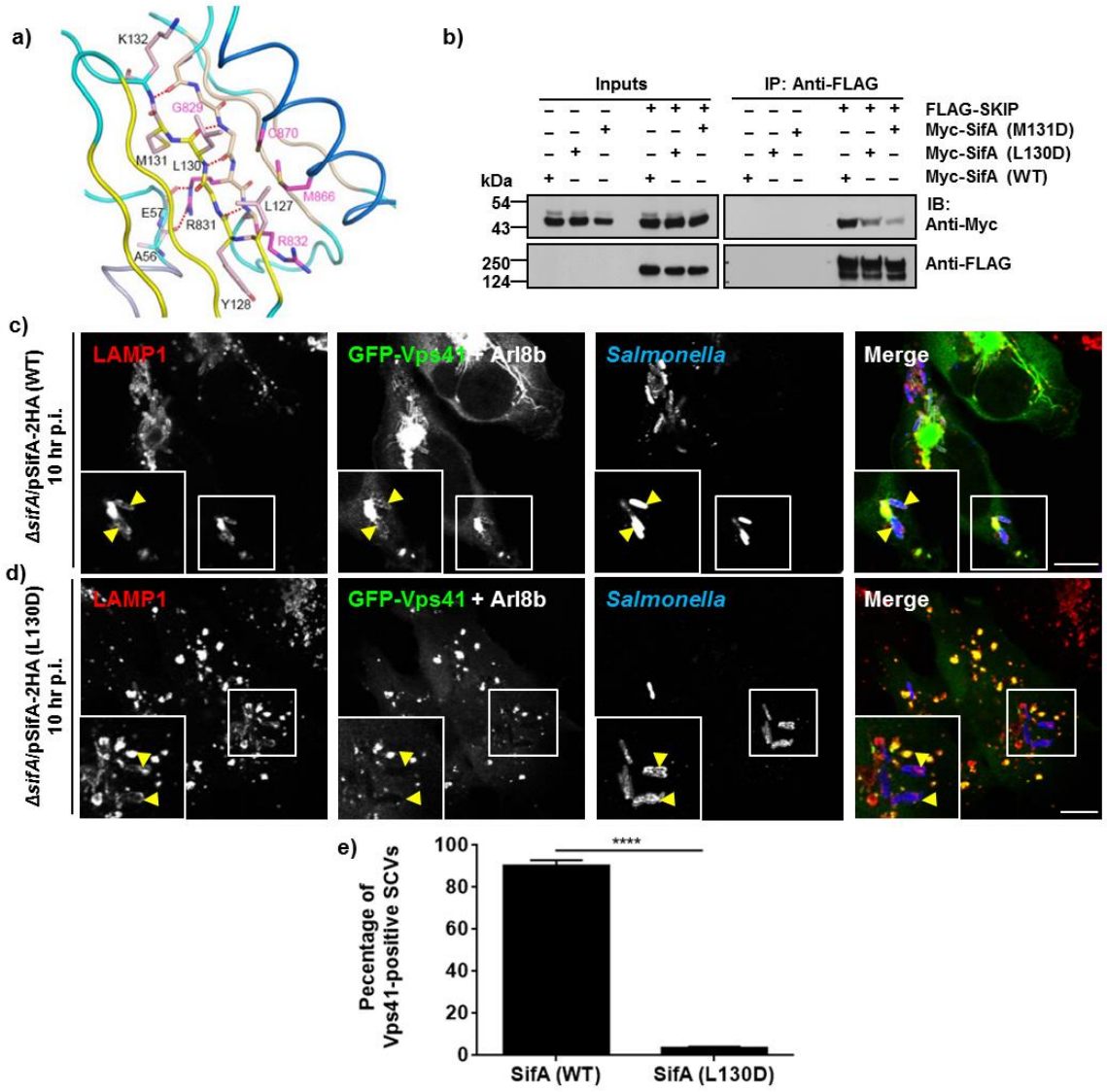


Figure 4.6: SifA (L130D) fails to recruit HOPS subunit Vps41 onto SCV membranes. a) A close-up of the side chains in SifA (black labels) and PHD (pink labels) at the interface is shown in stick representation. Hydrogen bonds are shown (dashed lines) *Adapted from: Ohlson MB, Huang Z, Alto NM, Blanc MP, Dixon JE, et al. (2008)*. **b)** Lysates from HEK293T cells co-transfected with plasmids expressing FLAG-SKIP and either Myc-SifA (WT), Myc-SifA (L130D) or Myc-SifA (M131D) were immunoprecipitated using anti-FLAG antibodies-conjugated resins. The precipitates were resolved on SDS-PAGE and immunoblotted with indicated antibodies. **c and d)** Representative confocal micrographs of HeLa cells infected with *Salmonella* strains *sifA*/pSifA (WT)-2HA **(c)** or *sifA*/pSifA (L130D)-2HA **(d)**, followed by co-transfection with plasmids

expressing GFP-Vps41 (green) and untagged-Arl8b. Cells were fixed 10 hr p.i., and immunostained with antibodies to *Salmonella (blue) and LAMP1 (red).* Arrowheads in the insets depict localization of Vps41 around SCV membranes (marked by LAMP1). **e)** Quantification of HA-Vps41-positive SCVs at 10 hr p.i. in HeLa cells infected with indicated *Salmonella* strains. Data represent mean ± S.D. of ~100 SCVs scored in each experiment for three independent experiments (****, P < 0.0001; Student's *t* test).

These findings were further corroborated by the live-cell imaging experiments in cells infected with either of these *Salmonella* strains, expressing either GFP-Vps41 alone or in presence of Arl8b that regulates the membrane recruitment of this HOPS subunit (**S15-S18 Movies**; **4.7a** and **4.7b Figs**).

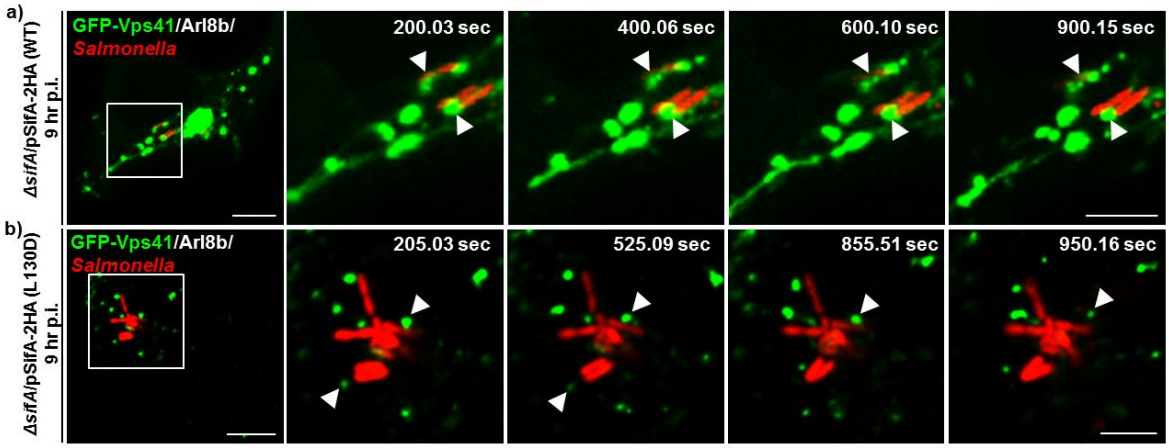


Figure 4.7: a and b) Recruitment of HOPS subunit Vps41 to SCVs requires SifA-SKIP interaction. Time-lapse microscopy was performed on HeLa cells infected with DsRed-expressing *Salmonella* strains *sifA*/pSifA (WT)-2HA **(a)** or *sifA*/pSifA (L130D)-2HA **(b)** and co-transfected with the plasmids expressing GFP-Vps41 and untagged-Arl8b. Time-lapse series were recorded 9 hr p.i., and still images correspond to movies shown as S16 and S18 Movies. Different panels represent the time-lapse series of the boxed area. Arrowheads indicate individual SCVs. Bars: (main) 10 µm; (insets) 5 µm.

4.2.3 SKIP regulates the association of HOPS complex to *Salmonella* vacuolar membranes

Next, to determine whether SKIP is indeed responsible for the recruitment of this tethering factor to the SCV membranes, we visualized the localization of HOPS subunit Vps41 to SCVs in control- and SKIP-siRNA treated HeLa cells infected with wild-type *Salmonella* co-expressing GFP-Vps41 and Arl8b. As depicted in **Figs 4.8a** and **4.8b**, while Vps41 localized to the LAMP1-positive SCVs in control cells, little or association was observed in SKIP depleted cells at 10 hr p.i. Quantification of Vps41-positive SCVs

in control and SKIP-siRNA treated cells demonstrated that the recruitment of HOPS subunit Vps41 to *Salmonella* vacuoles is hindered in the absence of SKIP **(Fig 4.8g).** Live-imaging of control- and SKIP-siRNA treated cells expressing either GFP-tagged Vps41 alone or co-expressing Arl8b infected with *Salmonella* further reinforced our steady state imaging data (**S11-S14 Movies**; **4.8c** and **4.8d Figs**). In line with these observations and in accordance with previous studies (Boucrot et al., 2005b; Zhao et al., 2015b), we found a significant defect in bacterial replication in SKIP-depleted cells as compared to control (**4.8h Fig**; control siRNA: ~3 fold and SKIP siRNA: ~1.3 fold increase in bacterial burden from 2 hr to 10 hr p.i.).

Transfection of a plasmid encoding for siRNA-resistant SKIP into the SKIP-depleted cells co-expressing GFP-Vps41 and Arl8b infected with *Salmonella* restored the localization of this protein subunit onto the SCV membranes. This result indicates that SifA cannot initiate HOPS recruitment in the absence of endogenous SKIP protein and this effect can be rescued by addition of exogenous SKIP; confirming the specificity of the SKIP-siRNA treatment and the essential requirement of this protein in HOPS recruitment during *Salmonella* infection (**Figs 4.8e-g**).

