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Proteome analyses of host membranes modified by intracellular *Salmonella enterica* Typhimurium

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„Zwei Dinge sind zu unserer Arbeit nötig:
Unermüdliche Ausdauer und die Bereitschaft, etwas, in das man viel Zeit und Arbeit gesteckt hat, wieder wegzuwerfen.“

Albert Einstein

Für meine Eltern

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I Introduction

One of the leading causes of morbidity and mortality worldwide are infectious diseases (World Health Organization, 2008). Bacterial pathogens play a key role and are still a public health threat, especially considering the increasing antibiotic resistance (World Health Organization, 2014). It is necessary to gain a deeper understanding of pathogen-host interactions to identify new targets for therapies on molecular and cellular levels (Briken, 2008). In particular the complex host/pathogen relations of intracellular bacterial pathogens in eukaryotic cells are of special interest (Bumann, 2010). These microorganisms have evolved strategies to manipulate host membranes and host trafficking system to create a replication niche and evade the host immune response (Alonso and Garcia-del Portillo, 2004). Thus the understanding of pathogen's modification of the host deepen the knowledge of the basic cellular processes (Sherwood and Roy, 2013).

This work focuses on the foodborne bacterial pathogen *Salmonella enterica* subsp. *enterica* serovar Typhimurium that is used as model system for *S*. Typhi infections. *S.* Typhimurium is able to create a unique replicative niche. The host endosomal system is remodelled by forming a network of tubular, membranous structures connected with the *Salmonella*-containing vacuole (SCV).

To understand intracellular survival and replication strategies of bacterial pathogens such as *S*. Typhimurium, a deep knowledge about the cellular environment is necessary to figure out how the endosomal system is manipulated by intracellular bacteria. In the following paragraphs the endosomal system, important intracellular bacterial pathogens and in particular *S*. Typhimurium will be described in more detail.

I.1 Home inside the host cell – the endosomal system

The host cell is a complex structure as habitat for intracellular bacterial pathogens. It contains several membranous organelles which are connected by an extensive vesicle transport system which can be divided in the secretory and endocytic pathway. An overview of compartments, considering the endocytic and secretory system, with their pathways and marker proteins is shown in Figure I-1.

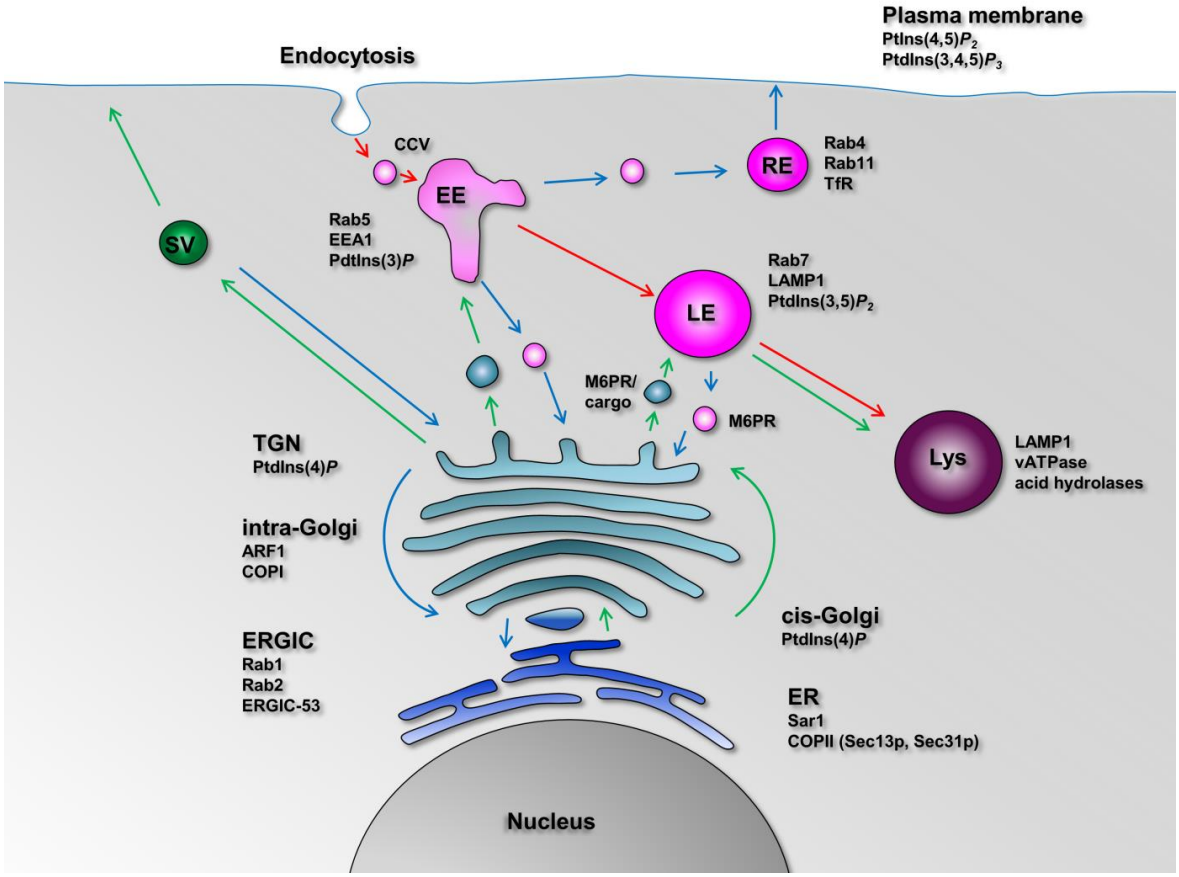

Figure I-1: Interaction of main compartments of endocytic and secretory pathways.
In the endocytic pathway (blue arrows) cargo is incorporated in vesicles derived from plasma membrane and is transported to early endosomes and via late endosomes to lysosomes. Proteins and lipids are delivered from the endoplasmic reticulum (ER) to the plasma membrane or to lysosomes in the secretory pathways (green arrows). Components of the early endosome are recycled by returning back to the plasma membrane, additional molecules from the early and late endosomes are transported back to the Golgi and to the ER (blue arrows). Marker proteins and lipids of the endocytic and secretory pathways are indicated. Although it should be taken into account that the majority of molecular markers are either transiently associated with vesicles or follow them through several steps during the maturation process. CCV, clathrin coated vesicle; EE, early endosome; ERGIC, ER-Golgi-intermediate compartment; LE, late endosome; Lys, lysosome; M6PR, mannose-6-phosphate receptor; TGN, trans-Golgi network; SV, secretory vesicle. Modified according to Hilbi and Haas (2012).

The secretory system transports proteins and lipids produced in the endoplasmic reticulum (ER) via ER-Golgi intermediate compartment (ERGIC), Golgi and vesicles to the plasma membrane or endosomes in a process called exocytosis (Hilbi and Haas, 2012). By the reversed process, endocytosis, the cargo (e.g. fluid, solutes, lipids, membrane proteins, a variety of nutrients and their carriers, receptor-ligand complexes, extracellular-matrix components, cell debris, bacteria, viruses) is incorporated by invagination of the plasma membrane and subsequent vesicle formation which undergoes a sorting, processing, recycling, storing or degrading processes (Huotari and Helenius, 2011). Different types of endocytosis are distinguished by the kind of cargo, which is transported. Receptor-mediated endocytosis allows internalization of particles with a size below 0.5 µm

and is driven by ligand-bound receptors and leads to a clathrin-dependent or clathrin-independent uptake (Mayor and Pagano, 2007). Internalization of large amounts of fluids and small particles like growth factors with vesicle size from 0.2 µm up to 5 µm is called macropinocytosis (Lim and Gleeson, 2011) whereas phagocytosis is the uptake of large particles (>0.5 µm) like bacteria or apoptotic cells in a receptor-mediated and actin-dependent way (reviewed in Flannagan *et al.*, 2012; Haas, 2007; Kinchen and Ravichandran, 2008; Rogers and Foster, 2008).

In spite of the complexity of these transport networks it is essential that integrity of organelles is maintained and the traffic is tightly regulated. To sum up, the endosomal system is an adaptable and dynamic continuum, where the content is continuously exchanged while its components are stepwise remodelled in their molecular and structural composition for a functional conversion (Saftig and Klumperman, 2009).

I.1.1 Rab GTPases as master regulators

Cellular traffic is coordinated by Rab GTPases and their effectors which coordinate formation, transport, tethering and fusion of vesicles (Zerial and McBride, 2001). Figure I-2 demonstrates the diversity of Rab GTPases involved in membrane trafficking. Rabs (Ras-like in rat brain (Touchot *et al.*, 1987)) are the largest family of small Ras-like GTPases. In the human genome 66 Rab GTPases are encoded inclusive different isoforms with distinct or overlapping functions (Klöpper *et al.*, 2012; reviewed in Wandinger-Ness and Zerial, 2014). Their size varies from 21 to 25 kDa (Bhuin and Roy, 2014). The majority of Rab GTPases are ubiquitous, however, also tissue specific expression occurs, e.g. Rab27a/b, which controls multivesicular body-exosome secretion in immune cells and melanocytes (Wandinger-Ness and Zerial, 2014).

Considering the protein structure, Rab GTPases contain, like other Ras superfamily members, several highly conserved regions. The nucleotide-binding motif is composed of a six-stranded β-sheet with five parallel strands and one anti-parallel strand, flanked by five α-helices (Pfeffer, 2005). Two regions termed switches I and II are the only elements in the Rab proteins which undergo conformational changes upon nucleotide exchange (Pfeffer, 2005). The hypervariable carboxy-terminal domain of Rab GTPase is posttranslational modified by prenylation of one, in most cases two, cysteine residues which are attached to a geranylgeranyl moiety to enable regulated membrane binding (Leung *et al.*, 2006; Maurer-Stroh *et al.*, 2003). This hypervariable region can be, depending on the Rab protein, dispensable for subcellular localization or in other cases crucial for specific interactions with effectors (Li *et al.*, 2014).

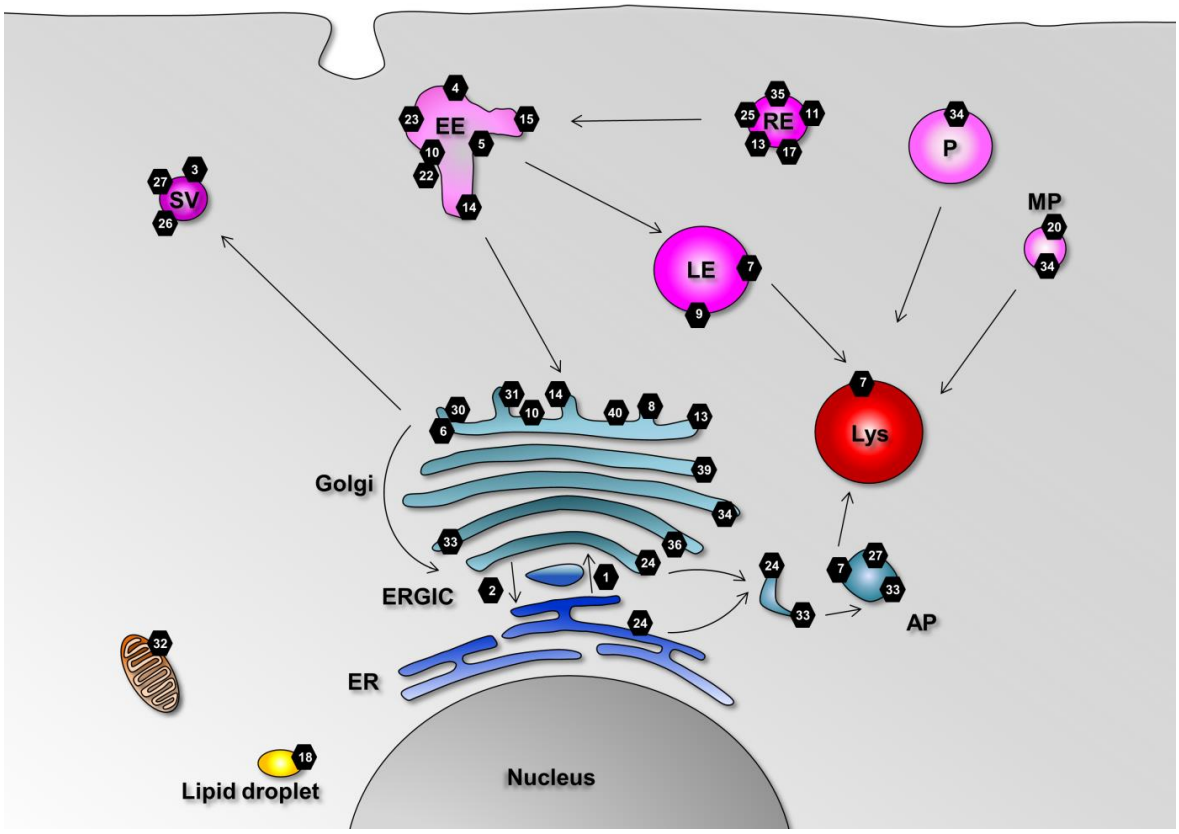

Figure I-2: Overview of Rab GTPases involved in intracellular membrane trafficking.
Localization (black hexagons) and associated vesicle transport pathways (arrows) of Rab GTPases are shown. Rab1 is responsible for the traffic from ER to Golgi, whereas Rab2 regulates the retrograde Golgi-ERGIC-ER traffic. The *intra*-Golgi traffic is regulated by Rab6. Rab8, Rab10 and Rab14 are involved in transport from the *trans*-Golgi network (TGN) to the plasma membrane. Exocytosis by secretory vesicles and granules is regulated by Rab3, Rab26, Rab27, and Rab37. A variety of Rabs are important for the endosomal system. The early endosome is the control centre, which determines the pathway. Rab5 mediates fusion with endocytic vesicles. One pathway is the direction to lysosomes for degradation with Rab7 as key Rab GTPase and the other pathway is the recycling endosome for the reuse of factors on the plasma membrane. Rab15 is responsible for membrane trafficking from the early endosome to the recycling endosome. Rab4 and Rab11 are involved in fast and slow recycling. Rab13, Rab23 and Rab25 mediate transport from the early endosome to the plasma membrane. Rab22 directs traffic from early endosomes to the TGN. Development of autophagosomes is controlled by Rab24 and Rab33 which regulate the formation of pre-autophagosomal structures to engulf cellular components to build autophagosomes which are targeted to lysosomes. Rab35 plays a role in plasma membrane recycling and Rab20 in apical membrane recycling and macropinocytosis. Rab31 is involved in M6P receptor transport to endosomes. Rab33 contributes to autophagosomes formation. The function of Rab34 mediates *intra* Golgi transport, formation of phagosomes and macropinosomes. Rab36 and Rab40 affect endosome/lysosome transport and localization Rab40 has influences on phagosomal acidification and secretion. Rab32 directs mitochondrial fission. AP, autophagosome, ERGIC, ER-Golgi intermediate compartment; ER, endoplasmic reticulum; EE, early endosome; LE, late endosome, Lys, lysosome, MP, macropinosome; P, phagosome; RE, recycling endosome; SV, secretory vesicle; modified according to (Hutagalung and Novick, 2011; Stein *et al.*, 2012; Wandinger-Ness and Zerial, 2014)

Rab proteins act as molecular switches with an active GTP-bound and an inactive GDP-bound state (Hutagalung and Novick, 2011). The newly synthesized Rab protein binds to a Rab escort protein (REP) which enables the interaction with the Rab geranylgeranyl transferase (RabGGT) for the prenylation (Andres *et al.*, 1993). The Rab protein with the geranylgeranyl tails and a bound GDP is transported by REP to its target membrane (Alexandrov *et al.*, 1994). The GDP displacement factor (GDF) assists in inserting the Rab GTPase into the correct membrane (Dirac-Svejstrup *et al.*, 1997). Afterwards the membrane-inserted Rab protein is activated by a guanine nucleotide exchange factor (GEF) which catalyses the exchange of GDP to GTP coupled with a conformational change (Blümer *et al.*, 2013). In this active state the Rab GTPase interacts with several effectors and regulates the traffic in its pathway.

For the inactivation of the Rab protein the GTPase accelerating protein (GAP) is stimulating the hydrolysis of GTP to GDP (Barr and Lambright, 2010). In this conformation the GDP dissociation inhibitor (GDI) binds to Rab protein and removes it from the membrane and the cycle can start from the beginning (Seabra and Wasmeier, 2004). To control the transition from one Rab GTPase to another Rab GTPase in the maturation process a cascade of GEFs and GAPs is proposed whereby an upstream Rab protein activates the GEF of a downstream Rab GTPase that in turn activates the GAP of the upstream Rab protein for its deactivation (Hutagalung and Novick, 2011).

I.1.2 Regulation of the endocytic pathway

Early endosomes (EE) are the starting point of the endocytic pathway. Cargo is delivered from the plasma membrane to the EEs by several mechanisms like the clathrin-mediated, caveolar-, glycosyl phosphatidylinositol-anchored protein enriched early endosomal compartments (GEEC)-, RhoA-, Cdc42-, or Arf6-dependent pathways (Mayor and Pagano, 2007). The EE is the main sorting station. On the one hand cargo is accumulated for the degradation via late endosomes in lysosomes, and on the other hand, membrane material and receptors are recycled back to the plasma membrane directly (fast) or indirectly (slowly) by recycling endosomes (Huotari and Helenius, 2011). EEs are tubular-vesicular structures with a luminal pH of ~6.0. They are located in the peripheral cytoplasm and move along microtubules during their maturation process (Maxfield and McGraw, 2004). The calcium concentration of these compartment is relatively low (Gerasimenko *et al.*, 1998). Rab5 is the key marker of the early endosome and is recruited by its effectors Rabex-5 and Rabaptin-5 in its active state to the endosome membrane (Hutagalung and Novick, 2011). The Rab5 effector VPS34/p150, a phosphatidylinositol 3-kinase, catalyses the phosphorylation of phosphatidylinositol to

PtdIns(3)*P* which is strongly enriched on early and late endosomes and mediates binding of FYVE zinc finger domain proteins (Wandinger-Ness and Zerial, 2014). In interplay with Rab5, PtdIns(3)*P* recruits further effectors, such as EEA1 (early endosome antigen 1), Rabenosyn-5, and Rabankyrin-5 which initiate a positive feedback loop for activation of Rab5 (Zerial and McBride, 2001). The early endosomes possess different membrane domains enriched in Rab5, Rab4, Rab11, adapter complex AP1, and a retromer complex located in the tubular structures which mediates molecular sorting for target vesicle transport (Roberts *et al.*, 1999; reviewed in Bonifacino and Rojas, 2006; Huotari and Helenius, 2011). While Rab4, Rab11 and AP1 play a role in recycling, the retromer complex is responsible for the retrograde TGN transport. This multisubunit protein complex consists of a sorting nexin dimer with a still-undefined combination of sorting nexins SNX1, SNX2, SNX5 or SNX6 and the cargo recognition trimer VPS26-VPS29-VPS35 (Bonifacino and Rojas, 2006; Huotari and Helenius, 2011).

In around ten to forty minutes EEs are converted to LEs (Huotari and Helenius, 2011). Intraluminal vesicles (ILVs) already arise in EEs by the incorporation of ubiquitinated proteins through an invagination of the endosome membrane mediated by the ESCRT complex and clathrin (Raiborg and Stenmark, 2009). For the conversion from Rab5 to Rab7, the key regulator of the late endosome (LE), Rab5 is indirectly switched “off” by a complex consisting of Mon1/Sand-1 and Ccz1 which binds to Rabex5 and promotes dissociation of Rabex-5 from the membrane and ends the positive feedback loop for Rab5 (Huotari and Helenius, 2011; Poteryaev *et al.*, 2010). Rab7 and HOPS bind to the Mon1/Sand-1-Ccz1 complex and Rab7 is activated. Rab7 is the key regulator of LEs. The CORVET complex, mediating homotypical fusions of EEs, is replaced by the HOPS complex during endosome maturation (Balderhaar and Ungermann, 2013), what facilitates tethering with LE and lysosomes (Kümmel and Ungermann, 2014). Apart from controlling the early-to-late endosomal maturation, Rab7 and its effectors direct microtubule movement and transport between endo- and lysosomes (Wang *et al.*, 2011).

The arising LEs are round or oval, have a luminal pH of 6.0-4.9, a size of 250-1,000 nm and are highly negatively charged on their surface, and containing numerous ILVs (Huotari and Helenius, 2011). Only the vacuolar part of the EE matures to LE. These vesicles move from the peripheral cytoplasm to the perinuclear region whereby they transiently (kiss-and-run) fuse with other EEs or LEs, until they finally fuse with endolysosomes or lysosomes for degradation (Luzio *et al.*, 2007). Marker proteins for LEs are integral lysosomal membrane proteins (LMP), such as lysosomal-associated membrane proteins 1 (LAMP1) and LAMP2, and acid hydrolases in the lumen (Saftig and Klumperman, 2009). Exchange with the TGN for hydrolase delivery and recycling of components of the LE, which are not determinate for degradation, such as M6PR

(mannose-6-phosphate-receptor; transport of hydrolases), tetraspanins and SNAREs, is enabled by Rab7, Rab9 and the retromer complex (Bonifacino and Hurley, 2008; Pfeffer, 2009). Furthermore PtdIns(3)*P* is converted to PtdIns(3,5)P_2 by a phosphatidylinositol 3-phosphate 5-kinase, PIKfyve, during endosome maturation (Shisheva, 2008). This conversion might be associated with endosome-TGN transport, acidification and fusion events (Huotari and Helenius, 2011).

The lysosomes are the final step of the endocytic pathway as point-of-no-return. To distinguish them from LEs, lysosomes are defined as LMP-rich with a pH below five but lack M6PRs (Saftig and Klumperman, 2009). The pH of the lumen is acidified to a pH 4.6-5.0 by proton-pumping vacuolar ATPases (vATPases) (Luzio *et al.*, 2007). In this compartment the whole cargo from endo-, and phagocytosis or autophagy is degraded. The shape is heterogeneous, mostly globular but also tubular. The lumen is often filled with membrane sheets and some ILVs (Saftig and Klumperman, 2009). Integral lysosomal membrane protein and lysosomal acid hydrolases are important for the function of these structures. The most abundant LMPs are LAMP1, LAMP2, LIMP2 (lysosome integral membrane protein 2) and tetraspanin CD63 (Saftig and Klumperman, 2009). The acid hydrolases are transported to the lysosomes from the TGN via LEs. The best studied pathway is the M6PR (mannose-6-phosphate-receptor) pathway. Newly synthesized acid hydrolases are tagged with mannose-6-phosphat in the *cis*-Golgi and bind to M6PRs which mediate delivery to endosomes in clathrin-coated vesicles (Luzio *et al.*, 2007). Further M6PR-independent pathways may play additional roles in transport of hydrolases to lysosomes, where the VPS10 family or LIMP2 are involved (Saftig and Klumperman, 2009). LMPs are delivered either via the plasma membrane in the endo-lysosomal system or directly from the TGN (Luzio *et al.*, 2007).

I.1.2.1 Function of the cytoskeleton in the endosomal system

Movement of the endocytic vesicles mediated by the cytoskeleton is essential for viability of the endosomal system. Endosomes mature during their transport along microtubules from the cell periphery to the perinuclear region (Huotari and Helenius, 2011). Microtubules consist of α,β-tubulin heterodimers that polymerize head-to-tail into protofilaments, bundled in parallel formation with a plus-end (β-subunit exposed) and minus-end (α-subunit exposed) and arise from the MTOC (microtubule-organizing centre) (Horgan and McCaffrey, 2011). The movement of vesicles depends on the motor proteins kinesin and dynein whereby kinesin mainly mediates plus-end directed and dynein minus-end directed transport (Hunt and Stephens, 2011). Both kinds of motor proteins are not

only involved in endosome movement and fusion, but also they are required for fission of EEs and cargo sorting (Horgan and McCaffrey, 2011). The kinesin-3 motor protein KIF16B (kinesin family member 16B) is required for plus-end-directed transport of EEs which is important for sorting and recycling or KIF3A with a similar role in LEs (Hunt and Stephens, 2011; Huotari and Helenius, 2011). For a movement to the perinuclear region a dynein-dependent transport is necessary. The binding to dynein is either direct or through the dynactin adaptor protein complex. A well-known mechanism is the minus-end directed traffic regulated by Rab7. Rab7-GTP interacts with its effectors RILP (Rab interacting lysosomal protein) and ORP1L (Oxysterol-binding protein-related protein 1) which bind the dynactin/p150(glued) subunit via the dynactin receptor βIII spectrin (Jordens *et al.*, 2001; Johansson *et al.*, 2007; reviewed in Huotari and Helenius, 2011; Wang *et al.*, 2011).
Additional to microtubules the actin cytoskeleton plays a role in endosome maturation. Actin nucleation driven by Arp2/3 is required for fission processes at early endosomes and annexin A2 is important for EE biogenesis (Derivery *et al.*, 2009; Duleh and Welch, 2012; Gomez and Billadeau, 2009; Morel and Gruenberg, 2009; reviewed in Girao *et al.*, 2008).

I.1.3 Phagocytosis

Phagocytosis is an important process for removal of apoptotic cells and invading bacteria (Kinchen and Ravichandran, 2008; Rogers and Foster, 2008). Phagosomes, vacuoles formed around a particle, mature in a severely regulated process parallel to endosome maturation with early, intermediate, late, and phagolysosome (Haas, 2007). During this process the phagosome will be acidified and enriched in various hydrolases and antimicrobial proteins due to an interaction with the endosomal and secretory system either by fusion or “kiss-and-run” processes with these vesicles (Flannagan *et al.*, 2012; Fairn and Grinstein, 2012). Beside the key Rab GTPases Rab5 and Rab7 a network of Rab proteins, which overlaps and differs from endosome maturation, is involved in phagosome maturation. Many Rab GTPases (e.g. Rab1, Rab2, Rab10, Rab14) of the ER-Golgi, *intra*-Golgi or Golgi-endosome traffic seem to be important (Kinchen and Ravichandran, 2008). Taken all together an enormous set of at least 34 Rabs were identified that may play a role in phagosome maturation (Fairn and Grinstein, 2012; Gutierrez, 2013; Kinchen and Ravichandran, 2008; Stein *et al.*, 2012). However, the function of the majority of identified Rabs in this process has to be clarified (Gutierrez, 2013). Thereby proteomic analysis and studies with intracellular pathogens were most critical for their identification (Stein *et al.*, 2012). Especially the capability of intracellular pathogens to circumvent phagosomal maturation by vacuole lysis or creating pathogen-

specific membrane-bound compartments allows new insights into the process of phagocytosis and phagosome maturation (Meresse *et al.*, 1999b).

I.2 Lifestyles of intracellular pathogens

Cell entry is the first step in intracellular lifestyle. There are two ways to get inside the host cell. First, bacteria are passively taken up by phagocytic cells like macrophages, neutrophils and dendritic cells (e.g. *Legionella* spp., *Coxiella burnetii*, *Mycobacterium* spp., *Brucella* spp. and *Salmonella enterica*) or second, they actively invade non-phagocytic cells like intestinal epithelial cells by inducing their own uptake (e.g. *Shigella* spp., *Listeria* spp. and *Salmonella enterica*) (Alonso and Garcia-del Portillo, 2004; Cossart and Roy, 2010).

For the invasion processes two morphologically distinct mechanisms are described which differ in the kind of membrane rearrangements, called zipper or trigger mechanism (Alonso and Garcia-del Portillo, 2004; Cossart and Roy, 2010). In the first mechanism the pathogen is taking advantage of a normal cellular function for its entry. The process is initiated by an interaction of a bacterial surface protein and its host cell receptor which causes a signal cascade for the formation of an internalization vacuole with a tightly membrane-enclosed pathogen (Asrat *et al.*, 2014; Cossart and Roy, 2010). As an example *Listeria monocytogenes* is using this mechanism by producing two important invasion proteins called internalin InlA and InlB which promote adhesion and uptake by an actin-mediated process (Bonazzi *et al.*, 2008; Ireton *et al.*, 1999; reviewed in Bierne *et al.*, 2007). The second mechanism is triggered by effectors of type III secretion systems (T3SS) that are directly injected into the host cytosol. These effectors are able to manipulate host signalling cascades by the activation of small guanosine triphosphatases (GTPases) of the Rho subfamily like Rho, Rac1 and Cdc42 to form large membrane ruffles which engulf the bacterium in a process similar to macropinocytosis (Alonso and Garcia-del Portillo, 2004; Cossart and Roy, 2010). Good examples for this type of uptake are *Shigella flexneri* and *Salmonella enterica* serovar Typhimurium. They translocate effector proteins into host cells to alter the actin cytoskeleton or membrane composition (Friebel *et al.*, 2001; Hänisch *et al.*, 2011; High *et al.*, 1992; Niebuhr *et al.*, 2002; Yoshida *et al.*, 2002; Zhou *et al.*, 2001; reviewed in Patel and Galán, 2005). The process of uptake of *Salmonella* will be described in more details in Chapter I.3.2.1. The main differences between both mechanisms are in cytoskeletal contribution and the starting point. The triggered mechanism needs dramatic cytoskeletal rearrangements compared to the zipper mode and effector proteins activate the mechanism from inside the cell whereby the

starting point of the zipper mechanism comes from the extracellular side of the cell (Alonso and Garcia-del Portillo, 2004).

After internalization two ways of intracellular lifestyle have been evolved. To survive degradation by the endo-lysosomal pathway, the pathogen either escapes from the phagosome to divide in the cytosol, or it establishes a replicative niche inside their hosts enclosed in a membrane-bound vacuole, called pathogen-containing vacuole (PCV), which is interacting with various host organelles (Ham *et al.*, 2011). In the following paragraphs examples for both kinds of parasitic lifestyle will be explained and an overview of intracellular bacteria is shown in Figure I-3.

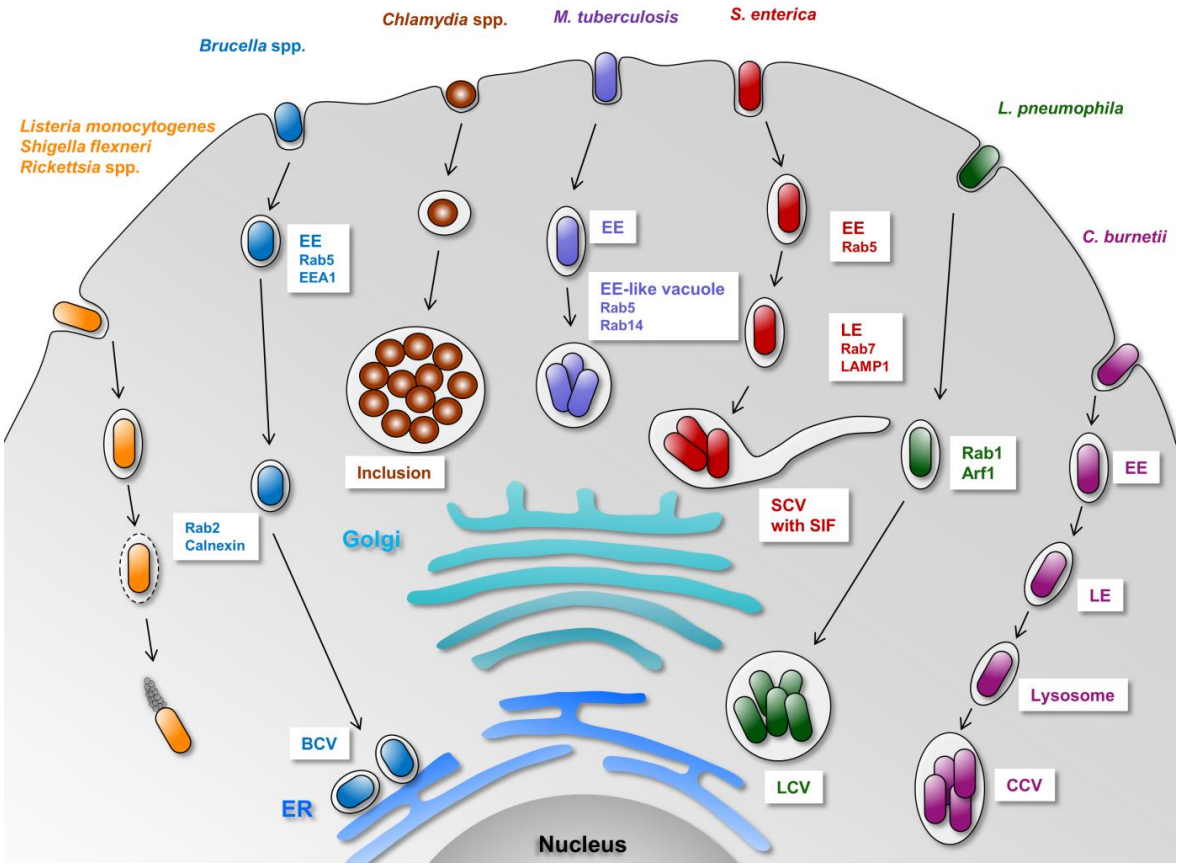

Figure I-3: Lifestyles of intracellular pathogens.
Intracellular pathogens create unique niches in host cells. *Burkholderia pseudomallei*, *Listeria monocytogenes*, *Shigella flexneri* and *Rickettsia* spp. are able to lyse the phagosome membrane and replicate in the cytosol. They are motile by an actin-driven process (actin-tail formation). For more details see chapter I.2.1. In contrast *Brucella* spp., *Chlamydia* spp., *Mycobacterium tuberculosis*, *S. enterica*, *Legionella pneumophila* and *Coxiella burnetii* replicate in vacuoles that are remodelled according to their requirements. Compartments of the endo-lysosomal/secretory system and host proteins involved in maturation of the bacteria-containing vacuoles are indicated in white boxes. The biogenesis is described in detail in chapter I.2.2. EE, early endosome; LE, late endosome; Lys, lysosome; BCV, *Brucella*-containing vacuole; CCV, *Coxiella*-containing vacuole; LCV, *Legionella*-containing vacuole; SCV, *Salmonella*-containing vacuole; SIT, *Salmonella*-induced tubules; modified after Alonso and Garcia-del Portillo(2004) and Cossart and Roy (2007).

I.2.1 Escape from the phagosome – cytosolic lifestyle

The intracellular lifecycle of cytosolic pathogens can be described generalized by three different steps (Ray *et al.*, 2009): The pathogen is enclosed in a primary vacuole after uptake and secretes effectors to enable a disruption of the vacuolar membrane. Replication starts in cytosol and, with the exception of *F. tularensis*, all cytosolic pathogens are motile through the polymerization of actin at one pole of the cell (Asrat *et al.*, 2014). If the bacterium is spread to a neighbouring cell it will be engulfed by a secondary double-membrane vacuole. Then the process starts again with secretion of proteins, lysis of the membrane and replication in the cytosol.
Following, extensively studied bacteria will be described in more detail as examples for intracellular cytosolic bacteria: *Listeria monocytogenes*, *Shigella flexneri*, *Burkholderia pseudomallei*, *Francisella tularensis* and *Rickettsia* spp.

I.2.1.1 Listeria monocytogenes

The Gram-positive bacterium *L. monocytogenes* has two lifestyles as saprophyte in soil or as an intracellular foodborne pathogen (Freitag *et al.*, 2009). In healthy individuals the pathogen causes self-limiting gastroenteritis, but in immunocompromised persons systemic infections are possible, which lead to meningitis or encephalitis, and pregnant women risk an infection of the foetus, which causes abortion (Cossart, 2011).
After invasion the vacuole is preserved from fusion to EE by a modulation of the activity of Rab5a (Alvarez-Dominguez *et al.*, 2008). Lysis of the vacuole is mainly mediated by the pore-forming toxin listeriolysin O (LLO) which causes transient changes in the pH and calcium concentration resulting in membrane disruption (Cossart, 2011). Additional two bacterial phospholipases, the phosphatidylinositol-specific phospholipases C (PI-PLC) and the broad-range phospholipase C (PC-PLC) are crucial for membrane rupture (Smith *et al.*, 1995). Inside the cytosol *Listeria* is motile through an actin-tail formation mediated by ActA which prevent autophagy and allows the pathogen to spread from one cell to another in a monolayer (Pizarro-Cerdá *et al.*, 2015).

I.2.1.2 Shigella flexneri

Shigellosis caused by *Shigella* spp. is usually a self-limiting acute intestinal infection which can range from mild diarrhoea to severe inflammatory bacillary dysentery, but it can be life-threatening for immunocompromised persons or inadequate medical care (Schroeder and Hilbi, 2008). *Shigella*, a member of the family *Enterobacteriaceae*, enters the intestine

via the faecal-oral route through contaminated food or water (Ogawa and Sasakawa, 2006). *S. flexneri* translocates effector protein through the Mxi-Spa T3SS. The effectors IpaB, IpaC, and IpaD are involved in the disruption of the vacuole membrane, supported by IpaH7.8 (Schroeder and Hilbi, 2008). Similar to *Listeria, Shigella* is motile by the IcsA-driven actin-polymerization on one pole of the bacterium which enables the bacterium to escape from the autophagy machinery by evading into surrounding cells (Jennison and Verma, 2004).

I.2.1.3 *Rickettsia* spp.

Rickettsia spp. are obligate intracellular α-proteobacteria. Typhus and spotted fever rickettsioses are transmitted by tick and flea bites or inhalation of their faeces (Lacz *et al.*, 2006). Phospholipase A_2 and the secreted phospholipase D seem to play a role in phagosome escape but the exact mechanism remains unknown (Hybiske and Stephens, 2008). *Rickettsia* spp. use a RickA-dependent actin-nucleation mechanism similar to that of other pathogens (Gouin *et al.*, 2004; Jeng *et al.*, 2004).

I.2.1.4 *Burkholderia pseudomallei*

The Gram-negative β-proteobacterium *B. pseudomallei* is a saprophyte in surface water and moist soil in tropical and subtropical regions and cause melioidosis, whereby the symptoms can range from localized abscess formation up to acute pneumonia and sepsis in patients with weakened immune system (Wiersinga *et al.*, 2006). Humans acquire it by inhalation, cutaneous infection or ingestion.
Two secretion systems are responsible for its pathogenesis. The T3SS3 is crucial for phagosome escape and the second secretion system T6SS1 is required for the formation of multinucleated giant cells (MNGC) by membrane fusion with bordering cells, a unique ability among intracellular bacteria (Stone *et al.*, 2014). Actin-based motility relies on the virulence factor BimA in a comparable mechanism to *Listeria* (Allwood *et al.*, 2011). The bacterial protein BopA protects cytosolic *B. pseudomallei* from autophagy (Stone *et al.*, 2014).

I.2.1.5 Francisella tularensis

Tularemia, caused by *F. tularensis*, is transmitted by arthropod bites, contact with infected animals, ingestion of contaminated food or water, or inhalation (Barel and Charbit, 2013).

Depending on the route of infection inflammation of the skin and lymph nodes, the eye, the intestine or pneumonia until sepsis are possible (Steiner *et al.*, 2014).

The γ-proteobacterium differs in its intracellular lifestyle from the pathogens described before. After uptake, the pathogen resides for one to four hours in a *Francisella*-containing phagosome (FCP), which undergoes phagolysosomal maturation with markers of the EE and LE, such as Rab5, EEA-1, CD63, LAMP1, LAMP2, and Rab7, but does not fuse with lysosomes (Celli and Zahrt, 2013). Then through an unknown process *F. tularensis* escapes from the FCP with the involvement of genes from the *Francisella*-Pathogenicity Island (FPI) and starts to proliferate, which leads to cell death, release of bacteria and reinfection (Jones *et al.*, 2014). In murine bone-marrow-derived macrophages (BMMs) it has been shown that a fraction of cytosolic pathogens is enclosed in large vacuoles with autophagic elements (*Francisella*-containing vacuoles, FCV) at late time points, but their importance for the infection process stays undefined because no FCVs were found in human macrophages (Akimana *et al.*, 2010; Edwards *et al.*, 2010; Checroun *et al.*, 2006; reviewed in Celli and Zahrt, 2013).

I.2.2 Modification of the phagosome – vacuolar lifestyle

In contrast to cytosolic pathogens (see Chapter I.2.1), bacteria which are adapted to a life inside a vacuole or PCV, are faced with the challenges of the phagolysosomal pathway. These pathogens evade the protection mechanisms of the cell to degrade bacteria by acidification and/or fusion with lysosomes. Bacteria such as *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, *Legionella pneumophila*, and *Salmonella enterica* serovar Typhimurium circumvent the maturation process by modifying the PCV in such a way that acidification is blocked. Thereby *Brucella* spp. and *Coxiella burnetii* are able to tolerate an acidic milieu in the vacuole (Asrat *et al.*, 2014).

I.2.2.1 Brucella spp.

Brucella spp. are α-proteobacteria, and in particular *B. abortus*, *B. melitensis*, and *B. suis*, are causative agents of brucellosis, a disease with diffuse symptoms such as fever and malaise which can affect internal organs like liver, spleen, the skeletal system or the male reproductive tract (Atluri *et al.*, 2011). The pathogen is transmitted by consuming unpasteurized dairy products or inhalation of contaminated aerosols or dust (Gorvel, 2008).

The *Brucella*-containing vacuoles (BCVs) undergo the phagosomal maturation process to lysosomes with acidification and are transiently positive for the typical markers of EE

(Rab5, EEA1, TfR), and LE (Rab7, LAMP1), but missing lysosomal markers such as cathepsin D (Gorvel, 2008). *Brucella* cyclic β-1,2-glucan may affect phagosomal maturation by manipulation of the PCV (Celli, 2006). Expression of the VirB type IV secretion system proteins depend on acid pH (Atluri *et al.*, 2011). This secretion system is essential for the biogenesis of the *Brucella* ER-derived vacuole, in which replication of *Brucella* takes place. From four to eight hours post infection (p.i.) a loss of LAMP1 on the BCV is observed accompanied by an accumulation of ER markers like calnexin, calreticulin and Sec61β (Celli, 2006). The pathogen manipulates the secretory pathway from the ERESs (ER-exit sites) and interacts with the Sar1-COPII complex to mediate BCV-ER fusion events (Gorvel, 2008). Additionally, ER-vesicles of the retrograde Golgi to ER traffic with Rab2 and GAPDH contribute to BCV biogenesis, whereby Rab2 and the *Brucella* effector RicA (Rab2 interacting conserver protein A) are essential for BCV establishment (Atluri *et al.*, 2011).