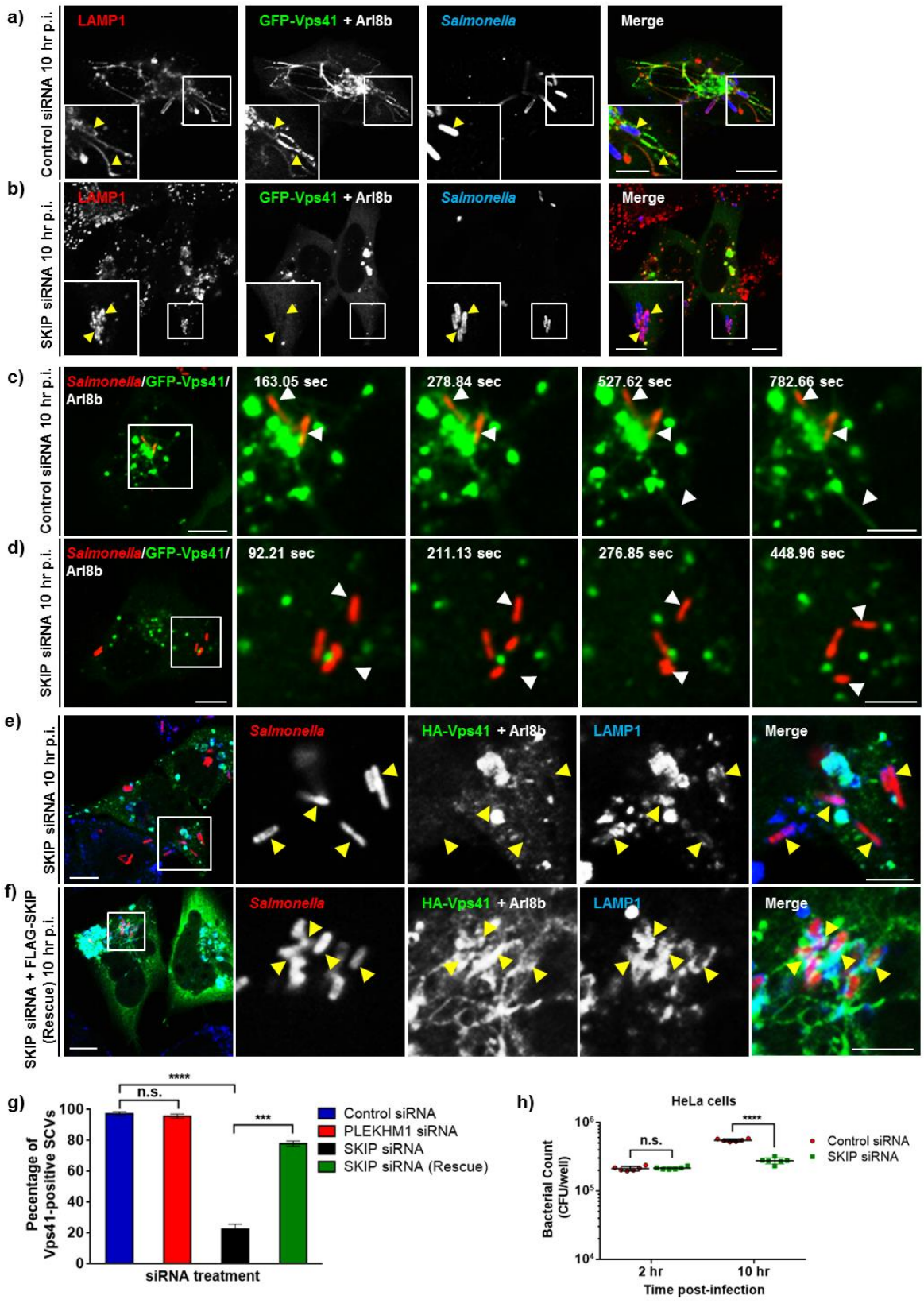


Figure 4.8: SKIP is required for Vps41 recruitment to SCV membranes. a and b) Representative confocal micrographs of control siRNA- or SKIP siRNA-treated HeLa cells infected with *Salmonella*, followed by co-transfection with plasmids expressing GFP-Vps41 (green) and untagged-Arl8b. Cells were fixed at 10 hr p.i., and immunostained with antibodies to *Salmonella* (blue) and LAMP1 (red). Arrowheads in the insets depict localization of Vps41 around SCV membranes (marked by LAMP1). **c and d)** Live-cell imaging was performed on control siRNA- or SKIP siRNA-treated HeLa cells infected with DsRed-expressing *Salmonella* (red) and co-transfected with plasmids encoding GFP-Vps41 and untagged-Arl8b. Time-lapse series were

recorded 10 hr p.i., and still images correspond to movies shown as S12 and S14 Movies. Different panels represent the time-lapse series of the boxed area. Arrowheads indicate individual SCVs. Bars: (main) 10 µm; (insets) 5 µm. **e and f)** Representative confocal micrographs of HeLa cells treated with SKIP siRNA, infected with DsRed-expressing *Salmonella*, followed by transfection with plasmids expressing HA-Vps41, untagged-Arl8b and vector **(e)** or siRNA-resistant FLAG-SKIP (rescue construct) **(f)**. Cells were fixed 10 hr p.i., and immunostained with antibodies to HA tag (green) and LAMP1 (blue). Different panels represent a higher magnification of the boxed areas, indicating Vps41 localization around SCV membranes (marked by arrowheads). Bars: (main) 10 µm; (insets) 5 µm. **g)** Quantification of HA-Vps41-postive SCVs at 10 hr p.i. in indicated siRNA treated HeLa cells. Data represent mean ± S.D. of ~50 SCVs scored in each experiment for three independent experiments (n.s., not significant; ***, P < 0.001; ****, P < 0.0001; Student's *t* test). **h)** Control siRNA- and SKIP siRNA-treated HeLa cells were infected with *Salmonella* for the indicated times and the number of CFU per well were determined and shown as dot plot. Data represent mean ± S.D. (n.s., not significant; ****, P < 0.0001; Student's t test).

Recently PLEKHM1, a protein with domain architecture similar to SKIP was reported to interact with both SifA and HOPS subunits Vps39 and Vps41 (McEwan et al., 2015a; McEwan et al., 2015c). While it was predicted that SifA recruits HOPS complex in a PLEKHM1 dependent manner, no experimental evidence was shown to prove the same. To test the role of PLEKHM1 in HOPS recruitment we checked for the localization of Vps41 in control- and PLEKHM1-siRNA treated cells infected with *Salmonella*. Contrary to the previous report, we observed that Vps41 continued to associate with the SCV membranes in PLEKHM1-depleted cells at 10hr p.i. indicating that PLEKHM1 does not facilitate the recruitment of this tethering protein to *Salmonella* phagosomal membranes. This effect was also quantified by calculating the percentage of Vps41-positive SCVs in both control- and PLEKHM1-siRNA treated *Salmonella* infected cells (**Figs 4.9a** and **4.9b**; **Fig 4.8g).**

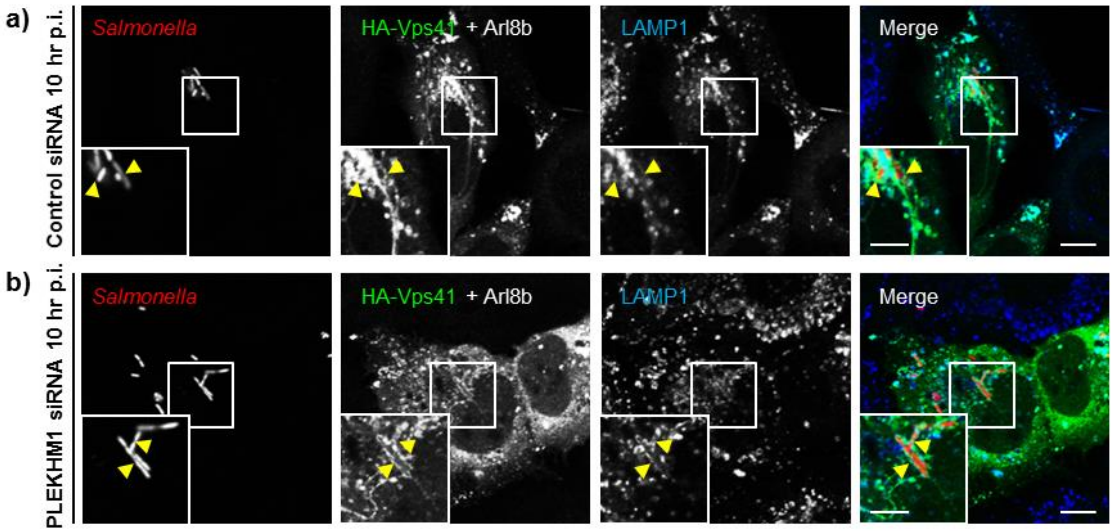


Figure 4.9 a and b) PLEKHM1 does not regulate the recruitment of HOPS complex onto SCV or SIF membranes. Representative confocal micrographs of HeLa cells treated with either control siRNA (a) or PLEKHM1 siRNA (b), and infected with DsRed-expressing *Salmonella* (red) followed by co-transfection with HA-Vps41 and Arl8b. Cells were fixed 10 hr p.i., and immunostaining was performed using anti-HA (green) and anti-LAMP1 (blue) antibodies. Insets depict higher magnification of the boxed areas (SCVs are indicated by arrowheads). Bars: (main) 10 µm; (insets) 5 µm.

4.2.4 SKIP acts as a linker between SifA and HOPS subunit; Vps39

SKIP directly interacts with Vps39 subunit of HOPS complex to mediate its association with the Arl8b-positive lysosomes and promote their kinesin1-driven anterograde motility (Khatter et al., 2015a). Since, SifA-SKIP interaction is obligatory for the recruitment of HOPS complex to *Salmonella* containing phagosomes; we speculated that the association of this multi-subunit tether to these structures might be mediated via its membrane anchoring subunit, Vps39.

To verify this, we co-expressed epitope-tagged Vps39 and SifA either in presence or absence of SKIP. Indeed, while little or no colocalization of Vps39 and SifA was observed (both proteins were cytosolic); the colocalization between the two proteins was remarkably enhanced upon co-expression of SKIP and the three proteins were localized on the peripheral pool of lysosomes (compare **Figs 4.10a** and **4.10d**). Besides, Vps39 was found to colocalize with SKIP on peripheral structures shown to be lysosomes, which are transported in an anterograde manner by direct binding between Arl8b-SKIP complex

to the microtubule motor protein kinesin-1 (**Figs 4.10a** and **4.10b)** (Boucrot et al., 2005a; Dumont et al., 2010b; Rosa-Ferreira and Munro, 2011b).

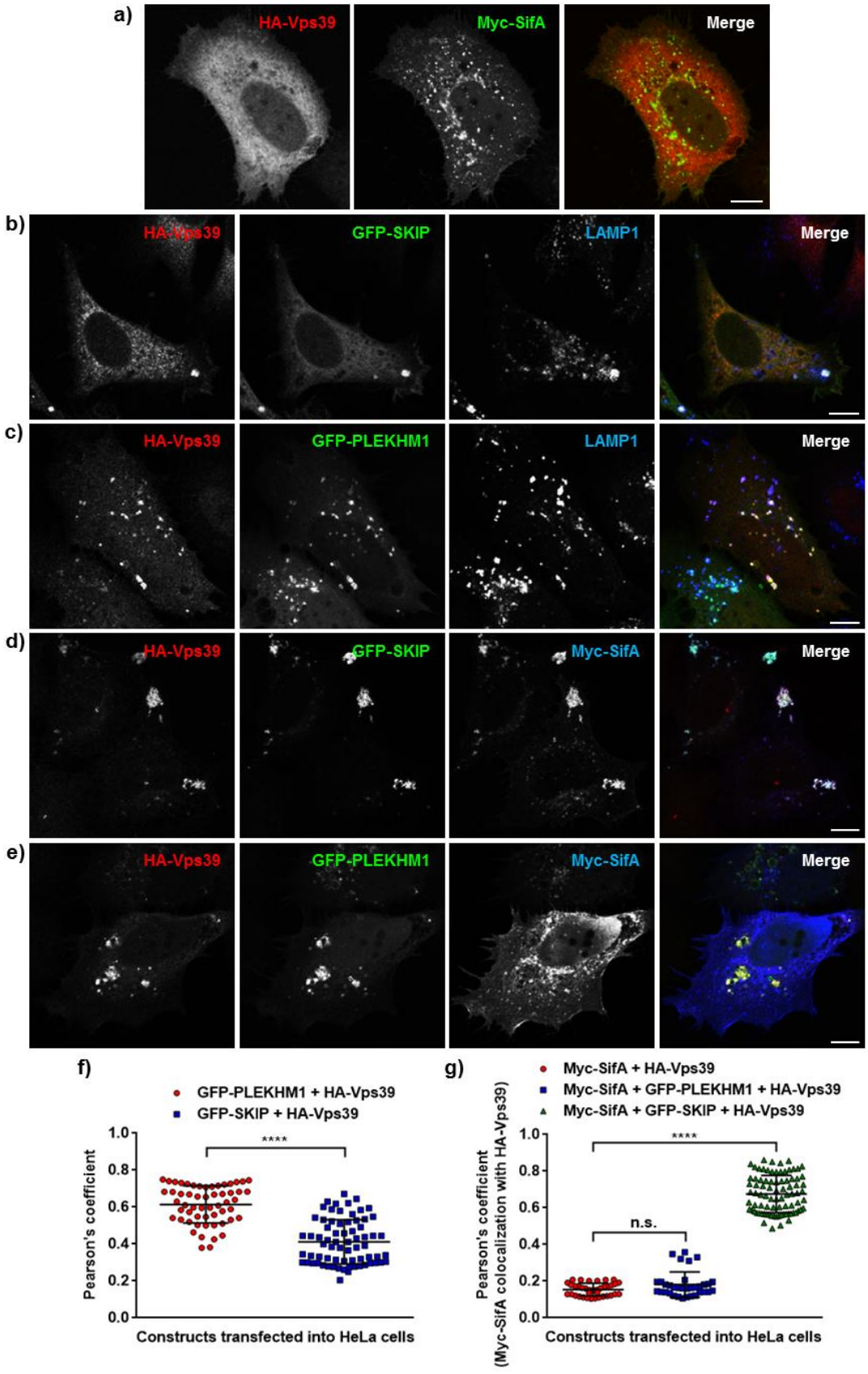


Figure 4.10: Bacterial effector SifA localizes with HOPS subunit Vps39 in a SKIP-dependent manner. a-e) Representative confocal micrographs of HeLa cells co-transfected with HA-Vps39 (red) and Myc-SifA (green) **(a)**, HA-Vps39 (red) and GFP-SKIP (green) **(b)**, HA-Vps39 (red) and GFP-PLEKHM1 (green) **(c)**, HA-Vps39 (red), GFP-SKIP (green) and Myc-SifA (blue) **(d)**, or HA-Vps39 (red), GFP-PLEKHM1 (green) and Myc-SifA (blue) **(e)**. Cells in (b) and (c) were also stained for lysosomes using with anti-LAMP1 (blue) antibodies. Bars: 10 μm**. f and g)** Pearson's correlation coefficient was calculated for the indicated protein pairs in transfected cells as labeled. Data represent mean ± S.D. over three independent experiments where ~25-30 transfected cells were analyzed in each experiment (n.s., not significant; ****, P < 0.0001; Student's *t* test).

The other HOPS subunits-Vps18 and Vps41, also showed a significantly higher co-localization with SifA in presence of SKIP (compare **4.11a** and **4.11c Figs**; compare **4.11b** and **4.11d Figs**). Quantification of Pearson's Correlation Coefficient (PCC) from 25-30 transfected cells over three independent experiments demonstrated a significant increase in colocalization of HOPS subunits with SifA in presence of SKIP (**Figs 4.10g** and **4.11e)**.

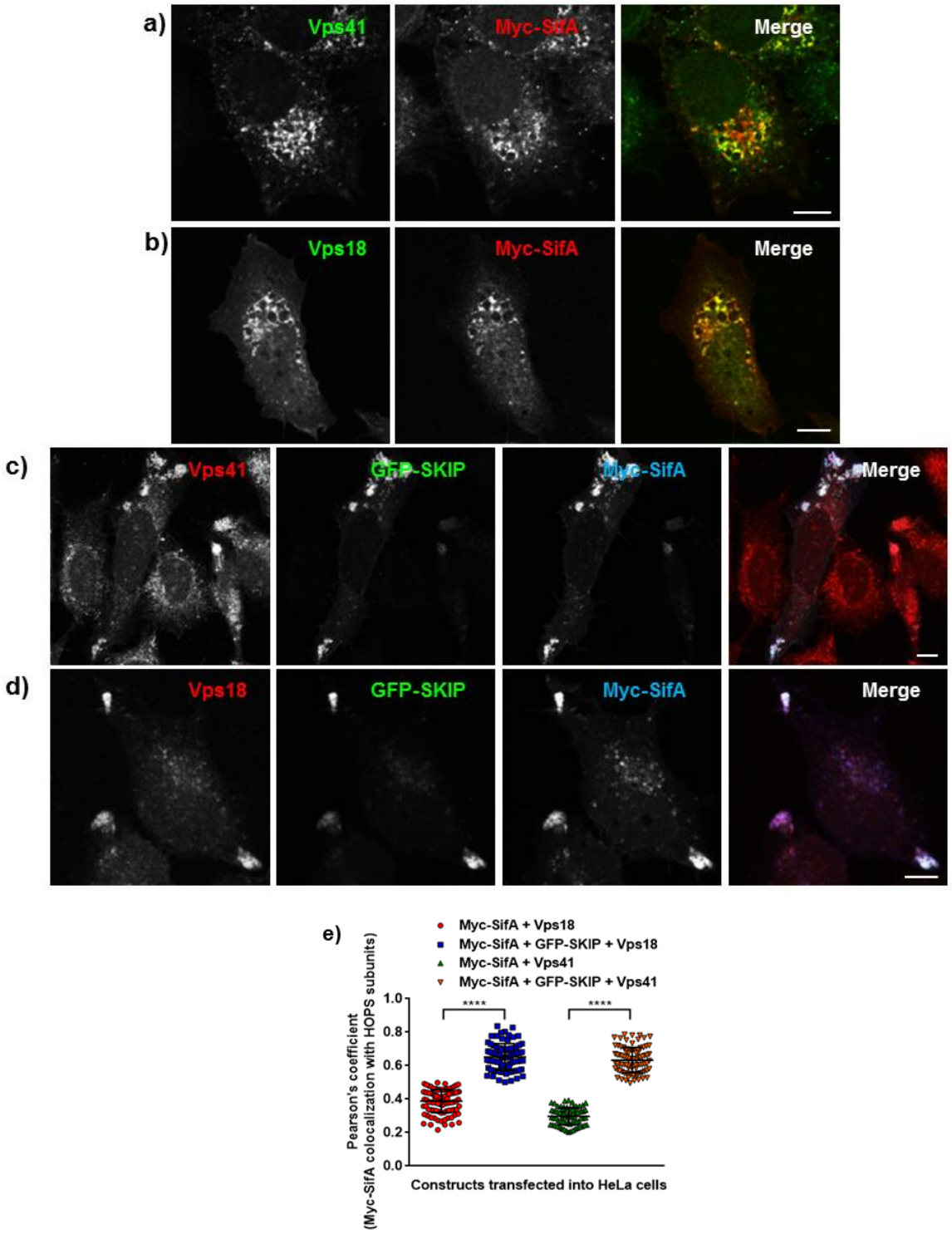


Figure 4.11: a-d) Enrichment of HOPS subunits to SifA-SKIP positive peripheral aggregates. Representative confocal micrographs of HeLa cells transfected with Myc-SifA alone (red), or co-transfected with Myc-SifA (blue) and GFP-SKIP (green). Cells were fixed and stained with antibodies against Vps41 (red, a and c) and Vps18 (red, b and d). Bars: 10 µm. **e)** Pearson's correlation coefficient was calculated for the indicated protein pairs in transfected cells as labeled. Data represent mean ± S.D. over three independent experiments where ~25-30 transfected cells were analyzed in each experiment (n.s., not significant; ****, $P < 0.0001$; Student's *t* test).