I.2.2.2 Coxiella burnetii

Coxiella burnetii is the causative agent of Q-fever, an acute, debilitating self-limiting influenza-like disease (van Schaik *et al.*, 2013; Omsland and Heinzen, 2011). The γ-proteobacterium is mainly transmitted via inhalation of contaminated aerosols.

After entry into the cell the *Coxiella*-containing vacuole (CCV), also called parasitophorous vacuole (PV), matures via the phagolysosomal pathway with EE and LE markers and, important for CCV-biogenesis, fusions with lysosomes are not avoided (van Schaik *et al.*, 2013). Lysosomal membrane markers as well as enzymes like cathepsin D, and lysosomal acid phosphatase are detectable. The luminal pH of the CCV is ~4.8.

The obligate intracellular pathogen has a biphasic developmental cycle. The non-replicating small cell variant is metabolic inactive, with condensed chromatin and resistant to harsh environmental conditions (Omsland and Heinzen, 2011). During acidification of the CCV the small cell variant is changed to the replicating large cell variant under contribution of the type IV Dot/Icm secretion system (Beare *et al.*, 2011; Carey *et al.*, 2011). In a timescale from eight hours to two days the CCV is enlarged in size by fusion with smaller CCVs or endosomes, lysosomes or autophagosomes, so that it almost completely fills the cytoplasm of the host (van Schaik *et al.*, 2013). The next four days *Coxiella* is replicating until it forms again the small cell variant (Omsland and Heinzen, 2011). The actin cytoskeleton, Rho GTPase, protein kinase C/A and myosin light chain kinase are implicated in the establishment and maintenance of the large CCV (Aguilera *et al.*, 2009; Hussain *et al.*, 2010; reviewed in van Schaik *et al.*, 2013). The vacuole is

interacting with the early secretory pathway and accumulates Rab1b (Campoy *et al.*, 2011).

I.2.2.3 Mycobacterium tuberculosis

Tuberculosis is caused by *M. tuberculosis*, affects the lungs and is transmitted from person to person through aerosols (World Health Organization, 2015). This acid-fast member of the Actinomycete family is a facultative intracellular pathogen of human mononuclear phagocytes (Clemens and Horwitz, 1995; Parish and Stoker, 1999). This bacterium blocks the biogenesis of the phagolysosome in an early stage by preventing the exchange of Rab5 by Rab7, the acidification through the exclusion of vATPase, and finally the fusion with lysosomes (Via *et al.*, 1997; Sturgill-Koszycki *et al.*, 1994; Deretic and Fratti, 1999). The exclusion of the EE marker EEA1 and as a consequence thereof the reduced PtdIns(3)*P* level as well as the influence of ManLAM (mannose lipoarabinomannan) on Ca^{2+} fluxes contribute to the arrest of the phagosomal maturation (Vergne *et al.*, 2004; Vergne *et al.*, 2005). Furthermore, the ESX-1 secretion system has additional importance for the stop in phagosome maturation (MacGurn and Cox, 2007).

I.2.2.4 Chlamydia spp.

Chlamydia causes a variety of diseases such as blinding trachoma or urogenital tract infections (*C. trachomatis*) or pneumonia (*C. pneumonia*) (Dautry-Varsat *et al.*, 2005). The Gram-negative obligate intracellular pathogen displays a unique biphasic developmental cycle. The elementary body (EB) is the small, metabolically inactive and infectious form which differentiates after entering epithelial cells within a few hours in lager metabolic active reticulate body (RB) (Dautry-Varsat *et al.*, 2005). Replication of RBs takes place in a parasitophorous vacuole called inclusion which separates shortly after internalization from endocytic pathways and is moved by a dynein-dependent manner to the MTOC in a *peri*-Golgi region (Bastidas *et al.*, 2013). After two to three days the pathogens differentiated back to EBs and are released in the extracellular medium by cell lysis and/or extrusions (Dautry-Varsat *et al.*, 2005). The inclusion interacts with the trafficking system of its host cell including Rab GTPases of recycling endosomes (Rab4, Rab11, and Rab14) and of ER-Golgi trafficking (Rab1, Rab6, and Rab10). These Rab proteins are detectable at the inclusion membrane whereby Rab4 and Rab11 are implicated in iron acquisition and Rab6 and Rab11 in sphingomyelin traffic and seems to be involved in the regulation of the Golgi-fragmentation into ministacks (Bastidas *et al.*, 2013). Proteins of the

PdtIns(4)*P* metabolism are important for infection (Moorhead *et al.*, 2010). Manipulation of the vesicle trafficking to the inclusion is maybe additionally mediated by proteins of *Chlamydia* with SNARE-like motifs, e.g. IncA, (Delevoye *et al.*, 2008; Paumet *et al.*, 2009).

I.2.2.5 Legionella pneumophila

Legionella pneumophila is the causative agent of a severe pneumonia termed Legionnaires' disease (Isberg *et al.*, 2009). This Gram-negative bacterium is ubiquitous in freshwater environments often as parasites of amoebae; however, in human-made water systems it is generally located in biofilms (Haneburger and Hilbi, 2014; Newton *et al.*, 2010).

Transmission normally occurs by inhalation of contaminated water droplets as an accidental infection of alveolar macrophages with point-of-no-return for *Legionella* (Newton *et al.*, 2010). Rapidly after entry into the cell *L. pneumophila* hijacks the ER-to-Golgi traffic to the *Legionella*-containing vacuole (LCV) and creates a membrane with ER characteristics (Xu and Luo, 2013). The bacterial Dot/Icm type 4 secretion system (T4SS) is essential for avoiding the endosomal maturation and establishment of the LCV (Sherwood and Roy, 2013). Effectors of *Legionella* interact with host phosphoinositide (PI) lipids, especially PtdIns(3)*P* or PtdIns(4)*P*, and modify the PI composition of the LCV (Haneburger and Hilbi, 2014). As an example, SidC binds to PtdIns(4)*P* and interacts with ER-derived vesicles (Ragaz *et al.*, 2008). Rab1, a Rab GTPase responsible for ER to Golgi trafficking, is recruited to the LCV and is activated by the *Legionella* effector SidM (DrrA) which associates with PtdIns(4)*P* and promotes nucleotide exchange of Rab1 (Isberg *et al.*, 2009).

Further modifications of the host trafficking system are involved in LCV establishment. The SNARE protein Sec22b, located in ER-derived vesicles, or Arf1, which contributes to the formation of COPI vesicles from the ER is associated with the LCV (Newton *et al.*, 2010). The translocated *Legionella* effector GEF RalF serves a GEF for Arf1 (Nagai *et al.*, 2002).

I.3 *Salmonella* as model organisms

Salmonella enterica is a rod-shaped, motile, member of the Gram-negative family *Enterobacteriacaea* with more than 2,500 serovars that are differentiated according to their flagellar and lipopolysaccharide (LPS) antigens (LaRock *et al.*, 2015). It is a facultative intracellular pathogen which is able to survive in a bacterial-containing vacuole (Malik-Kale *et al.*, 2011). The majority of our knowledge about *Salmonella*-host interactions is based on *S. enterica* subsp. *enterica* serovar Typhimurium (abbreviated as *Salmonella* in following text), that is used as model organism for typhoid fever and gastroenteritis in the mice model (Kuhle and Hensel, 2004; Valdez *et al.*, 2009).

I.3.1 Pathogenesis

According to their pathogenesis *Salmonella* strains are distinguished in typhoidal (*S. enterica* subsp. *enterica* serovars Typhi and Paratyphi) and non-typhoidal (*S. enterica* subsp. *enterica* serovars Enteritidis and Typhimurium) strains (Ramos-Morales, 2012). The typhoidal strains are human-restricted pathogens and cause the systemic disease enteric (typhoid) fever. In contrast to this the non-typhoidal strains infect a wider range of hosts (cattle, swine, poultry and humans) and cause a mild gastroenteritis (Salmonellosis), however, dehydration in very young or elderly patients, or systemic infections in immunosuppressed individuals can be life-threatening (LaRock *et al.*, 2015; Valdez *et al.*, 2009; World Health Organization, 2013). Salmonellosis is one of the most common foodborne diseases with tens of millions of cases in humans yearly (World Health Organization, 2013). *S. enterica* is a foodborne pathogen, generally transmitted by consumption of contaminated food or water, also person-to-person infections are possible (Ramos-Morales, 2012; World Health Organization, 2013).

After oral ingestion *Salmonella* has to pass the acidic milieu of the stomach and enters the small intestine where it circumvents the host immune response to get access to the epithelium. It is able to invade non-phagocytic epithelial cells, is phagocytosed by dendritic cells or taken up by microfold (M) cells (Haraga *et al.*, 2008; Jantsch *et al.*, 2011). M cells of Peyer's patches are the preferentially cell type for *Salmonella*'s entry and translocate it through the epithelium to lymphoid T and B cells (Haraga *et al.*, 2008). Depending on the *Salmonella* serovar, systemic illness is spread by infected macrophages to the reticuloendothelial system. Non-typhoidal strains induce an early local inflammation resulting in the infiltration of PMNs (polymorphonuclear leukocytes) into the intestinal lumen and diarrhoea (Valdez *et al.*, 2009).

I.3.2 Cellular aspects of *Salmonella*'s pathogenesis

I.3.2.1 Getting into host cells – invasion and phagocytic uptake

Salmonella is able to enter many types of phagocytic and non-phagocytic cells (enterocytes, macrophages, dendritic cells, neutrophils, and M cells) (Malik-Kale *et al.*, 2011). Actin remodelling, formation of membrane ruffling, and lamellipodial extensions at the contact site, which mediate the bacterial entry through membrane-bound vacuoles, are in common for phagocytosis or *Salmonella*-driven invasion (Patel *et al.*, 2005). Invasion of non-phagocytic cells is dependent of the type III secretion system 1 (T3SS1) encoded on *Salmonella* Pathogenicity Island 1 (SPI1) (Ly and Casanova, 2007; Patel and Galán, 2005; Zhou and Galán, 2001). The needle complex of the *Salmonella* T3SS consists of a basal body and a filamentous needle. The basal body is composed of two pairs of rings that span the inner and the outer membrane, linked by an rod in the periplasmic zone (Cornelis, 2006). A functional T3SS additionally needs a cytosolic regulatory element, connected with the basal body, an inner rod as base for needle and a translocon as pore-forming complex in the host membrane at the tip of the needle (Moest and Méresse, 2013). Injection of a subset of SPI1 effectors (SipA, SipC, SopB, SopE, and SopE2) via the T3SS1 mediates the *Salmonella*-triggered invasion through rapid and extensive membrane ruffles on the surface of the host followed by formation of phagosomes or macropinosomes (Malik-Kale *et al.*, 2011). These effectors direct a massive localized rearrangement of actin as well as host membranes and manipulate signalling pathways (McGhie *et al.*, 2009). SipC is involved in actin nucleation and bundling (Chang *et al.*, 2005; Chang *et al.*, 2007; Hayward and Koronakis, 1999; Myeni and Zhou, 2010). SipA binds and stabilizes actin, thereby promotes SipC functions (McGhie *et al.*, 2001; McGhie *et al.*, 2004; Popp *et al.*, 2008; reviewed in Hayward and Koronakis, 2002). The effector SopE mimics host guanine exchange factors and manipulates the actin cytoskeleton by activation of the Rho GTPase Rac-1 and Cdc42 via the Arp2/3 complex (Hardt *et al.*, 1998). SopE2 has similar functions like SopE but activates only Cdc42 (Friebel *et al.*, 2001). SopB (also known as SigD) is involved in a wide range of different processes during and after invasion. As an inositol phosphatase it influences the lipid compositions of the SCV and the host membrane. It remodels the actin cytoskeleton by interaction with Cdc42 and the SH3-containing guanine nucleotide exchange factor (SGEF), which is an exchange factor for RhoG. Furthermore it has a strong impact on bacteria-induced signalling (Bakowski *et al.*, 2010; Braun *et al.*, 2010; Burkinshaw *et al.*, 2012; Knodler *et al.*, 2009; Mallo *et al.*, 2008; Rodríguez-Escudero *et al.*, 2011; Rogers *et al.*, 2008).

SPI1 effectors are involved in intestinal inflammation by stimulating the production of the pro-inflammatory cytokine interleukin-8 (IL-8), which is mediated by mitogen-activated protein kinase (MAPK) and NF-κB pathways (Hapfelmeier *et al.*, 2004; Zhang *et al.*, 2002; reviewed in LaRock *et al.*, 2015). SptP, as antagonist of SopE, restores the actin cytoskeleton and switches off the MAPK signalling to return the normal state of the host cell (Fu and Galán, 1999; Kubori and Galán, 2003; reviewed in Haraga *et al.*, 2008).

I.3.2.2 Biogenesis of *Salmonella*-containing vacuole (SCV)

Directly after internalization *Salmonella* modifies its compartment to avoid the phagosomal maturation process. The SCV continuously matures through interplay with the host endocytic system into a unique compartment with special properties of LEs, including acidified lumen, lysosomal membrane glycoproteins like LAMP1, but no lysosomal hydrolases (Steele-Mortimer *et al.*, 1999; reviewed in Figueira and Holden, 2012). In the early phase of SCV biogenesis EE markers (such as Rab5, EEA1, TfR) are present, which are subsequently replaced by LE/Lys markers (e.g. Rab7, LAMP1, vATPase) (Smith *et al.*, 2005; Steele-Mortimer *et al.*, 1999; reviewed in Brumell and Grinstein, 2004; LaRock *et al.*, 2015). During the maturation process the SCV acidifies and moves from a periphery to a juxtanuclear positon (1-2 h p.i.), where replication and formation of tubular structures start three to six hours p.i. (Abrahams *et al.*, 2006; Garcia-del Portillo *et al.*, 1993; Drecktrah *et al.*, 2007; Rathman *et al.*, 1996; reviewed in Ramsden *et al.*, 2007b; Schroeder *et al.*, 2011). T3SS1 effectors are important for the invasion and early steps of the SCV biogenesis. The expression of T3SS2 effectors is induced after invasion and they control furthers stages of SCV maturation (Malik-Kale *et al.*, 2011). The SPI1 effector SopB modifies the early SCV membrane by removal of PtdIns(4,5)P_2 and indirectly controls an increase of PtdIns(3)*P* by recruitment of Rab5 (Bakowski *et al.*, 2010; Hernandez *et al.*, 2004; Mallo *et al.*, 2008).

Two sorting nexins SNX1 and SNX3 contribute to early SCV biogenesis (Braun *et al.*, 2010; Bujny *et al.*, 2008). SNX1 is localized on the SCV (15 min post invasion) and forms highly dynamic tubular structures, called spacious vacuole-associated tubules (SVATs) (Bujny *et al.*, 2008). This process is associated with a reduction in vacuole size and elimination of the cation-independent mannose-6-phosphate receptor (CI M6PR) (Garcia-del Portillo and Finlay, 1995). The exclusion of CI M6PR from the SCV was often interpreted as hint that fusion of SCVs and lysosomes does not occur, but fast removing of CI M6PR by SNX1 is more likely (Malik-Kale *et al.*, 2011), so that the SCVs is able to

fuse with lysosomes considering that lysosomal content markers are verifiable in the SCV (Drecktrah *et al.*, 2007; Oh *et al.*, 1996).

The second sorting nexin SNX3 is transiently located on the SCV (10 min) and subsequently (30-60 min) associated in tubular structures named SNX tubules which are important for the recruitment of Rab7 and LAMP1 to the SCV (Braun *et al.*, 2010). During biogenesis the intermediate SCV migrates to a juxtanuclear position close to the MTOC (Ramsden *et al.*, 2007b). The Rab-interacting lysosomal protein (RILP), an interaction partner of Rab7, mediates the movement along microtubules depending on the dynein-dynactin motor complex (Harrison *et al.*, 2004). In summary, SPI1 (SipA, SopE) and SPI2 (SseF, SseG, and SifA) effectors participate in this process of migration and stabilization of the SCV in the perinuclear position (Abrahams *et al.*, 2006; Brawn *et al.*, 2007; Deiwick *et al.*, 2006; Salcedo and Holden, 2003; Wasylnka *et al.*, 2008).

Additionally, a meshwork of F-actin, called vacuole-associated actin polymerizations (VAP), surrounds the SCV and is important for bacterial growth (Meresse *et al.*, 2001). The SPI2 effector SteC regulates the actin rearrangement through its kinase activity (Odendall *et al.*, 2012; Poh *et al.*, 2008). Two further SPI2 effectors SspH2 and SseI co-localize with polymerizing actin cytoskeleton but SpvB is sufficient to inhibit the formation of the VAP (Miao *et al.*, 2003).

I.3.2.3 Establishment of *Salmonella*'s replicative niche

In the late phase of infection (> 4 hours) *Salmonella* has established its replicative niche in the host cell with an active T3SS2 and starts to replicate. This unique niche is marked by an extensive network of *Salmonella*-induced tubules (SITs) (reviewed in Liss and Hensel, 2015; Schroeder *et al.*, 2011). In addition to the early SITs (SNX tubules and SVAT) three types of late SITs are distinguishable: *Salmonella*-induced filaments (SIF), *Salmonella*-induced SCAMP3 tubules (SISTs) and LAMP1-negative tubules (LNTs) (Schroeder *et al.*, 2011). SIFs are marked by presence of SPI2 effectors, LAMPs and other late endocytic markers, and SCAMP3 (Drecktrah *et al.*, 2007; Garcia-del Portillo *et al.*, 1993; Mota *et al.*, 2009; Rajashekar *et al.*, 2008). The SIF membrane is composed of similar components like the SCV membrane: lysosomal glycoproteins (lgps, such as LAMP1), vATPase, Rab7, lysobiphosphatidic acid, cholesterol and low levels of cathepsin D (Brumell *et al.*, 2001b; Drecktrah *et al.*, 2008; Garcia-del Portillo *et al.*, 1993; reviewed in Schroeder *et al.*, 2011). They are highly dynamic tubular structures arising from the SCV from four to six hours after invasion forming a more rigid complex network throughout the cell after eight hours (Rajashekar *et al.*, 2008; Drecktrah *et al.*, 2007). The SCV and the SIF membrane stay interconnected and enable continuous exchange (Krieger *et al.*, 2014). SIFs form double

membrane tubules and show cytosolic luminal content with cytoskeletal filaments (Krieger *et al.*, 2014). Unlike SIFs SISTs do not contain LE markers such as LAMPs and vATPase, but effectors and SCAMP3, a protein of *trans*-Golgi, endocytic and exocytic membranes. They are in close association with SIFs (Mota *et al.*, 2009) and indicate interactions of the SCV and its tubules with the secretory pathway (Kuhle *et al.*, 2006; Salcedo and Holden, 2003). LNTs are characterised by the presence of effectors and vATPases. They were identified in HeLa cells infected with a *sifA sopD2* deletion mutant (Schroeder *et al.*, 2010).

The physiological functions of SIFs, SIST and LNTs are not clear to date. Many different hypotheses exist: i) promotion of SCV membrane stability, ii) recruitment of membrane material, iii) dilution of lysosomal enzymes, iv) to nutrition of *Salmonella* within the SCV (Drecktrah *et al.*, 2008; Rajashekar *et al.*, 2008; reviewed in Haraga *et al.*, 2008; Liss and Hensel, 2015; Schroeder *et al.*, 2011). In the following all membranes which are associated with *Salmonella* including SCV and SIT membranes are referred to as *Salmonella*-modified membranes (SMMs).

The exact mechanism of SIF formation is not clear; however, the importance of many effectors in this process has been shown. SifA is essential for the maintenance of SCV integrity and SIF formation (Ruiz-Albert *et al.*, 2002; Brumell *et al.*, 2002b). The SPI1 effector SipA mediates the localisation of SifA to the SCV (Brawn *et al.*, 2007). SifA interacts with the host kinesin-binding protein SKIP (SifA and kinesin-interacting protein) and is involved in SIF formation in interplay with kinesin-1, a plus-end directed microtubule motor protein (Boucrot *et al.*, 2005; Dumont *et al.*, 2010). Recruitment of kinesin to the SCV is mediated by PipB2 which contributes to centrifugal extension of tubules (Boucrot *et al.*, 2005; Henry *et al.*, 2006). Deletion mutants of T3SS2 effector *pipB2* show shorter SIFs (Knodler and Steele-Mortimer, 2005). Furthermore, a complex formed of PLEKHM1 (Pleckstrin homology domain-containing protein family member 1), Rab7, and the HOPS tethering complex is used by an interaction with SifA for hijacking lysosomal membranes to the SCV (McEwan *et al.*, 2015).

The SPI2 effectors SopD2, SteA, and SseJ are also important for SIF formation. The number of SIFs induced by *sopD2* or *steA* deletion mutants is reduced (Domingues *et al.*, 2014; Jiang *et al.*, 2004). SopD2 is located in SITs and late endosomes (Brumell *et al.*, 2003; Jiang *et al.*, 2004). Cells infected with a *steA* deletion mutant strain display increased clustering of SCVs, and abnormal vacuoles with more than one bacterium (Domingues *et al.*, 2014).

SseJ is equipped with a phospholipase A1, deacylase and glycerophospho-lipid:cholesterol acyltransferase (GCAT) activity (Christen *et al.*, 2009; LaRock *et al.*, 2012; Lossi *et al.*, 2008; Ohlson *et al.*, 2005; reviewed in Kolodziejek and Miller, 2015).

The signalling state of RhoA controls the enzymatic activity of SseJ. Binding of RhoA, especially GTP-bound RhoA, enhances the activity of SseJ (Christen *et al.*, 2009). How the modification of lipids by SseJ, e.g. enrichment of cholesterol esters, directly or indirectly influences the protein recruitment is not clear (Kolodziejek and Miller, 2015). However, it is known that SseJ interacts with GTP-bound RhoA and SifA, whereas SifA preferentially binds GDP-bound RhoA (Christen *et al.*, 2009; Diacovich *et al.*, 2009; Ohlson *et al.*, 2008). Therefore SifA, SKIP, SseJ, and RhoA are supposed to promote SIF formation by membrane tubulation via movement along microtubules (Ohlson *et al.*, 2008; Ruiz-Albert *et al.*, 2002; reviewed in Schroeder *et al.*, 2011).

Additionally, two further effectors SseF and SseG play a role in SIF formation (Guy *et al.*, 2000; Kuhle and Hensel, 2002). SIFs of host cells infected with mutant strains defective in *sseF* or *sseG* are altered in their morphology, e.g. thinner, and display the phenotype of SIF called “pseudo-SIF” in cells fixed with *para*-formaldehyde (Krieger *et al.*, 2014; Kuhle and Hensel, 2002; Rajashekar *et al.*, 2014). Ultrastructural analysis of these SIFs reveals single membrane tubules with an electron-dense content and presumes a function of SseF and SseG in double membrane formation of SIFs (Krieger *et al.*, 2014). The intracellular replication rate is moderately reduced in deletion mutants of *sseF* (Abrahams *et al.*, 2006; Hensel *et al.*, 1998; Kuhle and Hensel, 2002; Müller *et al.*, 2012). SseF requires SscB as chaperone to facilitate secretion and functionality inside the host cell (Dai and Zhou, 2004). SseF and SseG contribute to perinuclear positioning of SCVs whereby SseF and SseG or the SseF-SseG complex are involved in association with the microtubule cytoskeleton and transport via the motor protein dynein (Abrahams *et al.*, 2006; Deiwick *et al.*, 2006; Kuhle *et al.*, 2004). Partially redundant functions of both proteins are supposed because of their similar phenotypes in SCV positioning and SIF formation, and their functional translocation as fusion proteins (Abrahams *et al.*, 2006; Deiwick *et al.*, 2006; Kuhle and Hensel, 2002; Müller *et al.*, 2012). SseF has a chaperone binding site (amino acids 12-49) and two hydrophobic regions. Transmembrane (TM) domain 1 (aa 63-110) plays a role in secretion, translocation and targeting to SCV and SIFs. The second hydrophobic region TM2 (aa 128-212) mediates the effector functions of SCV positioning and dynein recruitment (Abrahams *et al.*, 2006). A short hydrophobic motif (AIGAVL, aa 200-205) inside TM2 is functionally essential for SseF (Müller *et al.*, 2012). Furthermore this effector is an integral membrane protein after translocation and its C-terminus is exposed to the host cell cytoplasm assumed as possible interaction region with host proteins or effectors (Abrahams *et al.*, 2006; Müller *et al.*, 2012). Additionally SseF tolerates C-terminal fusions of various tags and *Salmonella* vaccine carriers utilise it for the delivery of recombinant antigens (Husseiny *et al.*, 2007).

To get an overview about the complex interplay between *Salmonella* and its host to establish the replicative niche, known *Salmonella* effectors and host proteins, which are involved in biogenesis of the SCV and SITs, are summarized in Figure I-4.

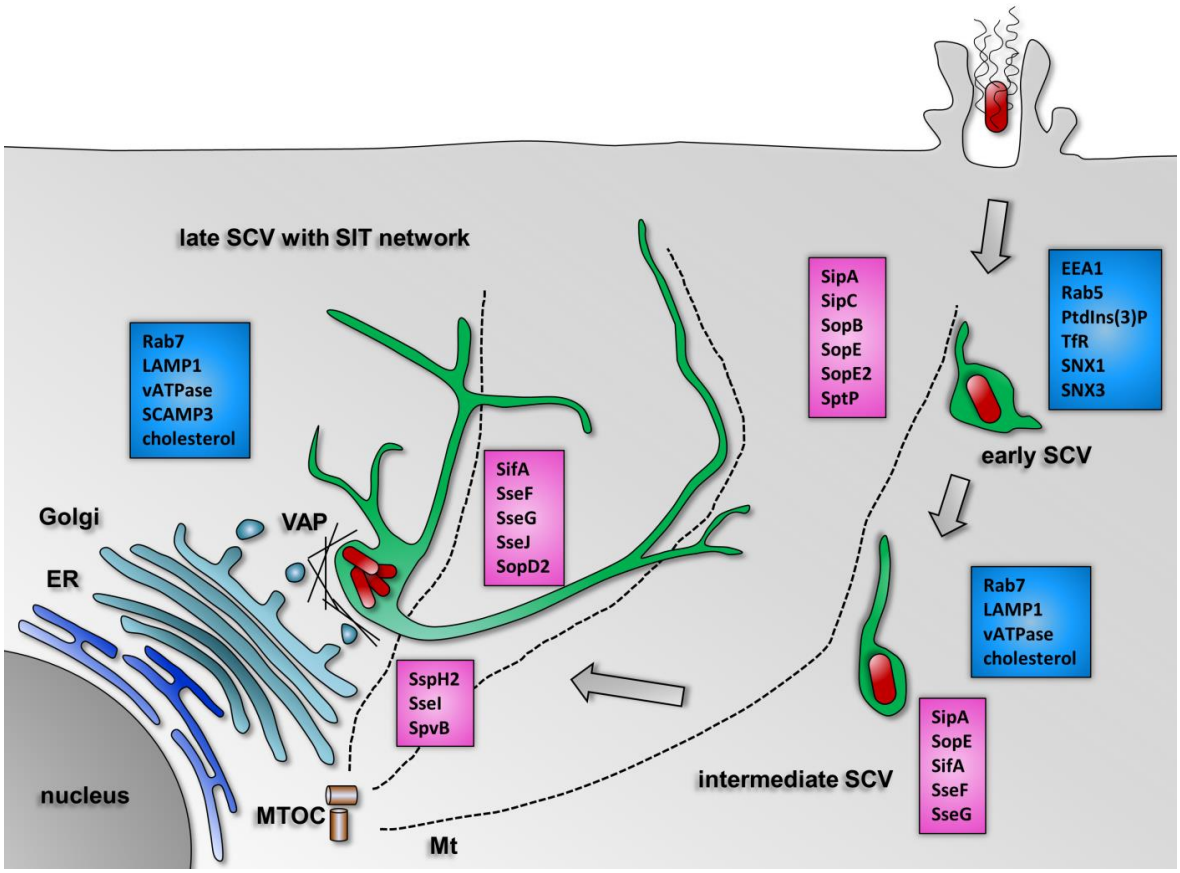

Figure I-4: Model of the *Salmonella*-containing vacuole biogenesis.
After uptake, *Salmonella* modifies its vacuole to escape phagocytic degradation by the host through the action of *Salmonella* effectors. The early SCV (0-60 min p.i.) has characteristics of EEs. It is spacious with a dynamic tubular network. The intermediate SCV (1-4 h p.i.) migrates to a juxtanuclear position and recruits proteins of LEs and Lys. At late time points (<4 h p.i.) *Salmonella* form tubular structures and start to replicate. *Salmonella* effectors (pink boxes) and host proteins/lipids (blue boxes) participate in maturation steps of the SCV. SCV, *Salmonella*-containing vacuole; *Salmonella*-induced tubules; MTOC, microtubule organization centre; Mt, microtubule; ER, endoplasmic reticulum; VAP, vacuole-associated actin polymerizations.

However, a subpopulation of *Salmonella* escapes from their membrane-bound compartment in epithelial cells and replicate in the cytosol, so that *Salmonella* seems to have a bimodal lifestyle (Malik-Kale *et al.*, 2012). In around 10 to 20% of infected epithelial cells hyper-replicating (defined as ≥100 bacteria/cell) *Salmonella* are detectable (Knodler *et al.*, 2010; Knodler *et al.*, 2014; Malik-Kale *et al.*, 2012). Escape of cytosolic bacteria into the extracellular environment is discussed as an additional mechanism for dissemination (Knodler, 2015).

I.4 Proteomics of intracellular pathogens

Development of new vaccines or antibiotics is depending on a detailed knowledge of the host-pathogen interplay and proteomics is one important element for the global understanding of this interaction (Bumann, 2010; Hartlova *et al.*, 2011).

The proteome is the complete set of proteins expressed by a genome, cell, tissue or organism at defined conditions. The discipline that investigates the proteome is called proteomics (Wasinger *et al.*, 1995; Wilkins, 1997). Blackstock and Weir (1999) applicably define proteomics as “the study of protein properties (expression level, post-translational modification, interactions etc.) on a large scale to obtain a global, integrated view of disease processes, cellular processes and networks at the protein level”.

The core technique for large-scale protein analysis is high-throughput mass spectrometry (MS) (Zhang *et al.*, 2014). MS is an analytical technique to determine the mass of atoms or molecules (de Hoffmann, 2000). A mass spectrometer consists of an ion source (to produce ions in the gas phase), a mass analyser (to separate the ions according to their mass to charge ratio (*m/z*)), a detector (to count ions for every *m/z* ratio) and a computer (to convert data from the analyser (*m/z*) and the detector (intensity) to a mass spectrum) (de Hoffmann, 2000). Three different methods are available for mass separation: separation on the basis of time-of-flight (TOF), by quadrupole electric fields generated by metal rods (quadrupole) or by selective ejection of ions from a three-dimensional trapping field (ion trap or Fourier transform ion cyclotron) (Mann *et al.*, 2001). Upstream a device is necessary for introducing the sample directly or by separation (gas chromatography (GC), liquid chromatography (LC) or capillary electrophoreses (CE)) (de Hoffmann, 2000). Addition of several analysers allows MS/MS or MS^n experiments (de Hoffmann, 2000).

A difficulty in proteomics with high throughput on a large scale is to separate analysts with identical *m/z.* To detect low-abundance species they have to be separated from higher abundance species to impede their dominance in the spectrum (Zhang *et al.*, 2014). The traditional method is to separate proteins by two dimensional polyacrylamide gel electrophoresis (2D-PAGE) but this is time consuming, labour intensive and problematic for membrane proteins or proteins with extreme pI or molecular weight (Gygi *et al.*, 2000; reviewed in Monteoliva and Albar, 2004; Otto *et al.*, 2014). In contrast, liquid chromatography (LC) facilitates the continuous separation of complex protein/peptide mixtures for a higher throughput (Zhang *et al.*, 2014).

Two methods for ionization of molecules are developed: electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) (Fenn *et al.*, 1989; Mann *et al.*, 2001). MALDI is used for the gel-based process whereas ESI is typically coupled with LC and a tandem mass spectrometer (LC-MS/MS) to perform high-throughput proteomics

(Aebersold and Mann, 2003; Mann *et al.*, 2001). Additionally, two approaches for protein identification exist in proteomics: i) the top-down approach with intact proteins, and ii) the more general used bottom-up approach on peptide level with proteins enzymatically digested to peptides (Zhang *et al.*, 2014).

Shotgun proteomics to gain a global protein profile of a complex mixture normally is gel-free and the peptides are separated by high performance LC (HPLC), commonly reversed-phase liquid chromatographic (RPLC) is used (Link *et al.*, 1999; McCormack *et al.*, 1997; Yates *et al.*, 1997; reviewed in Zhang *et al.*, 2013). Additionally, MS allows the characterization of the post-translation modifications (PTMs) and protein quantifications. The label-free quantitative mass spectrometry is based on intensity-based quantitation (measurement of the extracted peptide MS peak intensities) on the one hand or spectral counting (determination of the total number of MS/MS acquired for the same peptide) on the other hand (Zhang *et al.*, 2014). The other possibility for quantification is stable-isotopic-labelling. The metabolic labelling approach uses 'stable-isotope labelling with amino acids in cell culture' (SILAC) or chemically modifications by isotopic-labelled tags like post-digestion isotope-coded affinity tag (ICAT), isobaric tags (iTRAQ) or tandem mass tags (TMT) (reviewed in Hoedt *et al.*, 2014; Ong *et al.*, 2002; Zhang *et al.*, 2014).

Investigators have to be aware of the limitations of proteomics, especially for work on intracellular bacteria. Biological samples are complex and can exceed the analytical sensitivity of MS instruments, especially in the case of analysing the bacterial proteome in background of host proteins (reviewed in Hartlova *et al.*, 2011; Schmidt and Völker, 2011). High abundance of host proteins obscures the identification of bacterial proteins because of the low ratio of identified bacterial peptides to overwhelming number of host peptides (Li *et al.*, 2010). One possibility to overcome this problem is the analysis of bacterial proteome of *in vitro* cultures grown under *in vivo*-mimicking conditions and their comparison with cultures grown under standard conditions (Cash, 2011). For a more complete view of the host-pathogen interaction infection of cells and tissues is absolutely essential. Methods for separation by subcellular fractionation or specific enrichment of the desired compartment have to be established (Hartlova *et al.*, 2011). Subcellular fractionation by density gradient centrifugation is a standard method used for a variety of different intracellular pathogens to enrich the intracellular bacterium with or without its compartment (e.g. *C. burnetii*, *M. tuberculosis*, *S. enterica* serovar Typhimurium, *L. pneumonia* (Coleman *et al.*, 2007; Lamontagne *et al.*, 2009; Rao *et al.*, 2009; Shevchuk *et al.*, 2009; Shi *et al.*, 2006). Another approach to eliminate possible contamination is a more selective purification by immunoprecipitation with magnetic beads, shown e.g. for *F. tularensis* or *L. pneumophila* (Hoffmann *et al.*, 2012; Hoffmann *et al.*, 2014; Twine *et al.*, 2006; Urwyler *et al.*, 2009; Urwyler *et al.*, 2010). Enrichment of the fluorescent pathogen

by flow cytometry is also suitable and protocols for *S. enterica* and the intracellular protozoan parasite *Leishmania mexicana* are published (Becker *et al.*, 2006; Paape *et al.*, 2008)

Proteomics of bacterial-host interaction illuminate different aspects of this interplay, e.g. host and/or bacterial proteins, the compartment of the pathogen in the host, or changes in the proteome over time during the infection. The development of sophisticated protocols and the use of sensitive MS-instrument allow a deep view for a comprehensive understanding in the host-pathogen interactions.

I.4.1 Proteomics of *Salmonella* and the host

Proteomic as analytical tool to gain deeper insights into pathogenesis of *Salmonella* is widely used (Rodland *et al.*, 2008). First hints how *Salmonella* response to intracellular stress are coming from the analysis of the proteome from *in vitro* cultures grown under *in vivo*-mimicking conditions in comparison with standard growth conditions (Adkins *et al.*, 2006; Sherry *et al.*, 2011; Shi *et al.*, 2009a; Sonck *et al.*, 2009). However, to analyse the proteome of intracellular *Salmonella* and/or the host *in vitro* cell culture models are more suitable and are extensively used. Data for macrophage-like RAW264.7 (Imami *et al.*, 2013; Shi *et al.*, 2006; Shi *et al.*, 2009a), U937 (Abshire and Neidhardt, 1993), and HeLa cells (Auweter *et al.*, 2011; Imami *et al.*, 2013; Santos *et al.*, 2015; Vogels *et al.*, 2011) are available. In addition, proteomes of *in vivo* models with infected tissues were investigated like the caecum and spleen of BALB/c mouse (Becker *et al.*, 2006; Claudi *et al.*, 2014; Steeb *et al.*, 2013), the mucosa of piglets (Collado-Romero *et al.*, 2012) or the porcine mesenteric lymph nodes (Martins *et al.*, 2012). Furthermore SILAC-based quantitative proteomics of *Salmonella*-infected HeLa cells (Auweter *et al.*, 2011; Vogels *et al.*, 2011) and phosphoproteomics of infected RAW264.7 and HeLa cells were preformed (Imami *et al.*, 2013). Separation of *Salmonella* from the host cell/tissue is a mayor challenge, especially if the intracellular compartment of *Salmonella* is the aim of investigation (Rogers and Foster, 2008). Therefore, different protocols are published for the separation of *Salmonella* from its host. Fractionation based on differences in density and size (Liu *et al.*, 2015; Hashim *et al.*, 2000; Marsman *et al.*, 2004; Mills and Finlay, 1998; Santos *et al.*, 2015; Shi *et al.*, 2006; Vogels *et al.*, 2011), the treatment with detergents (Shi *et al.*, 2009b), or a combination of both (Shi *et al.*, 2006) are commonly used. Sorting of fluorescent *Salmonella* by flow cytometry with mechanical or detergent-mediated cell lysis is additional an appropriate method for the isolation of *Salmonella* from tissues/cells (Becker *et al.*, 2006; Claudi *et al.*, 2014; Meunier *et al.*, 2014; Meresse *et al.*, 1999a; Steeb *et al.*, 2013; Steele-Mortimer *et al.*, 1999; Thöne *et al.*, 2007). Until now research

was focused on global changes of the *Salmonella* and/or host proteome, but proteome data for the *Salmonella*-influenced compartments like the SCV or the SITs are limited (Becker *et al.*, 2006; Imami *et al.*, 2013; Collado-Romero *et al.*, 2012; Shi *et al.*, 2006; Santos *et al.*, 2015; Vogels *et al.*, 2011; reviewed in Rogers and Foster, 2008). This knowledge will help to get a better understanding of biogenesis and function of this unique cellular compartments and the influence on *Salmonella*'s pathogenesis.

I.5 Aim of this work

The facultative intracellular, foodborne pathogen *Salmonella enterica* serovar Typhimurium is able to avoid phago-/lysosomal degradation by remodelling its intracellular compartment, the *Salmonella*-containing vacuole (SCV). Responsible for this remodelling are secreted effector proteins which also initiate the formation of an extensive network of *Salmonella*-induced tubules (SITs) arising from the SCVs. The cellular origin, the composition and function of these *Salmonella*-modified membranes (SMM), which are defined as all host cell membranes modified by activities of intracellular *Salmonella*, are only partially understood. This unique compartment has properties of late endosomes, including acidified lumen, or lysosomal membrane glycoproteins like LAMP1. However, further cellular origins of SMM are assumed. Which proteins, from which origins, contribute to SMM biogenesis? To address this question the determination of SMM protein composition was aim of this work.

First aim was the development of a method for a systematic proteome-wide analysis of SMM because until recently protein components were mainly identified by fluorescence imaging (immunostaining, GFP-fusions). However, classical methods for subcellular fractionation like density gradient centrifugation are not suitable for purification of these fine tubular structures connected with SCVs. Thus, a special enrichment of the SMM is necessary which could be realized by immunoprecipitation of the membrane-integral SPI2 effector SseF as bait protein and subsequently analysis by mass spectrometry.
The successful development allows further questions:
Are there differences in the protein composition of the SMM from different cells, e.g. human epithelial cells HeLa or murine macrophage-like cells RAW264.7? Does the cell status influence the SMM protein composition, e.g. interferon γ activation of RAW264.7?
Additionally, has the bait protein used for immunoprecipitation an impact on the protein composition?

By comparing the resulting SMM proteomes with published proteomes of pathogen-containing vacuoles, e.g. the *Legionella*-containing vacuole, is it possible to draw conclusions about general strategies of intracellular pathogens to manipulate their host for their own survival and proliferation?