Furthermore, we also visualized the localization of SifA and Vps39 in presence of PLEKHM1, the other interaction partner of SifA. Notably, although Vps39 and PLEKHM1 colocalized on punctuate structures, SifA was not recruited to these punctae (**Figs 4.10e**; quantification of PCC shown in **Fig 4.10g**) indicating that PLEKHM1 does not promote the association of HOPS subunit Vps39 with SifA, consistent with our previous data. We also found significant colocalization of Vps39 with PLEKHM1, which was higher than its colocalization with SKIP (**Figs 4.10b** and **4.10c**; quantification of PCC shown in **Fig 4.10f**).

Next, we did biochemical analyses of Vps39 recruitment by GST-tagged SifA in presence of endogenous or overexpressed levels of SKIP using a GST pulldown assay. Consistent with our colocalization data, we observed a dramatic increase in the levels of Vps39 pulldown with SifA upon SKIP overexpression (**Figs 4.12a** and **4.12b**). This striking increase in pulldown of HOPS subunits was also reflected upon probing for endogenous Vps11, which directly binds to Vps39 during the assembly of the HOPS complex (**Fig 4.12a**). Vps41 pulldown with SifA was also increased upon SKIP overexpression, although this was less striking as compared to Vps39 and Vps11 (**Fig 4.12a**). Notably, we did not observe any increase in pulldown of HOPS subunit Vps39 with GST tagged-SifA upon PLEKHM1 overexpression (**Figs 4.12a** and **4.12b**); which supports our previous results that PLEKHM1 does not regulate SifA interaction with HOPS complex.

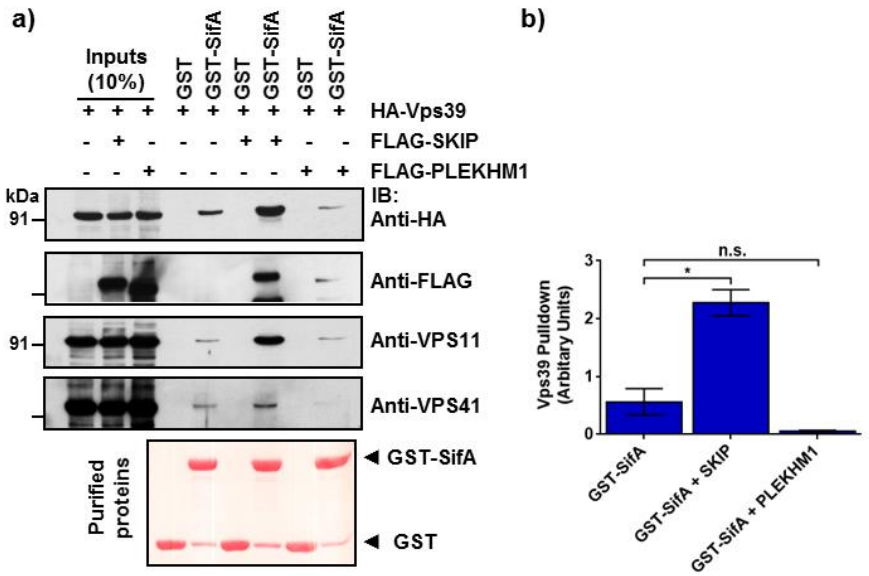


Figure 4.12: SKIP enhances the recruitment of HOPS subunits by SifA. a) GST or GST tagged-SifA were immobilized on resin and incubated with lysates prepared from HEK293T cells transfected with HA-Vps39 alone or co-transfected with HA-Vps39 and FLAG-SKIP or FLAG-PLEKHM1. The precipitates were resolved by SDS-PAGE and immunoblotted with indicated antibodies. Ponceau S stain was done to visualize purified protein. **b)** Densitometric analysis of immunoblots of HA-Vps39 pulldown (normalized to input signal band intensity) by GST tagged-SifA in presence of FLAG-SKIP or FLAG-PLEKHM1.

We also noted that colocalization and interaction of SifA with PLEKHM1 was significantly weaker than with SKIP, as revealed by colocalization coefficient quantification and growth curve analysis of yeast two-hybrid assay using SifA as a bait, and SKIP and PLEKHM1 as prey proteins (**Figs 4.13a-d**).

These findings were corroborated by GST pulldown assay where pull down of PLEKHM1 with GST tagged-SifA was found to be much lower as compared to SKIP from transfected cell lysates (**Figs 4.13f** and **4.13g**). Additionally, qRT-PCR analysis revealed that SKIP mRNA levels in HeLa cells were ~2.5 fold higher than PLEKHM1 levels (**Fig 4.13e**). Taken together, these results imply that at least in this cell line, more amount of the secreted bacterial effector SifA must be bound to SKIP as compared to PLEKHM1.

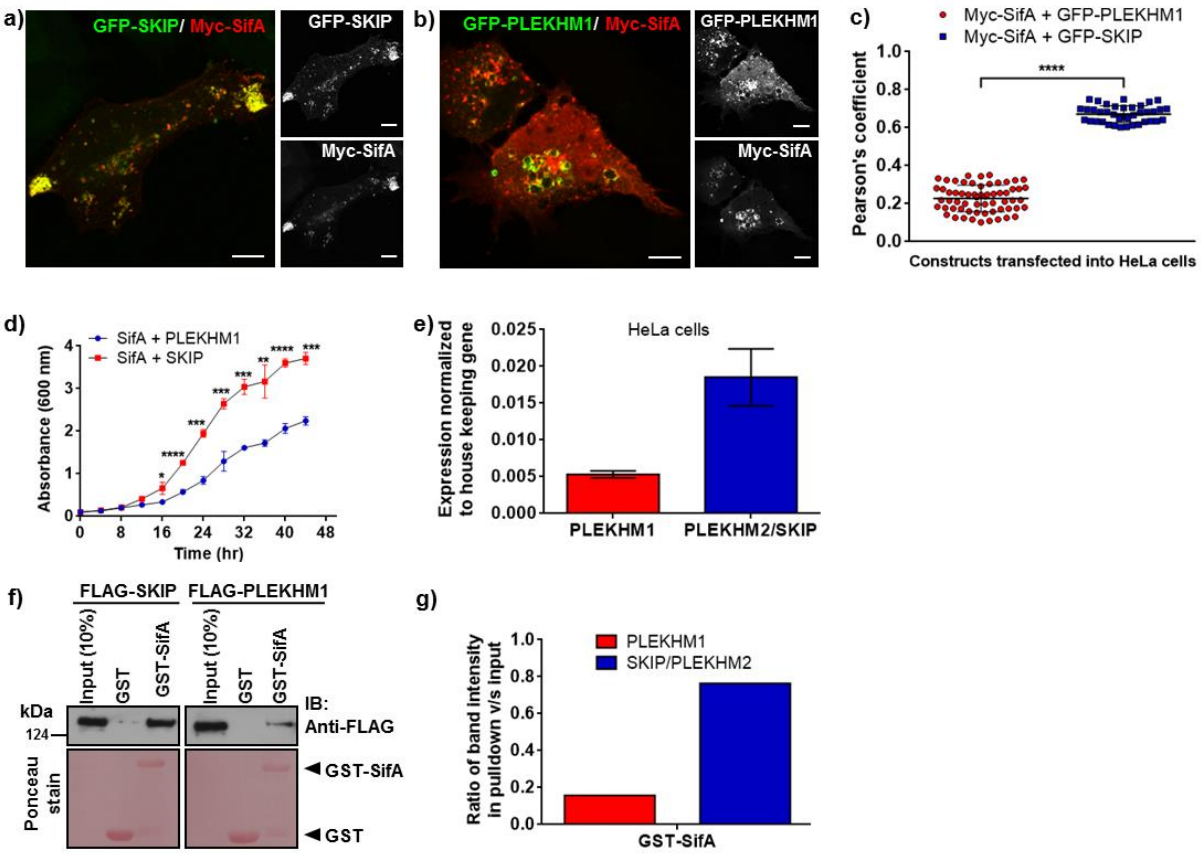
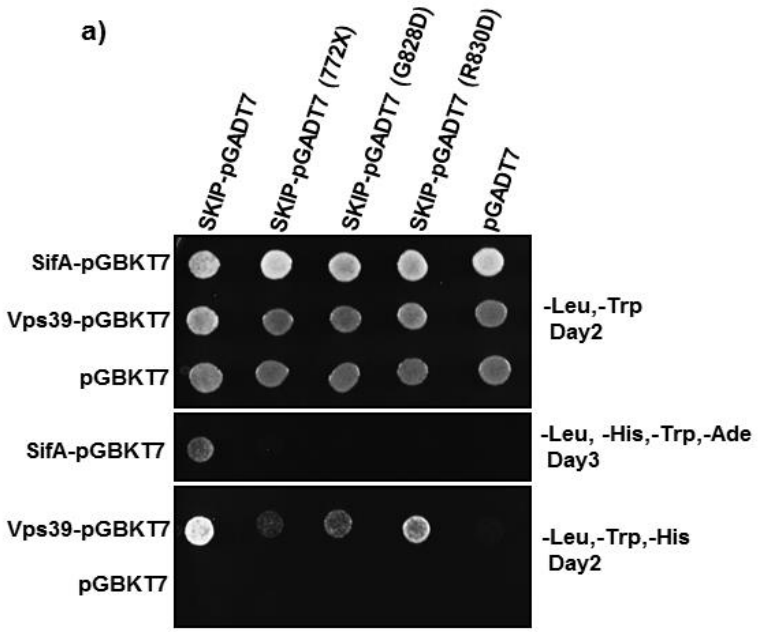


Figure 4.13: Characterization of SifA interaction with SKIP and PLEKHM1. a and b) Representative confocal micrographs of HeLa cells co-transfected with GFP-SKIP and Myc-SifA (red) or GFP-PLEKHM1 and Myc-SifA (red). Bars: (main) 10 µm. **c)** Pearson's correlation coefficient was calculated for the indicated protein pairs in transfected cells as labeled. Data represent mean ± S.D. over three independent experiments where ~25-30 transfected cells were analyzed in each experiment (****, $P < 0.0001$; Student's t test). **d)** Primary yeast cultures were seeded in SD/-leucine/-tryptophan broth from single colonies of S. cerevisiae AH109 strain co-transformed with indicated plasmids, and grown overnight at 30°C to saturation. The resulting cultures were diluted to approximately 0.1 OD (at 600 nm) in SD/-leucine/-tryptophan/-histidine broth and culture growth was monitored every 4 hr for 48 hr. **e)** qRT-PCR analysis to evaluate expression level of PLEKHM1 and SKIP in HeLa cells. **f)** Immunoblot of a GST pulldown assay using HEK293T cell lysates expressing FLAG-PLEKHM1 incubated with GST or GST tagged-SifA. Purified proteins were visualized by Ponceau S staining. **g)** Densitometric analysis of immunoblots of FLAG-PLEKHM1 or FLAG-SKIP pulldown with GST tagged-SifA (normalized to their respective input band intensity).

The crystal structure of SifA-SKIP complex revealed the interacting residues in the PH-domain of SKIP that mediate intimate association with N-terminus of SifA during *Salmonella* infection (Ohlson et al., 2008a; Zhao et al., 2015a). The mutational analyses of the SifA-PHD complex have confirmed the significance of the hydrogen bonding residues R831, C870, G828, and R832 in the PH domain of SKIP. The mutated version of PH domain with any of these mutations exhibits abrogated or reduced interaction to wild-type SifA *in vitro*.

To prove that SKIP forms the liaison of SifA with HOPS complex; we next analysed the interaction of these SifA-binding mutants of SKIP with Vps39 in a yeast-two hybrid assay where Vps39 or SifA were used as bait proteins against wildtype SKIP or SKIP mutants (772X, G828D, R830D). As expected, SifA was unable to interact with any of these mutants of SKIP. Interestingly, the binding of Vps39 to these mutants was also diminished as compared to wildtype SKIP protein indicating that the mutants of SKIP that are unable to interact with SifA are also defective in binding to Vps39 **(Fig 4.14a)**. To gain further insight into this we co-expressed Vps39 and SifA in presence of either wildtype SKIP or SKIP mutant (G828D). Although Vps39 could localize to the peripheral SKIP (G828)-positive aggregates, SifA was not present on these structures indicating that the formation of SifA-SKIP-Vps39 tripartite complex is abrogated in the presence of this mutation in the linker protein SKIP **(Figs 4.14b-f).**



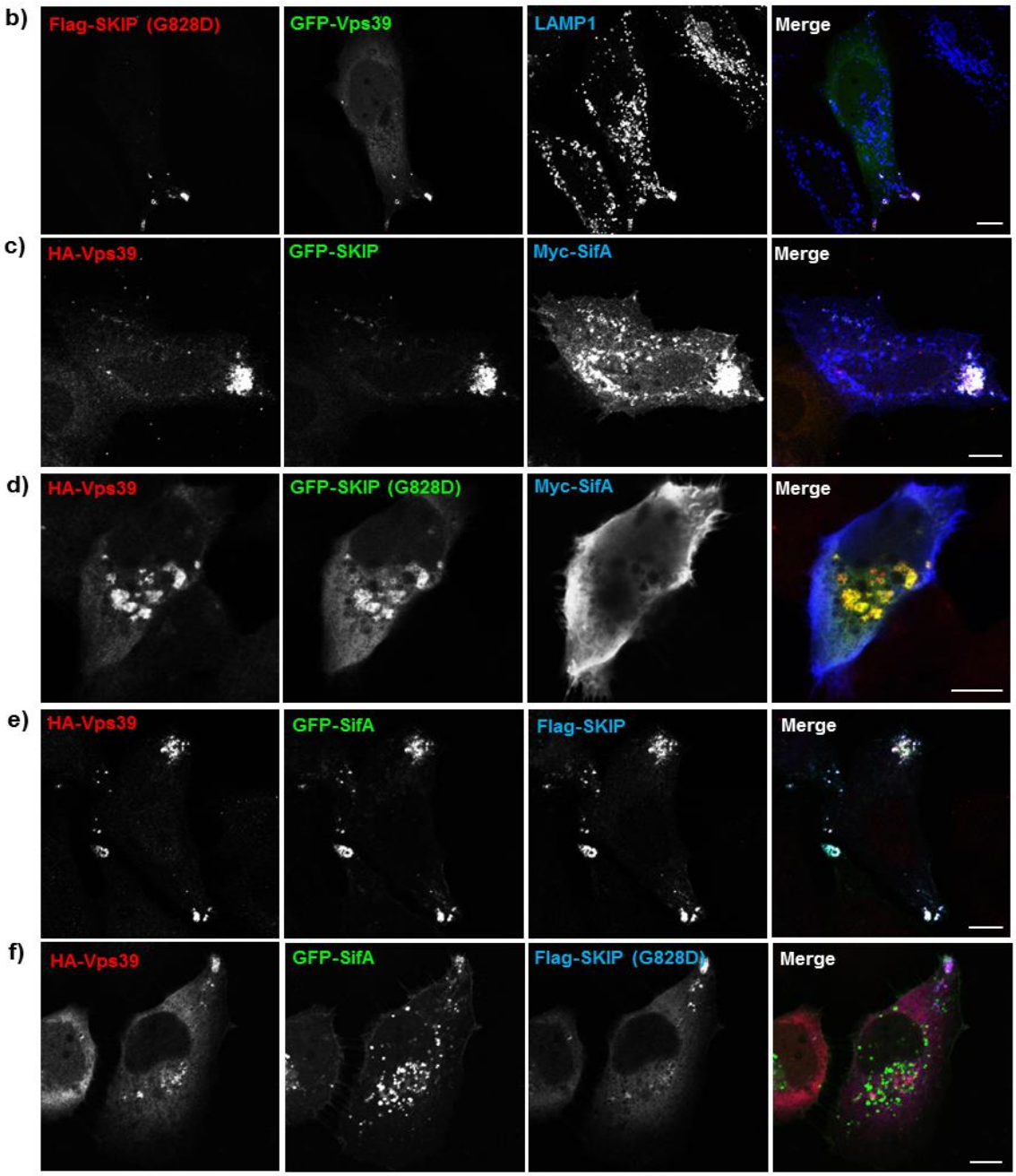


Figure 4.14: SKIP mutants defective for interaction with SifA show reduced interaction with HOPS subunit Vps39 and are unable to mediate the association of SifA to Vps39-positive aggregates. a) Yeast two hybrid interaction of SKIP-PH domain mutants was tested with either SifA or Vps39 by assaying the growth of co-transformants on selective medium to determine the interactions and non-selective medium to confirm viability. **b-f)** Representative confocal micrographs of HeLa cells co-transfected with either Flag-SKIP(G828D) and GFP-Vps39 (b); HA-Vps39, Myc-SifA and GFP-SKIP/GFP-SKIP(G828D) (c and d); HA-Vps39, GFP-SifA and FLAG-SKIP/FLAG-SKIP(G828D) (e and f). Bars:10 µm.

To conclusively determine whether SKIP is a linker between SifA and HOPS subunit-Vps39, we employed yeast three-hybrid assay to test interaction of SifA and Vps39 in the presence of either SKIP or PLEKHM1 as well as a SifA binding-defective mutant of SKIP (SKIP G828D). In this assay, linker protein is under the control of the Met25 promoter that remains repressed in the presence of methionine in the growth media. As depicted in **Fig 4.15a**, under methionine-deficient conditions, SifA showed interaction with Vps39 only in the presence of SKIP, but not SKIP G828D mutant or PLEKHM1. *This experiment was carried out in collaboration with Harmeet Kaur at IMTECH, Chandigarh.*

Further, to establish that endogenous levels of SKIP are sufficient to drive this interaction, we performed co-IP of SifA and Vps39 in control and SKIP depleted cells (**Fig 4.15b**; >90% gene silencing efficiency observed). As shown in **Figs 4.15c** and **9d**, co-IP of Vps39 with SifA was significantly reduced upon SKIP depletion, and was restored upon expression of the siRNA-resistant SKIP construct, suggesting that SKIP acts as a linker to facilitate interaction between SifA and HOPS complex. Similarly, no effect on the levels of co-IP Myc-tagged SifA with HA-Vps39 was observed upon PLEKHM1 depletion (**Fig 4.15e**; >90% silencing efficiency observed; **Fig 4.15f**), suggesting that PLEKHM1 does not facilitate interaction between SifA and HOPS complex.