II Results and Publications

II.1 Proteomes of host cell membranes modified by intracellular activities of *Salmonella enterica*

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Research

Proteomes of Host Cell Membranes Modified by Intracellular Activities of *Salmonella enterica**

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Intracellular pathogens need to establish a growth-stimulating host niche for survival and replication. A unique feature of the gastrointestinal pathogen *Salmonella enterica* serovar Typhimurium is the creation of extensive membrane networks within its host. An understanding of the origin and function of these membranes is crucial for the development of new treatment strategies. However, the characterization of this compartment is very challenging, and only fragmentary knowledge of its composition and biogenesis exists. Here, we describe a new proteome-based approach to enrich and characterize *Salmonella*-modified membranes. Using a *Salmonella* mutant strain that does not form this unique membrane network as a reference, we identified a high-confidence set of host proteins associated with *Salmonella*-modified membranes. This comprehensive analysis allowed us to reconstruct the interactions of *Salmonella* with host membranes. For example, we noted that *Salmonella* redirects endoplasmic reticulum (ER) membrane trafficking to its intracellular niche, a finding that has not been described for *Salmonella* previously. Our system-wide approach therefore has the potential to rapidly close gaps in our knowledge of the infection process of intracellular pathogens and demonstrates a hitherto unrecognized complexity in the formation of *Salmonella* host niches. ***Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.041145, 81–92, 2015.***

Bacterial pathogens have evolved sophisticated mechanisms enabling them to invade, reside in, and proliferate in a large range of eukaryotic hosts. This often involves hijacking the host phagosomal system, interfering with the host cell signaling and trafficking machinery, and establishing a replication niche to avoid clearance (1). Whereas some pathogens escape phagosomes and replicate in the host cytoplasm, most of the described pathogens replicate in membrane-bound, vacuole-like compartments (2). Such intracellular niches of various pathogens are diverse, and biogenesis often depends on the delivery of bacterial effector proteins into the host cell cytoplasm.

Salmonella enterica, the causative agent of localized gastroenteritis and the life-threatening systemic infection known as typhoid fever, forms so-called *Salmonella*-containing vacuoles (SCVs)[1] inside host cells (3). SCVs mature through continuous interactions with endocytic and recycling pathways, accompanied by a spatial shift from the side of internalization to the juxtanuclear position close to the microtubule-organizing center (4, 5). Whereas the initial maturation steps are similar to the canonical phagosome biogenesis, the formation of an extensive tubular membrane network extending from the mature SCV is unique to *Salmonella*-infected host cells. This network contains various tubular structures such as *Salmonella*-induced filaments (SIFs), sorting nexin tubules, *Salmonella*-induced secretory carrier membrane protein 3 tubules, and lysosome-associated membrane protein 1-negative tubules (5–7), distinguishable by individual organelle marker proteins. For instance, tubules decorated with lysosome-associated membrane protein 1 (LAMP1) are known as SIFs (8, 9). In this paper we refer to all host membranes modified by intracellular *Salmonella* as *Salmonella*-modified membranes (SMMs).

In general, the appearance of SMMs coincides with the onset of bacterial replication, and both phenomena are dependent on the translocation of effector proteins of the *Salmonella* Pathogenicity Island 2 (SPI2)-encoded type III secretion system (T3SS) (10, 11). These effector proteins manipulate a large number of host cell processes and force

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[1] The abbreviations used are: SCV, *Salmonella*-containing vacuole; CLSM, confocal laser-scanning microscopy; ER, endoplasmic reticulum; GEMN, Golgi–endoplasmic reticulum–microtubule organizing center–nucleus; IP, immunoprecipitation; LAMP, lysosome-associated membrane protein; LC, liquid chromatography; MS/MS, tandem mass spectrometry; SIF, *Salmonella*-induced filament; SMM, *Salmonella*-modified membrane; SPI, *Salmonella* Pathogenicity Island; T3SS, type III secretion system; WT, wild type; m.o.i., multiplicity of infection; p.i., post-infection; LCV, *Legionella*-containing vacuole; COP, coat protein complex.

the host cell to create a suitable microenvironment for *Salmonella* (7, 12, 13). Although many *Salmonella* effector proteins have been described (14), much less is known about the host proteins that are manipulated to foster bacterial growth.

A systematic proteome-wide analysis would be an important step toward understanding the mechanisms used by *Salmonella* to reorganize the host cell endosomal system during intracellular proliferation. However, one major challenge is the need to distinguish host proteins directed toward the *Salmonella*-induced compartments from those that are present independent of an infection.

In this report we describe a novel method for the enrichment of SMMs and utilize a comparative strategy to identify proliferation-relevant host proteins. This systematic characterization of the SMM proteome provides new insights into the cellular origin and biogenesis of SMMs and identifies host cell proteins modified by the activity of intracellular *Salmonella*.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals used in this study were obtained from Sigma Aldrich, unless otherwise indicated.

Cell Lines, Bacterial Strains, and Their Cultivation—Human epithelial cell line HeLa (ATCC No. CCL-2) and stable lentiviral-transfected HeLa LAMP1-GFP cells (15) were maintained in DMEM containing 4.5 g/l glucose, 4 mM L-glutamine, and sodium pyruvate (Biochrom, Berlin, Germany) supplemented with 10% inactivated FCS in an atmosphere of 5% CO_2 and 90% humidity at 37 °C. Cells from the murine macrophage-like cell line RAW264.7 (ATCC No. TIB-71) were cultured in DMEM containing 4.5 g/l glucose and 4 mM stable glutamine (Biochrom) supplemented with 6% FCS at 37 °C in an atmosphere containing 5% CO_2 and 90% humidity. *S. enterica* serovar Typhimurium strains NCTC12023 (wild type (WT)) or HH107 and P2D6 harboring p3711 for the synthesis of SseF-2TEV-2M45 were used. Strain P2D6 is defective in the SPI2-encoded T3SS due to *ssaV* mutation. For live cell imaging, strains harboring plasmids p3589 or pFPV-mCherry/2 were used for constitutive expression of mCherry. Strain characteristics are summarized in supplemental Table S1*A*. *Salmonella* strains were routinely cultured in Luria–Bertani broth containing 50 μg/ml carbenicillin (Roth, Karlsruhe, Germany) or 12.5 μg/ml chloramphenicol if required for the selection of plasmids.

Construction of Recombinant DNA Molecules—For construction of the plasmid p3711 encoding the bait protein SseF, the following sequence was synthesized by GeneArt (Invitrogen): CCCGGGGGATCCGCCATG**GAGAATCTTTATTTTCAGGGC**GGCGACGTC**GAAAACCTTTATTTCCAAGGA**GGGTCCGGCGATCGG*AGTAGGGATCGCCTACCTCCTTTTGAGACAGAGACGC*GCATCCTCGGCTCGGGC*AGCAGAGACCGTCTGCCGCCGTTCGAAACCGAGACGC*GCATCCTCTAGAGCGGCCGC. The sequence encodes two recognition sites for the tobacco etch mosaic virus protease (bold) and two sites encoding the M45 epitope for antibody binding (italic). The synthetic DNA molecule was inserted into pSK via SmaI/NotI to generate p3673. Next, a fragment containing Pro_{sseA} *sscB* $sseF_{1–258}$ was released from p2810 (16) via digestion with KpnI/EcoRV and inserted into p3673. Subsequently, the insert was released via KpnI/NotI digestion and subcloned into pWSK29 to generate p3711.

To generate a plasmid expressing LAMP1-mCherry, we amplified *mcherry* from pFPV-mCherry using mCherry-For-BamHI and mCherry-Rev-XbaI-NotI and cloned in pEGFP-N1 (Clontech, Mountain View, CA) to replace eGFP (supplemental Table S1*B*). Next, human *lamp1* was amplified from cDNA clone IRAU p969C0275D (ImaGenes, Berlin, Germany) using hLAMP1-For-EcoRI and hLAMP1-Rev-BamHI (supplemental Table S1*B*), EcoRI/BamHI restricted, and inserted into the pmCherry-N1 transfection vector to generate p3451.

Infection of HeLa Cells—Host cell infections were performed as described previously (17). In short, HeLa cells were infected with 3.5 h subcultures of *Salmonella* with a multiplicity of infection (m.o.i.) of 50 or 75. The bacteria were centrifuged onto the cells at 500 × *g* for 5 min, and the cells were then incubated for 25 min at 37 °C in an atmosphere of 5% CO_2 before extracellular bacteria were removed by three washes with pre-warmed PBS. Subsequently, host cells were maintained in cell culture media containing 100 μg/ml gentamicin (AppliChem, Darmstadt, Germany) for 1 h. Afterward, cells were cultivated in media with a decreased gentamicin concentration of 10 μg/ml for the rest of the experiment.

Intracellular Replication Assays—Gentamicin protection assays were performed according to Ref. 18. Briefly, *Salmonella* strains were grown to stationary phase. The A_{600} values of the cultures were adjusted with PBS to 0.2, and cultures were added with an m.o.i. of 1 to the seeded RAW264.7 cells. After centrifugation for 5 min at 500 × *g* and incubation for 25 min at 37 °C in an atmosphere of 5% CO_2, macrophages were washed three times with pre-warmed PBS before being incubated in cell culture medium containing 100 μg/ml gentamicin for 1 h. The medium was replaced with medium containing 10 μg/ml gentamicin, and the macrophages were kept in this medium for the remaining time of the experiment. To determine the amount of intracellular bacteria, we washed macrophages three times with PBS and lysed them with 0.1% Triton X-100 for 10 min at room temperature at 2 h and 16 h post-infection (p.i.). Serial dilutions of the lysates were plated on Müller-Hinton agar plates. Statistical analyses were performed using one-way analysis of variance with SigmaPlot 11.0 (Sysstat Software, San Jose, CA).

Confocal Laser-scanning Microscopy—Fluorescence imaging was partially performed using a Leica SP5 confocal laser-scanning microscope with live cell periphery equipped with an HCX PL APO CS ×100 (numerical aperture 0.7–1.4) oil immersion objective (Leica, Wetzlar, Germany). Images were acquired using the LAS AF (Leica Application Suite Advanced Fluorescence) software and the following filter combinations: GFP/Alexa Fluor 488 and mCherry/Alexa Fluor 568 with polychroic mirror DD 488/543 or the combination of GFP/Alexa Fluor 488, mCherry/Alexa Fluor 568, and Cy5 with the polychroic mirror TD 488/543/633. All images obtained were processed by Leica LAS AF. Scale bars were added with ImageJ (National Institutes of Health), and figures were arranged in Photoshop CS6 (Adobe, San Jose, CA).

Live cell imaging was performed as described elsewhere (15). SIF formation was monitored from 4 h to 16 h p.i. For SMM validation, HeLa cells were co-transfected using FuGENE® HD Transfection Reagent (Promega, Madison, WI) with plasmids encoding GFP-fusion proteins Rab2a, Rab5c, Rab7a, Rab10a, Rab11a, Rab14, and UtrCH and pLAMP1-mCherry (supplemental Table S1*A*) and then subsequently infected with *Salmonella* WT harboring pFPV-mCherry/2 at an m.o.i. of 75. Confocal laser-scanning microscopy (CLSM) images were taken at 8 h p.i.

Immunostaining was performed as described elsewhere (19). Briefly, infected HeLa LAMP1-GFP cells (m.o.i. 50) were fixed with 3% paraformaldehyde at 8 h p.i., washed, and incubated for 30 min in blocking solution (2% goat serum, 2% BSA, and 0.1% saponin in PBS) before being stained with primary antibodies — anti-M45 (1:500), *Salmonella* O antiserum (1:1000), anti-human EEA1 (1:500), or anti-human TfR (1:100) for 1 h at room temperature and anti-human CopA (1:17), anti-human CopG1 (1:12.5), anti-human Sec23a (1:17), anti-human Sar1a (1:12.5), or anti-human Mitofilin (1:250) overnight at 4 °C (supplemental Table S1*A*). Secondary antibodies were selected accordingly (supplemental Table S1*A*), and samples were incubated for 1 h at room temperature.

Spinning Disk Microscopy—Fluorescence imaging was partially performed using a Zeiss Cell Observer Spinning Disk microscope with Yokogawa Spinning Disc Unit CSU-X1a 5000, an Evolve EMCCD camera from Photometrics (Tucson, AZ), and live cell periphery, equipped with an Alpha Plan-Apochromat ×63 (numerical aperture 1.46) oil immersion objective (Zeiss, Jena, Germany). Images were acquired using the ZEN (Zeiss) software and the following filter combinations: GFP with BP 525/50, mCherry with LP 580, and mTurquoise2 with BP 485/30. All images obtained were processed by ZEN2012 software. Micrographs and live cell images were prepared as described before. HeLa cells were co-transfected using FuGENE® HD Transfection Reagent (Promega) with plasmids encoding GFP-fusion proteins Rab2a, Rab7a, Rab10a, and UtrCH, as well as pm-Turquoise2-Golgi, pmTurquoise2-ER, pLifeAct-mTurquoise2, and pLAMP1-mCherry.

Quantitation via Flow Cytometry Analysis—HeLa cells were infected with either HH107 [p3711] or P2D6 [p3711] at an m.o.i. of 50 for 25 min. At 4, 8, 12, and 16 h p.i. cells were fixed, permeabilized, and stained with primary anti-M45 (1:1000) and secondary anti-mouse IgG Alexa Fluor 488 (1:1000) for subsequent flow cytometry analysis using FACSCalibur (BD Biosciences). Experiments were performed in triplicate at least three times. Data were analyzed with FACS Express 4 (De Novo Software, Los Angeles, CA). Statistical analyses were performed using Student's *t* test with SigmaPlot 11 (Sysstat Software).

Enrichment of GEMN Fraction—Roughly 7×10^7 HeLa LAMP1-GFP cells were used per immunoprecipitation (IP) and biological replicate. Before cell homogenization, the infected host cells were rinsed thrice with PBS. Scraped cells were resuspended in osmostabilizing homogenization buffer (250 mM sucrose, 20 mM HEPES, 0.5 mM EGTA, pH 7.4), centrifuged at 1000 × *g* for 10 min, and resuspended in 1 ml of 4 °C pre-cooled homogenization buffer with 1× protease inhibitor mixture (Serva, Heidelberg, Germany). Host cells were mechanically disrupted with 0.5-mm glass beads (Scientific Industries, New York, NY) using a Vortex-2 Genie with Turbomix (Scientific Industries; three 1-min strokes) with intermediate cooling. The lysate was centrifuged at 100 × *g* for 10 min at 4 °C, and the resulting GEMN pellet was washed twice with pre-cooled 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 DNaseI (50 μg/ml) for 30 min at 37 °C. The protein concentration was determined via Bradford assay (Bio-Rad).

Immunoprecipitation—For IP, 25 μl of Protein G magnetic beads (GE Healthcare) were coated with 40 μg of purified anti-M45 antibody on a rotary shaker at 4 °C overnight. The beads were washed twice with PBS, cross-linked according to the manufacturer's instructions, and blocked for 30 min with 1% BSA in PBS at 4 °C. A total of 500 μg of GEMN proteins were adjusted to a final volume of 200 μl in resuspension mix (1.5 mM $MgCl_2$, 10 mM KCl, 0.1% Nonidet P-40) and then incubated with 25 μl of cross-linked anti-M45 antibody-labeled Protein G magnetic beads on a rotary shaker at 4 °C overnight. To remove unbound proteins, we washed the sample five times with 0.1% Nonidet P-40 in PBS. Finally, bound proteins were eluted in 25 μl of 1× SDS sample buffer (12.5% glycerol, 4% SDS, 2% mercaptoethanol, 50 mM Tris, pH 6.8).

SDS-PAGE and Western Blotting—Proteins were separated on NuPAGE Novex 4–12% gradient gels. For Western blot analysis, 2 μl of the protein sample were used per lane. Proteins were transferred to 0.2-μm nitrocellulose membranes (Protran, Whatman, Dassel, Germany), blocked with 5% w/v BSA, 0.1% v/v TWEEN in TBS, and then incubated in TBS containing 1% w/v BSA with primary and secondary antibodies as follows: anti-M45 (1:5000) and peroxidase-conjugated anti-mouse IgG (1:20000) (supplemental Table S1*A*). Proteins were detected by chemiluminescence with ECL detection reagent (Pierce, ThermoScientific, Rockford, IL) and blue-light-sensitive film (Agfa Healthcare NV, Morstel, Belgium).

Protein Digest, RP-LC Separation, MS, and Data Analysis—In total we performed four IP proteome experiments. For each profiling, the precipitated proteins were one-dimensionally separated via SDS-PAGE and immediately Coomassie Blue–stained (20). Gel lanes of each biological replicate were sliced into 36 gel pieces. Each gel slice was subjected to standard in-gel de-staining and trypsinolysis procedures (21). Afterward the digest was transferred into vials, resulting in a total of 144 digested samples.

The LC-MS/MS analysis was performed using an UltiMate 3000 NCS-3500 nano-HPLC system (Dionex, Sunnyvale, CA) controlled by Chromeleon chromatography software coupled to an amaZon speed ETD ion trap mass spectrometer with a CaptiveSpray source (Bruker Daltonics, Bremen, Germany). The UltiMate 3000 NCS-3500 nano-HPLC system (Dionex) was configured with a 2-cm PepMap 75-μm inner diameter C_{18} sample trapping pre-column (Thermo Fisher Scientific) and a 15-cm PepMap 75-μm inner diameter C_{18} microcapillary column (Thermo Fisher Scientific). Samples of 7 μl each were applied to the columns and separated by a 60-min linear gradient from 5% to 50% solvent B (80% acetonitrile, 0.1% v/v formic acid) with a flow rate of 300 nl/min. For each MS scan, up to eight abundant multiply charged species in the *m/z* 400–1600 range were automatically selected for MS/MS but excluded for 30 s after having been selected twice. The HPLC system was controlled using Compass 1.5 (Bruker).

Acquired MS/MS data were processed by the ProteinScape 3.1 software (Bruker) and searched against the UniProt human database (October 2013; 20272 entries) using ProteinExtraktor (Bruker). Spectral data are available in PeptideAtlas (ftp://PASS00480:SU9795nb, ftp.peptideatlas.org). Data analyses were conducted according the published guidelines (22). Mass tolerance values for MS and MS/MS were set at 200 ppm and 0.5 Da. Fixed search parameters were semi-tryptic digestion and up to 1 missed cleavage. Variable search parameters used for the search were deamidation (NQ) and oxidation (M). Proteins were considered as identified with a ProteinScape score of >40 and two unique peptides with >95% confidence. Peptide Decoy (Mascot) and false discovery rates were adjusted to 1% at the protein and peptide levels for all experiments.

All identified proteins were searched against the UniProt-GO Annotation database (23). Only proteins identified in two biological replicates were considered as candidates in the SMM proteome (supplemental Table S2*A*). Protein abundance was estimated using the exponentially modified protein abundance index approach (24). Identified proteins were converted using UniProt IP mapping (25) into STRING numbers and searched against the STRING database (version 8.3; minimal confidence score of 0.4 (26, 27)) to identify potential protein–protein interactions.

To compare the compositions of pathogen-modulated host compartments from different pathogens and hosts, we used the MGI vertebrate homology database to extract human–mouse homologues and the inparanoid tool (28) to determine human–*Dictyostelium* homologues.

RESULTS

Enrichment of SMMs Using a Salmonella *Translocated Membrane-integral Effector Protein*—Enrichment of pathogen-containing compartments is conventionally based on subcellular fractionation after disruption of infected cells. However, a lack of specificity, dilution effects, and fragmentation of the complex and extensive membrane structures minimize the success achievable in determining the intrinsic SMM protein composition. Therefore, we developed an alter-

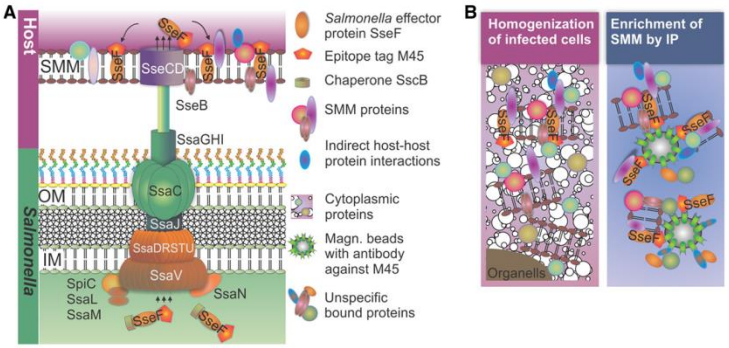

FIG. 1. **Schematic representation of *Salmonella* SPI2-T3SS injectosome (*A*) and approach for enrichment of *Salmonella*-modified membranes (SMMs) (*B*).** *Salmonella* translocates effector proteins into the host cell cytosol via the SPI2-encoded T3SS. A subset of SPI2-T3SS effectors are associated with or integral to SMMs. Modified effector proteins, such as M45-tagged SseF, are efficiently translocated by intracellular *Salmonella* into the host cell and integrate into membranes. After lysis of infected cells, immunoprecipitation of M45-tagged SseF using magnetic beads coated with anti-M45 antibody enriches the SMM decorated with SseF-2TEV-2M45.

native approach for enriching SMMs (Fig. 1). This approach utilizes SPI2-T3SS effector proteins that are embedded in SMMs as bait (Fig. 1*A*). IP of a tagged version of a membrane-integral SPI2-T3SS effector protein allows the enrichment of SMMs and additional associated proteins (Fig. 1*B*). A similar approach was recently used by the Hilbi group (29, 30) to analyze the composition of *Legionella*-containing vacuoles (LCVs). We selected effector protein SseF as bait for the IP. After translocation by the SPI2-T3SS, SseF has characteristics of an integral membrane protein in SCVs (19). As indicated by previous work, SseF is translocated in high amounts into host cells, is present within SIFs, and shows a long half-life (18). Furthermore, SseF is amenable as a fusion partner for various heterologous antigens and tags (31), rendering SseF as attractive bait for SMM enrichment.

We generated a low-copy-number vector for co-expression of *sseF* and its cognate chaperone *sscB* under control of the promoter Pro_{sseA} (supplemental Fig. S1*A*). Two TEV cleavage sites and a tandem M45 tag were fused to the C terminus of SseF, thus allowing immunoprecipitation with anti-M45 monoclonal antibodies. The resulting plasmid for synthesis of SseF-2TEV-2M45 was designated as p3711. We first tested the translocation and function of SseF-2TEV-2M45 in the *sseF*-deficient mutant strain HH107. The strain HH107 is attenuated in intracellular proliferation and induces an altered SIF network relative to the *Salmonella* WT (18, 32). Complementation with SseF-2TEV-2M45 restored intracellular proliferation of HH107 to WT levels (supplemental Fig. S2).

To exclude potential influences of the SseF-2TEV-2M45 construct on SIF network formation, we infected lentiviral-transfected HeLa LAMP1-GFP cells with HH107 translocating SseF-2TEV-2M45 and analyzed SIF formation via CLSM. LAMP1 is a prominent membrane-integral component of SIFs, and the fusion with GFP enabled us to continuously monitor their development. LAMP1-GFP transfection of HeLa cells had no influence on the infection process of *Salmonella* as previous experiments demonstrated (15, 33). As expected, the tagged effector protein SseF was present in SIFs and co-localized with LAMP1-GFP (Fig. 2). Furthermore, HH107 expressing SseF-2TEV-2M45 showed no impairment in SIF

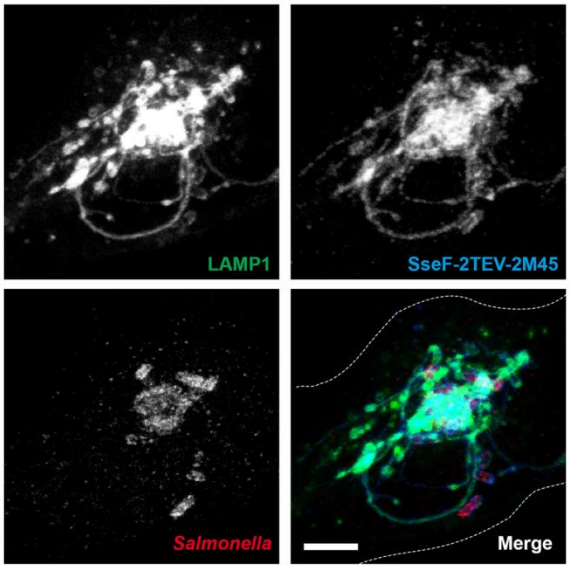

FIG. 2. **Epitope-tagged *Salmonella* effector protein SseF is an integral component of SMMs, indicated by its co-localization with LAMP1.** HeLa cells constitutively expressing LAMP1-GFP (green) were infected with *Salmonella* HH107 expressing SseF-2TEV-2M45 fixed at 8 h p.i., immunostained against *Salmonella* O antiserum (red) and the M45 tag (blue), and imaged using CLSM. Images are shown as maximum-intensity projections. Scale bar: 10 μm.

development (Fig. 3*A*). SIF formation, dynamics, and positioning were comparable to what was observed in HeLa cells infected with WT *Salmonella* (supplemental Fig. S1*B*).

To improve the capture yield of SMMs for a proteomics survey, we next screened for infection conditions in which the extension of the SMM network and the bait concentration were optimal. CLSM revealed an extensively widespread network of the LAMP1-GFP-positive SMM in *Salmonella* HH107 SseF-2TEV-2M45-infected HeLa cells around 8 h p.i. (Fig. 3*A*), which is consistent with previous findings from HeLa cells infected by WT *Salmonella* (34). Furthermore, we quantified the number of SseF-2TEV-2M45-positive *Salmonella*-infected HeLa cells at 4, 8, 12, and 16 h p.i. using flow cytometry. As

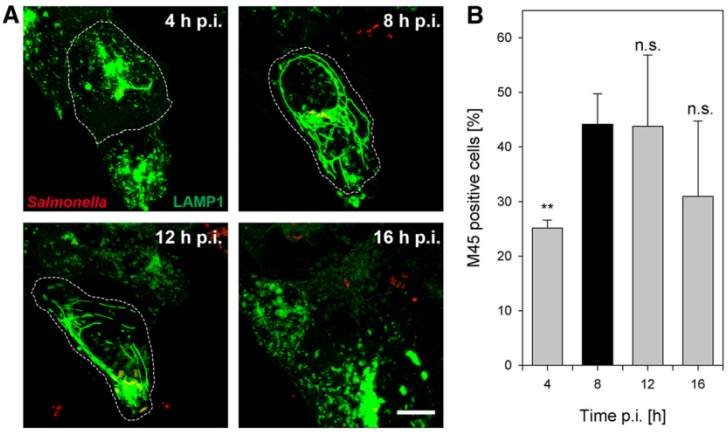

FIG. 3. **Extension of the SMM network (*A*) and the number of SseF-positive host cells (*B*) are maximal at 8 h p.i.** *A*, HeLa cells expressing LAMP1-GFP (green) were infected with *Salmonella* HH107 expressing SseF-2TEV-2M45 and mCherry (red). Continuous live cell imaging was performed and representative images of *Salmonella*-infected cells at various time points p.i. show the extent and variation in SIF morphology. Images are shown as maximum intensity projections. Scale bar: 10 μm. *B*, infected cells were fixed at the indicated time points p.i., immunostained for SseF-2TEV-2M45, and analyzed via flow cytometry. The percentage of M45-positive cells at various time points p.i. is indicated. Shown are the mean values (+ S.D.) of three independent experiments. Student's *t*-test relative to 8 h p.i. values: $^{**}p < 0.01$. n.s., not significant.

indicated in Fig. 3*B*, the cell number positive for translocated SseF-2TEV-2M45 was maximal at 8 h p.i., which is in good agreement with the CLSM analyses of the SMM network.

In summary, the data suggest that at 8 h p.i. the SMM network is fully developed and decorated with SseF. Therefore, this time point is optimal for the extraction of the SMM proteome for subsequent analyses.

Proteome Profiling of SMM—To determine the SMM proteome, we used the illustrated procedure integrating subcellular fractionation and IP with LC-MS/MS (Fig. 4). Because the SMM network of infected HeLa cells (8 h p.i.) is strongly intertwined with the host cells' GEMN complex, we decided to increase the amount of SMM-located proteins by enriching the GEMN fraction. To this end we first isolated the GEMN fraction using a combination of gentle mechanical lysis and centrifugation (supplemental Fig. S3*B/C*). Subsequently, the GEMN pellet was solubilized using mild detergents. The resulting mixture was then used for IP experiments. To elucidate *Salmonella* proteins specific to SMM and necessary for intracellular survival, we conducted comparative proteome analysis using the same methods with HeLa cells infected by *Salmonella* strain P2D6 harboring p3711. This strain is able to infect HeLa cells, but it is deficient in the translocation of SPI2-T3SS effector proteins and unable to induce SIFs, and it shows attenuated proliferation in host cells (supplemental Fig. S1*C*). We first used Western blots to confirm that the immune-precipitated fraction contained the bait protein SseF (supplemental Fig. S4*B*). Afterward the IP proteomes were profiled via LC-MS/MS. In total we performed 144 LC-MS/MS runs and identified 583 host cell proteins. Of these hits, 336 proteins were also detected in the immune-precipitated fraction of cells infected with control strain P2D6 [p3711]. Thus, 247 host cell proteins were found to be unique in the SMM proteome (supplemental Table S2*A*). The 20 most abundant proteins are listed in supplemental Table S3.

Classification by subcellular location of the 247 identified host cell proteins using the UniProt database (23) revealed

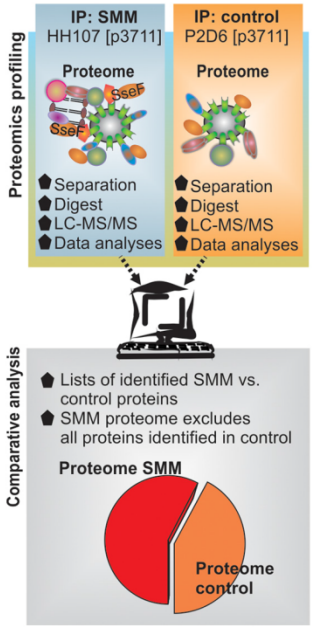

FIG. 4. **Schematic overview of experimental approach and data analysis.**

that a significant proportion of our identified SMM proteins are predicted to be of cytoplasmic origin (Fig. 5*A*). This might seem surprising considering that the enrichment primarily targeted the SMM. However, proteins that form tight interactions with SMM proteins are also likely to be co-isolated (Fig. 1*A*). This notion was supported using the STRING database (35), which revealed a highly intertwined protein–protein interaction network of the identified SMM proteins (supplemental Fig. S5). In total, 86% of all identified proteins showed potential physical and functional protein–protein interactions.

Origin and Function of the Identified SMM Proteome—It is still not clear how precisely SMMs are formed within the host

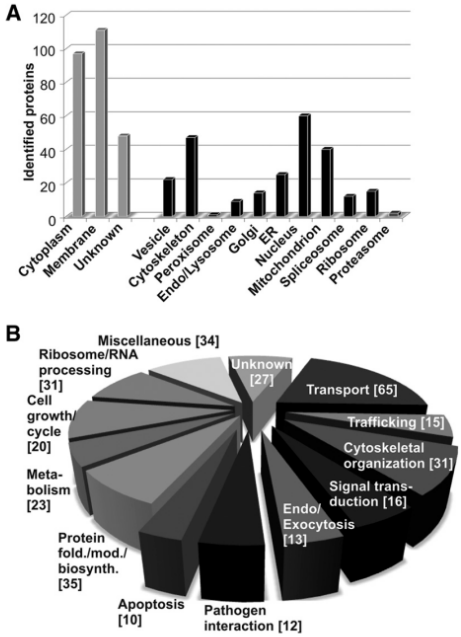

FIG. 5. **Classification of the identified SMM proteome according to Gene Ontology subcellular localization (*A*) and biological processes (*B*), based on the annotations in the UniProt database.** The term "cytoskeleton" includes proteins involved in stress fibers, intermediate filaments, microtubules, focal adhesion, and tight and cell junctions. Some proteins have more than one functional annotation. Numbers of identified proteins are indicated in brackets.

cell. However, several compartments and cellular structures have been implicated in their generation. *Salmonella* actively recruits host membranes for SIF formation from endosomes, lysosomes, and the trans-Golgi network (9, 36, 37). In addition, the cytoskeleton stabilizes SIFs and guides their elongation (38). In line with these observations, a number of cytoskeleton proteins (47 proteins), endosomal and lysosomal proteins (9), Golgi proteins (14), and vesicle-transport-related proteins (24) have been identified (Fig. 5*A*). Interestingly, proteins were also found to originate from other compartments such as the ER, nucleus, and mitochondria. This might hint at interplay between these compartments during the formation of SMMs.

The identified SMM proteins were further grouped according to their experimentally determined or predicted biological function (UniProt (23)) (Fig. 5*B*). A considerable number of SMM proteins were implicated in transport (65 proteins), vesicle trafficking (15 proteins), endo-/exocytosis (13 proteins), cytoskeletal organization processes (31 proteins), and signal transduction processes (16 proteins). Selected candidates are summarized in Table I. A subset of host cell proteins identified in our SMM proteome survey have previously been reported as associated with or involved in the biogenesis of SCVs or SIFs. These include GTPases Rab7a, Rab5c, Rab11a, and Rab14 (39, 40); filamin (41); myosin II (42); dynein (36); desmoplakin (43); and actin (44).

The majority of identified proteins were not reported to be located within SIFs, although some are known to be involved in the *Salmonella* infection process. These include, for instance, annexin A1 (45), α-actinin (46), actin-related protein 2/3 complex (47), BAG chaperone regulator 2 (43), cullin family proteins (48), coronin (49), tropomyosin (46), serine/threonine-protein phosphatase 2A (50), catenin α-1 (51), dynamitin (52), endoplasmin (50), coatomer I protein (48), elongation factors (50), T-plastin (53), small GTPase Ral-A (54), protein disulfide-isomerases (50), ezrin (55), spectrin (56), and ubiquitin-like modifier-activating proteins (50).

However, to the best of our knowledge, most of the identified proteins have not been mentioned before in relation to the intracellular lifestyle of *Salmonella*. Interesting candidates include trafficking-related components (coatomer II–associated small GTPase Sar1a, GTPase Rab2a, ADF-ribosylation factor 4, signal recognition particle subunits Srp72 and SrpR, Ras GTPase-activating protein-binding protein 2, B-cell receptor–associated protein 31, transmembrane emp24 domain–containing proteins 9 and 10, vesicle-associated membrane protein–associated proteins A and B, protein transport protein Sec23a, vacuolar protein sorting-associated protein 26B), nutrient transporter parts (tricarboxylate transport protein, phosphate transport protein, aspartate glutamate carrier 2, 4F2 cell-surface antigen), signaling components (Ras-related protein Rap1b, serine/threonine-protein phosphatase PGAM5), and cytoskeleton-associated elements (utrophin, protein spire homolog 2 Arf-GAP with Rho-GAP domain-ANK repeat and PH domain-containing protein 2, caldesmon). These host proteins, or organelles containing these proteins, are likely hijacked by *Salmonella* to provide the SCVs with nutrients, to avoid antimicrobial activities, to modify the normal endosomal maturation, and to support further intracellular proliferation. However, further validations are required to assess their impact on the survival of *Salmonella* inside host cells.

Intracellular Salmonella *Redirects Host Traffic to SMM*—Small Rab GTPases perform a fundamental role in membrane dynamics and are known key targets of intracellular bacterial pathogens (1, 57). In the presented SMM proteome analysis we identified six small Rab GTPases (Rab2a, Rab5c, Rab7a, Rab10a, Rab11a, and Rab14), of which only Rab7a was previously linked to SIF formation (1). To determine whether the other proteins are also involved in SIF formation and associated with the SMM network, we transiently co-transfected HeLa cells for synthesis of pLAMP1-mCherry and fusion proteins of GFP to Rab2a, Rab5c, Rab7a, Rab10a, Rab11a, or Rab14. Subsequently, cells were infected with WT *Salmonella* expressing mCherry and imaged via CLSM at 8 h p.i. All identified and analyzed Rab GTPase GFP-fusion proteins were localized at SCV and SIF membranes (Fig. 6), as indicated by co-localization with the marker protein LAMP1. Similarly, we analyzed the localization of the F-actin binding pro-

TABLE I
MS-identified SMM proteins involved in trafficking, transport, or cytoskeleton

Name (*_human)	Protein description	Localization
Transport (amino acids, sugar calcium, phosphate, and electrons)		
4F2	4F2 cell-surface antigen, amino acid transport	Plasma membrane
CTP	Tricarboxylate transport protein	Membrane, mitochondrion
CMC2	Calcium-binding mitochondrial carrier protein Aralar2	Membrane, mitochondrion
MPCP	Phosphate carrier protein	Membrane, mitochondrion
MTCH2	Mitochondrial carrier homolog 2	Membrane, mitochondrion
VCP	Transitional endoplasmic reticulum ATPase	ER
ATP5C/D/O	F_0F_1-ATPase complex	Membrane, mitochondrion
GTPases		
RAB2A	Rab GTPase Rab2a	ER, Golgi
RAB5C	Rab GTPase Rab5c	Vesicle, endosomes
RAB7A	Rab GTPase Rab7a	Vesicle, endosome, lysosome
RAB10	Rab GTPase Rab10	Vesicle, endosome, ER
RB11A	Rab GTPase Rab11a	Vesicle, endosome, lysosome
RAB14	Rab GTPase Rab14	Vesicle, endosome, lysosome
RALA	Multifunctional GTPase RalA	Vesicle, membrane
RAP1B	Ras GTPase Rap1b	Cell junctions, membrane
OPA1	Dynamin-like 120-kDa protein	Membrane, mitochondrion
SAR1A	COPII-associated GTPase Sar1A	ER
Clathrin, coatomer I and II–mediated transport		
AP2A1/B1	AP-2 complex subunits α and β	Coated pits, membrane
TFR	Transferrin receptor protein 1	Coated pits, vesicle, membrane, endosome
COPA/G1	Coatomer subunits α and γ	Vesicle, Golgi
BAP31	B-cell receptor–associated protein 31	Membrane, ER
TMED9/10	Transmembrane emp24 domain-containing proteins 9 and 10	Vesicle, Golgi, ER
SC23A	Protein transport protein Sec23a	Vesicle, Golgi, ER
VAPA/B	Vesicle-associated membrane protein-associated proteins A and B	Vesicle, membrane, microtubule, ER
ARF4	ADP-ribosylation factor 4	Membrane, Golgi
Cytoskeleton and membrane linker		
MYH9/10	Myosin II	Membrane, cytoskeleton
MYO1B/C	Myosin I	Cytoskeleton
ACTS	Actin	Cytoskeleton
DYHC1/7	Dynein	Cytoskeleton, microtubule
ARPC4	Arp2/3 complex	Cytoskeleton
DYST	Dystonin	Cytoskeleton, microtubule, ER
FLNA	Filamin-A	Cytoskeleton, endosome
CAPZB	F-Actin capping protein	Cytoskeleton
SYN1/2	Nesprin 1 and 2	Cytoskeleton, Golgi
PLST	Plastin-3	Cytoskeleton
ANAXA1	Annexin A1	Membrane, nucleus, cell projection
TCPE/G/Z	T-complex protein 1 subunit $\epsilon/\gamma/\zeta$	Cytoskeleton, microtubule
UTRO	Utrophin	Cell junction, cytoskeleton
COR1C	Coronin	Membrane, cytoskeleton
TPM2/3	Tropomyosin α and β	Cytoskeleton

tein utrophin (UtrCH). Here, we observed co-localization of UtrCH along SIFs, as well as an accumulation of UtrCH surrounding individual *Salmonella* assumed to be part of the vacuole-associated actin polymerization (Fig. 6). Controls verifying that the protein localizations in uninfected cells were not affected by overexpression of GFP fusion proteins are presented in supplemental Fig. S6.

Components of COPI- and COPII-mediated transport processes including CopA, CopG1 (both COPI), Sec23A, and Sar1A (both COPII) are also part of the SMM proteome. Although these proteins have been implicated in the replication niche formation of other pathogens (58), they have not been shown to be involved in *Salmonella*. We therefore immunostained these marker proteins in *Salmonella* WT mCherry-infected HeLa LAMP1-GFP and revealed localizations of COP vesicles along SIF membranes (Fig. 7). Similarly, we also observed the association of a representative mitochondrial marker protein, mitofilin, along SIFs. The association of proteins of the SMM proteome with SIFs further validates the usefulness of the SMM proteome approach.

In summary, these findings confirm that the presented SMM proteome enrichment and analysis strategy identified proteins located to the *Salmonella* compartment. In addition, the results clearly indicate the redirection of proteins of various host trafficking pathways (endosomal, recycling, Golgi–ER, and ER–ER), some of which were not known to be connected to SIFs, the formation of the replication niche inside the host, and *Salmonella* infection in general. Our findings of host transport processes

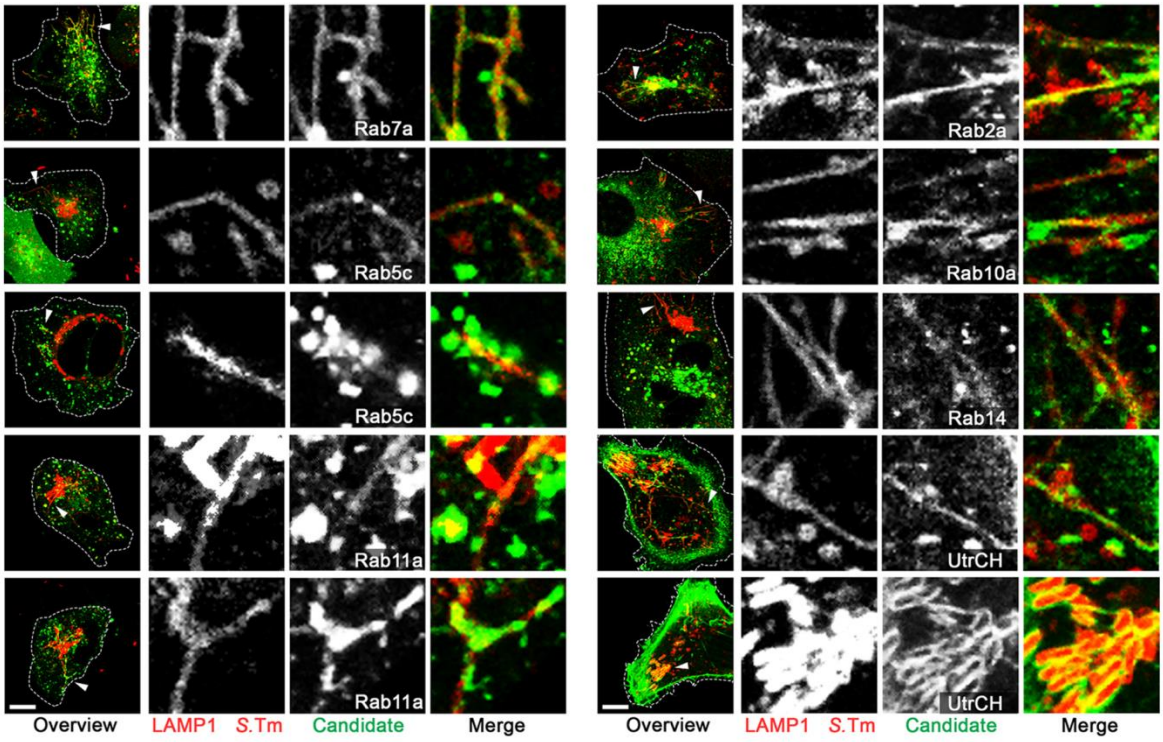

FIG. 6. **Co-localization of identified Rab GTPases and the F-actin binding protein utrophin with SMMs.** HeLa cells were co-transfected with p3451 for expression of LAMP1-mCherry (red) and various plasmids for the synthesis of fusion proteins of Rab2a, Rab5c, Rab7a, Rab10a, Rab11a, Rab14, or UtrCh to GFP (green) (supplemental Table S1*A*). Afterward, cells were infected with *Salmonella* WT (red) and imaged via CLSM at 8 h p.i. The lysosomal glycoprotein LAMP1 served as a marker for SIFs and SCVs. Controls verifying that the protein localizations were not affected by overexpression of GFP fusion proteins are presented in supplemental Fig. S6. Overview images are shown as maximum intensity projections. White arrowheads indicate the structures of interest shown as magnifications for each channel. Scale bar: 10 μm.

redirected by *Salmonella* are summarized in Fig. 8. Overall, the identified SMM proteins provide compelling insights into host processes that are usurped by *Salmonella* to ensure its survival.