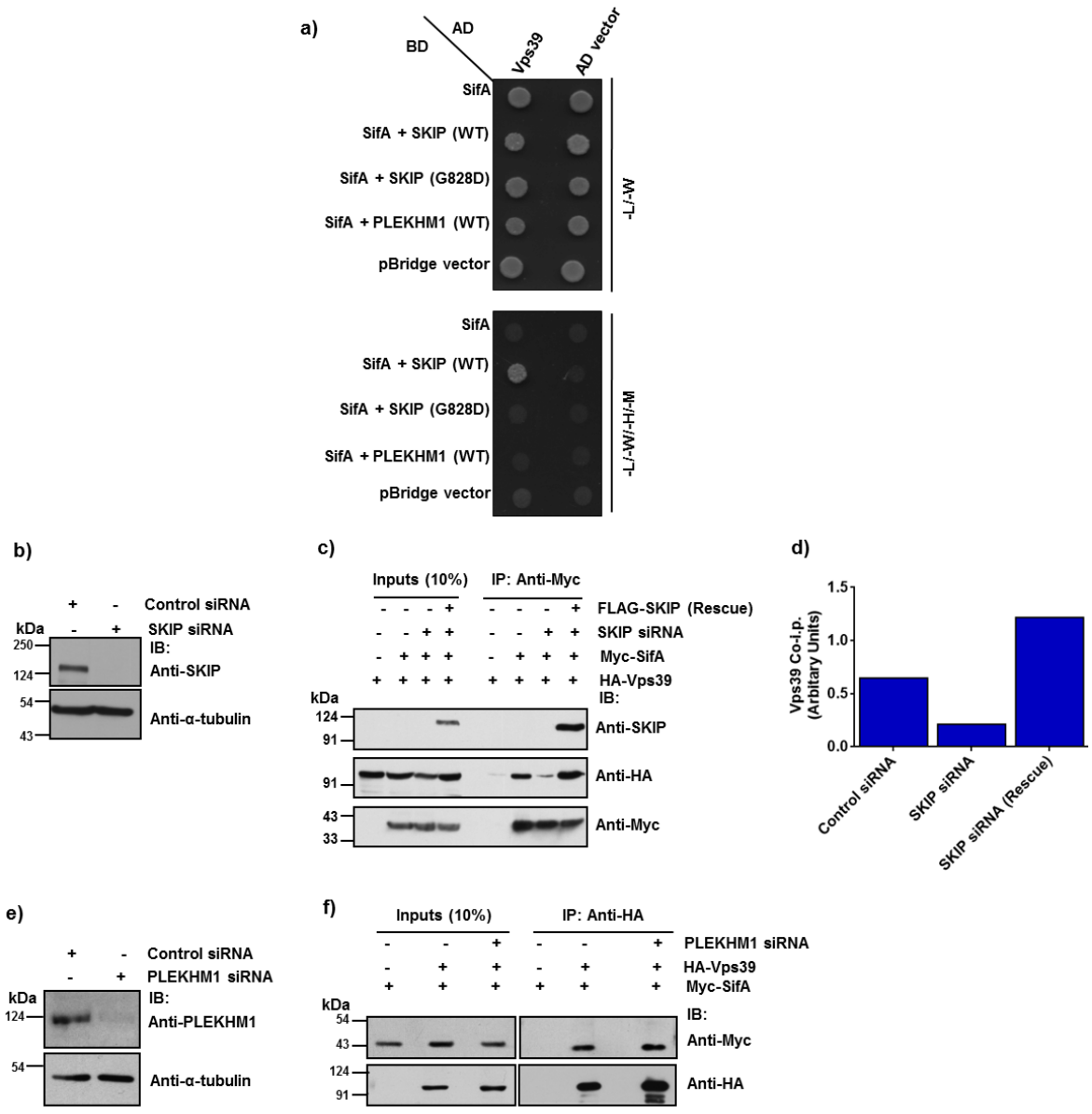


Figure 4.15: SKIP is required for interaction between bacterial effector SifA and HOPS complex. a) Yeast three-hybrid assay. Co-transformants were spotted on -Leu-Trp medium to check for viability, and on -Leu-Trp-His-Met media to test the interaction between SifA and Vps39 in the presence of SKIP (WT), SKIP (G828D) mutant or PLEKHM1. **b)** Control siRNA- or SKIP siRNA-treated HeLa cell lysates were resolved by SDS-PAGE, and immunoblotted with anti-SKIP antibody for assessing the knockdown efficiency and with anti-α-tubulin antibody as the loading control. **c)** Lysates from HEK293T cells treated with control- or SKIP-siRNA and transfected with indicated plasmids were immunoprecipitated using anti-Myc antibodies-conjugated resin. The cell lysates (inputs) and immunoprecipitates were resolved by SDS-PAGE and immunoblotted by Western blotting with the indicated antibodies. **d)** Densitometric analysis of immunoblots of HA-Vps39 co-immunoprecipitated (normalized to input signal band intensity) with Myc-SifA in control siRNA-, SKIP siRNA- or SKIP siRNA rescue construct-transfected HEK293T cell lysates. **e)** Lysates from control siRNA- or PLEKHM1 siRNA-treated HeLa cells were resolved by SDS-PAGE, and immunoblotted with anti-PLEKHM1 antibody for assessing the knockdown efficiency and with anti-α-tubulin antibody as the loading control. **f)** Lysates from HEK293T cells treated with control- or PLEKHM1-siRNA and expressing HA-Vps39 and Myc-SifA were immunoprecipitated with anti-HA antibodies-conjugated resin and precipitates were resolved on SDS-PAGE and immunoblotted with indicated antibodies.

4.2.5 SKIP but not Arl8b links SifA with HOPS subunit; Vps39

Since Arl8b extensively localizes to SCV or SIF membranes and mediates the recruitment of Vps41 subunit to these compartments, we speculated that Arl8b can also regulate the recruitment of HOPS complex to SifA-positive lysosomal clusters. To check this, we co-expressed SKIP and SifA alongwith Vps39 in control and Arl8b knockout HeLa cells. Interestingly, we observed that the tripartite complex (SifA-SKIP-Vps39) was intact even in the absence of this lysosomal GTPase and localized to similar globular compartments that are induced in the presence of SifA **(Figs 4.16a and 4.16b)**. This data clearly suggests that although Arl8b might be crucial for the localization of HOPS subunit Vps41 to SifA-positive structures in presence of endogenous SKIP but the recruitment of HOPS complex to these compartments is mediated by SifA in a SKIP-dependent manner.

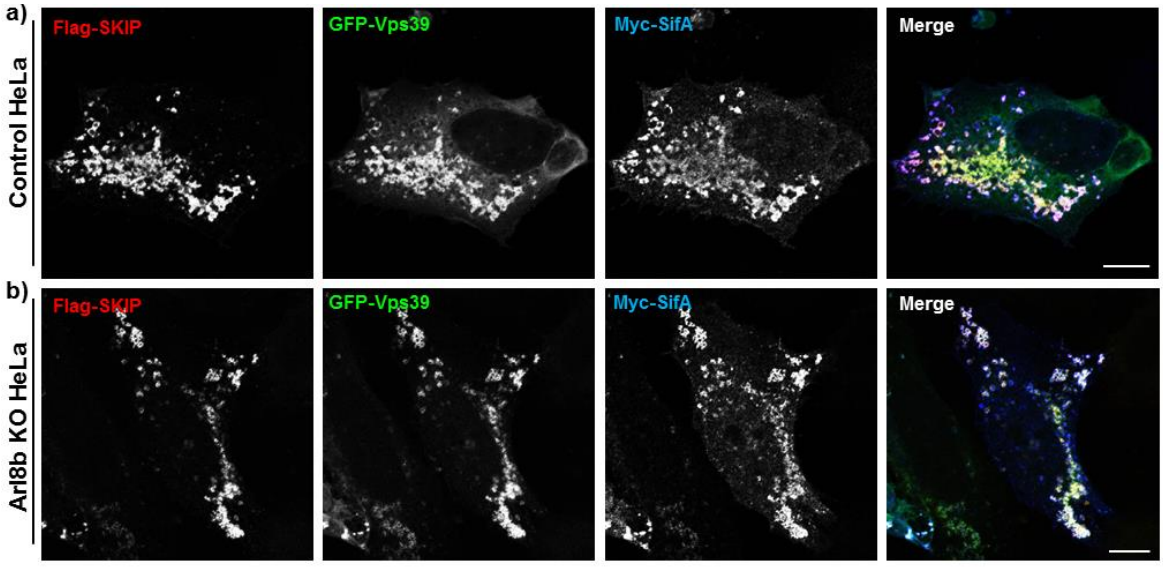


Figure 4.16: Absence of Arl8b does not affect the localization of Vps39 to SifA-SKIP positive globular aggresomes. a and b) Representative confocal micrographs of Control or Arl8b Knock-out HeLa cells co-transfected with Flag-SKIP, GFP-Vps39 and Myc-SifA. Bars:10 µm.

4.2.6 Depletion of HOPS subunit Vps39 prevents SifA-mediated vacuolation of LAMP1-positive endosomes

Taking into consideration that HOPS subunits localize to the fusion vertices of globular LAMP1-positive compartments formed upon ectopic expression of SifA, we further contemplated that SifA mediated clustering and vacuolation of late endosomes and lysosomes requires the presence of SKIP and HOPS complex. To validate this, we transfected epitope-tagged SifA in control-, Vps39- and SKIP-siRNA treated HeLa cells and analysed the particle size of LAMP1-positive compartments in each case. As compared to control-siRNA treated cells which showed the extensive vacuolation phenotype consistent with previous reports; the LAMP1-positive compartments in HOPS and SKIP-depleted cells exhibited a diffused punctate distribution. Accordingly, the particle size quantification data was also in line with these observations signifying that SifA-mediated increase in lysosomal particle size depends upon the expression of SKIP and HOPS subunit-Vps39 (**Figs 4.17a-d**).

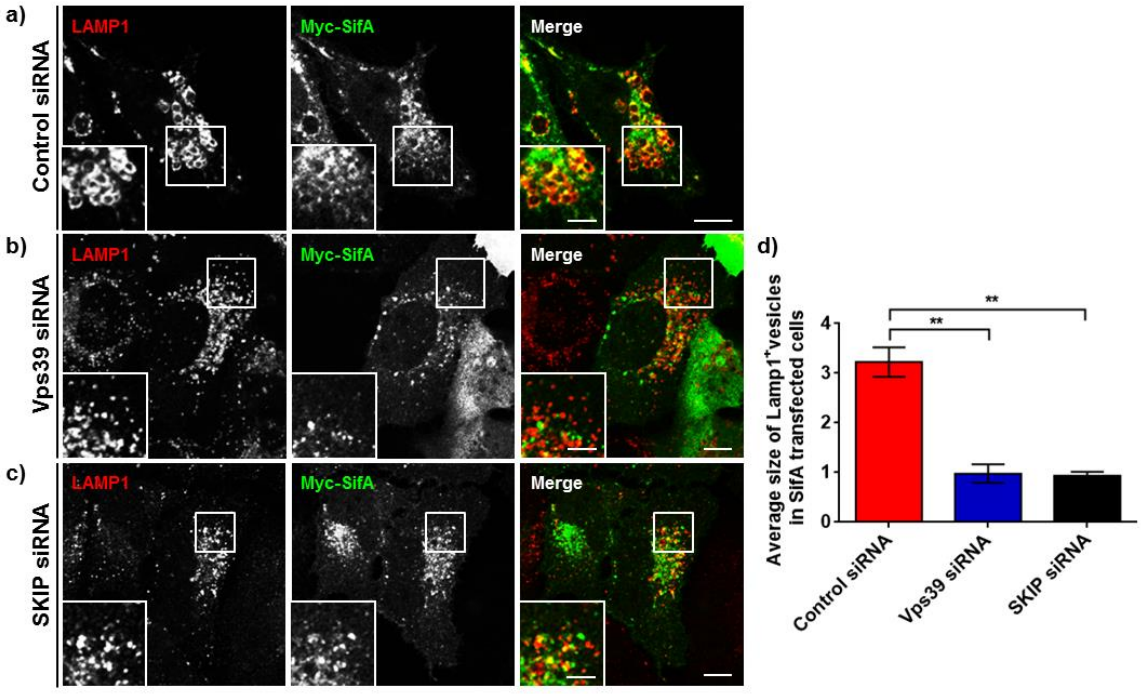


Figure 4.17: SifA-dependent lysosome clustering requires both SKIP and Vps39. a-c) HeLa cells treated with indicated siRNA were transfected with Myc-SifA expressing plasmid. The cells were fixed and co-stained using anti-Myc (green) and anti-LAMP1 (red) antibodies. Insets show higher magnification of the boxed areas clustered lysosomes induced by SifA expression, which is dependent upon Vps39 and SKIP expression. Bars: (main) 10 µm; (insets) 5 µm. **d)** Average size of LAMP1-positive compartments calculated in cells treated with indicated siRNA and transfected with Myc-SifA plasmid. Data represent mean ± S.D. of ~25-30 transfected per experiment over three independent experiments (**, P < 0.01; Student's t test).

Taken together, our findings indicate that *Salmonella* virulence factor SifA in complex with the host protein, SKIP, recruits the vesicle fusion machinery of the host including the tethering factor HOPS complex to SCV membranes, thereby, enabling SCV fusion with late endosomes and lysosomes (**Fig 4.18**).

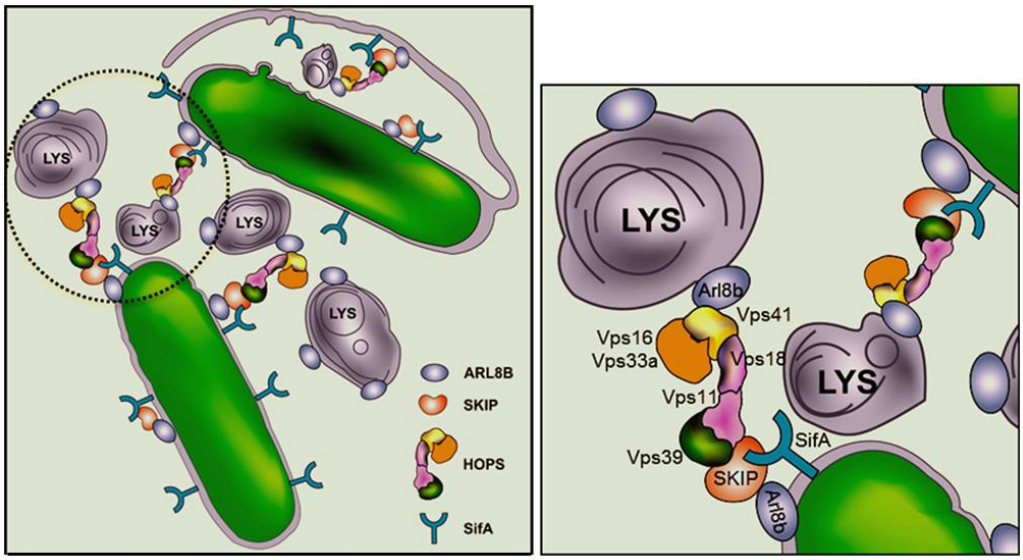


Figure 4.18: Working Model. Schematic depicting the molecular machinery required for SCV fusion with late endosomes and lysosomes. Multi-subunit tethering factor HOPS complex is a target for *Salmonella* effector SifA, which associates with its known binding partner-SKIP to recruit HOPS complex to SCV membranes, thereby enabling SCV fusion with Arl8b-positive lysosomes. *Adapted from: (Sindhwani et al., 2017).*

CHAPTER V

Conclusion and Perspective

Parts of this section have been derived from (Sindhwani et al., 2017)

Discussion

Salmonella Typhimurium is a successful intracellular pathogen that has developed an array of sophisticated strategies to massively remodel the host endosomal system for its own survival and propagation. Previous studies have shown that SCV biogenesis involves extensive interactions with the host endocytic pathway including late endosomes/lysosomes (Liss and Hensel, 2015b). However, little is known about how *Salmonella* mediates these interactions and whether it co-opts the late endosomal-lysosomal vesicle fusion machinery of the host cell for building its replicative niche. Conflicting reports have shown that while *Salmonella* inhibits activation of the small GTPase Rab7 (D'Costa et al., 2015; Meresse et al., 1999), it actively recruits Arl8b on SCV and SIFs (Kaniuk et al., 2011b) wherein both Rab7 and Arl8b are components of protein machinery required for late endosome-lysosome fusion (Marwaha et al., 2017). Intriguingly, Arl8b-positive lysosomes are less acidic and have reduced proteolytic activity than Rab7-positive endosomes (Johnson et al., 2016). It is interesting to speculate that Arl8b- but not Rab7-positive lysosomes act as source of membrane for SCV biogenesis and SIF formation during later time points of infection. This would ensure membrane and cargo delivery to SCVs without increasing the proteolytic activity within *Salmonella*'s replicative niche.

In this study, we have investigated the role of HOPS complex, a multisubunit tethering factor required for vesicle fusion with lysosomes, in regulating *Salmonella* survival and replication inside its vacuole. Our results reveal that HOPS complex is a target for *Salmonella* effector SifA, which in collaboration with its known binding partner SKIP and the host GTPase, Arl8b, recruits HOPS complex to SCV membranes, thereby enabling SCV fusion with lysosomes (**Fig 11g**). As late endocytic compartments are a source for both membrane and fluid-phase cargo, including nutrients for *Salmonella* residing in the vacuole (Liss and Hensel, 2015b; Liss et al.,

2017a), silencing of HOPS subunits inhibited *Salmonella* replication under both *in vitro* and *in vivo* conditions.

Unlike the defence strategies used by intracellular pathogens such as *M. tuberculosis* and *C. burnetti* (Ghigo et al., 2002; Vergne et al., 2004), *Salmonella* does not block the maturation of its phagosome, which rapidly (~30-60 min p.i.) acquires several (but not all) characteristics of the late endocytic compartments but does not become bactericidal (Liss and Hensel, 2015b). The acidic pH of the SCV (~<5) is required for the induction of the SPI-2 effectors, which in turn facilitate *Salmonella* replication inside the host cell (Yu et al., 2010b). At 1-2 hr p.i., we found weak but consistent localization of HOPS subunits on mature SCVs, which correlated with the recruitment of the lysosomal marker, LAMP1. While HOPS complex localized to the mature SCVs, we did not find an essential role of HOPS subunits, Vps41 and Vps39, in SCV maturation as indicated by a modest delay but not a block in LAMP1 acquisition in HOPS depleted cells. Our results support previous studies suggesting that SCV maturation is akin to an early to late endosome maturation event, regulated by proteins including PI(3) kinase and Rab7 (acquired upon HOPS depletion, **Fig 4**) that act upstream of the HOPS complex in endo-lysosome fusion (Brumell et al., 2002c; Meresse et al., 1999).

Previous live-cell imaging studies of *Salmonella*-infected HeLa cells and RAW264.7 macrophages have shown that at 6-8 hr p.i., 90% of SCVs interact with dextran-loaded terminal lysosomes, and acquire not only membrane but also fluid-phase cargo from these compartments (Drecktrah et al., 2007a; Rajashekar et al., 2008a). Besides delivering membranes for SCV biogenesis, fusion with late endosomes/lysosomes provides access to nutrients for bacterial replication (Liss et al., 2017a; Popp et al., 2015b). Intravacuolar *Salmonella* can access nutrients from the host endolysosomal compartments by direct fusion of SCV with these membranes or from cytosol by recruitment of nutrient transporters on SCV and SIF membranes. In both of these scenarios, extensive membrane network will be required, which

is delivered by host vesicle fusion machinery including HOPS complex. Accordingly, the ability of proline auxotrophic *Salmonella* strain to acquire proline from the extracellular media was also abrogated in HOPS-depleted cells. Besides their role as a tethering factor, HOPS subunits bind to SNARE proteins, which mediate membrane fusion (Jiang et al., 2014a; Solinger and Spang, 2013b). We found a comparable defect in *Salmonella* intracellular replication when we depleted other components of the vesicle fusion machinery including small GTPases-Rab7 and -Arl8b, as well as SNARE proteins: Vti1b, Syntaxin 8, Syntaxin 17 and VAMP7 that are known to regulate late endosome-lysosome fusion (Pryor et al., 2004). These results indicate that *Salmonella* co-opts the host vesicle fusion machinery for survival and replication within its intravacuolar niche.