DISCUSSION

The success of an intracellular pathogen depends on its ability to establish a niche for proliferation within its host. Although the infection process of *Salmonella* has been intensively studied (7, 50, 59–63), less is known about the host niche of this bacterium.

Salmonella-infected cells are characterized by a complex network of SCVs linked to various types of tubular membrane compartments, collectively termed the SMM. The extensive and highly dynamic nature of SMMs prevented classical subcellular fractionation, and we developed a new procedure for the enrichment of SMMs. In this paper we describe immunoproteomic analyses that allowed, for the first time, a survey of the proteome of the SMM.

It is assumed that the host niche of *Salmonella* is formed during its continuous interactions with the host endosomal and recycling pathway (4, 5). We identified several components of these host pathways such as Rab7a, Rab5c, Rab11a, and Rab14 that are known to be involved in the formation of SCVs or SIFs (36, 39–44). These findings demonstrate that our approach is well suited to identify infection-relevant host proteins from the SMM.

In addition we observed proteins indicative of other intracellular host membrane trafficking pathways. For instance, we identified proteins associated with the anterograde and retrograde transport system, responsible for trafficking between the ER and Golgi (58). This includes components of the COPI and COPII (COPI: coatomer subunits α and γ, transmembrane emp24 domain–containing proteins 9 and 10, vacuolar protein sorting–associated protein 26B, ADF-ribosylation factor 4, small GTPase Rab2a, and Rab2a effector glyceraldehyde-3-phosphate dehydrogenase (64); COPII: small coatomer II–associated GTPase SAR1, protein transport protein Sec23). Salcedo and Holden (65) stated in an earlier report that only *Salmonella* cells closely associated with the Golgi network are able to multiply. However, no vesicle trafficking or direct association or fusion with either the Golgi or the ER system was

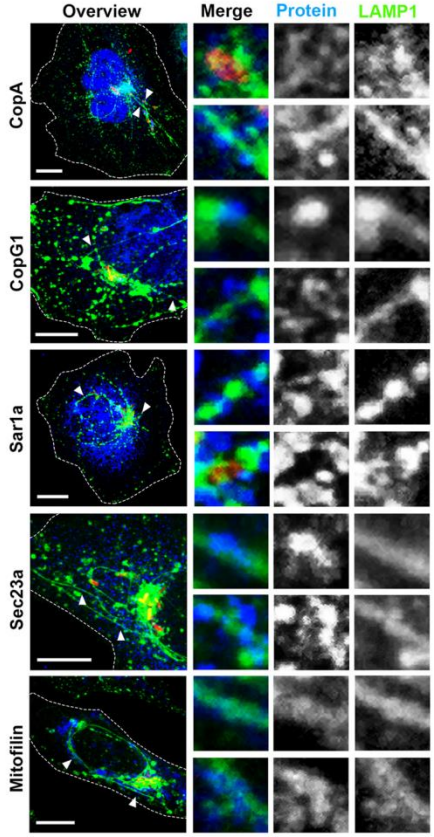

FIG. 7. **Localization of COPI, COPII, and mitochondria in *Salmonella*-infected cells.** HeLa LAMP1-GFP cells were infected with *Salmonella* WT mCherry (red) and fixed at 8 h p.i. Immunostaining was performed for COPI (CopA, CopG1), COPII (Sar1a, Sec23a), or mitochondria (Mitofilin) (blue), after which cells were imaged using a Leica SP5 CLSM. Images are shown as maximum intensity projections. White arrowheads indicate the structures of interest shown as magnifications for each channel. Scale bar: 10 μm.

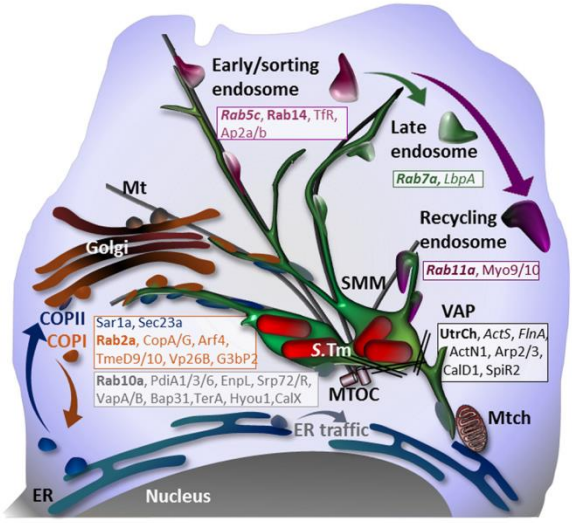

FIG. 8. **Model for the origin of SMMs.** SMM networks contain relocated host proteins from endocytic and intracellular host trafficking routes, as well as components of mitochondrial, ribosomal, ER, and nuclear origin identified in this study. Proteins known to be involved in SIF or SCV formation are italicized or bolded, respectively. Mt, microtubule; Mtch, mitochondrium; VAP, vacuole-associated actin polymerization; *S.*Tm, *Salmonella* Typhimurium.

observed. Our study thus provides the first evidence of direct interaction of SMMs with the ER system.

In addition to COPI and COPII components, we further identified the small GTPase Rab10a involved in ER dynamics (66), several ER chaperones (protein disulfide-isomerase PDIA1, PDIA3, PDIA6, endoplasmin, hypoxia up-regulated protein 1), SNARE proteins (VAMP-associated proteins A and B), vesicle recognition particles (subunit Srp72 and receptor SrpR), B-cell receptor–associated protein 31, and diverse ER membrane proteins (transitional ER ATPase, calnexin, estradiol 17-β dehydrogenase, dolichyl-diphosphooligosaccharide-glycosyltransferase, transmembrane protein 43).

In summary, the data indicate that *Salmonella* at the stage of efficient intracellular proliferation specifically intercepts intracellular membrane trafficking pathways and recruits membranes and proteins from endosomes (Fig. 8). However, this is a common strategy among other intracellular pathogens (58). For example, *Legionella pneumophilia* tethers and subsequently fuses transitional ER-derived vesicles to its vacuolar host compartment (LCV) (1). Similarly, *Brucella* spp. intercept COPI and COPII vesicle trafficking and target the ER to create a replicating niche termed the *Brucella*-containing vacuole (67). Interaction of the *Brucella*-containing vacuole with the ER leads, for instance, to the presence of several ER marker proteins such as calnexin, Sec61, Rab2a, and the Rab2a effector glyceraldehyde-3-phosphate dehydrogenase in the *Brucella*-containing vacuole. Interestingly, we observed many of these proteins in the SMM.

This raises the interesting question of whether there might be more commonalities between the host niche composition and mechanisms of intracellular survival of these bacteria. Recently, the proteome of LCVs has been profiled (29, 30). Unfortunately, these studies did not distinguish between proteins that are relevant to bacterial persistence and nonspecific host proteins. Nonetheless, this offers the opportunity to compare these host niches of different intracellular pathogens and identify common host proteins. One interesting finding of the comparative analyses was that 45% (109 of 247 proteins) of the SMM proteome isolated from HeLa was also found in LCVs isolated from *Dictyostelium* cells or mouse macrophages (supplemental Table S4). This is quite remarkable considering that the studies were based on different pathogens and host systems. Thus it appears that although *Salmonella* and *Legionella* utilize distinct mechanisms to manipulate

their host cells, the pathogen-containing compartments share a remarkable degree of similarity.

The 109 proteins common to the SMM proteome and LCVs are of diverse origin, including ER, mitochondria, nucleus, ribosomes, and endocytic pathways (supplemental Table S4). Again, we found that a large number of the proteins identified in both studies were involved in transport processes (43 proteins), which indicates that these transport processes might be essential for survival for a wide range of intracellular pathogens. Further notable groups of proteins were involved in vesicle trafficking (12 proteins), endo-/exocytosis (10 proteins), protein folding/modification and biosynthesis (17 proteins), and metabolism (16 proteins). In contrast, only relatively few (eight) proteins with cytoskeletal functions were found in LCVs and SMMs. This includes actin; two motor proteins (myosin 9 and myosin 10); and actin-binding proteins coronin-1C, α-actinin, F-actin capping protein, Arp2/3 complex, and desmoplakin. Actin is involved in the formation of a stabilizing meshwork around pathogen-containing compartments such as SCVs (41, 68). Motor proteins are important mediators of vesicular transport, and interference with host cell motor protein function enables intracellular pathogens to segregate their own membrane-bound compartments from the default endosomal maturation pathway (59, 69). For example, SCVs maintain a juxtanuclear position and are restricted in motility (4, 44, 70). However, dynein, a motor protein identified here, was found only on SMMs. This is consistent with the finding that the SMM network extension is dynein mediated (52, 71). Interestingly, tubular networks are not known for *Legionella*-infected host cells. Thus, despite the fact that similar host proteins are recruited, the bacteria appear to elicit different intracellular phenotypes. This might hint at a novel mechanism that needs further exploration. Together, these findings suggest the interesting possibility that despite the differences in the structures of the niches occupied by the pathogens, they might commandeer similar host proteins to establish their intracellular niches.

A general limitation of proteome studies is that typically the separation of proteins or peptides prior to MS is insufficient to fully resolve complex protein mixtures. As a consequence, low-abundance proteins in particular might be absent from an individual run. However, the use of immunoprecipitation appears to reduce the complexity to a manageable degree, as we found from our combined dataset. Nonetheless, the main purpose of this analysis was to provide priority targets to be investigated in further detail using targeted methods. Although validating all targets would be beyond the scope of this study, we found that at least 11 proteins identified via the proteome approach were indeed associated with SMMs, including a number of new proteins previously not related to the intracellular lifestyle of *Salmonella*.

In summary, our proteomic study provides global insights into the molecular machinery involved in the formation and maintenance of SMMs. The data indicate that more membrane trafficking systems than hitherto realized are involved, and we have provided numerous targets for further investigation. It is also interesting to note that despite the unique structure of SMMs, the host-hijacking mechanisms are markedly similar to those of other intracellular pathogens.

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II.2 How *Salmonella* modifies its killer – proteomic analysis of *Salmonella*-modified membranes in macrophages

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II.2.1 Abstract

The intracellular pathogen *Salmonella enterica* serovar Typhimurium remodels the eukaryotic endosomal system to create a replicative niche inside host cells. Translocation of effector proteins by *Salmonella* facilitates the establishment of an extensive interconnected tubular membrane network arising from the *Salmonella*-containing vacuole (SCV). However, the cellular origin, the composition and function of these membrane structures are only partially understood. Here, we enriched *Salmonella*-modified membranes (SMM) either from interferon γ (IFNγ) activated and non-activated infected RAW264.7 macrophages using an affinity-based proteome approach. Proteomic analysis reveals cytoskeletal, endo- and lysosomal proteins as well as components of the endoplasmic reticulum to Golgi trafficking route, enabling us to develop new theories about the origin of the *Salmonella*-induced tubules. Comparisons of host compartment proteomes gained from different cell types and pathogens provide communalities of pathogen induced host manipulation strategies.

II.2.2 Introduction

Macrophages are immune cells with central importance for the defence against and clearance of pathogens. However, some pathogenic bacteria, such as *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Brucella* spp., and *Salmonella* Typhimurium are even able to survive and replicate inside these cells (Celli, 2006; Duclos and Desjardins, 2000; Linehan and Holden, 2003; Holden, 2002; Isberg *et al.*, 2009; Newton *et al.*, 2010; Stanley and Cox, 2013; Vergne *et al.*, 2004; Xu and Luo, 2013). These intracellular pathogens evolved strategies to avoid intracellular killing by modifying their vacuoles in which they reside after cell entry (Alonso and Garcia-del Portillo, 2004; Asrat *et al.*, 2014; Cossart and Roy, 2010).

Salmonella enterica serovar Typhimurium is a facultative intracellular foodborne pathogen which can cause diverse diseases ranging from mild gastroenteritis up to life-threatening systemic infections in immunosuppressed individuals (Malik-Kale *et al.*, 2011; LaRock *et al.*, 2015). Colonization of the intestine is followed by breaching the epithelial barrier. In the submucosa *Salmonella* enters resident macrophages and is disseminated through the reticuloendothelial system (Haraga *et al.*, 2008; Ibarra and Steele-Mortimer, 2009; Salcedo *et al.*, 2001). Directly after internalization *Salmonella* starts to modify its residing compartment, the *Salmonella*-containing vacuole (SCV), and avoids the typical phagosomal maturation process. Interplaying with the host endocytic system, the SCV continuously matures into a unique compartment with properties of late endosomes (LE) to some extent, including acidified lumen, lysosomal membrane glycoproteins like LAMP1, but without lysosomal hydrolases (Figueira and Holden, 2012; Steele-Mortimer *et al.*, 1999). Remodelling is directed in early stages by effector proteins of the *Salmonella* Pathogenicity Island-1 (SP1) which are substituted by SPI2 effectors (Kuhle and Hensel, 2004). These effectors are responsible for the formation of an extensive network of *Salmonella*-induced tubules (SITs) arising from the SCV (Liss and Hensel, 2015; Schroeder *et al.*, 2011). Three types of SITs are distinguishable at the late stage of infection (>4 h): *Salmonella*-induced filaments (SIF), *Salmonella*-induced SCAMP3 tubules (SISTs) and LAMP1-negative tubules (LNTs) (Schroeder *et al.*, 2011). Many *Salmonella* effectors are known which are involved in remodelling of the endosomal system, but the host repertoire of proteins is much less elucidated (Haraga *et al.*, 2008; Liss and Hensel, 2015; Rajashekar and Hensel, 2011). Of special interest is the origin and composition of these *Salmonella*-modified membranes (SMMs) which are defined as all host cell membranes modified by activities of intracellular *Salmonella*. In a systematic proteome-wide analysis (Chapter II.1) we were able to identify 247 proteins of SMM in HeLa cells (Vorwerk *et al.*, 2015). We demonstrated that proteins of SMM arise from various origins, including ER, mitochondria, nucleus, and ribosomes, besides known components of the endosomal system and Golgi. We developed a new method for the enrichment of SMMs by immunoprecipitation on the basis of integral membrane-protein SseF, a SPI2 effector which was tagged with a M45 epitope. Here, we adapted this method for the murine macrophage-like cell line RAW264.7 and determined the SMM proteome of macrophages which were either activated with IFNγ before infection or non-activated. The inflammatory cytokine IFNγ, alone or in interplay with microbial products (e.g. lipopolysaccharide, LPS) and further cytokines (e.g. tumour necrosis factor, TNF) induce the classical activation of macrophages through the IFNγ-receptor or Toll-like receptor TLR2/TLR4, respectively, which recognizes PAMPs (pathogen-associated-

molecular-patterns) (Mantovani *et al.*, 2004; Muraille *et al.*, 2014). T helper 1 (T_H1) cells, as well as $CD8^+$ T cells produce IFNγ during adaptive immune response or by natural killer (NK) cells during the innate immune response (Mosser and Edwards, 2008). Antigen-presenting cells (APCs) provide TNF. These activated cells, also called M1 macrophages, are characterised by an increased microbicidal capacity, enhanced secretion of cytokines and mediators, and higher expression of co-stimulatory molecules (Mosser and Edwards, 2008). Characteristics are an increased secretion of pro-inflammatory cytokines such as TNF, interleukin-1 (IL-1), IL-6/12/23, but low levels of IL-10, and a high antigen production (Arango Duque and Descoteaux, 2014; Martinez and Gordon, 2014). Antimicrobial activity is mediated by production of nitric oxide synthase-2 (NOS-2/iNOS)-dependent reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) (Muraille *et al.*, 2014). IFNγ-regulated transcription is mediated by the IFNγ-receptor which recruits Janus kinase 1 (Jak1) and Jak2 that activate STAT1 (signal transducers and activators of transcription 1) and interferon regulatory factors (IRF) like IRF-1 and IRF-8 (Hu and Ivashkiv, 2009; Schroder *et al.*, 2004). Response to LPS is controlled by TLR4 which activates MyD88 and MaL/Tirap (Toll-interleukin 1 receptor domain containing adaptor protein). Transcription regulation relies on nuclear factor of kappa light polypeptide gene enhancer (NF-κB), activator protein 1 (AP-1), IRFs, STAT1, and EGR (early growth response) family members with overlaps to the IFNγ pathway (Martinez and Gordon, 2014). Activation of macrophages serves as defence mechanism against pathogens. However, *Salmonella* is able to escape from these mechanisms (Langermans *et al.*, 1991; Lindgren *et al.*, 1996; Muraille *et al.*, 2014; Wick, 2004). Therefore, we analysed the influence of IFNγ activation on the composition of the SMM.

In this proteomic survey, we identified 262 proteins of the proteome of *Salmonella*-modified membranes in macrophages and 142 proteins in the SMM proteome of IFNγ-activated RAW264.7. In agreement with our previous results (Vorwerk *et al.*, 2015) many proteins are involved in host membrane trafficking. In this study we compared the SMM proteome from macrophage with the one from HeLa cells (Vorwerk *et al.*, 2015) and the proteome of *Legionella*-containing vacuoles (LCVs) (Hoffmann *et al.*, 2013) resulting in 32 identical proteins. This set of proteins facilitates the goal to get a deeper view into the origin of pathogen-modified compartments.

II.2.3 Results

II.2.3.1 Enrichment of *Salmonella*-modified membranes from infected macrophages

To elucidate the composition of the SMM network formed in mouse macrophage-like cells (RAW264.7), the SMM enrichment protocol previously developed for HeLa cells (Chapter II.1, Vorwerk *et al.*, 2015) required adjustments to cope with the variations of the infection process in RAW264.7 cells. To obtain maximal protein amounts necessary for proteomics, the time point with the largest extension of the SMM network was selected by analysing the development of the SIF network after infection by live cell imaging. Therefore, stably transfected RAW264.7 LAMP1-GFP cells with LAMP1-GFP as marker for SIFs and the *Salmonella* strain Δ*sseF::aph* [*sseF*::tev::tev::M45::M45] constitutively expressing mCherry were used.

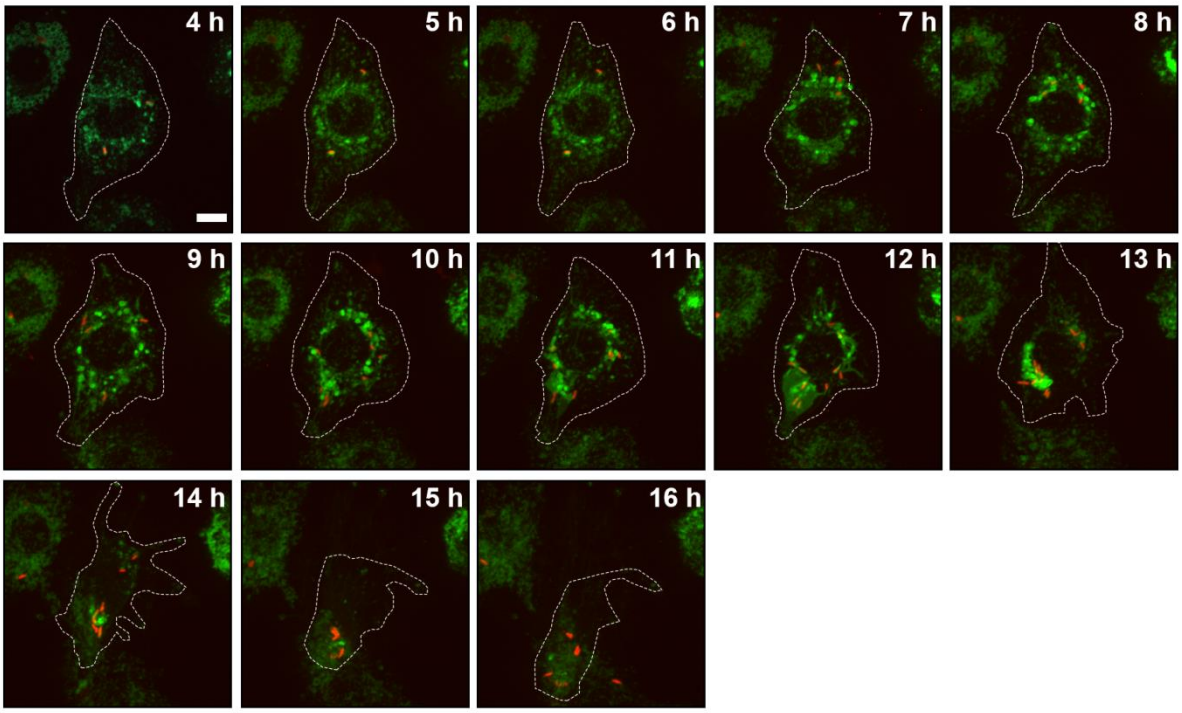

Figure II.2-1: SIF network extension reaches its maximum at 12 h p.i. in IFNγ-activated RAW264.7 LAMP1-GFP cells.
RAW264.7 cells expressing LAMP1-GFP (green) were infected with *Salmonella* Δ*sseF::aph* expressing SseF-2TEV-2M45 and mCherry (red). Cells were activated with IFNγ 24 h before infection. Continuous live cell imaging was performed every hour from four to 16 h p.i. Representative images of a *Salmonella*-infected cell demonstrate the extension and variation of the SIF network over time. Images are shown as maximum intensity projection. Scale bar: 10 µm.

At early time points of infection (4-5 h p.i.) only thin lysosomal tubular structures, typical for macrophages (Knapp and Swanson, 1990; Krieger *et al.*, 2014), were detectable (Figure II.2-1). They were substituted by thicker SIFs which expanded to large networks throughout the cell with huge vacuoles at 11-12 h p.i. In the late phase (14-16 h p.i.) cells detached from the surface and SIFs were only marginally detectable (Figure II.2-1).

Immunostaining was used to observe the translocation of the SPI2 effector SseF tagged with the epitope tag M45 in non-activated or IFNγ–stimulated RAW264.7 cells (Figure II.2-2). Non-activated and IFNγ–stimulated stably transfected RAW264.7 LAMP1-GFP cells were infected with the *S.* Typhimurium strain Δ*sseF::aph* [*sseF*::tev::tev::M45::M45] and constitutively expressed mCherry. Cells were fixed at 4, 8, 12 and 16 h p.i. and stained against epitope tag M45. An increase of SseF effector translocation could be observed over time in non-activated (Figure II.2-2A) as well as IFNγ–activated (Figure II.2-2B) cells. At 4 h p.i. SseF was not detectable under either condition, while at 8 h p.i. SseF was translocated in a subset of the infected cells. In contrast to non-activated cells, the amount of translocated SseF effector protein seems to be reduced in IFNγ–activated RAW264.7 cells. Maximal effector translocation was reached under both conditions at 12 h p.i. At later time points the amount of effector protein was further increased; however, the number of intact infected cells was significantly reduced. Co-localisation of tagged SseF with LAMP1 as a marker for the SMM was observable.

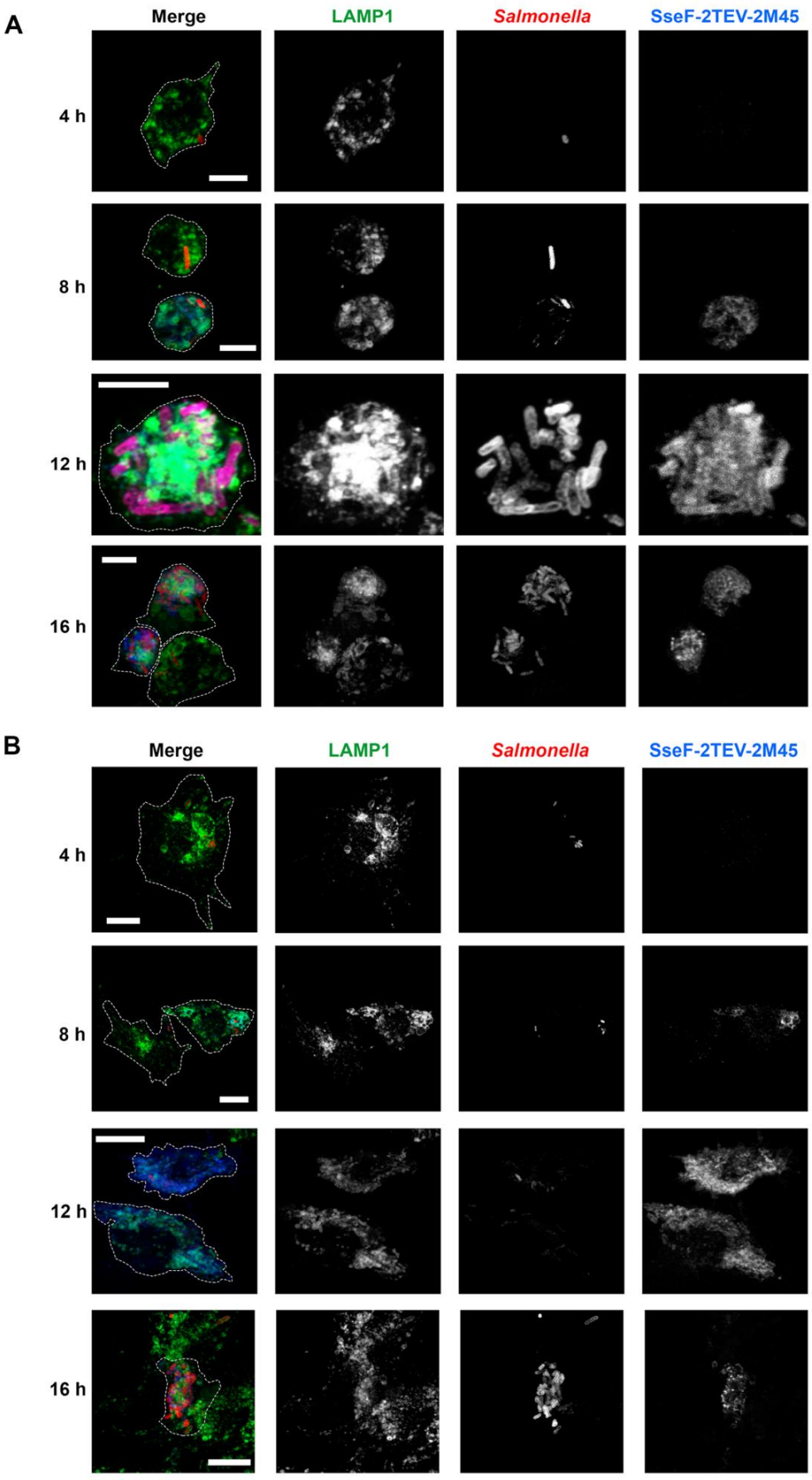
A
Merge
LAMP1
Salmonella
SseF-2TEV-2M45
4 h
8 h
12 h
16 h
B
Merge
LAMP1
Salmonella
SseF-2TEV-2M45
4 h
8 h
12 h
16 h

Figure II.2-2: Detection of translocated effector SseF-2TEV-2M45 in non-activated and IFNγ-stimulated RAW264.7 LAMP1-GFP cells at various time points p.i. by immunostaining.
Stably transfected RAW264.7 cells expressing LAMP1-GFP (green) were infected with S. Typhimurium Δ*sseF::aph* expressing SseF-2TEV-2M45 (blue) and mCherry (red), fixed 4, 8, 12, and 16 h p.i., and stained with anti-M45 antibody and anti-mouse-IgG-Cy5. (A) Non-activated cells were used for infection. Scale bar: 5 µm (B) Cells were IFNγ-activated 24 h before infection. Scale bar: 10 µm. Representative micrographs of each time point were chosen and images are shown as maximum intensity projection.

To quantify the amount of effector positive cells and the effector level of the cells, infected non-activated and activated RAW264.7 cells were fixed 4, 8, 12, and 16 h p.i., stained against the epitope tag M45 and subsequently analysed by flow cytometry (Figure II.2-3). At 4 h p.i. the amount of effector-positive cells was only around 2%. The rate increased to roughly 20% at 8 h p.i. and reached its maximum of around 30% at 12 h p.i., before it declined to roughly 20% at 16 h p.i. There were no significant differences between non-activated and activated RAW264.7. Measuring the relative fluorescence intensity (RFI) of the M45 signal enabled us to determine the relative amount of effector in proportion to M45-positive cells or all cells. The highest values for the M45-positive cells were obtained at 12 and 16 h p.i. (Figure S II.2-1A). In addition RFI measurement of the M45 signal in the complete cell population displayed the highest RFI values at 12 h p.i. (Figure S II.2-1B). Along with the results of live cell imaging and immunostaining, 12 h p.i. was chosen as optimal time for harvesting to generate cell material for the proteome profiling.

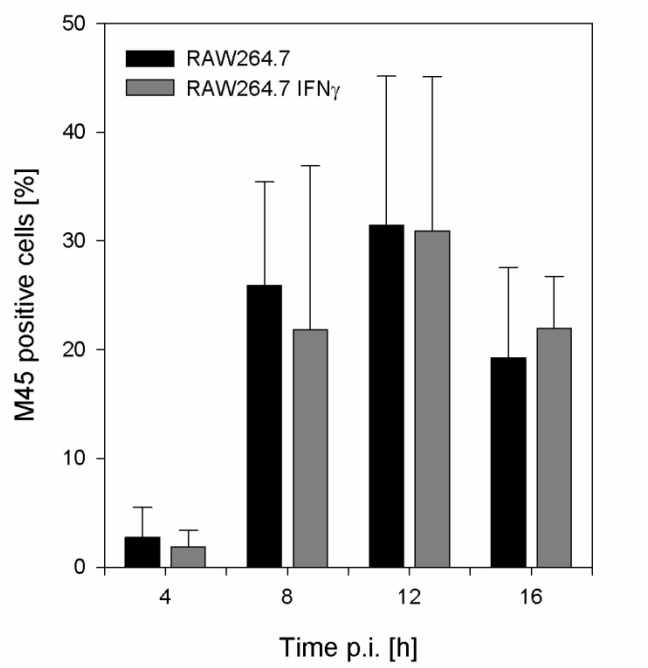

Figure II.2-3: Flow cytometry analyses indicate the highest amount of translocated effector protein positive cells at 12 h p.i.
RAW264.7 cells activated with IFNγ 24 h before infection or without activation were infected with *S.* Typhimurium Δ*sseF::aph* expressing SseF-2TEV-2M45, fixed at 4, 8, 12 and 16 h p.i., immunostained against SseF-2TEV-2M45 and analysed by flow cytometry. The percentage of cells positive for M45 at various time points p.i. is indicated. Shown are the mean values (+ standard deviation) of at least three independent experiments. The values of 4 and 12 h p.i. show a significant difference with $P < 0.05$ (Student's *t*-test).

II.2.3.2 Proteome analysis of *Salmonella*-modified membranes

To prepare cell material for MS-analysis ~1.6×10^8 RAW264.7 cells were infected with *S.* Typhimurium Δ*sseF::aph* [*sseF*::tev::tev::M45::M45] or *S.* Typhimurium *ssaV::*mTn*5* [*sseF*::tev::tev::M45::M45] as negative control and harvested at 12 h p.i. After homogenisation and enrichment of the SMM fraction the material was used for immunoprecipitation (IP). Western blots (Figure S II.2-2) demonstrated the availability of the tagged effector SseF-2TEV-2M45 in the SMM-enriched fraction subsequently used for IP and liquid-chromatography mass spectrometry (LC-MS/MS). We included only proteins as part of the SMM proteome, if the proteins were observed in at least two biological replicates. Altogether, 262 proteins were identified in the immune-precipitated fraction of infected non-activated RAW264.7 (Table S II.2-1) and 142 proteins of IFNγ-activated RAW264.7 cells (Table S II.2-2), respectively. All proteins that were observed in the negative control, IPs with cells infected with *S.* Typhimurium *ssaV::*mTn*5* [*sseF*::tev::tev::M45::M45], were eliminated from the SMM proteome. In total 239 proteins were therefore excluded (Table S II.2-3). Comparisons of the identified SMM proteomes

from non- and IFNγ–activated macrophages revealed a common set of 111 proteins (Table S II.2-4). Furthermore 151 proteins were specific for non-activated RAW264.7 cells (Table S II.2-5) and 31 proteins for activated RAW264.7 cells (Table S II.2-6), respectively (Figure II.2-4).

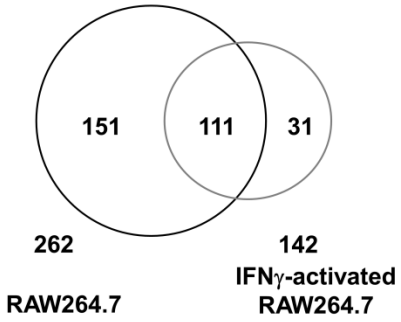

Figure II.2-4: SMM proteomes of resting and IFNγ–activated RAW264.7 cells.
Venn diagram shows proteins identified under both conditions, as well as proteins specific for resting or IFNγ-activated RAW264.7 cells.

The identified SMM proteomes were further grouped according their predicted or experimentally determined subcellular location or biological processes using the UniProt database (Dimmer *et al.*, 2012) or the PANTHER classification system (Mi *et al.*, 2013a; Mi *et al.*, 2013b). Both classification schemes show no drastic differences between SMM proteome of non- and IFNγ–activated RAW264.7 cells (Figure II.2-5A). Around 30% are membrane proteins. Similar percentages of proteins are localised at the cytoskeleton (approx. 15%), at vesicles (5%) and ribosomes (approx. 15%). Differences between non- and IFNγ–activated RAW264.7 cells are obvious with proteins of endo-/lysosomal origin (8% of the proteins in non-activated RAW264.7 cells to 1% in activated cells) and ER-associated ones (11% of proteins from IFNγ–activated cells, to 3% of non-activated cells). The percentage of nucleus associated proteins is higher in IFNγ–activated cells (31%) in comparison to non-activated cells (23%), whereas the percentage of proteins with mitochondrial origin is higher in non-activated (23%) macrophages to activated (14%) cells.

Classification according to GO biological processes (Figure II.2-5B; descriptions of gene ontologys (GOs) are listed in II.2.5) revealed that most proteins are involved in metabolic processes (58/54/60% of the identified proteins; non-activated, activated as well as overlap, respectively), mainly protein metabolism (32/30/37%). Regulation of biological processes and cellular component organization are represented with about 12% or 15%, respectively. Furthermore, cytoskeletal (11/15/17%) and transport-related components

(20/16/15%), especially vesicle-mediated transport (10/9/9%), were identified. Only 2% of the proteins play a role in apoptotic processes.

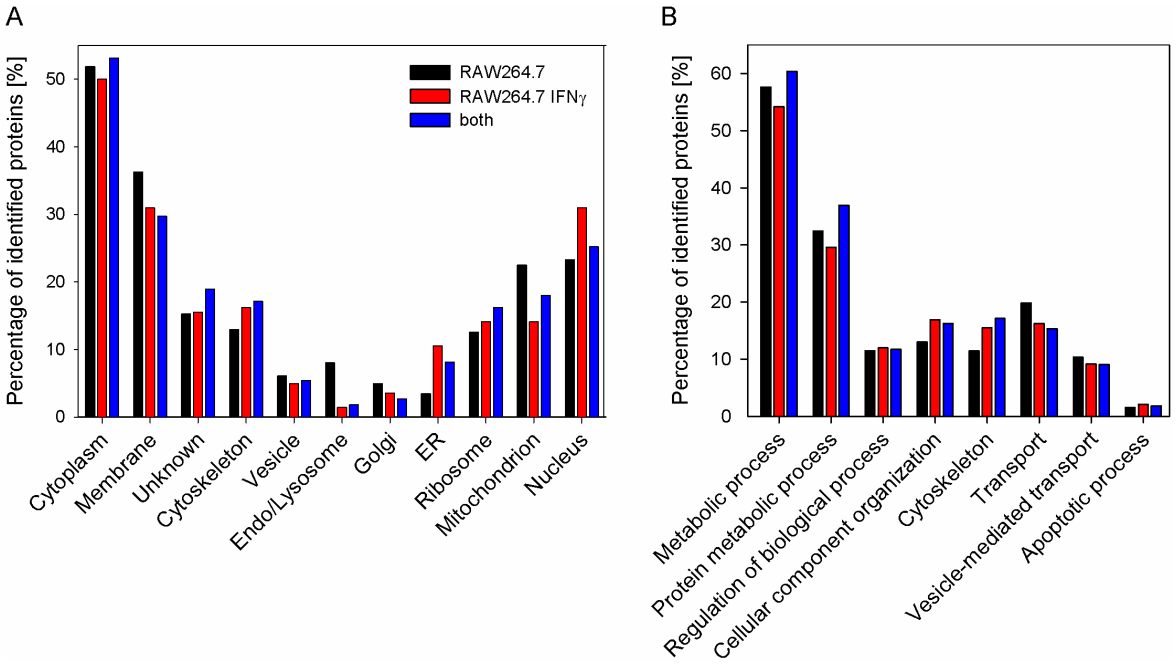

Figure II.2-5: Subcellular localization and biological processes of SMM proteins.
SMM proteins originating from RAW264.7, IFNγ-activated RAW264.7, and both were classified according to subcellular localization (A) and GO biological processes (B) referring to UniProt or PANTHER. Proteins can be grouped in more than one category due to their annotations, not all proteins could be annotated via PANTHER.

Proteins of endo-/lysosomal origin as well as Golgi apparatus are known to be components of the *Salmonella*-modified membranes or participate in their formation like proteins of cytoskeleton (Brumell *et al.*, 2001b; Garcia-del Portillo *et al.*, 1993; Meresse *et al.*, 2001; Mota *et al.*, 2009). In Table II.2-1 promising MS-identified proteins are listed which are associated with transport, trafficking, Golgi/ER, or cytoskeleton. Numerous elements of the cytoskeleton are part of the SMM proteome, e.g. proteins involved in actin crosslinking (α-actinin-1/4) and formation of actin filaments (Arp2/3, Arp2/3 complex 1B, 2-5), or microtubules (tubulin α-1A, tubulin β-4B/5) and myosin motor proteins (myosin regulatory light chain 12B, myosin-9, myosin light polypeptide 6). Many proteins of the endocytic and secretory vesicle transport were identified, including five distinct Rab GTPases (Rab1b, Rab5c, Rab8b, Rab14, and Rab18), proteins involved in the COPI-mediated pathway (α/β/β2/Δ/ε/γ1-COP) or vATPases subunits A and B2. Additionally, Golgi and ER proteins are detected which contribute to the protein transport between these organelles (e.g. Sec22b, Sec61-α1/β, p24δ1, VAP-A, Arf1/3, SNAP-α).

The identification of IFNγ-induced proteins as well as proteins of the major histocompatibility antigen (MHC) class I and II and the antigen peptide transporter was

prevalently possible in activated macrophages (Table S II.2-1/2). The proteins β_2-microglobulin, interferon-activatable protein 202 (Ifi202), interferon-inducible GTPase1, antigen peptide transporter (APT1), H2 class I histocompatibility antigen, D-D and L-D α chain, and γ chain were only detectable in the samples of IFNγ-activated cells. However, only the interferon-induced transmembrane protein 3 (IFITM3) was shown in both, non-activated and activated macrophages.

Moreover, proteins involved in protein translation such as eukaryotic translation initiation factors, ribosomal proteins, t-RNA ligase, translation regulation, modification as well as folding (T-complex protein 1, TCP-1-$\alpha/\beta/\Delta/\varepsilon/\gamma/\eta/\theta/\zeta$) were detected (Table S II.2-1/2).

In addition, mitochondrial proteins, for instance ATP synthase and NADH dehydrogenases subunits, were identified in untreated cells whereas cytochrome c oxidase subunits were detected in both samples (Table S II.2-1/2).