One of the hallmarks of *Salmonella* intracellular lifestyle is presence of striking tubular membranes or SIFs that emanate from the juxtanuclear SCVs (Garcia-del Portillo et al., 1993b). The ability to form SIFs was found to directly correlate with *Salmonella*'s ability to replicate both under *in vitro* and *in vivo* conditions, as supported by the replication defect observed in *Salmonella* strains defective in SIF formation (Brumell et al., 2001b). Recent studies have now shown that SIF formation allows *Salmonella* to convert the host cell endosomal system into a continuum with the SCV, not only providing SCVs access to the endocytosed material but the extensive SIF network is proposed to rapidly dilute the antimicrobial activities transferred to the vacuole upon its fusion with the host late endosomes and lysosomes. As a result, SCVs competent to form SIFs have bacteria with significantly higher metabolic activity than one that cannot form SIFs (Liss et al., 2017a). Using live-cell imaging we found that depletion of HOPS subunits completely inhibited SIF formation by *Salmonella,* supporting the strong replication defect observed in these cells.

Although SIFs are the major tubular networks found in *Salmonella* infected cells, there have several reports have evidenced the formation of other kinds of SITs (SISTs, LNTs etc.) that

originate from the SCVs at different times post-invasion. Here, we have shown that HOPS complex is indispensable for SIF formation but we did not look at the morphological profile of tubules other than SIFs upon HOPS depletion. Further, studies would be needed to determine whether HOPS complex and other components of the vesicular fusion machinery (Arl8b, SKIP, SNAREs) also play a role in the formation of these distinct tubular networks.

The proteomic analysis of the SCV membranes and SIT network has revealed the enrichment of host proteins of the endolysosomal system and trans-Golgi network in the isolated SIT and SCV fractions alongwith proteins derived from compartments such as endoplasmic reticulum, nucleus and mitochondria (Vorwerk et al., 2014). The presence of such diverse eukaryotic proteins in SIF fraction prompts a question that how does intravacuolar *Salmonella* associate with these organelles and acquires these proteins. In this study we have demonstrated that *Salmonella* hijacks the components of the endolysosomal fusion machinery and HOPS complex to mediate tethering and fusion of the endocytic vesicles with SCV and SIF membranes to recruit membrane for expansion of the tubular network and acquisition of nutrients for the replicating *Salmonella* population. On these lines, one can further speculate that *Salmonella* might also interact and seize several other tethering factors that regulate the association and fusion of vesicles at these organellar membranes.

SifA is the most well characterized *Salmonella* effector named for its essential role in mediating SIF formation (Garcia-del Portillo et al., 1993b). Accordingly, *Salmonella* strains lacking SifA show a strong replication defect, as they fail to induce SIF formation and escape into the cytosol (Brumell et al., 2001b). SifA has been shown to interact with two host proteins namely SKIP/PLEKHM2 and PLEKHM1 via pleckstrin homology (PH) domains of these proteins (Boucrot et al., 2005b; McEwan et al., 2015d). We found that SKIP, but not PLEKHM1, acts as a linker to mediate interaction of HOPS complex with SifA by simultaneously binding to HOPS subunit-Vps39. These results were surprising given the fact that previously PLEKHM1

was implicated in recruitment of HOPS complex to mediate SCV fusion with detoxified lysosomes (McEwan et al., 2015d). However, the role of PLEKHM1 as a linker was never directly tested in this study and it was speculated based on the fact that PLEKHM1 binds to both HOPS complex and SifA (McEwan et al., 2015b; McEwan et al., 2015d). A direct comparison of PLEKHM1 and SKIP's linker role and their relative binding affinities for SifA as well as comparison of expression levels of both proteins in HeLa cells led us to conclude that SifA-SKIP promotes recruitment of HOPS subunits to SCV compartment. It will be interesting to determine whether SifA and Vps39 have overlapping binding sites on PLEKHM1, preventing SifA recruitment to PLEKHM1 and Vps39-positive compartment. Our study also suggests a novel role for SKIP in promoting *Salmonella* intracellular replication, besides its known function in preventing kinesin-1 accumulation on SCVs and regulating vacuolar integrity (Boucrot et al., 2005b; Dumont et al., 2010a; Leone and Meresse, 2011). This study has provided a clearer picture of the proteins interacting at this host-pathogen interface however, the order in which these proteins are recruited and the interacting domains of the binding partners still remains enigmatic.

HOPS complex localization to SCVs and SIFs also required small GTPase Arl8b, which is highly enriched on these compartments and regulates lysosomal localization of both of its effectors-SKIP and Vps41 subunit of the HOPS complex (Kaniuk et al., 2011b; Khatter et al., 2015b; Rosa-Ferreira and Munro, 2011a). Recently, we have uncovered that PLEKHM1, like SKIP, binds to Arl8b via its RUN domain and is a shared effector of Rab7 and Arl8b, which simultaneously binds to both GTPases to promote cargo trafficking to lysosomes (Marwaha et al., 2017). Since *Salmonella* has devised a strategy to inhibit Rab7 activation, on the other hand Arl8b is enriched on SCVs and SIFs; it will be relevant to determine whether PLEKHM1 role in SCV fusion with lysosomes is dependent upon its interaction with Arl8b.

Unlike *Salmonella Typhimurium,* much less is known about the intracellular lifestyle of the human-restricted pathogen-*Salmonella typhi*, the typhoid-causing strain of the same serovar. Intracellular *S. typhi* secretes the typhoid toxin inside its SCV, which is then packaged into vesicular carriers that are then transported into the extracellular space to mediate its effect in an autocrine and paracrine manner on the host cells (Chang et al., 2016; Spano et al., 2008). Interaction of *S. typhi* vacuole with the host endocytic machinery and mechanisms regulating formation and transport of the typhoid toxin-containing vesicular carriers are only beginning to be understood (Fowler et al., 2017; Galan, 2016). Indeed, like *S. Typhimurium*, intracellular replication of *S. typhi* was impaired in Rab7-depleted cells, suggesting that *S. typhi* might also manipulate host late endosomes and lysosomes to regulate biogenesis of its SCV and growth inside the host cells (Spano et al., 2011). Future studies are required to address whether the host endocytic machinery regulates *S. typhi* replication and biogenesis of the typhoid toxin vesicular carriers that will reveal novel targets for development of antimicrobial molecules.

Apart from *Salmonella* other bacterial species that manipulate the endolysosomal system for their survival inside the host cells might target this multisubunit tethering factor to either bypass the canonical phagosome maturation pathway or to allow interaction with the lysosomes which was observed in the case of *Salmonella*. In the former case, the pathogens might interfere and inhibit HOPS assembly or HOPS localization to prevent fusion with the degradative lysosomal compartments which is a much obvious strategy. However, like *Salmonella*, certain pathogens such as *Coxiella burnetti* and *Helicobacter pylori* continue to interact with late endosomes and lysosomes and survive in giant vacuoles that are highly fusogenic with these acidic compartments. *Coxiella burnetti* is a gram-negative intracellular pathogen that is the causative agent of Q fever in humans (flu-like illness). Upon internalization into the host the nascent or small vacuoles sequestering these bacteria undergo maturation through interactions with different membranous organelles (endosomes, lysosomes, autophagosomes and Endoplasmic reticulum) into larger vacuoles which are conducive for bacterial proliferation. Recently, it has

been shown that the interaction of HOPS complex and RILP is essential for the homotypic fusion of smaller non-replicative CCVs (nrCCVs) and heterotypic fusion of CCVs with lysosomes that results in the biogenesis of characteristic replication-competent giant CCVs for propagation of bacterial infection. This process of CCV development also requires the coordinated actions of the late endosomal and lysosomal GTPases Rab7 and Arl8b, which mediate the anterograde transport and fusion of lysosomes with the numerous smaller nrCCVs or larger CCVs (Flores RMO. et. al. (2019). PloS one, 14(1), e0209820). Similarly, another successful bacterial pathogen, *Helicobacter pylori* disrupts the normal phagosome maturation process to build a hybrid phagosome-endosome-lysosome compartment known as megasomes in human phagocytic cells, which is essential for the establishment of persistent infection in the host. Likewise, *Salmonella* phagosomes, the H. pylori megasomes are also trafficked towards the perinuclear region and acquire late endosomal and lysosomal markers such as Rab7, CD63, LAMP1 and LAMP2. It has been reported that these vacuolar compartments retain these endosomal markers, indicating that the increase in size is due to continued fusion events between the endocytic/lysosomal vesicles with the megasomes harbouring bacteria. However, nothing is known about the molecular players involved in the formation of these giant megasomes observed during *H. pylori* infection which is crucial for persistent infections. Therefore, it would be appropriate and interesting to explore the role of HOPS complex and other components of the fusion machinery during *H. pylori* infection (Ref: Borlace GN., et al. Gut pathogens 3.1 (2011): 3). Apart from the bacterial pathogens, there have been few reports regarding enveloped viruses that exploit the proteins involved in endosomal maturation or late endosomal-lysosomal fusion to insert their genome into the host cell. For instance, mutations in HOPS complex subunits have been reported to impair infection by Ebola virus (Carette JE. et. al. Nature. 2011 Sep;477(7364):340) and RNAi-mediated downregulation of HOPS subunits affects the entry of mouse hepatitis coronavirus (MHV) into host cells (Burkard C. et.al., 2014. PLoS pathogens, 10(11), p.e1004502).

CHAPTER VI

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