Table II.2-1: Selected *Salmonella*-modified membrane proteins of non- and IFNγ-stimulated RAW264.7 cells identified by MS[a]

Name (*_MOUSE)	Protein description	Localization	Detection in RAW264.7	RAW264.7 IFNγ	both
GTPases and associated proteins					
RAB1B	Ras-related protein Rab1b	Vesicle		×	
RAB5C	Ras-related protein Rab5c	Vesicle, cell membrane, EE	×	×	×
RAB8B	Ras-related protein Rab8b	Vesicle, cell membrane, phagosome	×		
RAB14	Ras-related protein Rab14	Vesicle, RE, EE	×		
RAB18	Ras-related protein Rab18	Cell membrane	×		
RAC1	Ras related C3 botulinum toxin substrate 1	Cell membrane, EE	×		
RAP1B	Ras-related protein Rap1b	Cell membrane, cell junction	×	×	×
G3BP1	Ras GTPase-activating protein-binding protein 1	Cytoplasm	×	×	×
G3BP2	Ras GTPase-activating protein-binding protein 2	Cytoplasm	×		
Cytoskeleton					
ACTN1	α-actinin-1	Cytoskeleton, cytoplasm, cell junction	×		
ACTN4	α-actinin-4	Nucleus, cytoplasm, cell junction	×	×	×
ACTS	Actin, α skeletal muscle	Cytoplasm, cytoskeleton		×	
ACTZ	α-centractin	Cytoplasm, cytoskeleton		×	
ARC1B	Actin-related protein2/3 complex subunit 1b	Cytoplasm, cytoskeleton	×	×	×
ARP2	Actin-related protein 2	Cytoplasm, cytoskeleton	×	×	×
ARP3	Actin-related protein 3	Cytoplasm, cytoskeleton	×	×	×
ARPC2	Actin-related protein2/3 complex subunit 2	Cytoplasm, cytoskeleton	×		
ARPC3	Actin-related protein2/3 complex subunit 3	Cytoplasm, cytoskeleton	×	×	×
ARPC4	Actin-related protein2/3 complex subunit 4	Cytoplasm, cytoskeleton	×	×	×
ARPC5	Actin-related protein2/3 complex subunit 5	Cytoplasm, cytoskeleton	×	×	×
CALM	Calmodulin	Cytoplasm, cytoskeleton, spindle	×	×	×
CAP1	Adenylat cyclase-associated protein 1	PM, cytoskeleton	×	×	×
CAPZB	F-actin-capping protein subunit β	Cytoplasm, cytoskeleton	×	×	×
CAZA2	F-actin-capping protein subunit α2	Cytoplasm, cytoskeleton	×	×	×
CKAP4	Cytoskeleton-associated protein 4	ER, cytoplasm, cytoskeleton	×		
COR1A	Coronin-1A	Cytoplasm, cytoskeleton	×	×	×
COF1	Cofilin-1	Nucleus, cytoplasm, cytoskeleton	×		
DC1I2	Cytoplasmic dynein 1 intermediate chain 2	Cytoplasm, cytoskeleton	×		
DCTN2	Dynactin subunit 2	Cytoplasm, cytoskeleton		×	
K2C8	Keratin, type II cytoskeletal 8	Cytoplasm	×		
MARE1	Microtubule-associated protein rP/EP family member 1	Cytoplasm, cytoskeleton	×		
ML12B	Myosin regulatory light chain 12B	Cytoplasm, cytoskeleton	×		
MYH9	Myosin-9	Cytoplasm, cytoskeleton	×	×	×
MYL6	Myosin light polypeptide 6	Cytoplasm, cytoskeleton	×	×	×
PKHO2	Pleckstrin homology domain-containing family O member 2	Cytoplasm, cytoskeleton, cell junctions	×	×	×
PLEC	Plectin	Cytoplasm, cytoskeleton	×	×	×
PLEK	Pleckstrin	Cytoplasm, cytoskeleton	×		
PLSL	Plastin-2	Cytoplasm, cytoskeleton	×	×	×
RADI	Radixin	Cell membrane, cytoskeleton		×	
SEP11	Septin-11	Cell membrane, cytoskeleton	×		
SEPT2	Septin-2	Cell membrane, cytoskeleton	×		
TBA1A	Tubulin α-1A chain	Cytoplasm, cytoskeleton	×		
TBB4B	Tubulin β-4B	Cytoplasm, cytoskeleton	×		
TBB5	Tubulin β-5	Cytoplasm, cytoskeleton	×	×	×
TLN1	Talin-1	Cell projection, ruffle membrane	×		
VASP	Vasodilator-stimulated phosphoprotein	Cytoplasm, cytoskeleton	×		
Endocytic and secretory vesicle transport					
AP2B1	AP-2 complex subunit β	Coated pit, PM	×		
ARF1	ADP-ribosylation factor 1 (ARF1)	Golgi, cytoplasm, perinuclear region	×		
ARF3	ADP-ribosylation factor 3 (ARF3)	Golgi, vesicle		×	
CLH1	Clathrin heavy chain 1	Vesicle, coated pit	×	×	×
COPA	Coatomer subunit α	COPI-coated vesicle, Golgi	×	×	×
COPB2	Coatomer subunit β'	COPI-coated vesicle, Golgi	×		
COPB	Coatomer subunit β	COPI-coated vesicle, Golgi, ERGIC	×		
COPD	Coatomer subunit Δ	COPI-coated vesicle, Golgi	×		
COPE	Coatomer subunit ε	COPI-coated vesicle, Golgi	×	×	×

Name (*_MOUSE)	Protein description	Localization	Detection in RAW264.7	RAW264.7 IFNγ	both
COPG1	Coatomer subunit γ-1	COPI-coated vesicle, Golgi	×		
EHD1	EH domain-containing protein 1	RE, EE, PM	×		
EHD4	EH domain-containing protein 4	RE, EE, PM	×		
IFM3	Interferon-induced transmembrane protein 3	Cell membrane, LE, lysosome	×	×	×
NSF	Vesicle-fusing ATPase	Cytoplasm, Golgi, LE, PM	×		
SC22B	Vesicle-trafficking protein Sec22b	ER, ERGIC, Golgi	×		
SSNA	α-soluble NSF attachment protein (SNAP-α)	Cell membrane			
TMEDA	Transmembrane emp24 domain-containing protein 10 (p24δ1)	ER, Golgi, vesicle	×		
VAPA	Vesicle-associated membrane protein-associated protein A (VAP-A)	ER, Golgi, vesicle	×	×	×
VATA	V-type proton ATPase catalytic subunit A	Cytoplasm, LE, mitochondrion, PM	×		
VATB2	V-type proton ATPase subunit B, brain isoform	Cytoplasm, LE	×		
VPS35	Vacuolar protein sorting-associated protein 35 (VPS35)	Cytoplasm, LE, EE	×		
Endoplasmic reticulum and Golgi					
AT2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	ER	×		
EMC2	ER membrane protein complex subunit 2	Nucleus, ER, cytoplasm	×		
ESYT1	Extended synaptotgmin-1	ER		×	
HYOU1	Hypoxia up-regulated protein 1 (GRP-170)	ER	×		
IIGP1	Interferon-induced GTPase 1	ER, Golgi		×	
IRGM1	Immunity-related GTPase family M protein	Golgi, vesicle, LE	×		
LYRIC	Protein LYRIC	ER, nucleus, cytoplasm	×		
PDIA4	Protein disulphide-isomerase A4	ER		×	
RSAD2	Radical S-adenosyl methionine domain-containing protein 2	ER		×	
RTN4	Reticulon-4	ER	×	×	×
S61A1	Protein transport protein Sec61 subunit α isoform 1	ER	×		
SC61B	Protein transport protein Sec61 subunit β	ER	×	×	×
SQSTM	Sequestosome-1 (STONE14) (Ubiquitin-binding protein p62)	Cytosol, ER, LE, nucleus, lysosome	×		
SSRD	Translocon-associated protein subunit Δ	ER	×	×	×
TAP1	Antigen peptide transporter 1 (APT1)	ER		×	

[a]Full lists of proteins identified by mass spectrometry are catalogued in Table S II.2-1 (infected RAW264.7) or Table S II.2-2 (infected and IFNγ-activated RAW264.7).
(EE, early endosome; ER, endoplasmic reticulum; RE, recycling endosome; PM, plasma membrane; LE, late endosome)

II.2.4 Discussion

How *Salmonella* is able to remodel the host endosomal system to establish a niche which allows survival and replication inside host cell, especially inside its natural enemy, the macrophage, is partly understood (Buchmeier and Heffron, 1989; Haraga *et al.*, 2008; Langermans *et al.*, 1991; LaRock *et al.*, 2015; Malik-Kale *et al.*, 2011). It is known that the biogenesis of this replication niche is bound to the formation of an extensive tubular membrane network (Liss and Hensel, 2015; Schroeder *et al.*, 2011), requiring supply of host membrane material. Identification of host components of this compartment was until recently, mainly executed by fluorescence imaging (immunostaining, GFP-fusions) (Brumell *et al.*, 2001a; Drecktrah *et al.*, 2007; Garcia-del Portillo and Finlay, 1995; Rathman *et al.*, 1996; Smith *et al.*, 2005; Smith *et al.*, 2007; Steele-Mortimer *et al.*, 1999). In our previous study (Chapter II.1) we elucidated the general composition of those membrane structures modified by *Salmonella* by using a systematic proteomic approach. We identified components of the cytoskeleton, host endosomal/recycling pathway, and intracellular host membrane trafficking (Vorwerk *et al.*, 2015). Remarkably, we showed the interaction and recruitment of endoplasmic reticulum components to those SMM networks, which strengthens the ER involvement during *Salmonella's* niche establishment.

In this study we identified 15 proteins involved in Golgi to ER transport (Table II.2-1). Manipulation of ER-Golgi trafficking would allow *Salmonella* to gain membrane material for the establishment of a *Salmonella*-induced tubular membrane network. SISTs (*Salmonella*-induced SCAMP3 tubules), as part of this tubular network, are an example for the recruitment of TGN-derived membrane material containing a *post*-Golgi protein SCAMP3 (Mota *et al.*, 2009). In agreement with our previous work (Vorwerk *et al.*, 2015) we were able to identify several ER and Golgi proteins (Table II.2-1) and a considerable amount of proteins involved in anterograde as well retrograde ER-Golgi vesicle transport as part of the macrophage SMM proteome (Table II.2-1). Proteins of the COPI-pathway (α/β/β2/Δ/ε/γ1-COP) and other proteins involved in ER-Golgi trafficking (Sec22b, Sec61 α1/β, p24δ1, VAP-A, Arf1/3, SNAP-α) seems to be hijacked by *Salmonella*. A contribution of ER membranes to phagocytosis in macrophages seems to be an important aspect for the biogenesis of the SCV (Gagnon *et al.*, 2002; Knodler and Steele-Mortimer, 2003; Santos *et al.*, 2015) but the responsible components have to be elucidated in more detail.

One interesting candidate is the v-SNARE (soluble *N*-ethylmaleimide-sensitive factor [NSF] attachment protein receptor) Sec22b. It is part of the anterograde and retrograde vesicular transport between the ER and the Golgi and is located on vesicular structures in the periphery as well as Golgi and ERGIC (Zhang *et al.*, 1999). Sec22b is involved in membrane supply from the ER to the plasma membrane during phagocytosis in

macrophages (Becker *et al.*, 2005) as a negative regulator (Hatsuzawa *et al.*, 2009). This SNARE protein is also important for the biogenesis of the *Legionella*-containing vacuole (Derre and Isberg, 2004; Kagan *et al.*, 2004). It has been shown that Sec22b interacts with NSF on LCVs. The authors assumed that *Legionella* stimulates endogenous pathways to promote the fusion of ER-derived vesicles with phagosomes by enhancing interaction between Sec22b and plasma membrane localized t-SNAREs whereby Sec22b interacts with NSF (Arasaki and Roy, 2010; Arasaki *et al.*, 2012). NSF was also identified in our SMM proteome (Table II.2-1). Additionally, Sec22b is important for the biogenesis of the *Leishmania* parasitophorous vacuole, an eukaryotic intracellular parasite (Canton *et al.*, 2012; Ndjamen *et al.*, 2010). Another interesting candidate is Sec61, responsible for protein transport into the ER (Dudek *et al.*, 2015; Park and Rapoport, 2012). Sec61β was identified at the ER-derived *Brucella*-containing vacuole (de Bolle *et al.*, 2012; Pizarro-Cerda *et al.*, 1998).

Again many proteins of the host cytoskeleton and membrane trafficking pathways were identified (Table II.2-1). Although the majority of identified proteins are not known to be linked with *Salmonella* infection, several of these proteins were characterized in *Salmonella* host interaction processes, e.g. α-actinin (Finlay *et al.*, 1991), Arp2/3 (Criss and Casanova, 2003; Hänisch *et al.*, 2010; Hänisch *et al.*, 2011; Unsworth *et al.*, 2004; Shi *et al.*, 2005), dynein (Abrahams *et al.*, 2006; Boucrot *et al.*, 2005; Domingues *et al.*, 2014; Guignot *et al.*, 2004; Harrison *et al.*, 2004; Marsman *et al.*, 2004; Ramsden *et al.*, 2007b; Ramsden *et al.*, 2007a), Rac1 (Shi *et al.*, 2005), coatomer I protein (Misselwitz *et al.*, 2011), cofilin (Dai *et al.*, 2004; McGhie *et al.*, 2004), myosin II (Hänisch *et al.*, 2011; Wasylnka *et al.*, 2008), *N*-ethylmaleimide-sensitive fusion protein (Mukherjee *et al.*, 2000), ubiquitin-binding protein p62 (Tattoli *et al.*, 2012), α- and β-tubulin (Abrahams *et al.*, 2006; Brumell *et al.*, 2002a; Finlay *et al.*, 1991; Guignot *et al.*, 2004; Rajashekar and Hensel, 2011), and vATPase (Steele-Mortimer *et al.*, 1999).

All identified Rab GTPases (Rab1, Rab5c, Rab8, Rab14, and Rab18; Table II.2-1) are known to localize at *Salmonella*-containing vacuoles or *Salmonella*-modified membranes (Brumell and Scidmore, 2007; Hashim *et al.*, 2000; Kuijl *et al.*, 2013; Madan *et al.*, 2012; Smith *et al.*, 2007). In particular, Rab1b is an interesting candidate for further research. Rab1b was only identified in the SMM proteome of IFNγ-activated RAW264.7 cells. This isoform plays a well-defined role in ER-to-Golgi protein transport and is involved in COPI and COPII vesicle formation and trafficking (Garcia *et al.*, 2011). Rab1 is important for the LCV biogenesis (Kagan *et al.*, 2004) and it was shown that *Legionella* protein DrrA (SidM) constitutively active Rab1b by adenosine monophosphorylation (AMPylation) (Müller *et al.*, 2010). Rab1b is also recruited to the *Coxiella*-replicative vacuole at later infection times (Campoy *et al.*, 2011). Additionally, Ypt1, the yeast homolog of Rab1, is required for

autophagy in yeast which is also discussed for mammalian Rab1 (Ao *et al.*, 2014; Chua *et al.*, 2011; Lynch-Day *et al.*, 2010; Zoppino *et al.*, 2010). Especially in context of *Salmonella* Rab1 seems to be an important factor, since it contributes to autophagy and a knockdown decreases replication of *Salmonella* in HeLa cells (Huang *et al.*, 2011). Furthermore, it has been recently shown that autophagy facilities cytosolic replication of *Salmonella* (also called hyper-replication) (Yu *et al.*, 2014). Thus the role of Rab1, and especially of its isoform Rab1b, has to be elucidated in more detail for *Salmonella* replicating in the SCV or in the cytosol and, in particular, for its function in activated macrophages.

To find differences or similarities between the newly identified proteome of *Salmonella*-modified membranes of macrophages to previous results (Chapter II.1) from the proteome of the SMM of the epithelial HeLa cell line (Vorwerk *et al.*, 2015) we compared SMM proteomes of non-activated macrophages (Table S II.2-1) and HeLa. We found 19% (46 proteins) identical RAW and HeLa SMM proteins (Table S II.2-7). The distribution of subcellular localization and biological processes is similar to the previous analysed proteomes. However, 31% of the overlapping proteins are involved in transport processes whereas 22% are in vesicle-mediated transport (Figure II.2-6B). Furthermore, we compared the SMM proteome with the published *Legionella*-containing vacuole (LCV) proteome obtained from RAW264.7 cells (Hoffmann *et al.*, 2014). Here, we observed an overlap of 87 proteins, representing 58% of SMM proteins and 10% of the LCV proteome (Table S II.2-8).

The overlap of SMM proteomes (RAW264.7, HeLa) and LCV (RAW264.7) is of special interest. It consists of 32 proteins grouped according to their function in cytoskeleton and adhesion proteins, metabolism, protein metabolism, and trafficking (Table II.2-2). 59% of the identical proteins are membrane proteins (Figure II.2-6A). 34% of the proteins are of mitochondrial origin, 12% Golgi, 15% ER and 6% nucleus components. Classified according biological processes, 55% of proteins are involved in metabolic processes and 34% are transport-associated (Figure II.2-6B). In particular components of the vesicle mediated transport (25%) seem to be prominent in the common host compartment proteome suggesting partially similar hijacking strategies of both pathogens.

Table II.2-2: Proteins commonly identified in the proteomes of the LCV from RAW264.7 macrophages and SMM from HeLa cells and RAW264.7 macrophages.

Name	Description	Localization
Cytoskeleton and adhesion protein		
ANXA1	Annexin A1	Nucleus, cytoplasm, cell projection, cilium
ARPC4	Actin-related protein 2/3 complex subunit 4	Cytoplasm, cytoskeleton, cell projection
CAPZB	F-actin-capping protein subunit β	Cytoplasm, cytoskeleton
MYH9	Myosin-9	Cytoplasm, cytoskeleton
Metabolism		
AT5F1	ATP synthase F_0 complex subunit B1, mitochondrial	Mitochondrion
ATP5H	ATP synthase subunit d, mitochondrial	Mitochondrion
ATPG	ATP synthase subunit γ, mitochondrial	Mitochondrion
ATPO	ATP synthase subunit O, mitochondrial	Mitochondrion
C1TM	Monofunctional C1-tetrahydrofolate synthase, mitochondrial	Mitochondrion
NDUS1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	Mitochondrion
NDUS3	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	Mitochondrion
TXTP	Tricarboxylate transport protein, mitochondrial	Mitochondrion
Protein metabolism		
EF1B	Elongation factor 1-β	Unknown
EF1G	Elongation factor 1-γ	Unknown
HYOU1	Hypoxia up-regulated protein 1 (GRP-170)	ER
RM19	39S ribosomal protein L19, mitochondrial	Mitochondrion
RM48	39S ribosomal protein L48, mitochondrial	Mitochondrion
RPN2	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2	ER
SRPR	Signal recognition particle receptor subunit α	ER
SYRC	Arginine-tRNA ligase, cytoplasmic	Cytoplasm
TCPE	T-complex protein 1 subunit ε (TCP-1-ε)	Cytoplasm
TCPG	T-complex protein 1 subunit γ (TCP-1-γ)	Cytoplasm
TCPZ	T-complex protein 1 subunit ζ (TCP-1-γ)	Cytoplasm
TRAP1	Heat shock protein 75 kDa, mitochondrial	Mitochondrion
Trafficking		
COPA	Coatomer subunit α	Cytoplasm, Golgi, COPI-vesicle
COPG1	Coatomer subunit γ-1	Cytoplasm, Golgi, COPI-vesicle
EHD4	EH domain-containing protein 4	EE, RE
RAB14	Ras-related protein Rab14	EE, RE, Golgi
RAB5C	Ras-related protein Rab5c	Cell membrane, EE
SNAA	α-soluble NSF attachment protein (SNAP-α)	Membrane
TMEDA	Transmembrane emp24 domain-containing protein 10 (p24δ1)	ER, ERGIC, Golgi
VAPA	Vesicle-associated membrane protein-associated protein A (VAP-A)	ER

(EE, early endosome; ERGIC, ER-Golgi intermediate compartment; ER, endoplasmic reticulum; COPI, coat protein complex I)

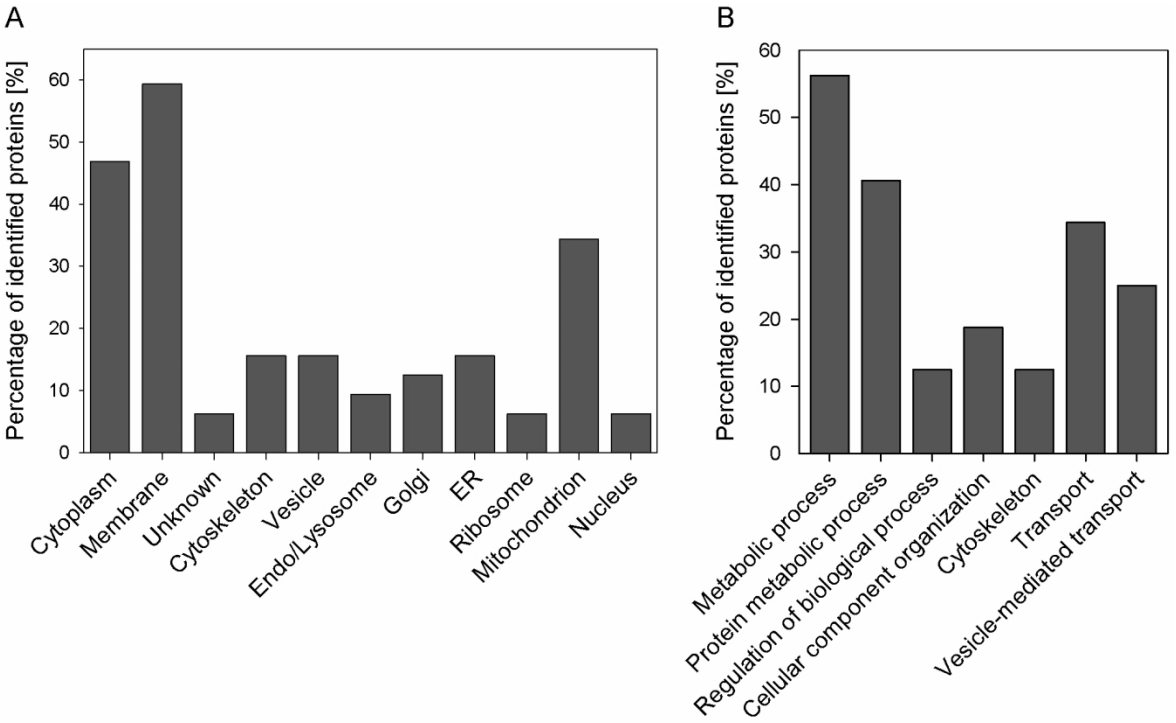

Figure II.2-6: Subcellular location and biological processes of proteins identified in common for the *Salmonella*-modified membrane proteome (RAW264.7/HeLa) and the *Legionella*-containing vacuole proteome (RAW264.7).
Proteins were classified according to UniProt (A) or PANTHER (B). Proteins can be assigned in more than one category.

Surprisingly, a high percentage of proteins is involved in metabolic processes (50%, especially protein metabolic processes with more than 30%) or they are proteins of mitochondrial and nucleus origin (<20%) in the macrophage SMM proteome (Figure II.2-5), as well as in the overlapping proteome of the HeLa SMM (Figure II.2-6). Likewise, this is comparable with values of the LCV proteome (Hoffmann *et al.*, 2014). It was assumed that LCVs are possibly associated with mitochondria (Finsel and Hilbi, 2015). A similar percentage of metabolic proteins were classified in microtubule-associated proteins during macrophage activation (Patel *et al.*, 2009). The authors explained this by contribution of microtubules in translation since microtubules serve as platform for mRNA translation and transport of mRNA transcripts to their cellular location (Hovland *et al.*, 1996; Litman *et al.*, 1994; Parton *et al.*, 2014; Patel *et al.*, 2009; Wickham *et al.*, 1999). This might be also possible for the complex network of SMM stabilized by cytoskeleton (Abrahams *et al.*, 2006; Harrison *et al.*, 2004; Meresse *et al.*, 2001; Rajashekar and Hensel, 2011).

Besides *Legionella* the identification of other metabolic proteins (ATP synthase β-chain and NADH-ubiquinone oxidoreductase) was also possible in *Francisella tularensis*-containing vacuoles (Kovářová *et al.*, 2002) or in the proteome of purified phagosomes originated from J744 mouse macrophage-like cells (ATP synthase) (Garin *et al.*, 2001).

Thus it seems that these representing cellular proteins can be accumulated from phagosomes during trafficking and are not simple contamination (Kovářová *et al.*, 2002). The presence of the T-complex proteins in the overlapping proteome might be explained by the function of the complex. It mediates as a chaperonin folding of actin and tubulin (Chen *et al.*, 1994; Kubota *et al.*, 1995; Spiess *et al.*, 2004; Sternlicht *et al.*, 1993; Ursic and Culbertson, 1991; Vinh and Drubin, 1994) and is located in the *trans*-Golgi (Willison *et al.*, 1989). The cytoskeleton plays an important role for *Salmonella* (Abrahams *et al.*, 2006; Knodler and Steele-Mortimer, 2003; Rajashekar and Hensel, 2011) as well as for *Legionella* (Finsel and Hilbi, 2015) during the establishment of their compartment in the cell. Therefore the isolation of the components of the cytoskeleton such as actin or tubulin might be connected with the co-purification of their chaperonin or have additional unknown functions. Additionally, it has been shown that the *Salmonella* effector SipB, induces autophagy by disturbing mitochondria (Hernandez *et al.*, 2003) which could contribute to the high percentage of mitochondrial proteins in SMM proteome.

Proteins involved in trafficking and commonly identified should be in special focus for further investigations. Proteins of the COPI vesicle (α/γ-COP), the EH-domain-containing protein 4, the Rab GTPases Rab5c and Rab14, the α-soluble NSF attachment protein (SNAP-α), the transmembrane emp24 domain-containing protein 10 (p24δ1), and the vesicle-associated membrane protein-associated protein A (VAP-A) offer possible explanations for general strategies used by vacuolar pathogens to survive inside the host cell.

A further aim of this study was to analyse the effect of IFNγ-activation on the proteome of *Salmonella*-modified membranes. Considering the subcellular localization of the identified proteins of the SMM a lower amount of mitochondrial proteins in activated macrophages was shown (Figure II.2-5) which could be explained by the anaerobic metabolic shift toward the anaerobic glycolytic pathway providing rapid energy and biosynthetic precursors for synthesis of pro-inflammatory proteins (Zhu *et al.*, 2015). Additionally, a shift from endo-/lysosomal to ER proteins was obvious (Figure II.2-5). The percentage of ER-associated proteins is higher and the amounts of endo-/lysosomal and mitochondrial proteins are lower in the SMM proteome of IFNγ–activated cells in comparison to non-activated macrophages. Apparently, the ER gains in importance as origin for SMM in contrast to the endo-/lysosomal system in activated macrophages. The IFNγ-induced process of cytokine production and antigen presentation might be an additional protein trafficking route which is hijacked by *Salmonella* to obtain membrane material for the SMMs. This would also explain the large number of IFNγ-induced proteins

(β_2-microglobulin, Ifi202, interferon-inducible GTPase1, APT1, H2 class I histocompatibility antigen, D-D and L-D α chain and γ chain) identified in IFNγ-activated macrophages.

With only one exception, the IFN-induced transmembrane protein 3 (IFITM3), proteins involved in IFNγ-response are predominantly detected in activated macrophages. IFITM3 was detected in both, non-activated and activated macrophages. IFITM3 has fascinating functions as membrane organizer influencing clathrin and v-ATPase during the innate immune response (Wee *et al.*, 2012) which also could be important for SCV maturation. The IFN-inducible GTPase1 (IIGP1 or IRGA6) was identified only in activated macrophages (Table S II.2-2). These immunity-related GTPases (IRG, also known as p47 GTPases) seem to be crucial factors in regulation of intracellular pathogen survival in IFNγ-activated host cells and is recruited from the ER and Golgi to the PCVs of various pathogens (Taylor, 2007). IIGP1 is an important resistance factor against *Chlamydia trachomatis*, because accumulation at the inclusion triggers rerouting and lysosomal degradation (Al-Zeer *et al.*, 2009). Similar effects have been shown with the protozoan parasite *Toxoplasma gondii* (Lubitz *et al.*, 2013; Martens *et al.*, 2005). IIGP1 plays a role in *Listeria monocytogenes* infections (Zerrahn *et al.*, 2002) and *Brucella abortus* activates expression of IRGs as IGP1 via type-I IFN secretion at late time points of infection, when *Brucella* has established its replication niche (Lapaque *et al.*, 2009). This GTPase could also be involved in SCV maturation in IFNγ-activated macrophages and is one interesting candidate for further investigations.

Interestingly, a delay of about four hours in effector translocation for IFNγ-activated macrophages in comparison to non-treated cells was detectable which was monitored by immunostaining and FACS analysis (Figure II.2-1, Figure II.2-2, Figure II.2-3). This could be connected with a delay in phagosomal maturation in activated macrophages which was observed through phagosomal proteome analysis of IFNγ-activated macrophages (Jutras *et al.*, 2008; Trost *et al.*, 2009). The authors assume that a delay in lysosomal proteolytic activities maximizes epitope generation and antigen presentation (Trost *et al.*, 2009). How this delay effects SCV biogenesis and *Salmonella* replication has to be investigated in activated macrophages. A delay in the maturation process of the SCV could lead to a retarded exchange of early to late endosomal/lysosomal marker proteins. In untreated RAW264.7 cells the segregation of the SCV takes places between 15 min and four hours and depends on bacterial protein biosynthesis (Garvis *et al.*, 2001; Holden, 2002). How this process of SCV biogenesis is changed in comparison to untreated macrophages and how the delayed phagosomal maturation affect the expression of effectors which are necessary for the establishment of the SCV (Cirillo *et al.*, 1998; Pfeifer *et al.*, 1999) should be analysed. Especially the influence of a possible decelerated acidification on the

expression and secretion of SPI2 effectors in IFNγ-treated macrophages might be important for the survival of intracellular *Salmonella* (Beuzon *et al.*, 1999; Deiwick and Hensel, 1999; Nikolaus *et al.*, 2001). Additionally, the inhibition of vacuolar acidification in IFNγ-treated RAW264.7, e.g. by effect of bafilomycin (Rathman *et al.*, 1997) might give hints to clear the influence on effector expression and replication changes in IFNγ-treated cells. Furthermore, it has been shown that IFNγ promotes stabilization of microtubules in macrophages (Binker *et al.*, 2007; Khandani *et al.*, 2007; Patel *et al.*, 2009). Stable microtubules in activated macrophages are important for cell spreading as well as binding of large particles (Binker *et al.*, 2007). This could also be important for *Salmonella* uptake or SCV biogenesis.

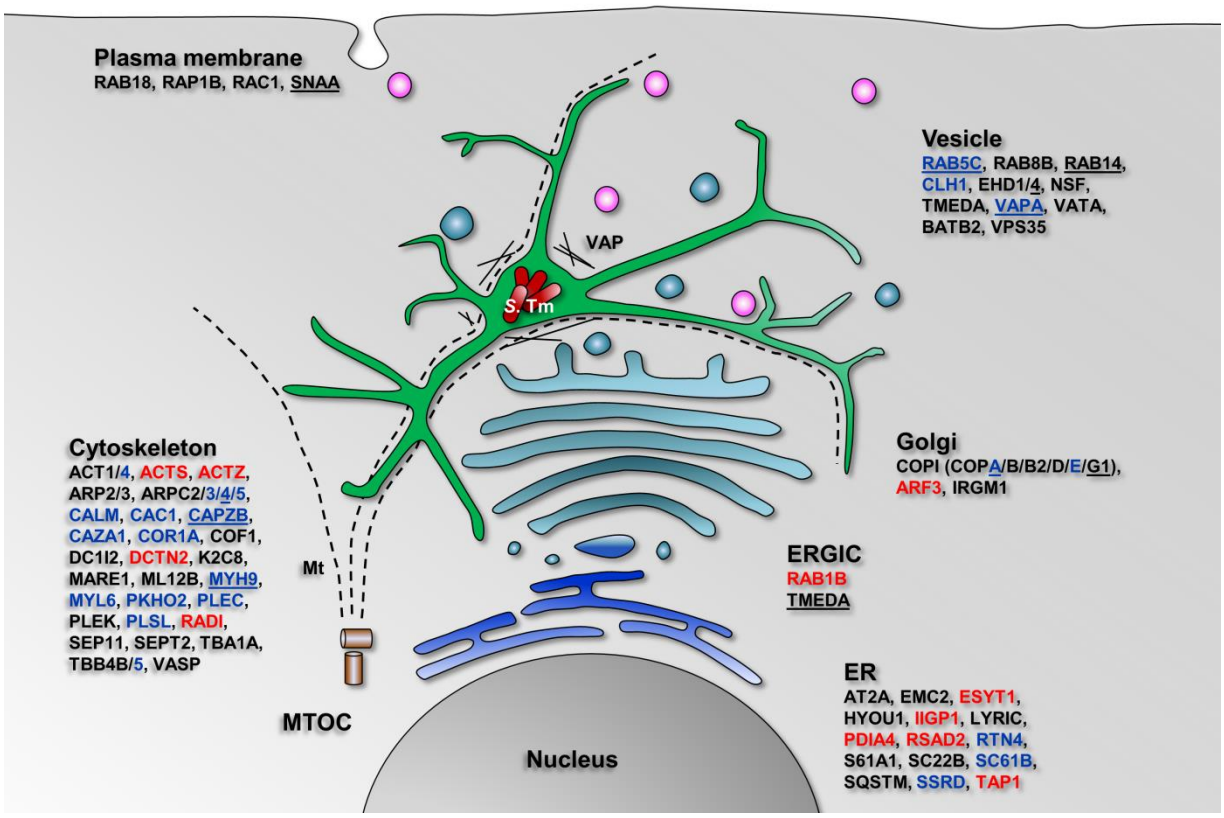

Figure II.2-7: Model for the origin of SMM proteins in non-activated and IFNγ-stimulated RAW264.7 cells.
S. Typhimurium recruits host proteins of the endocytic and secretory vesicle transport as well as cytoskeletal proteins to the SMM network. Additionally, proteins of the ER, ERGIC and Golgi contribute to formation of SMMs. Proteins identified in non-activated cells are shown in black, identified in IFNγ-stimulated cells are red and detected in both are blue. Underlined protein names in the overlap of proteome of the LCV from RAW264.7 macrophages and SMM from HeLa cells and RAW264.7 macrophages. *S*. Tm, *Salmonella* Typhimurium; MTOC, microtubule-organizing centre; Mt, microtubule; VAP, vacuole-associated actin polymerization; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment.

To sum up, this study provides new theories about the origin and influence of IFNγ-activation on SMM. Future work will be spent on validation of promising candidates, summarized in Figure II.2-7. The analysis of the protein-localization in infected cells by GFP-fusions or immunostainings will be the first step. Additionally, the effect of RNAi knockdown on *Salmonella* replication and SIF formation would be interesting to understand the importance of the protein for these processes. Subsequently, interaction studies are necessary to identify the *Salmonella* counterparts and further host proteins.

II.2.5 Material and Methods

Chemicals

All chemicals used in this study were obtained from Sigma Aldrich, if not further indicated.

Cell lines, bacterial strains and their cultivation

The murine macrophage-like cell line RAW 264.7 (ATCC no. TIB-71) as well as stably lentiviral-transfected RAW264.7 LAMP1-GFP cells (Krieger *et al.*, 2014) were cultured in DMEM containing 4.5 g × L^{-1} glucose and 4 mM stable glutamine (Biochrom) supplemented with 6% FCS at 37°C in an atmosphere containing 5% CO_2 and 90% humidity. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) strains NCTC12023 (wild type, WT), or Δ*sseF::aph* (HH107) and *ssaV*::mTn*5* (P2D6) harbouring the plasmid [*sseF*::tev:tev::M45::M45] for synthesis of SseF-2TEV-2M45 were used. The *ssaV*::mTn*5* strain is defective in the *Salmonella* Pathogenicity Island 2 (SPI2)-encoded type III secretion system (T3SS). For live cell imaging, strains harbouring plasmids for constitutive expression of mCherry (pFPV-mcherry/2 or p3589) were used. Strain and plasmid characteristics are summarised in Table II.2-3. *Salmonella* strains were routinely cultured in Luria-Bertani (LB) broth containing 50 µg × mL^{-1} carbenicillin (Roth) or 12.5 µg × mL^{-1} chloramphenicol if required for selection of plasmids.

Table II.2-3: Strains and plasmids used in this study.

Designation	Relevant characteristics	Reference
***S. enterica* strains serovar Typhimurium**		
NCTC 12023	wild type	NCTC, lab stock
HH107	Δ*sseF*::*aph*	(Hensel *et al.*, 1998)
P2D6	*ssaV*::mTn*5*	(Shea *et al.*, 1996)
Plasmids		
p3711	Pro_{sseA} *sscB* $sseF_{1-258}$::tev::tev::M45::M45	(Vorwerk *et al.*, 2015)
pFPV-mcherry/2	const. mCherry expression, Cm^R	(Drecktrah *et al.*, 2008)
p3589	const. mCherry expression, Amp^R	(Lorkowski *et al.*, 2014)

Infection of RAW264.7 cells

RAW264.7 or RAW264.7 LAMP1-GFP cells were infected with overnight cultures of *Salmonella* with a multiplicity of infection (MOI) of 50. The bacteria were centrifuged onto the cells at 500 × g for 5 min, incubated for 25 min at 37°C in an atmosphere of 5% CO_2. Extracellular bacteria were removed by washing thrice with PBS. Subsequently, host cells were maintained in cell culture media containing 100 µg × mL^{-1} gentamicin (AppliChem) for 1 h. Afterwards, cells were cultivated in media with a decreased gentamicin concentration of 10 µg × mL^{-1} for the rest of the experiment. For activation of RAW264.7 cells 7.5 ng x mL^{-1} IFNγ (BD Heidelberg) was added to the cell culture medium 24 h before infection.

Immunostaining

Immunostaining was performed as described before (Müller *et al.*, 2012). Briefly, infected RAW LAMP1-GFP cells (MOI 50) were fixed with 3% PFA at 4, 8, 12, and 16 h p.i., washed with PBS and incubated for 30 min in blocking solution (2% goat serum, 2% BSA and 0.1% saponin in PBS) before incubated with anti-M45 (1:500) as primary antibody and anti-mouse-Cy5 as secondary antibody (Table II.2-4) for 1 h at RT.

Table II.2-4: Antibodies used in this study.

Designation	Relevant characteristics	Reference, source
Anti-M45	Purified; mouse anti-M45 epitope tag	(Obert *et al.*, 1994)
Anti-mouse-IgG-Cy5	Goat anti-mouse IgG (H+L) Cy5	Jackson ImmunoResearch
Anti-mouse-IgG Alexa Fluor 488	Goat anti-mouse IgG (H+L) Alexa Fluor® 488	Life technologies
Anti-mouse-IgG-HRP	Peroxidase-conjugated goat anti-mouse IgG	Dianova

Fluorescence imaging was performed using the Leica SP5 CLSM, equipped with HCX PL APO CS 100× (NA 0.7–1.4) oil immersion objective (Leica). Images were acquired using the LAS AF (Leica Application Suite Advanced Fluorescence) software and the following filter combinations: GFP/Alexa Fluor488 and mCherry/Alexa Fluor568 with polychroic mirror DD 488/543 or the combination of GFP/AlexaFluor488, mCherry/AlexaFluor568 and Cy5 with the polychroic mirror TD 488/543/633. All images obtained were processed by Leica LAS AF. Scale bars were added with ImageJ (US National Institutes of Health) and figures arranged in Photoshop CS6 (Adobe).

Live cell imaging

Live cell imaging was performed as described before (Zhang and Hensel, 2013). SIF formation was monitored starting at 4 h until 16 h p.i. using the Zeiss Cell Observer microscope with a Yokogawa Spinning Disc Unit CSU-X1a 5000, Evolve EMCCD camera from Photometrics (USA) and live cell periphery, equipped with LD Plan-Neofluar 40× (NA 0.6) without immersion objective (Zeiss). Images were acquired using the ZEN (Zeiss) software and the following filter combinations: GFP with BP 525/50, mCherry with LP 580. All images obtained were processed by ZEN 2012 software.

Quantitation by flow cytometry analyses

RAW264.7 cells were infected as described before either with *Salmonella* Δ*sseF::aph* [*sseF*::tev::tev::M45::M45] or *ssaV::*mTn5 [*sseF*::tev::tev::M45::M45]. At 4, 8, 12 and 16 h p.i. cells were fixed with 3% PFA in PBS, permeabilized with 0.1% saponin in 10% iFCS/PBS, stained primary with anti-M45 (1:1,000) and secondary with anti-mouse IgG Alexa Fluor 488 (1:1,000) (Table II.2-4) for subsequent flow cytometrical analyses using FACSCalibur (BD Biosciences). Experiments were performed in triplicates at least three times. Data were analysed with FACS Express 4 (De Novo Software). Statistical analyses were performed using Student's *t*-test with SigmaPlot 11 (Systat Software).

Preparation of SMM-enriched fraction

About 1.6×10^8 RAW264.7 cells were used per IP and biological replicate. Before cell homogenization, the infected host cells were rinsed thrice with PBS. Scraped cells were resuspended in osmostabilizing homogenization buffer (250 mM sucrose, 20 mM HEPES, 0.5 mM EGTA, pH 7.4), centrifuged at 1,000 × g for 10 min and resuspended in 1 mL of 4°C pre-cooled homogenization buffer with 1 x protease inhibitor cocktail (Serva). Host cells were mechanically disrupted with 0.5 mm glass beads (Scientific Industries) using the Vortex-2 Genie with Turbomix (Scientific Industries; 5 × 1 min strokes) with intermediate cooling. The lysate was centrifuged at 100 × g for 15 min at 4°C. The supernatant was centrifuged again at 8,000 × g for 10 min at 4°C to give the SMM-enriched fraction. The pellet was washed twice with pre-cooled homogenization buffer with protease inhibitor cocktail. The final SMM-enriched fraction was resuspended in 500 µL homogenization buffer supplemented with 1.5 mM $MgCl_2$ and treated with DNaseI (50 µg × mL^{-1}) for 30 min at 37°C. Protein concentration was determined via Bradford assay (BioRad).

Immunoprecipitation

For IP, 25 µL Protein G magnetic beads (GE) were coated with 40 µg purified anti-M45 antibody on a rotary shaker at 4°C overnight. The beads were washed twice with PBS, cross-linked according to the manufacturer's instructions and blocked for 30 min with 1% BSA in PBS at 4°C. A total of 500 µg SMM-enriched fraction's proteins were adjusted to a final volume of 200 µL in resuspension mix (1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40) and then incubated with 25 µL cross-linked anti-M45 antibody (Table II.2-4) labelled Protein G magnetic beads on a rotary shaker at 4°C overnight. To remove unbound proteins, samples were washed five-times with 0.1% NP-40 in PBS. Finally, bound proteins were eluted in 25 µL 1 × SDS sample buffer (12.5% glycerol, 4% SDS, 2% mercaptoethanol, 50 mM Tris, pH 6.8).

SDS-PAGE and Western blotting

Proteins were separated in 12% SDS-PAGE for Western Blotting or in 4-12% gradient gels (NuPAGE Novex, Life) for MS analysis with NuPage MOPS buffer. For Western blot analysis, 2 µL of the protein sample were used per lane. Proteins were transferred to 0.2 µm nitrocellulose membranes (Protran, Whatman), blocked with 5% w/v BSA, 0.1% v/v TWEEN in TBS before being incubated in TBS containing 1% w/v BSA with primary and secondary antibodies (Table II.2-4) as follows: anti-M45 (1:5,000) and anti-mouse-IgG-HRP (1:20,000). Proteins were detected by chemiluminescence with ECL detection reagent (Pierce) and blue light-sensitive film (Agfa Healthcare NV) or the ChemiDoc^{TM} MP System (Bio Rad).

Protein digest, RP-LC separation, MS and data analysis

In total twelve IP proteome experiments were performed. For each profiling, precipitated proteins were separated by SDS-PAGE, Coomassie Blue-stained before sliced into 34 gel pieces (Hansmeier *et al.*, 2006), and subjected individually to standard in-gel de-staining and trypsinolysis (Chao *et al.*, 2010). Afterwards, digests were transferred into vials, resulting in a total of 408 digested samples.

The LC MS/MS analysis was performed using an UltiMate 3000 NCS-3500 nano-HPLC system (Dionex) controlled by Chromeleon chromatography software coupled to the AmaZon ETD speed ion trap MS with CaptiveSpray source (Bruker Daltonics). The UltiMate 3000 NCS-3500 nano-HPLC system (Dionex) was configured with a 2-cm PepMap 75 µm-i.d. C_{18} sample trapping pre-column (Thermo Fischer Scientific) and a 15-cm PepMap 75 µm-i.d. C_{18} microcapillary column (Thermo Fischer Scientific). Samples of 7 µL each were applied to the columns and separated by a 60-min linear gradient from 5 to 50% solvent B (80% ACN, 0.1% v/v formic acid) with 300 nL × min^{-1} flow rate. For each MS scan, up to eight abundant multiply charged species in the m/z 400–1600 range were automatically selected for MS/MS but excluded for 30 seconds after having been selected twice. The HPLC system was controlled using Compass 1.5 (Bruker).

Acquired MS/MS data were processed by the ProteinScape 3.1 software (Bruker) and searched against the UniProt mouse database (12/2014) using ProteinExtraktor (Bruker). Spectral data are available in PeptideAtlas (ftp://PASS00480:SU9795nb @ftp.peptideatlas.org/). Data analyses were conducted according the published guidelines (Taylor and Goodlett, 2005). Mass tolerance values for MS and MS/MS were set at 0.8 Da and 4 Da. Fixed search parameters were tryptic digestion and miss cleavage up to 1. Variable search parameters used for the search were deamidation (NQ), carboxymethyl (C) and oxidation (M). Proteins were considered as identified with

ProteinScape score >40 and two unique peptides with >95% confidence. Peptide Decoy (Mascot) and FDR was adjusted to 1% at protein and peptide level for all experiments.
All identified proteins were searched against the UniProt-GOA database (Dimmer *et al.*, 2012). Only proteins identified in two biological replicates were considered as a candidate of the SMM proteome (Table S II.2-1/2) and grouped with PANTHER Classification System (www.pantherdb.org) (Mi *et al.*, 2013a; Mi *et al.*, 2013b) according their Gene Ontology (GO). Following GO annotations are listed: metabolic process (GO:0008152; catabolic process; biosynthetic process; catalytic activity; secondary metabolic process; generation of precursor metabolites and energy; primary metabolic process; coenzyme metabolic process; vitamin metabolic process; sulphur compound metabolic process; phosphate-containing compound metabolic process; nitrogen compound metabolic process), cytoskeleton (GO:0005856; actin cytoskeleton; microtubule organizing centre; microtubule; intermediate filament cytoskeleton); protein metabolic process (GO:0019538; protein folding; protein complex assembly; cellular protein modification process; ferredoxin metabolic process; prosthetic group metabolic process; translation; proteolysis), regulation of biological process (GO:0050789; regulation of cellular amino acid metabolic process; regulation of nucleobase-containing compound metabolic process; regulation of phosphate metabolic process; regulation of vasoconstriction; regulation of carbohydrate metabolic process; regulation of gene expression, epigenetic; negative regulation of apoptotic process; regulation of cell cycle; regulation of translation), transport (GO:0006810; lysosomal transport; nucleobase-containing compound transport; protein transport; vesicle-mediated transport; peroxisomal transport; transporter activity; nuclear transport; vitamin transport; carbohydrate transport; ion transport; phosphate ion transport; mitochondrial transport; extracellular transport; amino acid transport; lipid transport), apoptotic process (GO:0006915; induction of apoptosis; negative regulation of apoptotic process), cellular component organization (GO:0016043; cellular component organization; cellular component biogenesis), and vesicle-mediated transport (GO:0016192; exocytosis; endocytosis).
To compare the composition of pathogen-modulated host compartments from different pathogens and hosts, the MGI vertebrate homology database to extract human-mouse homologues (www.informatics.jax.org/homology.shtml) was used (Eppig *et al.*, 2015).

II.2.6 Acknowledgements

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II.2.7 Supplementary Materials

Additional supporting information is listed on the attached DVD.

II.3 The SPI2-T3SS effector SseJ based *Salmonella*-modified membrane proteome

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II.3.1 Abstract

Effectors of the *Salmonella* Pathogenicity Island 2 type 3 secretion system modify the eukaryotic endosomal system to facilitate intracellular survival of the foodborne pathogen *Salmonella enterica* serovar Typhimurium. After translocation these effectors mediate formation of a replicative niche, the *Salmonella*-containing vacuole (SCV) connected with an extensive tubular membrane network. To deepen our understanding of cellular origin, composition and function of these membrane structures we used an affinity-based proteome approach with the *Salmonella*-modified membrane associated SPI2 effector SseJ as bait protein. Within this proteomic analysis host proteins as well as *Salmonella* proteins were identified. Numerous proteins are components of the cytoskeleton or involved in intracellular vesicle trafficking of endo- and lysosomes, or transport between Golgi and ER. *Salmonella* proteins indicate co-purification of the complete bacterium. Thus this study will be an important starting point for further investigations to increase our knowledge about the interplay between effectors and host protein which are involved in formation of *Salmonella*-induced structures.

II.3.2 Introduction

Salmonella enterica serovar Typhimurium is a facultative intracellular foodborne pathogen and a major cause for gastroenteritis. Inside its host, *Salmonella* resides in a special membrane compartment, the *Salmonella*-containing vacuole (SCV) (Bakowski *et al.*, 2008). Alteration of the host to achieve intracellular survival and proliferation is mediated by effectors, secreted into the host cytosol by type III secretion systems (T3SS) encoded by *Salmonella* Pathogenicity Island 1 and 2 (SPI1 or SPI2) (Haraga *et al.*, 2008). The host endosomal system is remodelled in a SPI2-dependent way that leads to tubular membranous extensions of the SCV called *Salmonella*-induced tubules (SITs) (Liss and Hensel, 2015). Different kinds of SITs occur in the *Salmonella*-infected cell (Schroeder *et al.*, 2011): at early points in time after infection spacious vacuole-associated tubules (SVAT) and sorting nexin 3 tubules (SNX3 tubules) (Braun *et al.*, 2010; Bujny *et al.*, 2008) are present. They are replaced by LAMP1-positive *Salmonella*-induced filaments (SIFs) (Garcia-del Portillo *et al.*, 1993); *Salmonella*-induced SCAMP3 tubules (SISTs) (Mota *et al.*, 2009), as well as LAMP1-negative tubules (LNT) which are marked by SPI2 effectors (Schroeder *et al.*, 2010). All membranes which are altered by activities of intracellular *Salmonella* including SCV and SIT membranes are named in the following as *Salmonella*-modified membranes (SMMs).

However, there are many open questions regarding origin, formation and function of these tubular structures arising from the SCV. The comprehension of the influence of *Salmonella* effectors translocated into the host cytosol will reflect the complex interplay between effectors and host proteins. In this work we selected the SPI2 effector SseJ for our proteomic study because of its location at the membrane of the SCV and SIFs (Freeman *et al.*, 2003). It is involved in SCV membrane dynamics by remodelling the lipid and protein content of the SCV membrane and contributes to membrane tubulation (Kolodziejek and Miller, 2015). Its importance for SCV membrane dynamics is displayed during the loss of vacuolar membrane integrity of the *sifA* mutant whereas the *sifA sseJ* double mutant remains within the SCV (Beuzon *et al.*, 2000; Ruiz-Albert *et al.*, 2002). Likewise to the *sifA* mutant the SCV integrity of *sseJ* mutants is destabilized if the actin-based motor myosin II is inhibited (Wasylnka *et al.*, 2008). Loss of SseJ activity reduces virulence of *Salmonella* in mice and attenuates the replication in cell cultures (Freeman *et al.*, 2003; Ohlson *et al.*, 2005; Ruiz-Albert *et al.*, 2002). These observations, together with the lack of a striking intracellular phenotype, argues for a highly specific, localized function in interplay with further proteins which may be able to compensate the lack of SseJ (Kolodziejek and Miller, 2015). SseJ acts as phospholipase A1, deacylase and glycerophospholipid:cholesterol acyltransferase (GCAT) and interacts with RhoA, a Rho

family GTPase (Christen *et al.*, 2009; LaRock *et al.*, 2012; Lossi *et al.*, 2008; Ohlson *et al.*, 2005). RhoA enhances SseJ enzymatic activity which regulates membrane alteration (Christen *et al.*, 2009; Ohlson *et al.*, 2008). Furthermore, SseJ indirectly interacts with SifA whereby SifA preferentially binds GDP-bound RhoA and SseJ GTP-bound RhoA (Christen *et al.*, 2009; Diacovich *et al.*, 2009; Ohlson *et al.*, 2008). The interplay of the bacterial proteins SifA and SseJ with host cell SKIP (SifA and kinesin interacting protein) and RhoA are supposed to promote SIF formation by membrane tubulation via movement along microtubules (Ohlson *et al.*, 2008; Ruiz-Albert *et al.*, 2002; Schroeder *et al.*, 2011). However, recent findings suggest that SifA and SseJ are involved in distinct pathways (Zhao *et al.*, 2015).

By using SseJ as bait protein, we were able to unravel the SseJ based SMM proteome and provide new interesting protein candidates possibly involved in SITs biogenesis which can be used as starting points for further research.

II.3.3 Results

To clarify the function of the SPI2 effector SseJ in the process of SMM biogenesis the SseJ SMM proteome was analysed by mass spectrometry (MS)-based immunoprecipitation. Therefore SseJ tagged with the M45 epitope was used. Visualization of the tagged effector protein revealed co-localization with LAMP1-GFP on the membrane of the SCV and SIF network (Figure II.3-1). Four IPs were conducted and Western Blot analyses proofed SseJ-enrichment (Figure S II.3-1), before samples of IP 1-3 were used for LC-ESI-MS/MS analyses.

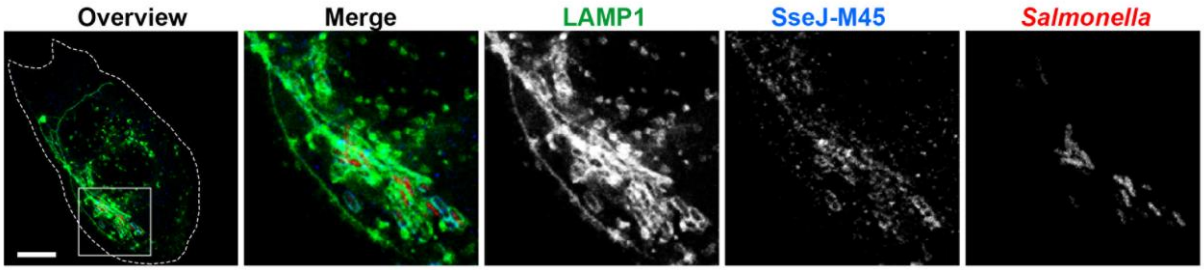

Figure II.3-1: Localization of SseJ-M45 in SMMs.
Stably transfected HeLa cells expressing LAMP1-GFP (green) were infected with *Salmonella* Δ*sseJ* synthesizing SseJ-M45 (blue) and mCherry (red), fixed 8 h p.i. and stained with anti-M45 antibody and anti-mouse-IgG-Cy5. Representative micrograph was chosen and images are shown as maximum intensity projection. The dashed line in overview indicates the host cell boundary. Scale bar: 10 µm.

II.3.3.1 SseJ host SMM

In total, 2,823 host proteins (Table S II.3-1) were identified in the IP eluates of three biological replicates. To determine the SseJ host SMM proteome all proteins were eliminated which were also detected in the control (2,226 proteins) (Table S II.3-2). Additionally, only proteins that were at least identified in two of three MS runs were further considered. These criteria led to 110 host proteins present in SMM positive for SseJ (Table S II.3-3).

The proteins were grouped according to their localization (Figure II.3-2A). A proportion of 37% (41 proteins) are membrane and 36% (40 proteins) cytoplasmic proteins. The proteins are associated with the nucleus (26%, 29 proteins), mitochondria (11%, 12 proteins), ribosomes (6%, 7 proteins), Golgi apparatus (11%, 12 proteins), ER (7%, 8 proteins), cytoskeleton (9%, 10 proteins), vesicles (5%, 6 proteins), and endo-/lysosomal pathway (3%, 3 proteins). Four percentages of the identified proteins (4 proteins) are secreted, and 27% (30 proteins) have no known subcellular localization.

Regarding the biological processes of the identified proteins (Figure II.3-2B) the majority of the proteins (43%, 48 proteins) are involved in metabolism. Further proteins participate

in protein metabolism (13%, 14 proteins), regulation of biological processes (6%, 7 proteins), cellular component organization (8%, 9 proteins) or can be linked with transport processes (19%, 21 proteins) and, especially, vesicle-mediated transport (10%, 11 proteins).

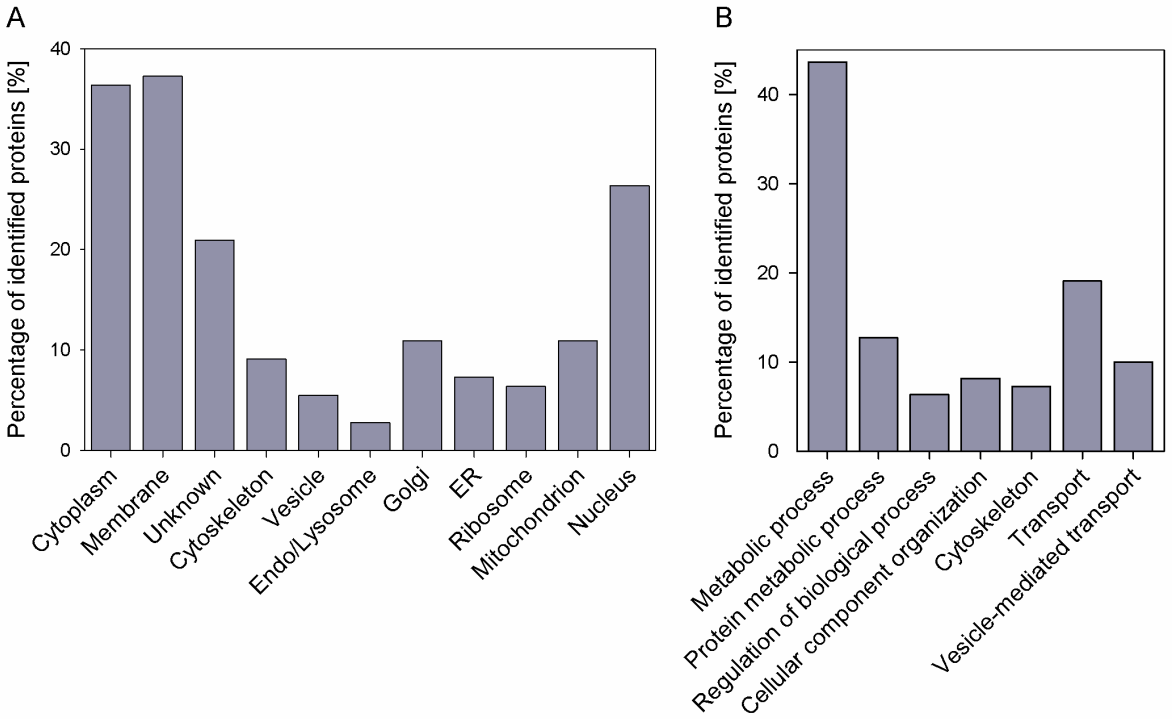

Figure II.3-2: Subcellular localization and biological processes of human SseJ based SMM proteome.
Human proteins were classified into (A) subcellular localization referred to UniProt and (B) GO biological processes according to the PANTHER classification system. Proteins can be grouped in more than one category due to their annotations, not all proteins were able to be annotated by PANTHER.

Table II.3-1: Selected host proteins identified by mass spectrometry[a]
(EE, early endosome; ER, endoplasmic reticulum; LE, late endosome)

Entry name (*_HUMAN)	Description	Localization
Trafficking		
COPE	Coatomer subunit ε	Cytoplasm, Golgi apparatus membrane, COPI-coated vesicle
COPZ1	Coatomer subunit ζ-1	Cytoplasm, Golgi, COPI-coated vesicle
P3C2A	Phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit α	Cell membrane, Golgi, clathrin-coated vesicle, nucleus, cytoplasm
PA24A	Cytosolic phospholipase A2	Cytoplasm, vesicle
RAB5B	Ras-related protein Rab5b	Cell membrane, EE
RAP1B	Ras-related protein Rap1b	Cell membrane, cytosol, cell junction
UBP5	Ubiquitin carboxyl-terminal hydrolase 5	Lysosome
VAMP8	Vesicle-associated membrane protein 8	Lysosome, EE, LE, cell membrane
Cytoskeleton		
ACTG	Actin, cytoplasmic 2 (γ-actin)	Cytoplasm, cytoskeleton
E7EVA0	Microtubule-associated protein	Cytoplasm, cytoskeleton
ML12B	Myosin regulatory light chain 12b	Cytoplasm, cytoskeleton
RHOA	Transforming protein RhoA	Cell membrane, cytoplasm, cytoskeleton
SYNPO	Synaptopodin	Cytoplasm, cytoskeleton, cell/tight junction
TBB2A	Tubulin β-2A chain	Cytoplasm, cytoskeleton
ZW10	Centromere/kinetochore protein zw10 homolog	Cytoplasm, ER, cytoskeleton, spindle
ER and Golgi apparatus		
ARF5	ADP-ribosylation factor 5	Golgi, cytoplasm
B4GT1	β-1,4-galactosyltransferase 1	Golgi, cell membrane, secreted
GALT1	Polypeptide N-acetylgalactosaminyltransferase 1	Golgi, secreted
MGST3	Microsomal glutathione S-transferase 3	ER
MYO6	Unconventional myosin-VI	Golgi, nucleus, cytoplasm, clathrin-coated pit
PDC10	Programmed cell death protein 10	Cytoplasm, Golgi, cell membrane
PLPL6	Neuropathy target esterase	ER
RASN	GTPase Nras	Cell membrane, Golgi
RER1	Protein Rer1	Golgi
S35B2	Adenosine 3'-phospho 5'-phosphosulfate transporter 1	Golgi
SPCS3	Signal peptidase complex subunit 3	ER
TOIP2	Torsin-1A-interacting protein 2	ER, nucleus
TPPC3	Trafficking protein particle complex subunit 3	Golgi, ER
TUSC3	Tumor suppressor candidate 3	ER

[a]The entire list of host SseJ SMM proteins identified by MS are catalogued in Table S II.3-3.

In Table II.3-1 proteins are listed which were MS-identified as components of the SseJ SMM proteome and associated with trafficking, cytoskeleton, or ER/Golgi apparatus. Proteins of these origins are assumed to be components of the SMMs or participate in their formation (Brumell *et al.*, 2001b; Garcia-del Portillo *et al.*, 1993; Meresse *et al.*, 2001; Mota *et al.*, 2009). Coatomer subunit ε/ζ1 (ε/ζ1-COP), vesicle-associated membrane protein 8 (Vamp-8), Ras-related protein Rab5b and Rap1b are worth to mention as trafficking proteins. A number of proteins of the cytoskeleton are components of the SMMs, e.g. actin, tubulin β-2A chain, myosin regulatory light chain 12B, microtubule-associated protein and the transforming protein RhoA. Furthermore, ER and Golgi proteins contribute to the SMMs, e.g. ADP-ribosylation factor 5 (Arf5), unconventional myosin-VI, GTPase Nras and trafficking protein particle complex subunit 3 (Tppc3).

II.3.3.2 Comparison to the SseF SMM proteome

The following proteins were observed in the SseJ and SseF based SMMs proteomes (Chapter II.1): bifunctional purine biosynthesis protein PURH, Ras-related protein Rap1b, 60S ribosomal protein L32 and L7-like 1 and the small nuclear ribonucleoprotein-associated protein N (snRNP-N).

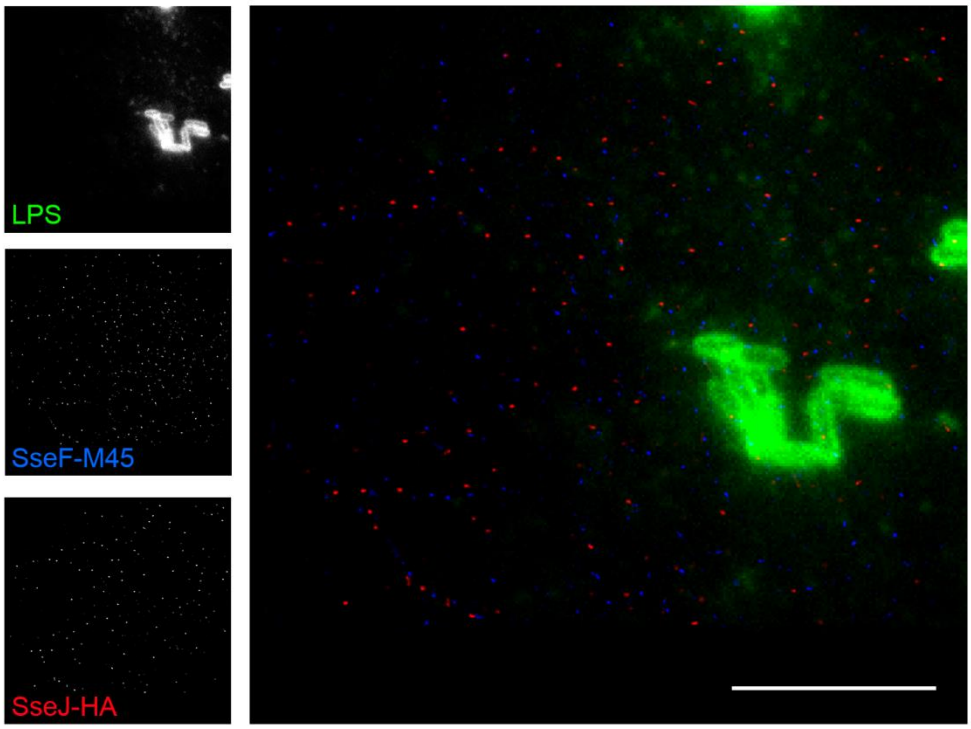

Figure II.3-3: Super resolution microscopy reveals spatial proximity of SseJ and SseF, but no co-localisation in high resolution.
HeLa cells infected with S. Typhimurium *sseF::*HA *aph* [*sseF*::M45] were fixed 8 h p.i. and stained with antibodies coupled to Cy3 (HA-tag, red) and Cy5 (M45-tag, blue). Additionally, LPS was stained with Alexa488 (green).
1,000 frames with 32 ms exposure time were acquired using excitation with a 561 nm and 640 nm laser. Images were taken at time points of steady state of fluorescence intensity, i.e. when blinking Cy3 and Cy5 molecules were observed. Image series were localized using a modulated MTT version (Serge *et al.*, 2008; Wilmes *et al.*, 2012). Scale bars: 5 µm.

To understand the low overlap between the identified proteins fished by SseF and SseJ we analysed the protein localisation of SseF and SseJ by super resolution microscopy (Figure II.3-3). Therefore, the localisation of the chromosomal HA-tagged SseF and plasmid-encoded M45-tagged SseJ were visualised in an infected HeLa cell. The high resolution revealed that the proteins are in close proximity but they do not co-localise with each other.

II.3.3.3 *Salmonella* proteins

In three independent biological replicates 572 *Salmonella* proteins were identified. However, only 228 proteins were detected in all three replicates. According to PSORTb 3.0.2 [www.psort.org; (Yu *et al.*, 2010)] most proteins have cytoplasmic (74%, 168 proteins) origin. Though, some identified proteins are also located in the periplasmic space or outer membrane (18%, 42 proteins), or are extracellular (2%, 4 proteins) (Figure II.3-4). The majority of identified proteins are involved in metabolism (27%, 61 proteins) and protein biosynthesis (22%, 51 proteins). In addition, proteins with a function in transport (11%, 25 proteins) and transcription (9%, 21 proteins) are represented. Proteins contributing to stress response (1%, 3 proteins), motility (2%, 4 proteins), cell assembly (4%, 8 proteins), and pathogenesis (1%, 2 proteins) were also identified (Figure II.3-4).

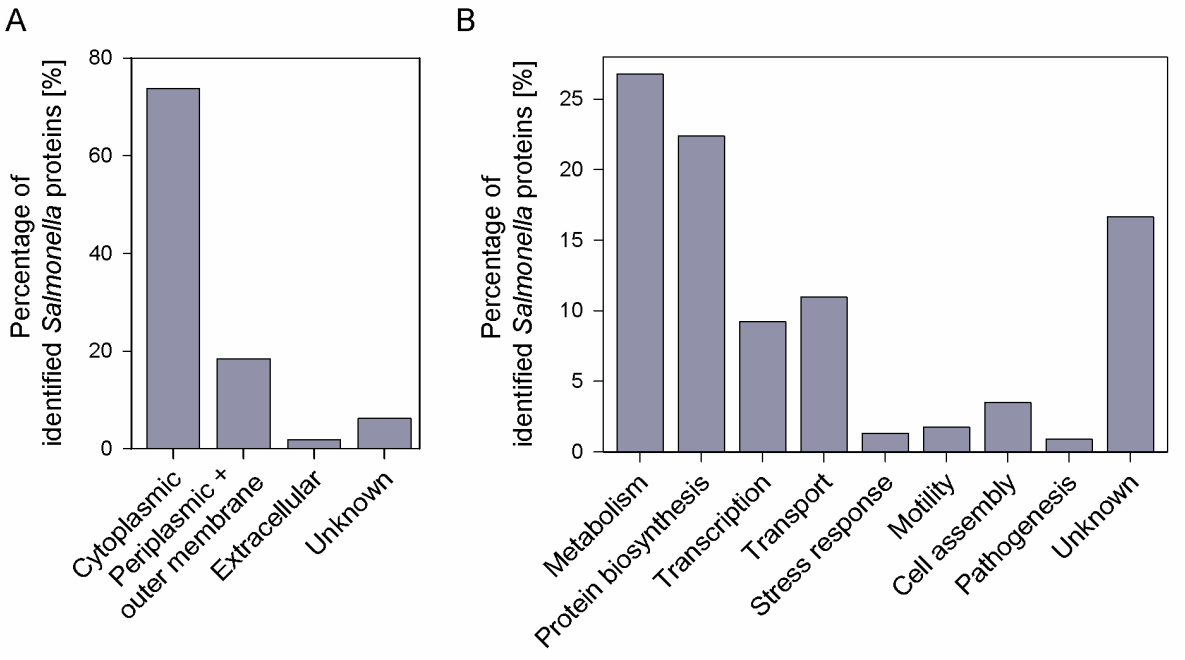

Figure II.3-4: Subcellular localization and biological processes of *Salmonella* proteins identified in the SseJ based SMM proteome.
Salmonella proteins were classified according to (A) subcellular localization (PSORTb) and (B) GO biological processes referred to the UniProt database. Proteins can be grouped in more than one category due to their annotations.

In addition to the bait protein SseJ, we were also able to identify effector protein SipB (Table II.3-2). In total, three components of the SPI1-T3SS system (SipB, InvG, PrgK) (Burkinshaw and Strynadka, 2014) were identified. Several proteins located in the periplasm, cytoplasmic or outer membrane and transport activity were detected, e.g. OmpC/D/F/X or iron transporter as SitA, FepA, or IroN. Further proteins as the outer membrane protein assembly factor BamA/B/C/D or flagellin (FliC) were identified.

Table II.3-2: Selected identified *S.* Typhimurium proteins by mass spectrometry[a]

Entry name (*_SALT1)	Protein names	Gene names	Biological process	Localisation
Proteins involved in pathogenesis				
D0ZI42	Secreted effector protein SseJ	*sseJ* STM14_1974	Hydrolase activity, acting on ester bonds; lipid metabolic process	
D0ZV22	Translocation machinery component SipB	*sipB* STM14_3484	Pathogenesis	Host associated
D0ZV35	Outer membrane secretin	*invG* STM14_3497	Protein secretion; protein transporter activity	Outer membrane
D0ZV08	Needle complex inner membrane lipoprotein	*prgK* STM14_3470	Protein secretion	T3SS
D0ZVH8	Virulence membrane protein PagC	*pagC* STM14_1501		Outer membrane
Transport activity				
D0ZX63	Biopolymer transport protein ExbB	*exbB* STM14_3825	Protein transporter activity	Cytoplasmic membrane
D0ZJR9	Chaperone SurA (Peptidyl-prolyl cis-trans isomerase SurA)	*surA* STM14_0111	Chaperone mediated protein folding requiring cofactor; Gram-negative-bacterium-type cell outer membrane assembly; protein transport	Periplasmic
D0ZNM5	Colicin I receptor	*cirA* STM14_2713	Transporter activity	Outer membrane
D0ZRE8	Glutamine ABC transporter periplasmic protein	*glnH* STM14_964	Transporter activity	Periplasmic
D0ZQK9	Histidine transport protein	*hisJ* STM14_2900	Transporter activity	Periplasmic
D0ZQQ1	Long-chain fatty acid outer membrane transporter	*fadL* STM14_2942		Outer membrane
D0ZJS0	LPS-assembly protein LptD	*imp lptD* STM14_0112	Gram-negative-bacterium-type cell outer membrane assembly; lipopolysaccharide transport	Outer membrane
D0ZX97	Outer membrane channel protein	*tolC* STM14_3859	Protein transport; transporter activity	Outer membrane
D0ZLY6	Outer membrane phosphoporin protein E	*phoE* STM14_0376	Ion transport; porin activity	Outer membrane
D0ZSK6	Outer membrane protein F	*ompF* STM14_1130	Ion transport; porin activity	Outer membrane
D0ZRF5	Outer membrane protein X	*ompX* STM14_971		Outer membrane
D0ZP82	Outer membrane receptor FepA	*fepA* STM14_0682	Siderophore transmembrane transporter activity	Outer membrane
D0ZTZ6	Outer membrane receptor FepA	*iroN* STM14_3348	Siderophore transmembrane transporter activity	Outer membrane
D0ZJ50	Outer-membrane lipoprotein LolB	*lolB* STM14_2149	Protein transporter activity	unknown
D0ZQG6	Protein TolB	*tolB* STM14_0870	Protein import	Periplasmic
D0ZI45	Putative periplasmic binding protein	STM14_1977	Transporter activity	Periplasmic
D0ZUZ6	Putative periplasmic binding protein	*sitA* STM14_3458	Cell adhesion; metal ion transport	Periplasmic
D0ZXY7	Preprotein translocase subunit SecG	*secG* STM14_3976	P-P-bond-hydrolysis-driven protein transmembrane transporter activity; protein secretion	Cytoplasmic membrane
Proteins with periplasmic, flagellar, or outer membrane localisation				
D0ZKY1	Chaperone protein skp	*hlpA* STM14_0267		Periplasmic
D0ZHY7	Conjugative transfer: surface exclusion	*traT* STM14_5621	Regulation of conjugation	Outer membrane
D0ZL85	Flagellin	*fliC* STM14_2378	Bacterial-type flagellum-dependent cell motility	Flagellar

Entry name (*_SALT1)	Protein names	Gene names	Biological process	Localisation
D0ZUJ2	Glucans biosynthesis protein G	*mdoG opgG* STM14_1317	Catalytic activity; glucan biosynthetic process	Periplasmic
D0ZQ59	LPS-assembly lipoprotein LptE	*lptE rlpB* STM14_0755	Gram-negative-bacterium-type cell outer membrane assembly	Outer membrane
D0ZP66	Outer membrane esterase	*apeE* STM14_0666	Hydrolase activity, acting on ester bonds; lipid metabolic process	Outer membrane
D0ZKY0	Outer membrane protein assembly factor BamA	*bamA yaeT* STM14_0266	Gram-negative-bacterium-type cell outer membrane assembly; protein insertion into membrane	Outer membrane
D0ZRU1	Outer membrane protein assembly factor BamB	*yfgL bamB* STM14_3091	Gram-negative-bacterium-type cell outer membrane assembly; protein insertion into membrane	Outer membrane
D0ZRQ2	Outer membrane protein assembly factor BamC	*nlpB bamC* STM14_3051	Gram-negative-bacterium-type cell outer membrane assembly; protein insertion into membrane	Outer membrane
D0ZTR7	Outer membrane protein assembly factor BamD	*yfiO bamD* STM14_3264	Gram-negative-bacterium-type cell outer membrane assembly; protein insertion into membrane	Outer membrane
D0ZQG7	Peptidoglycan-associated outer membrane lipoprotein	*pal* STM14_0871		Outer membrane

[a]The entire lists of *S.* Typhimurium proteins identified by MS as SseJ SMM are catalogued in Table S II.3-6.

II.3.4 Discussion

The identification of SseJ specific SMM proteins as well as known and novel interaction partner of the SPI2 effector SseJ will help to deepen our understanding of the interplay between effectors and host proteins involved in the progress of SIT formation and maintenances. In this proteomic survey we were able to detect beside the bait protein SseJ (Figure II.3-1, Table II.3-1) its known host interaction partner RhoA (LaRock *et al.*, 2012; Ohlson *et al.*, 2008). Furthermore, the interaction partner RhoC could not be confirmed (Auweter *et al.*, 2011; Ohlson *et al.*, 2008). SseJ and RhoA form a stable protein complex (Ohlson *et al.*, 2008). SseJ binds to the regulatory switch region of RhoA and influences in this way the function of RhoA as activator of endogenous binding proteins (LaRock *et al.*, 2012). Vice versa RhoA controls the enzymatic activity of SseJ which is enhanced by binding the GTP-bound RhoA (Christen *et al.*, 2009; Ohlson *et al.*, 2008). Its activity as phospholipase A1, deacylase and glycerophospholipid:cholesterol acyltransferase (GCAT) contributes to membrane dynamics of the SCV and membrane tubulation (Christen *et al.*, 2009; LaRock *et al.*, 2012; Lossi *et al.*, 2008; Ohlson *et al.*, 2005). In this context it seems interesting that a phosphoinositide 3-kinase (P3c2A) was identified which generates phosphatidylinositol 3-phosphate (PtdIns(3)*P*) and phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) and is involved in transport between TGN and clathrin-coated vesicles (Domin *et al.*, 2000; Falasca and Maffucci, 2007; Gaidarov *et al.*, 2001; Gaidarov *et al.*, 2005). This protein may also contribute to SCV biogenesis similar to Vps34, a phosphatidylinositol 3-kinase that is responsible for PtdIns(3)*P* formation of the early SCV and indirectly recruited by the activity of the SPI1 effector SopB via Rab5a (Bakowski *et al.*, 2010; Hernandez *et al.*, 2004; Mallo *et al.*, 2008). Additionally, the cytosolic phospholipase A2 can take part in SCV and SIT membrane modifications.

In addition, many proteins known to be involved in *Salmonella* infection were identified: actin (Meresse *et al.*, 2001), β-tubulin (Brumell *et al.*, 2002a; Finlay *et al.*, 1991; Guignot *et al.*, 2004; Kuhle *et al.*, 2004), myosin II (Hänisch *et al.*, 2011; Wasylnka *et al.*, 2008), coatomer I protein (Misselwitz *et al.*, 2011), Rab5a-c (here Rab5b) (Smith *et al.*, 2007), and Vamp-8 (Dai *et al.*, 2007) which is a promising candidate for further investigations. Vamp-8 is a v-SNARE protein that contributes to homotypic fusion of early and late endosomes (Antonin *et al.*, 2000), is involved in cytokinesis (Low *et al.*, 2003) and the regulation of exocytosis (Nagamatsu *et al.*, 2001; Wang *et al.*, 2007). During *Salmonella* infection it is recruited to *Salmonella*-induced membrane ruffles by accumulation with PtdIns(3)*P* which is generated by the inositol phosphatase activity of SopB (Dai *et al.*, 2007). Vamp-8 also seems to remain in association with SCVs (Dai *et al.*, 2007).

Furthermore, Vamp-8 is involved in the *Coxiella burnetii* vacuole biogenesis (Campoy *et al.*, 2013) and in combination with Vti1b it plays an important role in the fusion of antimicrobial and canonical autophagosomes with lysosomes, an essential process for autophagic degradation (Furuta *et al.*, 2010), whereby Vti1b is located in the SIFs (Figure S II.3.2). Altogether, these observations point out that Vamp-8 might be involved in SCV maturation and SIT biogenesis. By live cell analysis Vamp-8-GFP fusion shows weak co-localization with the SCV and SIF membrane (personal communication A. Kehl and V. Liss). However, its function in SCV biogenesis is so far unclear and has to be elucidated in further studies. The ADP ribosylation factor Arf5 primarily localizes to the ER/Golgi intermediate compartment and the *cis*-Golgi and is involved in regulation of ER to Golgi transport (Chun *et al.*, 2008; Volpicelli-Daley *et al.*, 2005). Furthermore, it plays a role in clathrin-mediated endocytosis of specific cargoes (Moravec *et al.*, 2012). Possibly its functions are similarly exploited by *Salmonella* as shown for Arf1 und Arf6 (Davidson *et al.*, 2015; Humphreys *et al.*, 2013). *Salmonella* seems to profit in a not negligible extent from the transport between Golgi and ER by hijacking vesicles such as COPI vesicles, recently pointed out in our previous publication (Vorwerk *et al.*, 2015). In this study the COPI components ε-COP and ζ1-COP (Beck *et al.*, 2009) were identified. In addition, the Golgi membrane protein Rer1, the trafficking protein particle complex subunit 3 (Tppc3), and the GTPase Rab1b are three further interesting candidates. Rer1 is required for the correct localization of ER membrane proteins during the retrograde transport (Sato *et al.*, 1997) and Tppc3 is involved in vesicle docking and fusion in the Golgi-ER transport (Sacher *et al.*, 1998). GTPase Rab1b also seems to be promising, although it is known to contribute to correct endothelial cell polarity and establishment of basal endothelial barrier function (de Rooij *et al.*, 1998; Pannekoek *et al.*, 2011; Rehmann *et al.*, 2008).

The overlap between the proteins identified by immunoprecipitation with SseF or SseJ is a small fraction of the identified proteins as mentioned before. To explain this we analysed protein localization of both effector proteins by super-resolution microscopy (Figure II.3-3) and revealed no co-localization of both effectors. Although they seem to be part of the same compartment, SIFs and SCV membrane, their exact localisation is not clear. SseF is an integral membrane protein (Müller *et al.*, 2012), whereas SseJ has no transmembrane domain but only a coiled-coil domain at the C-terminus (amino acid 308-325) which is necessary but not sufficient for membrane targeting (Knodler *et al.*, 2011). Its location seems to be at the cytoplasmic side of these structures (Freeman *et al.*, 2003; Ohlson *et al.*, 2005; Ruiz-Albert *et al.*, 2002). These conditions could lead to a more loose association of SseJ to SMMs and possibly impede co-purification of SMMs by SseJ in comparison to the integral effector SseF. This might result in identification of transient interaction partners changed during the purification procedure. Additionally, it was shown

that SIF are formed by a double membrane and SseF is involved in this process (Krieger *et al.*, 2014). The location of the effectors within the double membrane so far remains unknown. Maybe SseF is orientated to the inner lumen whereas SseJ is cytosolic which could explain the low overlap between both SMM proteomes. Furthermore the MS-platform was changed in comparison with the analysis of SseF proteome in HeLa (Chapter II.1) or macrophages (Chapter II.2). The number of identified proteins was increased by using a more sensitive MS-instrument, but the restrictive analysis criteria to avoid false-positives leads to unique 110 host proteins. These criteria maybe also eliminate possible SMM components. For further projects it will be necessary to analyse the SMM protein composition with such sensitive instruments quantitatively to distinguish between false-positives and false-negatives.

In addition to host proteins the SseJ SMM proteome survey revealed 228 co-purified *S*. Typhimurium proteins. More than the half of identified proteins was cytosolic. Proteins of the central metabolism or protein biosynthesis (Figure II.3-4) imply co-purification of the complete bacterium. Interestingly, identification of several presumably low abundance proteins with a regulatory role (RecA) or a function in pathogenesis (SpiB, InvG, PrgK) was possible (Table II.3-2). RecA is an activator of the SOS system and has a role in swarming motility (Mayola *et al.*, 2014). Different proteins involved in iron transport were identified, e.g. SitA, Feb and IronN (Table II.3-2). Though SitA is an iron transporter (Zhou *et al.*, 1999). FebA and IronN are the bacterial outer membrane receptors for the siderophores enterobactin and salmochelin (Rabsch *et al.*, 1999). Nagy *et al.* (2013) suggest that *Salmonella* intercepts ferritin-containing vesicles and utilizes enterobactin or salmochelin to remove Fe^{3+} from the cytosolic storage protein ferritin to deal with intracellular iron starvation. This hypothesis should be revised and the contribution of these siderophores receptors analysed in terms of SIT formation.

It is noticeable that besides SseJ only the SPI1 effector SipB was identified. SipB is important for *Salmonella* invasion of epithelial cells. SipB, together with SipC, is part of the translocon of the SPI1-T3SS (Burkinshaw and Strynadka, 2014; Kaniga *et al.*, 1995). SipB is involved in apoptosis by binding to caspase 1 (Hersh *et al.*, 1999). Expressed in cultured cells, SipB forms unusual multimembrane structures similar to autophagosomes with mitochondrial and ER markers. Translocated SipB is localised to mitochondria (Hernandez *et al.*, 2003). It might be that SipB is involved in SIT and double membrane structure formation of SIFs, as well as its mitochondrial localisation contributes to the association or recruitment of mitochondrial proteins to SCV or the SIT network. The influence of SipB on SIT formation should be analysed in future.

The presence of a SPI1 effector is surprising because it is assumed that the level of SPI1 effectors decline during the course of infection whereas the level of SPI2 effectors

increases. This was demonstrated for the SPI1 effector SipA, and the SPI2 effectors SifA and PipB2 (Szeto *et al.*, 2009). However, SipA is still located at the SCV 8 h p.i. and mediates efficient translocation of SPI2 effectors like SifA (Brawn *et al.*, 2007) that could also be the case for SipB. It might be that SP1 effectors are not only important for uptake and early SCV biogenesis, but also for late phases of infection. To gain a deeper understanding of the function and interplay between SPI1 and SPI2 effectors inside the host, quantitative analyses of the presence of effectors from the time point of entry over SCV biogenesis and establishment of a tubular network until *Salmonella* escape are required.

One approach should be the elucidation of the fate of effectors inside the cell on a single cell level by trackable effectors in live cell imaging. This should be combined with a quantitative proteomics approach to determine effector levels in a time series starting from the time of infection. Thereby the interplay between the effectors would be clarified. However, it has to be differentiated between host and pathogen to observe the level of translocated effector inside the cytosol in contrast to the expressed effector in the bacterium. Especially it would be necessary to distinguish between the host cytosol, integral and associated SMM proteins and proteins of the complete bacterium. This would allow identifying *Salmonella's* contributions to SMMs.

Altogether, we were able to identify a list of SseJ based SMM proteins whose functions in SCV and SIT biogenesis has to be clarified in future. After validation this can be a starting point for further research on proteins which were not linked to SCV or SIT biogenesis so far in order to clarify the unresolved questions how *Salmonella* effectors are able to modify the host endosomal system to form *Salmonella*-induced structures.

II.3.5 Material and Methods

Chemicals

All chemicals used in this study were obtained from Sigma Aldrich, if not further indicated.

Cell lines, bacterial strains and their cultivation

Human epithelial cell line HeLa (ATCC no. CCL-2) as well as stable lentiviral-transfected HeLa LAMP1-GFP cells (Zhang and Hensel, 2013) were maintained in DMEM containing 4.5 g × L^{-1} glucose, 4 mM L-glutamine and sodium pyruvate (Biochrom) supplemented with 10% inactivated FCS in an atmosphere of 5% CO_2 and 90% humidity at 37°C. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) strains NCTC12023 (wild type, WT), or HH107 and P2D6 harbouring p2129 for synthesis of SseJ-M45 were used. Strain P2D6 is defective in the *Salmonella* Pathogenicity Island 2 (SPI2)-encoded type III secretion system (T3SS) due to *ssaV* mutation. For immunostaining, strains harbouring plasmid p3589 were used for constitutive expression of mCherry. Strain characteristics are summarized in Table II.3-13. *Salmonella* strains were routinely cultured in Luria-Bertani (LB) broth containing 50 µg × mL^{-1} carbenicillin (Roth) or 12.5 µg × mL^{-1} chloramphenicol if required for the selection of plasmids.

Table II.3-3: Strains and plasmids used in this study.

Designation	Relevant characteristics	Reference
***S. enterica* strains serovar Typhimurium**		
NCTC 12023	wild type	NCTC, lab stock
MvP392	Δ*sseJ*::*aph*	(Kuhle *et al.*, 2004)
P2D6	*ssaV*::mTn*5*	(Shea *et al.*, 1996)
MvP396	*sseF*::HA *aph*	(Lober *et al.*, 2006)
Plasmids		
p2095	Pro_{sseA} *sscB sseF*::M45 in pWSK29	(Hansen-Wester *et al.*, 2002)
p2129	Pro_{sseJ} *sseJ*::M45	(Hansen-Wester *et al.*, 2002)
p3589	const. mCherry expression, Cm^R	(Lorkowski *et al.*, 2014)

Infection of HeLa cells

Host cell infections were performed as described previously (Rajashekar *et al.*, 2008). In short, HeLa cells were infected with 3.5 h subcultures of *Salmonella* with a multiplicity of infection (MOI) of 50. The bacteria were centrifuged onto the cells at 500 × g for 5 min, incubated for 25 min at 37°C in an atmosphere of 5% CO_2 before extracellular bacteria were removed by washing thrice with PBS. Subsequently, host cells were maintained in cell culture media containing 100 µg × mL^{-1} gentamicin (AppliChem) for 1 h. Afterwards, cells were cultivated in media with a decreased gentamicin concentration of 10 µg × mL^{-1} for the rest of the experiment.

Immunostaining

Immunostaining was performed as described before (Müller *et al.*, 2012). Briefly, infected HeLa-LAMP1-GFP cells (MOI 50) were fixed with 3% PFA at 8 h p.i., washed and incubated in blocking solution (2% goat serum, 2% BSA and 0.1% saponin in PBS) before stained with the following primary antibodies: mouse anti-M45 (1:500), and *Salmonella* O antiserum (1:1,000) for overnight at 4°C (Table II.3-4). Secondary antibodies were selected accordingly (Table II.3-4) and samples were incubated for 1 h at RT.

Immunostaining for super-resolution microscopy was performed with following antibodies in PBS with 0.1% saponin: anti-rat-HA (1:200), mouse anti-M45 (1:500), and *Salmonella* O antiserum (1:1000) as primary antibodies and suitable secondary antibodies anti-rat-IgG-Cy3 (1:2000), anti-mouse-IgG-Cy5 (1:2000) and anti-rabbit-IgG Alexa488. Fixed cells were incubated for 1 h at RT for each antibody.

Table II.3-4: Antibodies used in this study.

Designation	Relevant characteristics	Reference, source
Mouse anti-M45	Purified; mouse anti-M45 epitope tag	(Obert *et al.*, 1994)
Rat anti-HA	Rat anti-HA c3f10	Roche
Salmonella O antiserum	Rabbit anti-*Salmonella* O antiserum Group B Factors 1,4,5,12	BD Difco
Anti-rat-IgG-Cy3	Goat anti-mouse IgG Cy3	Jackson ImmunoResearch
Anti-mouse-IgG-Cy5	Goat anti-mouse IgG (H+L) Cy5	Jackson ImmunoResearch
Anti-mouse-IgG Alexa Fluor 488	Goat anti-mouse IgG (H+L) Alexa Fluor® 488	Life technologies
Anti-rabbit-IgG Alexa Fluor 488	Goat anti-rabbit IgG (H+L) Alexa Fluor® 488	Life technologies
Anti-mouse-IgG-HRP	Peroxidase-conjugated goat anti-mouse IgG	Dianova

Fluorescence imaging was performed using the Leica SP5 CLSM with live cell periphery, equipped with HCX PL APO CS 100× (NA 0.7 – 1.4) oil immersion objective (Leica). Images were acquired using the LAS AF (Leica Application Suite Advanced Fluorescence) software and the following filter combinations: GFP/Alexa Fluor488 and mCherry/Alexa Fluor568 with polychroic mirror DD 488/543 or the combination of GFP/AlexaFluor488, mCherry/AlexaFluor568 and Cy5 with the polychroic mirror TD 488/543/633. All images obtained were processed by Leica LAS AF. Scale bars were added with ImageJ (US National Institutes of Health) and figures arranged in Photoshop CS6 (Adobe).

Super-resolution microscopy

TIRF microscopy was performed with an inverted Olympus IX71 microscope equipped with a motorized quad-line total internal reflection (TIR) illumination condenser (Olympus),

with 488 nm (250 mW), 561 nm (150 mW) and 647 nm (250 mW) lasers (Olympus) as well as a back-illuminated EM-CCD camera (Andor iXon Ultra 897). A 150 x objective plus 1.6 x magnification with a numerical aperture of 1.45 (UAPON 150 x/1.45, Olympus) was used for TIR-illumination. The excitation beam was reflected into the objective by a quad-line dichroic beam splitter for reflection at 405 nm, 488 nm, 568 nm and 647 nm (Di01 R405/488/561/647, Semrock). 1000 frames were recorded with an exposure time of 32 ms for 561 nm and 640 nm laser with a power of 33 mW for fixed cells. For oxygen depletion 100 mM β-mercaptoethylamine, 4.5 mg x ml^{-1} D glucose, 0.04 mg x ml^{-1} catalase and 0.5 mg x ml^{-1} glucose-oxidase were added in 1 ml PBS. Localization of single molecules was carried out as previously described (Hess *et al.*, 2006; Serge *et al.*, 2008; Wilmes *et al.*, 2012).

Enrichment of GEMN fraction

Enrichment of the GEMN fraction was described before (Vorwerk *et al.*, 2015). In short, roughly 7×10^7 HeLa LAMP1-GFP cells were per IP and biological replicate used. Before cell homogenization, the infected host cells were rinsed thrice with PBS. Scraped cells were resuspended in osmostabilizing homogenization buffer (250 mM sucrose, 20 mM HEPES, 0.5 mM EGTA, pH 7.4), centrifuged at 1,000 × g for 10 min and resuspended in 1 mL of 4°C pre-cooled homogenization buffer with 1 x protease inhibitor cocktail (Serva). Host cells were mechanically disrupted with 0.5 mm glass beads (Scientific Industries) using the Vortex-2 Genie with Turbomix (Scientific Industries; 3 × 1 min strokes) with intermediate cooling. The lysate was centrifuged at 100 × g for 10 min at 4°C and the resulting GEMN pellet was washed twice with pre-cooled homogenization buffer with protease inhibitor cocktail. The final GEMN pellet was resuspended in 500 µL homogenization buffer supplemented with 1.5 mM $MgCl_2$ and treated with DNaseI (50 µg × mL^{-1}) for 30 min at 37°C. Protein concentration was determined via Bradford assay (BioRad).

Immunoprecipitation

For IP, 25 µL Protein G magnetic beads (GE) were coated overnight with 40 µg purified mouse anti-M45 antibody on a rotary shaker at 4°C. The beads were washed twice with PBS, cross-linked according to the manufacturer's instruction and blocked for 30 min with 1% BSA in PBS at 4°C. A total of 500 µg GEMN proteins were adjusted to a final volume of 200 µL in resuspension mix (1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40) and then incubated overnight with 25 µL cross-linked mouse anti-M45 antibody (Table II.3-4) labelled Protein G magnetic beads at 4°C on a rotary shaker. To remove unbound

proteins, the sample was five-times washed with 0.1% NP-40 in PBS. Finally, bound proteins were eluted in 25 µL 1 × SDS sample buffer (12.5% glycerol, 4% SDS, 2% mercaptoethanol, 50 mM Tris, pH 6.8).

SDS-PAGE and Western blotting

Proteins were separated in 12% SDS-PAGE for Western Blotting or in 4-12% gradient gels (NuPAGE Novex, life) for MS analysis with NuPage MOPS buffer. For Western blot analysis, 2 µL of the protein sample were used per lane. Proteins were transferred to 0.2 µm nitrocellulose membranes (Protran, Whatman), blocked with 5% w/v BSA, 0.1% v/v TWEEN in TBS before incubated in TBS containing 1% w/v BSA with primary and secondary antibodies (Table II.3-4) as follows: mouse anti-M45 (1:5,000) and anti-mouse-IgG-HRP (1:20,000). Proteins were detected by chemiluminescence with ECL detection reagent (Pierce) and blue light-sensitive film (Agfa Healthcare NV).

Protein digest, RP-LC separation, MS and data analysis

In total we performed eight IP proteome experiments whereby IPs 1-3 were used. The IP eluate were separated by 1D-SDS-PAGE. Gel lanes were divided into ten equidistant pieces and excised before subjected to tryptic digest. For subsequent LC-MS/MS measurements, digests were separated by reversed phase chromatography using an EASY-nLC II system (Proxeon, Odense, Denmark) with self-packed columns [Luna 3µ C18(2) 100A, Phenomenex, Germany]. Following loading/desalting in 0.1% acetic acid in water peptides were loaded onto the column at a constant flow rate of 700 nl min and separated by applying a binary non-linear gradient from 5–50% acetonitrile in 0.1% acetic acid over 70 min. MS and MS/MS data were acquired by a LTQ-Velos Orbitrap mass spectrometer (Thermo Fisher, Bremen, Germany) at a spray voltage of 2.4 kV. After a survey scan at a resolution of = 30 000) with activated lock mass option, the twenty most intensive precursor ions were selected for fragmentation. Singly charged ions were excluded from MS/MS analysis.

Database searches were performed with Sorcerer Sequest 4 (version 27, revision 11; SageN, Milptas, CA, USA). UniProt reference database for *Salmonella enterica* sv. Typhimurium 14028s concatenated with human were used as target/decoy databases with a list of common contaminants (downloaded August, 2013). Search parameter included: fixed modification: trypsin; with a MS tolerance: 1.00 Da MS/MS tolerance: 10 ppm; variable modification: methionine oxidation. Proteins were only considered as identified if at least 2 unique peptides (Xcorr > 2.2, 3.3 and 3.8 for doubly, triply and higher

charged peptides with delta cN > 0.1) have been assigned resulting in a FDR of < 1% on the protein level. Scaffold (version 3.5.1, Proteome Software Inc., Portland, OR, USA) was used for data storage and display.

All identified proteins were searched against the UniProt-GOA database as of 08/2013 (Dimmer *et al.*, 2012). Only proteins identified in two (human) and three (*S*. Typhimurium) biological replicates were considered as a candidate of the SMM proteome (Table S II.3-3, Table S II.3-6). Identified proteins were converted using UniProt IP mapping (David and Yip, 2008) (assessed 01/2015).

Subcellular localization of *Salmonella* proteins were sorted by PSORTb 3.0.2 [www.psort.org; (Yu et al., 2010)]. Human proteins are grouped with PANTHER Classification System (www.pantherdb.org) (Mi et al., 2013a; Mi et al., 2013b) according their gene ontology (GO). Following GO annotations are listed: metabolic process (GO:0008152; catabolic process; biosynthetic process; catalytic activity; secondary metabolic process; generation of precursor metabolites and energy; primary metabolic process; coenzyme metabolic process; vitamin metabolic process; sulphur compound metabolic process; phosphate-containing compound metabolic process; nitrogen compound metabolic process), cytoskeleton (GO:0005856; actin cytoskeleton; microtubule organizing centre; microtubule; intermediate filament cytoskeleton); protein metabolic process (GO:0019538; protein folding; protein complex assembly; cellular protein modification process; ferredoxin metabolic process; prosthetic group metabolic process; translation; proteolysis), regulation of biological process (GO:0050789; regulation of cellular amino acid metabolic process; regulation of nucleobase-containing compound metabolic process; regulation of phosphate metabolic process; regulation of vasoconstriction; regulation of carbohydrate metabolic process; regulation of gene expression, epigenetic; negative regulation of apoptotic process; regulation of cell cycle; regulation of translation), transport (GO:0006810; lysosomal transport; nucleobase-containing compound transport; protein transport; vesicle-mediated transport; peroxisomal transport; transporter activity; nuclear transport; vitamin transport; carbohydrate transport; ion transport; phosphate ion transport; mitochondrial transport; extracellular transport; amino acid transport; lipid transport), apoptotic process (GO:0006915; induction of apoptosis; negative regulation of apoptotic process), cellular component organization (GO:0016043; cellular component organization; cellular component biogenesis), and vesicle-mediated transport (GO:0016192; exocytosis; endocytosis).

II.3.6 Acknowledgment

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II.3.7 Supplementary Materials

Additional supporting information is listed on the attached DVD.

II.4 Contributions to Co-authors

Proteomes of host cell membranes modified by intracellular activities of *Salmonella enterica*

Stephanie Vorwerk, Viktoria Krieger, Jörg Deiwick, Michael Hensel, and Nicole Hansmeier

V.K. performed experiments shown in Fig. 6 and Fig. S6. J.D. created the plasmid p3711. N.H. and S.V. performed mass spectrometry and data analysis.

How *Salmonella* modifies its killer – proteomic analysis of *Salmonella*-modified membranes in macrophages

Stephanie Vorwerk, Michael Hensel, and Nicole Hansmeier
N.H. performed mass spectrometry.

The SPI2-T3SS effector SseJ based *Salmonella*-modified membranes proteome

Stephanie Vorwerk, Britta Barlag, Nathalie Böhles, Dörte Becher, Michael Hensel, and Nicole Hansmeier

N.B. performed the experiment shown in Figure II.3-1 and B.B. in Figure II.3-4. Mass spectrometry was performed in the lab of D.B.

III Discussion

III.1 Proteomics as suitable tool for host-pathogen interaction analysis

Infectious diseases are a major burden for health and economy, especially due to the increasing resistance of pathogens to antibiotics (World Health Organization, 2014). This trend is also true for *Salmonella* (Koluman and Dikici, 2013). The development of new antibiotics and vaccines will be a major task in the future. To understand host-pathogen interactions and identify potential new targets, proteomics will be an important tool since it enables data mining using a system level approach (Bumann, 2010).

Proteomic research of host-pathogen interactions is closely related to phagosome proteomics. The protein composition of phagosomes has been targeted in many studies and is well characterized (Garin *et al.*, 2001; Gotthardt *et al.*, 2006; Jutras *et al.*, 2008; Rogers and Foster, 2008; Stuart *et al.*, 2007; Trost *et al.*, 2009). Proteomic analysis of interactions between host and intracellular pathogens is especially challenging because of overwhelming amounts of host proteins against low concentrations of bacterial proteins (Schmidt and Völker, 2011). Thus, the main focus of pathogen proteomics was placed on either pathogen or host proteomes. Intracellular pathogen proteomics have been reviewed with focus on bacterial proteomes (Schmidt and Völker, 2011; Bumann, 2010; Cash, 2011), host proteins (Hartlova *et al.*, 2011; Herweg *et al.*, 2015; Li *et al.*, 2010) or pathogen and host proteins (Coiras *et al.*, 2008; Li and Lostumbo, 2010). Table III-1 summarizes the proteomic studies conducted on the intracellular bacterial pathogens *L. monocytogenes*, *Shigella* spp., *Rickettsia* spp., *F. tularensis*, *Brucella* spp., *Chlamydia* spp., *Mycobacterium* spp., *Legionella* spp., and *S*. Typhimurium.

Remarkably, most proteomic surveys focused on the pathogen proteome and only a few addressed the host proteins (Table III-1). The pathogen-containing vacuoles (PCVs) have been only recently investigated and are mainly described for *B. abortus* (Fugier *et al.*, 2009), *C. trachomatis* (Aeberhard *et al.*, 2015), *M. tuberculosis* (Lee *et al.*, 2010; Li *et al.*, 2011; Rao *et al.*, 2009), and *L. pneumophila* (Bruckert and Abu Kwaik, 2015; Hoffmann *et al.*, 2014; Shevchuk *et al.*, 2009; Urwyler *et al.*, 2009).

Table III-1: Proteomic surveys of selected intracellular pathogens (*L. monocytogenes*, *Shigella* spp., *Rickettsia* spp., *F. tularensis*, *Brucella* spp., *Chlamydia* spp., *Mycobacterium* spp., *Legionella* spp., *S.* Typhimurium)

Pathogen	Cell line/tissue/animal	Target	Method for enrichment	MS	No. of identified proteins	Reference
Listeria monocytogenes						
EGD (serotype 1/2a)	THP-1 (human monocytic cell line)	soluble bacterial proteins	differential and density gradient centrifugation	2DE, MALDI-TOF MS	N/A	(Van de Velde *et al.*, 2009)
F2365 (serovar 4b), EGDe (serovar 1/2a), HCC23 (serovar 4a)	J774A.1 (murine macrophage like cell line)	soluble bacterial proteins		2D LC-MS/MS	N/A	(Donaldson *et al.*, 2011)
***Shigella* spp.** (reviewed in Suh *et al.*, 2014)						
S. flexneri M90T (virG)	HeLa CCL-2TM , iRiKO mouse embryonic fibroblasts (MEFs)	host proteins	no separation	nanoLC-MS/MS	1183 phosphorylated host proteins, 334 proteins with phosphorylation changes	(Schmutz *et al.*, 2013)
S. flexneri 2457T	Henle cells (intestinal 407 cells)	extracellular/ intracellular bacterial proteins	differential and density gradient centrifugation	LC-MS/MS, 2D MALDI-TOF, LC-iontrap-MS/MS	448 bacterial proteins (127 altered in abundance)	(Pieper *et al.*, 2013)
S. flexneri 2a 2457T *iscA*	HeLa cells	host/bacterial proteins	no separation	LC-MS/MS	N/A	(Kentner *et al.*, 2014)
***Rickettsia* spp.**						
R. prowazekii Madrid E		bacterial proteins	differential and density gradient centrifugation	2D LC-MS/MS	252 bacterial proteins	(Chao *et al.*, 2004)
R. conorii (Seven Malish, ATCC VR-613T)	Vero cells	bacterial proteins (crude extract, membrane-enriched)	density gradient centrifugation	2DE MALDI-TOF	N/A	(Renesto *et al.*, 2005)
R. prowazekii Breinl	egg yolk sacs	bacterial proteins	density gradient centrifugation	2D LC-MS/MS	251 bacterial proteins	(Chao *et al.*, 2007)
R. felis (California 2)	XTC-2 cells (*Xenopus laevis*)	bacterial proteins	differential and density gradient centrifugation	2DE MALDI-TOF, nanoLC-MS/MS	165 bacterial proteins	(Ogawa *et al.*, 2007)
R. parkeri Portsmouth	Vero E6 (African green monkey kidney cell line)	bacterial proteins and surface-exposed proteins	differential and density gradient centrifugation	2DE MALDI-TOF/TOF MS	N/A	(Pornwiroon *et al.*, 2009)
R. prowazekii Breinl, Rp22, clinical isolate, Madrid E, Evir	L929 (mouse fibroblast cell line)	bacterial proteins	differential and density gradient centrifugation	2D DIGE MALDI-TOF/TOF	N/A	(Bechah *et al.*, 2010)

Pathogen	Cell line/tissue/animal	Target	Method for enrichment	MS	No. of identified proteins	Reference
R. prowazekii Madrid E	EYS (embryonated hen egg yolk sacs), L929 (murine fibroblast), ISE6 (*I. scapularis* cell line (tick)), Sf21 (*S. frugiperda* insect cell line)	bacterial proteins (host proteins)	differential centrifugation	nanoLC-MS/MS	host/bacterial proteins: 33/128 (EYS/L929); 18/126 (EYS/ISE6); 20/116 (EYS/Sf21); 30/158 (L929/Sf21); 32/70 (ISE6/L929); 14/120 (ISE6/Sf21)	(Tucker *et al.*, 2011)
Francisella tularensis (reviewed in Kilmury and Twine, 2011)						
F. tularensis LVS	J774.2 (murine macrophage-like cell line)	bacterial proteins	metabolic ^{35}S-labelling, differential centrifugation	2DE MALDI-TOF/TOF	N/A (Edman degradation)	(Golovliov *et al.*, 1997)
F. tularensis FSC033	spleen of BALB/c mouse	bacterial proteins	immunomagnetic separation (IMS) with, rabbit-anti-*Francisella* antisera	2DE nanoLC-MS/MS	78 differentially expressed bacterial proteins	(Twine *et al.*, 2006)
F. tularensis subsp. *holarctica* FSC 200	J774.2 (murine macrophage-like cell line)	bacterial proteins	metabolic ^{35}S-labelling, differential centrifugation	2DE-MALDI-TOF/TOF	64 identified macrophage-induced bacterial proteins	(Pávková *et al.*, 2013)
F. tularensis subsp. *holarctica* (LVS)	J774.2 (murine macrophage-like cell line)	host proteins	SILAC, preparation of detergent-resistant membranes (lipid rafts), density gradient centrifugation	2D MALDI TOF/TOF	N/A	(Hartlova *et al.*, 2014)
***Brucella* spp.** (reviewed in de Figueiredo *et al.*, 2015; DelVecchio *et al.*, 2002)						
B. abortus 2308 GFP or 2308 Ds Red	BHK-21 (baby hamster kidney cells)	host proteins	subcellular fraction by density gradient	2DE MALDI TOF/TOF	N/A (proteins of BCV)	(Fugier *et al.*, 2009)
B. suis 1330 (ATCC 23444)	J774.2 (murine macrophage-like cell line)	bacterial proteins	differential and density gradient centrifugation	2D DIGE MALDI TOF MS	168 altered bacterial protein concentrations in comparison with extracellularly grown	(Al Dahouk *et al.*, 2008)
B. abortus 2308/S19	RAW264.7 (murine macrophage-like cell line)	bacterial proteins	osmotic shock, differential and density gradient centrifugation	LC-MS/MS	190 differentially expressed bacterial proteins during the infection time course	(Lamontagne *et al.*, 2009)
B. abortus 2308	HeLa, J774.2	bacterial proteins	differential and density gradient centrifugation	2DE MALDI TOF/TOF MS	2 bacterial proteins overexpressed	(Roset *et al.*, 2013)
B. abortus A19	THP-1 (human monocytic cell line)	host proteins	no separation	2DE MALDI TOF/TOF MS	44 altered host proteins	(Wu *et al.*, 2014)
Coxiella burnetii (reviewed in Kowalczewska *et al.*, 2011; Ihnatko *et al.*, 2012; Toman *et al.*, 2013; Vranakis *et al.*, 2013)						
Nine Mile strains,	egg yolk sacs	bacterial proteins	chemical treatment, differential	2D MALDI TOF; LC-	197 bacterial proteins	(Skultety *et al.*, 2005)

Pathogen	Cell line/tissue/animal	Target	Method for enrichment	MS	No. of identified proteins	Reference
virulent phase I (RSA 493)			centrifugation	MS/MS, MALDI MS/MS		
Nine Mile Crazy (RSA514)	Vero cells (CCL-81, ATTC)	bacterial proteins	density gradient centrifugation	2DE MALDI TOF/TOF MS	N/A	(Coleman *et al.*, 2007)
Nine Mile strains, virulent phase I (RSA 493)	egg yolk sacs	bacterial proteins	differential centrifugation	MALDI TOF MS, LC-MS/MS	N/A	(Hernychova *et al.*, 2008)
Nine Mile strains, virulent phase I (RSA 493), avirulent phase II (RSA 439)	egg yolk sacs	bacterial proteins	differential and density gradient centrifugation	LC-MS/MS	450 bacterial proteins (235 phase I, 215 phase II)	(Skultety *et al.*, 2011)
Nine Mile (RSA439) phase II reference strain	Vero cells (ATCC no. CCL-81)	secreted, bacterial, host proteins	density gradient centrifugation; differential centrifugation	COFRADIC, LC-MS/MS	547 bacterial proteins (53 altered)	(Vranakis *et al.*, 2011)
Nine Mile	MonoMac-1 (human monocytes)	host proteins	differential centrifugation	2DE LC-MS/MS	N/A	(Shipman *et al.*, 2013)
***Chlamydia* spp.**						
C. pneumoniae (VR 1310, ATTC)	HEp-2 cells	bacterial proteins	^{35}S-labeling, density gradient centrifugation	2DE MALDI TOF MS	263 bacterial proteins	(Vandahl *et al.*, 2001)
C. trachomatis A, D, L2	HeLa	bacterial proteins	^{35}S-labeling, density gradient centrifugation	2DE MALDI TOF MS	250 bacterial proteins	(Shaw *et al.*, 2002)
C. pneumoniae CWL029 (VR 1310, ATTC)	HEp-2 cells	bacterial proteins	^{35}S-labeling, differential and density gradient centrifugation	2DE MALDI TOF MS	N/A	(Wehrl *et al.*, 2004)
C. pneumoniae A-03 (ATCC VR-1452)	HEp-2 cells	bacterial proteins	^{35}S-labeling, no separation	2DE MALDI TOF MS	N/A	(Mukhopadhyay *et al.*, 2004)
C. trachomatis, biovar LGV, serovar L2, 434/Bu isolate	BGMK cells (Buffalo green monkey kidney)	bacterial proteins	density gradient centrifugation	2DE, MudPIT, LC-MS/MS	328 bacterial proteins	(Skipp *et al.*, 2005)
C. trachomatis, biovar LGV, serovar L2, 434/Bu isolate	HeLa 229	bacterial proteins	density gradient centrifugation, sarkosyl fractionation of EB	LC-MS/MS	329 bacterial proteins	(Liu *et al.*, 2010)
C. trachomatis, biovar LGV, serovar L2, 434/Bu isolate	HeLa CCL2	bacterial proteins	density gradient centrifugation	LC/LC-MS/MS	485 non-redundant bacterial proteins	(Saka *et al.*, 2011)
C. trachomatis L2 lymphatic isolate, 434/Bu	HeLa	host proteins	density gradient centrifugation, immune-magnetic separation with anti-IncA antibody, SILAC	LC-MS/MS	1400 inclusion host proteins, 351 inclusion-associated host proteins	(Aeberhard *et al.*, 2015)

Pathogen	Cell line/tissue/animal	Target	Method for enrichment	MS	No. of identified proteins	Reference
***Mycobacterium* spp.** (reviewed in de Souza and Wiker, 2011; Mehaffy *et al.*, 2012; Calder *et al.*, 2015; Gengenbacher *et al.*, 2014; He *et al.*, 2012)						
M. tuberculosis H37Rv	bone marrow-derived macrophages (C57BL/6 mouse)	bacterial proteins	density gradient centrifugation	2DE MALDI TOF/TOF MS	N/A	(Mattow *et al.*, 2006)
M. tuberculosis H37Rv, Δ*fbpA*, BCG Pasteur vaccine strain	BMA.A3 (mouse macrophage like cell line macrophages of C57BI/6 mouse)	host proteins	differential and density gradient centrifugation	nanoLC-MS/MS	322 host proteins	(Rao *et al.*, 2009)
M. bovis BCG	THP-1 (human monocytic cell line)	host proteins	density gradient centrifugation	LC-MS/MS	447 BCG phagosome host proteins; 289 latex bead phagosome host proteins	(Lee *et al.*, 2010)
M. tuberculosis H37Rv	guinea pig lungs	bacterial proteins	differential gradient centrifugation	nanoLC-MS/MS	545 bacterial proteins	(Kruh *et al.*, 2010)
M. tuberculosis H37Rv, *fbpA* mutant, BCG Pasteur vaccine strain	bone marrow derived macrophages and DCs from C57BL/6 mouse, BMA.A3 (mouse macrophage like cell line)	host proteins	differential and density gradient centrifugation	nanoLC-MS/MS	host proteins: 890 (BCG), 780 (KO), 680 (H37Rv), DCs: 830 (BCG), 860 (KO), 810 (H37Rv)	(Li *et al.*, 2011)
M. tuberculosis H37Rv, H37Ra, *M. smegmatis*	THP-1 (human monocytic cell line)	intracellular bacterial secretome	Myco-MetRS for selective proteome labelling through ANL incorporation	LC-MS/MS	15 (H37Ra), 31 (H37Rv) 22 (BND433) bacterial proteins	(Chande *et al.*, 2015)
***Legionella* spp.**						
L. pneumophila Corby, *L. heckeliae*	*Dictyostelium discoideum* AX2	host and bacterial proteins	density gradient centrifugation	2DE MALDI TOF MS	157 phagosome host proteins	(Shevchuk *et al.*, 2009)
L. pneumophila JR32	*D. discoideum*	host proteins	immune-magnetic separation with anti-SidC antibody, density gradient centrifugation	LC-MS/MS	566 host proteins	(Urwyler *et al.*, 2009)
L. pneumophila JR32	RAW264.7	host proteins	immune-magnetic separation with anti-SidC antibody, density gradient centrifugation	LC-MS/MS	1156 host proteins, 434 bacterial proteins	(Hoffmann *et al.*, 2014)
L. pneumophila AA100/130b WT	human U937 macrophages	host proteins	differential centrifugation	nanoLC-MS/MS	1193 (ubiquitinated: 24) host proteins	(Bruckert and Abu Kwaik, 2015)

Pathogen	Cell line/tissue/animal	Target	Method for enrichment	MS	No. of identified proteins	Reference
***Salmonella enterica* sv. Typhimurium**						
ATCC 14028	RAW264.7	host and bacterial proteins	differential and density gradient centrifugation; differential solubilisation by detergents	LC-MS/MS, FTICR MS	315 bacterial proteins, 371 host proteins	(Shi *et al.*, 2006)
SL1344 *hisG rpsL xyl*	BALB/c mouse: caecum, spleen	bacterial proteins	flow cytometry	LC-MS/MS	898 bacterial proteins	(Becker *et al.*, 2006)
ATCC 14028	RAW264.7	host and bacterial proteins	differential solubilisation by detergents	LC-MS/MS	115 bacterial proteins, 1006 host proteins	(Shi *et al.*, 2009b)
ATCC 14028	HeLa	host proteins	differential and density gradient centrifugation	SILAC, LC-MS/MS	765 (105 affected) host proteins	(Vogels *et al.*, 2011)
phagetype DT104 (isolate from pig)	piglet ileum/lymph nodes	host proteins	no separation	2D DIGE MALDI-TOF/TOF	N/A	(Collado-Romero *et al.*, 2012; Martins *et al.*, 2012)
SL1344, SL1344_*ssaR*	RAW264.7, HeLa	host proteins	differential centrifugation	nanoLC-MS/MS, phophoproteomic	RAW264.7: 442 (112 regulated), HeLa: 606 (57 regulated) host proteins	(Imami *et al.*, 2013)
SL1344 *sifB::gfp*	BALB/c mouse: spleen	bacterial proteins	flow cytometry	LC-MS/MS	1182/1589 bacterial proteins	(Claudi *et al.*, 2014; Steeb *et al.*, 2013)
SL1344	HeLa CCL2	host proteins	differential and density gradient centrifugation	LC-MS/MS	2522 host proteins (30 min: 173 proteins enriched, 3 h: 124, 95 at both time points)	(Santos *et al.*, 2015)
SL1344	HeLa	bacterial proteins	differential centrifugation	LC-MS/MS	1675 bacterial proteins	(Liu *et al.*, 2015)

N/A, number of identified proteins is not available; 2 DE, 2 dimensional electrophoresis; BCG, Bacille Calmette-Guérin; COFRADIC, combined fractional diagonal chromatography; DIGE, difference gel electrophoresis; FTICR, Fourier transform ion cyclotron resonance; iRiKO; inducible Rictor-knockout; LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MudPIT, multidimensional protein identification technology; SILAC, stable-isotope labelling with amino acids in cell culture; TOF, time-of-flight

PCVs are vacuoles which are modified by pathogens to avoid phagolysosomal degradation and enable formation of a replicative niche (Meresse *et al.*, 1999b). The proteomic analysis of these pathogen-containing host compartments requires special enrichment methods (Herweg *et al.*, 2015). The aforementioned studies have all in common that the pathogens remain in a vacuolar phagosomal compartment, which allows purification techniques such as differential and/or density gradient centrifugation, or these approaches in combination with immunomagnetic separation (IMS) (Table III-1).

The use of immunomagnetic separation (IMS) for proteomic analysis follows protocols developed for the isolation of intracellular *F. tularensis* (Twine *et al.*, 2006). This method showed its great capability for purification of PCVs derived from various intracellular pathogens. For instance, Twine *et al.* (2006) focused on the isolation of *F. tularensis* from the host cell cytosol, whereas the PCVs of *L. pneumophila* (Hoffmann *et al.*, 2014; Urwyler *et al.*, 2009) and *C. trachomatis* (Aeberhard *et al.*, 2015) (Table III-1) were the target for proteome analysis. *F. tularensis* were isolated *in vivo* from spleens of infected mice by using IMS with rabbit-anti-*Francisella* antisera and the bacterial proteins were separated by 2DE and selected spots were analysed by nanoLC-MS/MS (Twine *et al.*, 2006). In addition to this, the successful isolation of LCVs from *D*. *discoideum* and murine macrophage-like RAW264.7 cell line has been demonstrated by IMS with an antibody against SidC, an effector protein that binds via a 20 kDa PtdIns(4)*P*-binding domain to the LCV membrane (Hoffmann *et al.*, 2014; Urwyler *et al.*, 2009). The isolation method of the inclusions of *C. trachomatis* was similar to the one used for LCV purification. An antibody against the bacterial IncA transmembrane protein, located in the inclusion membrane, was utilized in IMS and additionally the host protein composition of the inclusion was quantitatively analysed with SILAC (Aeberhard *et al.*, 2015).

In contrast to these pathogens with phagosomal compartments, *S*. Typhimurium remains in a vacuole-like compartment, called early SCV, only for the first hours post infection. During replication on the other hand the SCV extends and forms a continuum of elaborate tubular membrane structures, which is an intrinsic feature among intracellular bacteria (Liss and Hensel, 2015; Schroeder *et al.*, 2011). Therefore the purification of intact SCV were considered as especially challenging (Rogers and Foster, 2008). Thus, until recently *Salmonella* investigations mainly focussed on the proteome of *Salmonella* (Becker *et al.*, 2006; Claudi *et al.*, 2014; Liu *et al.*, 2015; Shi *et al.*, 2006; Shi *et al.*, 2009b; Steeb *et al.*, 2013), changes in host proteome after infection (Imami *et al.*, 2013; Shi *et al.*, 2009b; Vogels *et al.*, 2011), or interaction partners of effectors (Auweter *et al.*, 2011). Recently the proteomes for early and maturing SCVs have been published (Santos *et al.*, 2015).

Facing the actual challenges, we developed a new approach. To analyse the protein composition of the established SMM network at later time points, specific enrichment of

these membranes without dilution effects or low specificity was required. The method is a combination of differential centrifugation and affinity purification using the epitope-tagged SPI2-T3SS effector SseF as bait protein (Chapter II.1, Fig. 1) (Vorwerk *et al.*, 2015). SseF was chosen because of its features as integral membrane protein, location in SCV and SIF membrane and amenability for addition of C-terminal tags (Abrahams *et al.*, 2006; Müller *et al.*, 2012). Use of SseF as bait enables the successful analysis of the protein composition of SMM for the first time (Vorwerk *et al.*, 2015). Furthermore, this approach was transferable from an epithelial cell line (HeLa) to macrophage-like cells (RAW264.7) (Chapter II.2) and should also be applicable to other cell lines (e.g. primary cells like bone marrow-derived macrophages).

Additionally, we tested the approach in combination with another SPI2 effector protein SseJ. It is located at the membrane of the SCV and SITS and involved in SCV membrane dynamics by remodelling the lipid and protein content of the SCV membrane and membrane tubulation (Kolodziejek and Miller, 2015). Purification of SMM using the effector SseJ showed a remarkably low overlap (5 proteins) with the SseF-based SMM proteome (Chapter II.3). Several possible explanations might be responsible for these differences in the resulting protein composition. SseJ and SseF were both located in SMM but super-resolution images indicate that they are not co-localized in low nanometre scale (Figure II.3-4). This could possibly result in the purification of different SMM parts. Furthermore, SseJ is not an integral membrane protein, so that during purification steps transiently changing interaction partners could be purified. Switching to a different, more sensitive MS-setup did not increase the number of identified proteins. The restrictive analysis criteria applied for identification of unique SMM proteomes by eliminating all proteins of the control, used in this work, reduced extremely the amount of specifically identified proteins. The enormous number of proteins in the negative control (2224 proteins) was completely excluded from 2823 identified human proteins in total. Nevertheless, the use of sensitive instruments will be necessary in the future to identify low abundant proteins of the SMM.

This aspect illustrates that the use of more sensitive instrument necessitates a quantitative approach to discriminate between false-positives and false-negatives. Many different approaches are available for quantitative proteomics (reviewed in context of microbiology in Otto *et al.*, 2014). A label free approach is spectral counting in which the acquisition of the total number of MS/MS for the same peptide enables to analyse the relative abundance of this peptide in the mixture (Zhang *et al.*, 2014). The other possibility is stable isotopic labelled quantitation. Two distinct approaches exist: proteins are labelled upstream of the sample preparation or peptides are labelled post-digestion. SILAC (stable isotope labelling with amino acids in cell culture) is a widely used metabolic/*in vivo*

labelling technique. During cell growth proteins are labelled with heavy amino acids added to the media and are mixed with protein sample of standard media with natural isotopic composition amino acids. The relative quantitation is determined by measuring the intensity ratio of the peptide pairs which differs in their mass because of the different isotope composition (Ong *et al.*, 2003). Another approach is *in vitro* or chemical labelling post-digestion with isobaric tags specifically labelled peptides containing primary amine groups. Isobaric mass tags (iTRAQ) or tandem mass tags (TMT) facilitate relative and absolute quantitation. Peptides which are labelled with different isobaric tags cannot be distinguished in MS, but they generate reporter ions with different masses in MS/MS (Zhang *et al.*, 2014). Their ratios of the peak intensities allow quantitation.

Label free and SILAC-based quantitations have been successfully performed in the context of intracellular pathogen compartments (Aeberhard *et al.*, 2015; Santos *et al.*, 2015).

In future the combination of quantitative proteomics with the novel, in this work developed, approach to purify SMMs will enlarge the possibilities of further applications. Comparisons of the protein composition of SMM between different time points of infections, various cell lines, *Salmonella* effector mutants with SIF-phenotypes as well as SMM proteomes fished by different effector proteins are some examples for further investigations that will be useful to gain new knowledge about the biogenesis of SITs.

III.2 The *Salmonella*-modified membrane proteome

The focus of this work was the analysis of the protein composition of *Salmonella*-modified membranes. These membranes are formed by the activity of *Salmonella* effectors to generate an extensive tubular network connected with SCVs (Schroeder *et al.*, 2011). Our newly developed methods utilizing the integral membrane effector SseF as target protein in IMS allowed the identification of 247 host SMM proteins of *Salmonella*-infected HeLa cells at late stage of infection (8 h p.i.) with fully established SIT network (Chapter II.1 , Vorwerk *et al.*, 2015). In contrast, the SMM proteome of RAW264.7 cells at a late phase of infection (12 h p.i.) consists of 262 and 142 proteins of resting or IFNγ-activated RAW264.7, respectively (Chapter II.2). Comparison of the SMM proteomes of HeLa cells and non-activated macrophages reveals 46 identical proteins (Table SII.2-7). This is particularly remarkable since highly diverse cell lines as host (human epithelial vs. mouse macrophage-like cells) and different time points after infection (HeLa: 8 h, RAW264.7: 12 h) were used for analysis. However, these distinct time points for harvesting were chosen because of the observation that *Salmonella* in RAW264.7 cells are delayed in effector expression and SIF network formation in contrast to HeLa cells (Figure II.2-1/2). The selected time points showed similar values in the amount of cells which are positive for the M45-tagged effectors (Chapter II.1 Fig. 3B, Fig. II.2-3). Furthermore, the SIF network is stable in both cases, at least visible in HeLa and IFNγ-activated RAW264.7 (Chapter II.1 Fig. 3A, Figure II.2-1) and the effector SseF is well detectable in co-localisation with LAMP1 by immunostaining (Chapter II.1 Fig. 2, Figure II.2-2). Thus, differences based on time variance cannot be excluded but these chosen time points allow comparable conditions between *Salmonella*-infected HeLa and RAW264.7 cells. Altogether 46 proteins are in common in the HeLa and RAW264.7 SMM proteome and therefore comprise the cell independent SMM proteins for late time points of infection (Table SII-2.7). Eighteen proteins especially notable are those associated with the cytoskeleton (α-actinin-1, actin-related protein 2/3 complex subunit 4, F-actin-capping protein subunit β, myosin-9), involved in vesicle trafficking (AP-2 complex subunit β1, coatomer subunit α/γ1, EH domain-containing protein 4, Ras-related protein Rab5c, Rab14, Rap1b, α-soluble NSF attachment protein, transmembrane emp24 domain-containing protein 10, vesicle-associated membrane protein-associated protein A), or located at the Golgi and ER (hypoxia up-regulated protein 1/GRP-170, dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2/ribophorin II, protein transport protein Sec61 subunit β, signal recognition particle receptor subunit α) (Table SII.2-7). An overview of selected identified proteins and their subcellular origins as possible source for SMM is shown in Figure III-1.

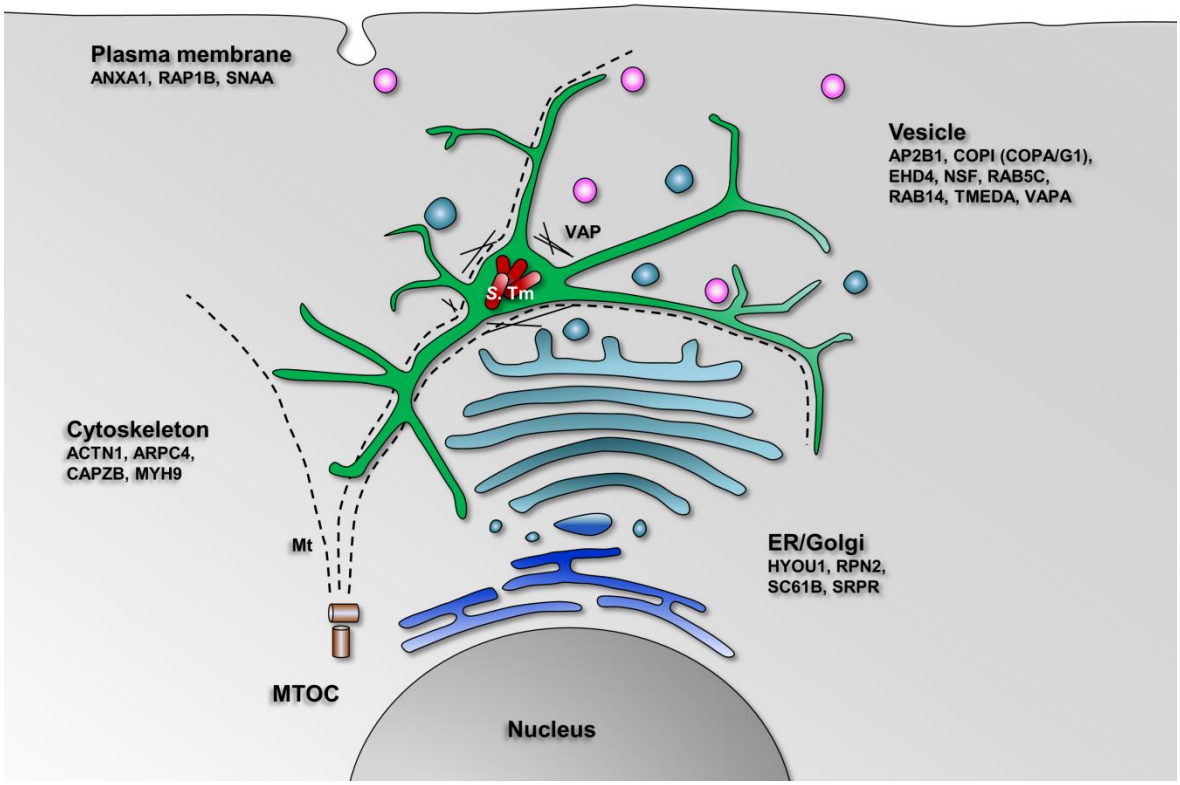

Figure III-1: Model for the source of host proteins recruited to *Salmonella*-modified membranes
Remodelling of the host facilitates establishment of a tubular membrane network throughout the cell by *S*. Typhimurium. Shown are selected proteins consistently identified in the SMM proteome from HeLa cells and RAW264.7 macrophages and involved in the endocytic (pink bubbles) and secretory vesicle (light blue bubbles) transport system, with the ER/Golgi apparatus, and the cytoskeleton. By activities of the bacterium they are recruited to the SMM network. *S*. Tm, *Salmonella* Typhimurium; MTOC, microtubule-organizing centre; Mt, microtubule; VAP, vacuole-associated actin polymerization; ER, endoplasmic reticulum.

These results demonstrate the importance of the cytoskeleton and its associated proteins for SCV and SIF formation. On the one side actin plays a role for VAP which could contribute to SCV integrity and for actin filament co-localization with SIFs for stabilization (Meresse *et al.*, 2001; Miao *et al.*, 2003).On the other side microtubules are important also serving as stabilizing scaffolds for SIFs (Brumell *et al.*, 2002a; Rajashekar and Hensel, 2011). While the cytoskeletal proteins build the scaffold and routes for the SMM network, vesicles are the transport vehicle to supply intracellular *S*. Typhimurium. It seems that the bacterium is able to redirect the endocytic and secretory vesicle trafficking for its own purpose. This is indicated by the presence of components of EE (Rab5c) (Bucci *et al.*, 1995), RE (Rab14) (Junutula *et al.*, 2004), COPI (α/γ1-COP, p24δ1) (Beck *et al.*, 2009) and clathrin-coated vesicles (AP-2 complex subunit β) (Nakatsu and Ohno, 2003).

Usurpation of the ER/Golgi vesicle transport could be used by *Salmonella* as additional source for membrane material and nutrients. Influence of the ER to intracellular *Salmonella* proliferation has not been described yet, but the identification of proteins

involved in ER/Golgi trafficking (α/γ1-COP, TMEDA, VAP-A) and ER proteins (GRP-170, ribophorin II, Sec61-B, SR-α) suggests a contribution which will be discussed in more detail in context with the proteins GRP-170 in Chapter III.3.2 and γ1-COP/p24δ1 in Chapter III.3.3.

Rab GTPases are the key players in vesicle trafficking and therefore important regulators for *Salmonella* to manipulate. In the SMM proteome of infected HeLa cells six Rab proteins (Rab2a, Rab5c, Rab7a, Rab10, Rab11a, Rab14) were identified (Chapter II.1), whereas Rab5c, Rab8b, Rab14, Rab18 were associated with the macrophage SMM and only Rab1b in IFNγ-activated RAW264.7 cells (Chapter II.2). These differences in recruitment of Rab GTPases suggest that *Salmonella* is manipulating the regulation of vesicle trafficking in a cell- and activation state-dependent way. Nevertheless, two small GTPases, i.e. Rab5c and Rab14 were identified in the SMM proteome common for HeLa and RAW264.7. Rab5 is the key player in early endosome maturation (Numrich and Ungermann, 2014). However, three isoforms can be distinguished with overlapping but also specialised functions (Bucci *et al.*, 1995; Chavrier *et al.*, 1992; Wilson and Wilson, 1992). First the role of Rab5a in early endocytosis has been well described (Bucci *et al.*, 1992; Gorvel *et al.*, 1991). All isoforms collaborate in endocytosis (Bucci *et al.*, 1995). In addition, they are differently expressed after cytokine treatment and especially the expression of Rab5c is increased (Bhattacharya *et al.*, 2006). The isoforms Rab5a and Rab5b, but not Rab5c, are involved in trafficking and degradation of epidermal growth factor receptor (EGFR): the degradation of EGFR was impeded in cells that were depleted in Rab5a and Rab5b, but only minimally in Rab5c depleted cells (Chen *et al.*, 2009). Rab5c is a binding partner of AMAP1, which is regulated by Arf6 and involved in integrin recycling (Onodera *et al.*, 2012). Only depletion of Rab5c altered cell morphology in HeLa cells. Such cells are spindle-shaped and the formation of membrane ruffles and cell motility is reduced. This is linked with a reduction of EGF-stimulated Rac1 activity (Chen *et al.*, 2014). These results illustrate that the different isoforms of Rab5 are regulatory parts of a fine-tuned endocytic network which is manipulated by *Salmonella* during infection. At the early phases of infection Rab5a function is important for SCV maturation (Mallo *et al.*, 2008; Mukherjee *et al.*, 2001). Co-localisation of all three isoforms has been detected at early SCVs until 60 min p.i. (Smith *et al.*, 2007). Interestingly, only Rab5c was identified in the SMM proteome at late phase of infection (Table SII.2-7) and additionally verifiably located at the SIFs (Chapter II.1 Fig. 6). Nonetheless, it is possible that all Rab5 isoforms are involved in maturation of SCVs from early to late SCVs. While the isoforms Rab5a and Rab5b seem to be removed during this process, Rab5c stays in a noticeable amount at SCVs to maintain some characteristics of an early endosome potentially to impede the maturation to lysosomes. Rab5c is differently regulated in contrast to the other

isoforms which has been demonstrated in the context of EGFR and AMAP1 (Chen *et al.*, 2009; Chen *et al.*, 2014; Onodera *et al.*, 2012), making it a primary target for manipulation by *Salmonella*. Interestingly, also Rac1 is part of the SMM proteome in macrophages (Chapter II.2). The different up- and down-stream effectors of the Rab5 isoforms are probably directly or indirectly affected by *Salmonella* and this leads to a continuous association of Rab5c with the SMM.

Another identified small GTPase in both HeLa and RAW264.7 SMM proteomes is Rab14. This GTPase has been shown to be recruited to maturating SCVs, particularly early SCVs (Smith *et al.*, 2007) and also SIFs (Chapter II.1 Fig. 6). Its contribution to SCV maturation has also been demonstrated elsewhere (Kuijl *et al.*, 2013) and will be discussed in detail in the following (Chapter III.3.3).

The determined protein composition of the SMM indicates that *Salmonella* hijacks many different transport pathways to establish its intracellular niche which ultimately allows the bacterial replication. It should be investigated how the presence and absence of proteins identified in proteomic studies affect intracellular *Salmonella*. The influence of these proteins should be analysed on single cell level to clarify their impact on proliferation inside the SCV and SIT formation.

Additionally, it seems that infection time, cell type, and cell status influence the protein composition. However, also general manipulation strategies exist that target the same proteins in different cells. Aim of following studies should be the identification of these differences and similarities. This knowledge will help to understand how *Salmonella* is able to manipulate so many different hosts and can pave the way for the development for new drugs or vaccines.

III.3 Comparison of PCV proteomes

The comparison of PCV proteomes is a suitable tool to identify commonalities of pathogen strategies for the manipulation of host cells, also lending itself for the identification of potential new drug targets.

The proteomes of the SMM (Chapter II.1, Chapter II.2) and the published proteomes of three other pathogens that reside in PCVs (*L. pneumophila*, *C. trachomatis* and *M. bovis*) were compared (Table S III-3) and proteins common to all PCV proteins are listed in Table III-2. An overview of proteins recruited to PCVs is shown in Figure III-2. Despite different hosts, different purification methods, and different MS analysis methodology it was possible to determine ten proteins that are congruent in all analysed proteomes (Table III-2). Proteome data of the PCVs were obtained from LCV purified by IMS and gradient centrifugation from *Dictyostelium discoideum* (Urwyler *et al.*, 2009) or mouse macrophage RAW264.7 cells (Hoffmann *et al.*, 2014). To identify *C. trachomatis* inclusion proteins of infected HeLa cells also IMS and gradient centrifugation combined with SILAC were used (Aeberhard *et al.*, 2015). *M. bovis* phagosomes were isolated from human macrophages (THP-1 cells) by density gradient centrifugation (Lee *et al.*, 2010).

Table III-2: The overlapping proteins of PCVs of *S*. Typhimurium (HeLa and RAW264.7) (Vorwerk *et al.*, 2015; Chapter II-1; Chapter II-2), *L. pneumophilia* (Hoffmann *et al*., 2014; Urwyler *et al*., 2009), *C. trachomatis* inclusion (Aeberhard *et al.*, 2015) and *M. bovis* (Lee *et al*., 2010)

Entry name	Protein names	Subcellular location
ANXA1	Annexin A1	Nucleus, cytoplasm, cell projection, cilium
ARPC4	Actin-related protein 2/3 complex subunit 4	Cytoplasm, cytoskeleton, cell projection
CAPZB	F-actin-capping protein subunit β	Cytoplasm, cytoskeleton
COPG1	Coatomer subunit γ-1	Cytoplasm, Golgi, vesicle, COPI-vesicle
HYOU1	Hypoxia up-regulated protein 1 (GRP170)	ER
RAB14	Ras-related protein Rab14	RE, EE, Golgi apparatus, vesicle, phagosome
TCPE	T-complex protein 1 subunit ε	Cytoplasm, cytoskeleton, MTOC
TCPG	T-complex protein 1 subunit γ	Cytoplasm
TCPZ	T-complex protein 1 subunit ζ	Cytoplasm
TMEDA	Transmembrane emp24 domain-containing protein 10 (p24δ1)	Golgi, ER, ERGIC, vesicle

COPI, coat protein complex I; EE, early endosome; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; MTOC, microtubule-organizing centre; RE, recycling endosome;

Two proteins are involved in actin cytoskeleton formation: Arp2/3 subunit 4 (ARPC4) and the F-actin-capping protein subunit β (CAPZB). Four proteins possess functions in protein folding, whereby three proteins belong to the T-complex protein 1 (TCPE/G/Z), apart from the hypoxia up-regulated protein 1 (HYOU1/GRP-170). The remaining proteins play a role in protein sorting (annexin A1, ANXA1; coatomer subunit γ-1, COPG1; Rab14; transmembrane emp24 domain-containing protein 10, TMEDA/p24δ1). It cannot be excluded that these identified proteins are abundant proteins causing a common contamination. However, as discussed the following text the proteins might have plausible functions for intracellular bacteria and PCV biogenesis.

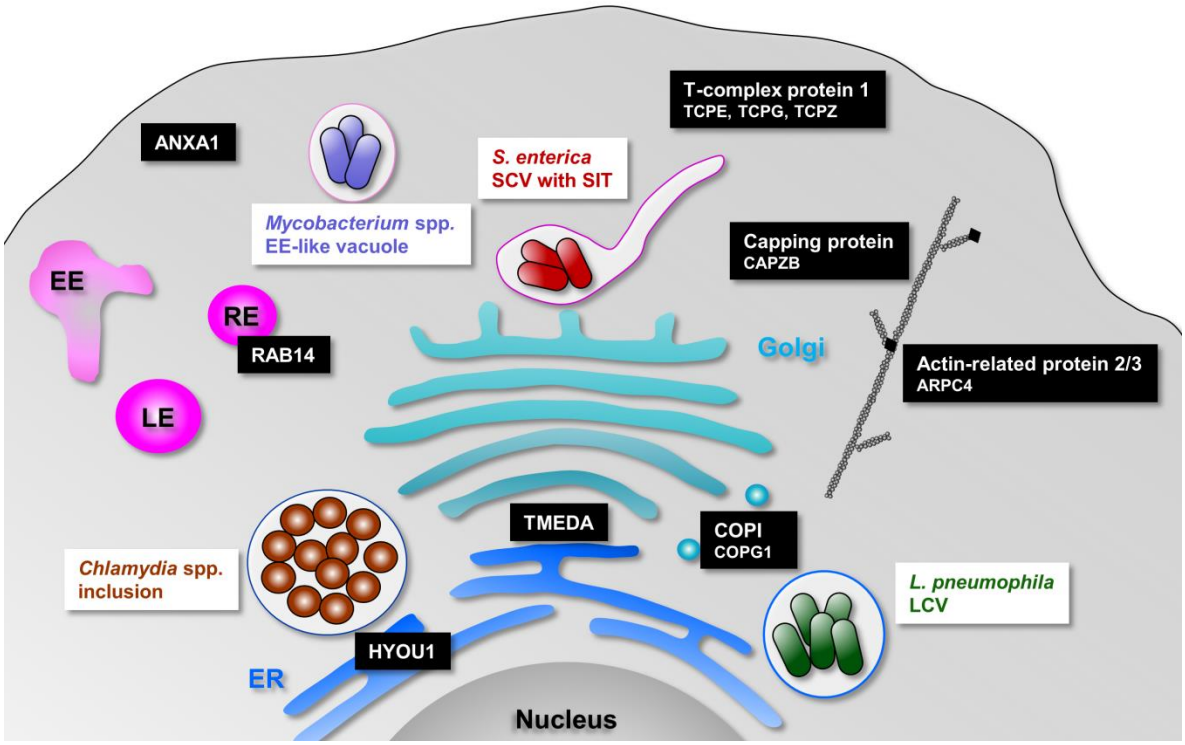

Figure III-2: Overview of the proteins recruited to PCVs of *S*. Typhimurium, *L. pneumophilia*, *C. trachomatis* and *M. bovis*.
Ten proteins were identified as common to the PCV proteomes: two actin cytoskeletal proteins (Arp2/3 subunit 4, ARPC4; F-actin-capping protein subunit β, CAPZB), four cytosolic proteins (T-complex protein 1, TCPE/G/Z; annexin A1, ANXA1), one ER protein (hypoxia up-regulated protein 1, HYOU1/GRP-170), two ER/Golgi trafficking proteins (coatomer subunit γ-1, COPG1; transmembrane emp24 domain-containing protein 10, TMEDA/p24δ1) and one endocytic vesicle protein Rab14. These proteins seems to be recruited by the activity of intracellular pathogens to their compartment and suggest a contribution of their respective organelle in biogenesis of PCVs. EE, early endosome; ER, endoplasmic reticulum; LE, late endosome; RE, recycling endosome.

III.3.1 Actin cytoskeletal proteins

Actin-related protein 2/3 complex (Arp2/3) – ARPC4

One of two cytoskeleton proteins of the overlapping list is the actin-related protein (Arp) 2/3 complex subunit 4 (ARPC4). The Arp2/3 complex generates actin branches. The subunits ARPC1-5 form the framework located at the mother filament for positioning of the subunits ARP2 and ARP3 as start point of the daughter filament with a characteristic ~70° angle of the branch to the mother filament (Pollard, 2007). Activators of the Arp2/3 complex are nucleation promoting factors (NPFs) which are separated in two classes. Class I NPFs possess a carboxy-terminal WCA domain and subcategorized into five groups: WASP (Wiskott-Aldrich syndrome protein) and neural WASP (N-WASP), WAVE (WASP-family verprolin homologue, also known as SCAR), WASH (WASP and SCAR homologue), and JMY (junction-mediating regulatory protein) (Campellone and Welch, 2010). Cortactin and HS1 belong to the second class of NPFs. Additionally, branching inhibitors exist: Type I coronins, cofilin, GMF (glia maturation factor), gadkin and protein interacting with C kinase 1 (PICK1) (Rotty *et al.*, 2013). The Arp2/3 complex is involved in many different cellular functions such as phagocytosis, endocytosis, endosomal fission, cytoplasmic streaming by polymerizing branched actin and at the leading edge of migrating cells. In all these processes Arp2/3-generated branched actin provides the driving force for the formation of protrusions (lamellipodia) (Rotty *et al.*, 2013).

For *S*. Typhimurium it is known that the Arp2/3 complex is recruited to SPI1-T3SS-induced membrane ruffles (Criss and Casanova, 2003; Stender *et al.*, 2000) but it is not essential for the invasion (Hänisch *et al.*, 2010). The translocated effectors SopE/E2 and SopB are responsible for cytoskeletal rearrangement during uptake (Stender *et al.*, 2000). SopE and SopE2 mimic cellular guanine exchange factors (GEFs) and are able to activate the Rho GTPases Cdc42, Rac1, and RhoG (Friebel *et al.*, 2001; Hardt *et al.*, 1998), and the inositol phosphatase SopB indirectly activates Cdc42 and RhoG (Norris *et al.*, 1998; Patel and Galán, 2006) which in turn activate the NPFs of Arp2/3. The NPFs N-WASP, WAVE and WASH are involved in this process (Hänisch *et al.*, 2010; Unsworth *et al.*, 2004) with Cdc42/WAVE and N-WASP again not being essential for invasion (Hänisch *et al.*, 2010). It has been demonstrated that ARP3, a protein of the Arp2/3 complex, is not recruited to *S.* Typhimurium microcolonies and that an overexpression of GFP-N-WASPΔWA, a truncated form of N-WASP with dominant-interfering effect on the activity of the wild type protein, reduced invasion but actin assembly of intracellular *Salmonella* was not effected (Unsworth *et al.*, 2004). However, in this comparison ARPC4, a protein

binding the mother filament, was identified (Table II.2-1). Its location is maybe altered by *Salmonella*. One possibility could be that complex formation is blocked by *Salmonella* effectors which would inhibit actin branching. This would be in agreement with observations that the actin meshwork around the microcolonies is predominantly consisting of filaments with less branches (Meresse *et al.*, 2001). It might be that the effectors SteC and SpvB are play a role this process. The SPI2 effector SteC is involved in the formation of the VAP (vacuole-associated actin polymerization) by activating the MAP kinases MEK and ERK, myosin light chain kinase (MLCK) and myosin IIB to accumulate actin filaments (Odendall *et al.*, 2012), whereas SpvB has converse function. This effector disrupts actin cytoskeleton of infected cells at late time points and inhibits VAP formation by depolymerisation of actin by ADP-ribosylation of monomeric actin (Miao *et al.*, 2003; Tezcan-Merdol *et al.*, 2001). Interestingly, actin filaments were identified inside or associated with SITs (Krieger *et al.*, 2014) and actin has a role in SIF formation (Chikkaballi, unpublished data).

To identify the impact of Arp2/3 to SCV and SIF formation the specific and reversible inhibition of this complex with the small-molecule CK-666 (Nolen *et al.*, 2009) would be interesting to analyse by live cell imaging of *Salmonella*-infected cells at different time points after infection. By using an inhibitor side effects observed with knockout or knockdown would be avoided (Rotty *et al.*, 2013).

Another interesting aspect is the observation that in cells which depleted of WASH, an NPF of Arp2/3 complex, endosomes form long and highly dynamic membrane tubules along microtubules (Derivery *et al.*, 2009) which could resemble SITs. It has been demonstrated that endosomes positive for Rab4, Rab5, Rab7, and Rab11 were tubulated upon RNAi-mediated knockdown of WASH. The authors suggest that WASH contributes to the fission of transport intermediates from sorting and recycling endosomes (Derivery *et al.*, 2009). Additionally, it has been demonstrated that WASH is located in microdomains of the endosomal membrane (Derivery *et al.*, 2009; Derivery *et al.*, 2012). Branched actin filaments with Arp2/3 as nucleation factor are a key player in endosomal sorting. In this context discrete actin patches seem to be a platform for signalling to induce localised force that could contribute to the formation and fission of endosomal tubules (Seaman *et al.*, 2013). This would also be a possible scenario for the formation of SITs and their dynamic behaviour by microtubule-dependent pulling forces and actin-dependent pushing forces.

Interestingly, it has been shown for *M. tuberculosis* that ARPC4 expressed in the bacterium severely affects growth and the immune response *ex vivo* (Ghosh *et al.*, 2013). ARPC4 interacts with an essential protein Rv1626 that is predicted to be a transcription anti-terminator. Further functions are proposed because of its property to enhance the

actin polymerization efficacy of the Arp2/3 complex (Ghosh *et al.*, 2013). This interaction allows drawing conclusions about the importance of ARPC4 for *M. tuberculosis* intracellular survival. An interaction with the Arp2/3 complex and its involvement in uptake and biogenesis of the PCV would be possible if proteins like Rv1626 were secreted by *M. tuberculosis*.

Also, during *C. trachomatis* invasion actin recruitment to the invasion site is based on Arp2/3 activity. Arp2/3 in conjunction with Rac and NPFs WAVE2 and Abi-1 is necessary for uptake (Carabeo *et al.*, 2007). Additionally Hybiske and Stephens (2007) have shown that the entry of *C. trachomatis* into non-phagocytic cells is dependent on clathrin-mediated endocytosis by analysing the effect of RNAi knockdown of the clathrin heavy chain, dynamin-2, heat shock 70-kDA protein 8, ARP2, cortactin, and calmodulin. Furthermore, the chlamydial translocated actin recruiting phosphoprotein (Trap) cooperates with the Arp2/3 complex to enhance actin filament formation (Jiwani *et al.*, 2012). The Arp2/3 complex is also utilized for intracellular motility by various intracellular bacterial pathogens (reviewed in Truong *et al.*, 2014; Welch and Way, 2013). *S. flexneri* uses an Arp2/3-dependent mechanism of actin-based motility by the activity of the secreted effector IcsA which mimics Cdc42 to activate N-WASP (Egile *et al.*, 1999). Also, for *Rickettsia* spp. Arp2/3 is in interplay with the effector RickA involved in actin tail formation (Gouin *et al.*, 2004).

As described here the Arp2/3 complex is an important host protein for intracellular survival for a variety of pathogens. It seems that they utilize the complex in different, similar, or coincident functions which are only partially understood. Obvious importance of this complex calls for further investigation in future.

F-actin-capping protein CapZ

The second actin-associated protein identified in comparison is the subunit β of CapZ. The F-actin-capping protein or short capping protein (CP) has an important role in actin assembly and actin-based processes as morphogenesis and differentiation (Cooper and Sept, 2008). CapZ binds, in contrast to Arp2/3 which is located at the pointed ends of actin filaments, barbed ends and stops filament growth, thus preventing addition and loss of actin monomers at the end (Casella *et al.*, 1987; Isenberg *et al.*, 1980). CapZ is an α/β-heterodimer (Yamashita *et al.*, 2003). Interestingly, F-actin-capping protein subunit α and β were identified as components of the WASH complex, an NPF of Arp2/3 (Derivery *et al.*, 2009). The identification of a subunit of the Arp2/3 complex and a subunit of the WASH

complex further support that both complexes are important for *Salmonella* not only for the cell entry (Hänisch *et al.*, 2010), but additionally for the PCV biogenesis for *Salmonella* and other intracellular pathogens. Derivery *et al*. (2009) demonstrated that the β-subunit of CapZ co-localises with WASH in the perinuclear region and is located without WASH in lamellipodial/ruffling regions of the plasma membrane. This is the region where most of the PCVs are located at later time points of infection. If this perinuclear localisation of the β-subunit is also true in infected cells and if this protein contributes to PCV biogenesis or maintenance should be analysed in future.

Related to *Salmonella* it has been investigated that the stapling activity of SPI1 effector SipA is not able to reanneal actin filaments capped by CapZ (Popp *et al.*, 2008). However, *L. monocytogenes* is able to redirect CapZ by recruiting it to actin filament tails (David *et al.*, 1998). This demonstrates that intracellular pathogens usurp the function of CapZ for their purpose, but such mechanism has not been investigated for pathogens that reside in vacuoles.

III.3.2 Protein folding

Hypoxia up-regulated protein 1 (GRP-170/ORP-150)

Hypoxia up-regulated protein 1 (HYOU1) can be predominantly found as 150 kDa oxygen-regulated protein (ORP-150) or 170 kDa glucose-regulated protein (GRP-170) in literature. It is involved as molecular ER chaperone in stress response and its expression is induced in consequence of environmental stress (glucose starvation, hypoxia, reductive reagents) and in the pathology of many diseases (e.g. ischemia, cancer, diabetes) (Easton *et al.*, 2000; Ikeda *et al.*, 1997). ORP-150 is also one major calcium-binding protein in the ER (Naved *et al.*, 1995). The expression of ORP-150 is highly induced under hypoxia and glucose deprivation in HeLa cells (Cechowska-Pasko *et al.*, 2005, 2006), the cell line which was used for part of experiments in this study.

Extracellular ORP-150 supports macrophage-mediated pathogen sensing and innate immunity (Zuo *et al.*, 2012). It has been demonstrated that extracellular ORP-150 binds CpG oligodeoxynucleotides, that mimic microbial DNA, and is sensed by Toll-like receptor 9 (TLR9). During cell entry the protein directly interacts with endosomal TLR9 which results in a synergistic activation of the MyD88-dependent signalling. Zuo *et al.* (2012) suggested that ORP-150 promotes sensing of pathogen-associated signals by intracellular receptors when it is released from damaged cells. Furthermore, ORP-150 is

in focus of cancer research. It has been demonstrated to induce antitumor immunity *in vivo* (Park *et al.*, 2006; Wang *et al.*, 2006; Wang *et al.*, 2001, 2003).

The presence of this ER chaperone in the list of overlapping proteins is surprising. Recently, it has been shown that the SCV membrane interacts with ER membrane in the early phase of SCV biogenesis (Santos *et al.*, 2015). Additionally, a strong interaction of the LCV with the ER is well known. *L. pneumophila* converts the vacuole membrane to a rough ER membrane until it fuses with the ER (Robinson and Roy, 2006; Swanson and Isberg, 1995). Furthermore, also chlamydial inclusions are in a very close contact to the ER and form ER-inclusion membrane contact sites (MCSs) (Derré, 2015). Maybe stress induced by the intracellular pathogen enhances the expression of ORP-150 for compensation and at this PCV/ER membrane sites this ER protein is directed to PCV. This protein has possibly additional intracellular functions for pathogen sensing and innate immunity as it has been demonstrated if extracellularly localised (Zuo *et al.*, 2012). Here it would be necessary to analyse the expression profile and localisation in infected cells. Additionally, the identification of bacterial interaction partners would be of high importance to understand ORP-150 impact on intracellular bacterial lifestyle.

T-complex protein 1

The T-complex protein 1 (TCP-1, CCT or TRiC) is a type II chaperonin and the mammalian complex contains the subunits TCP-1-α/β/γ/δ/ε/η/θ and CCTζ-1/2 (Brackley and Grantham, 2009). The subunits are arranged in a ring and two stacked rings form the complex with a central cavity (Hartl and Hayer-Hartl, 2002; Horwich *et al.*, 2007). As chaperonin, TCP-1 binds unfolded polypeptides and facilitates their folding inside the cavity in dependency of ATP whereby the built-in lid opens and closes the cavity (Lopez *et al.*, 2015). Its primary function seems to be *de novo* protein folding during normal cell growth since cellular stress does not increase expression of the subunits (Brackley and Grantham, 2009). This complex supports folding of approximately 10% of the eukaryotic proteome, including many cytoskeletal components and cell cycle regulators (Gómez-Puertas *et al.*, 2004; Yam *et al.*, 2008). Moreover, TCP-1 is essential for the folding of actin and tubulin (Sternlicht *et al.*, 1993; Yaffe *et al.*, 1992). Besides the cytosol, TCP-1 was reported to be located in *trans*-Golgi (Willison *et al.*, 1989).

An astonishing fact is that three of eight T-complex protein 1 subunits were identified (Table III-2). One possible explanation could be that this complex is co-purified with the PCVs because of extensive function as chaperonin for a great plenty of proteins, thus also proteins recruited to the PCVs. An argument for this co-purification is the fact that TCP-1

is a chaperonin for actin and tubulin which are closely associated with SMM, for instance. The SCVs are surrounded by actin in the VAP (Meresse *et al.*, 2001), and microtubules stabilize the SITs (Brumell *et al.*, 2002a). α- and β-tubulin have also been identified on isolated LCVs (Hoffmann *et al.*, 2014; Shevchuk *et al.*, 2009; Urwyler *et al.*, 2009). The *C. trachomatis* inclusion is stabilized by actin and intermediate filaments (Kumar and Valdivia, 2008) and *M. marinum* and *M. tuberculosis* trigger WASH-driven actin polymerization at the vacuole (Kolonko *et al.*, 2014). However, co-purification also due to the two other actin cytoskeleton associated proteins ARPC and CAPZB would be possible. Nevertheless, the identification of three of eight subunits indicates a direct or indirect active recruitment. This is maybe linked with the remodelling of the cytoskeleton during PCV biogenesis and needs further investigation to understand TCP-1 contribution to PCV biogenesis.

III.3.3 Protein sorting

Annexin A1

Annexins are Ca^{2+}-regulated proteins which are able to respond to intracellular Ca^{2+} changes and bind to membrane phospholipids where they organise membrane domains or recruit proteins for interaction (Gerke *et al.*, 2005). Annexin A1 is involved in many physiological processes, e.g. hormone secretion, apoptosis, differentiation. As a glucocorticoid-regulated protein it is involved in the regulation of the innate and adaptive immune response (Gavins and Hickey, 2012). Subcellular localization and protein binding partners of this annexin vary according to its physiological role whereby the phosphorylation status of different sites affect the localization, binding properties and functions of annexin A1 (D'Acunto *et al.*, 2014).

Hormone exocytosis is negatively regulated through a mechanism that reorganizes the actin cytoskeleton (McArthur *et al.*, 2009). Additionally, it has been suggested that annexin A1 contributes to inward vesiculation that takes place in multivesicular endosomes (Gerke *et al.*, 2005). In isolated MVBs this protein is associated in a calcium-independent way and phosphorylated by the EGF receptor kinase in a Ca^{2+}-dependent form for membrane association (Futter *et al.*, 1993). Together with findings that annexin A1 is implicated in vesicle aggregation and fusion (Blackwood and Ernst, 1990; Ernst *et al.*, 1991; Seemann *et al.*, 1996) its role in inward budding in MVBs is discussed (Futter *et al.*, 1993). It can be speculated whether these properties contribute to the formation of SITs. The origin of the extensive tubular network is not completely understood, e.g. how and why vesicles fuse

with, or bud off the tubules. In this process annexin A1 might be involved. Furthermore, small vesicles inside the SIFs were detected (Krieger *et al.*, 2014) and annexin A1 possibly participates in their formation.

A known connection of this protein in SCV or SIF biogenesis was not shown until now. Only in a study about dexamethasone, a glucocorticoide analogue, it has been shown that *S*. Typhimurium infections are modulated by dexamethasone *in vivo* independent of annexin A1 (Smyth *et al.*, 2008). The knockout of ANXA1 has no effect on *S*. Typhimurium infection in mice (Smyth *et al.*, 2008). However, the involvement of another family member of the annexin family is known for *S.* Typhimurium. Annexin A2 is used for invasion with participation of SopB (Jolly *et al.*, 2014). In contrast to *S.* Typhimurium annexin A1 is essential in immunity to *M. tuberculosis* infections. Tzelepis *et al*. (2015) demonstrated that *Anxa1*-deficient mice were highly susceptible to *M. tuberculosis* infections and impacted *M. tuberculosis* antigen-specific $CD8^+$ T cell response negatively. In human blood monocyte-derived DCs annexin A1 expression is strongly reduced. Altogether this reveals that annexin A1 as an engulfment ligand is notably facilitating DC cross-presentation to increase phagocytosis of apoptotic cells/vesicles (efferocytosis) and the DC antigen-presenting machinery during *M. tuberculosis* infections (Tzelepis *et al.*, 2015). For *C. trachomatis* no co-localisation of annexin A1 has been detected after entry of elementary bodies in epithelial cells by immunofluorescence staining whereas annexin A3, A4 and A5 directly associate with chlamydial aggregates after entry (Majeed *et al.*, 1994). Recently, annexin A1 has been identified as novel interaction partner of the *L. pneumophila* effector SidM (So *et al.*, 2016), a Rab1/Rab35 GEF/AMPylase (Finsel and Hilbi, 2015).

These diverse characteristics highlight annexin A1 as a promising candidate for further research. It would be especially interesting to understand if, and if so how the intracellular bacteria manipulate various functions of annexin A1 in the cell. This might occur directly by bacterial interaction partners, or indirectly by Ca^{2+} level changes. An impact during uptake of the bacterium as well as vacuole formation is reasonable and should be investigated.

COPI vesicle components – γ1-COP and TMEDA

COPI – γ1-COP

γ1-COP (COPG1) is part of the vesicular coat of the COPI vesicles which mediate retrograde trafficking of luminal and membrane proteins from *cis*-Golgi back to the ER and within the Golgi stack (Jackson, 2014). This complex consists of seven subunits (α, β, β′, ε, γ, δ, ζ) (Waters *et al.*, 1991). They form two subcomplexes. The B-complex (α, β′, ε) is proposed to be the outer layer and binds cargo (Hsia and Hoelz, 2010). Recruitment of the F-complex (β, γ, δ, ζ) by the GTP-bound small GTPase Arf1 which in turn is bound to γ- and ζ-COP (Yu *et al.*, 2012) leads to COPI polymerization and finally vesicle formation (Serafini *et al.*, 1991; Bremser *et al.*, 1999). The cargo is sorted on the basis of different signals, e.g. SNARE proteins, KDEL/HDEL receptor, type I transmembrane proteins with linear C-terminal dilysine-based motifs (retrograde trafficking motifs) and p24 family proteins with diphenylalanine motifs (FFxx) (Jackson, 2014).

Interestingly, γ-COP as well as ζ-COP exist in two isoforms (γ1-/γ2- and ζ1-/ζ2-COP) (Futatsumori *et al.*, 2000) which show different localisations inside the cell. γ1-COP is preferentially located in the early Golgi apparatus and γ2-COP in the late Golgi (Moelleken *et al.*, 2007). The isoforms may have different properties concerning interactions with cargo, other COPI proteins or auxiliary proteins (Beck *et al.*, 2009). Surprisingly, it has been demonstrated that γ-COP interacts with another candidate of the overlapping protein list p23 (Futatsumori *et al.*, 2000), also called transmembrane emp24 domain-containing protein 10 (TMEDA, p24δ1) that will be described in the following paragraph.

Transmembrane emp24 domain-containing protein 10

Transmembrane emp24 domain-containing protein 10 (TMEDA, also named p24δ1 or p23) belongs to the p24 family and is a major component of COPI-vesicles (Sohn *et al.*, 1996). It is involved in the cargo sorting and formation of COPI-vesicles (Beck *et al.*, 2009; Jenne *et al.*, 2002). The cytoplasmic tails of dimeric p23 binds the cytosolic inactive (GDP-bound) Arf1 (Gommel *et al.*, 2001). This leads to a simultaneous nucleotide exchange to GTP mediated by a member of the Sec7 family of GEFs (GBF1) (Beck *et al.*, 2009). Additionally, p23 is involved in cargo discrimination. In the case of secretory cargo p23 dimers bind to two independent binding sites in γ-COP, but as monomer it binds to other sites than γ-COP for ER-resident cargos (Béthune *et al.*, 2006).

The detection of these two COPI vesicle proteins indicates a contribution of the *intra*-Golgi and retrograde Golgi-ER trafficking to the formation of PCVs. Especially after establishing the pathogen's vacuole, supply with nutrients and membrane material is necessary for bacterial replication. In this case hijacking vesicles of the retrograde transport might be a general process of intracellular bacteria for maintenance of the PCV. The interplay of γ-COP and p23 as interaction partners in cargo sorting (Béthune *et al.*, 2006) might be a hint why these proteins are recruited to the PCVs in conjunction. The pathogens usurp the cellular functions of these proteins and utilize them for their own nutrition. Regarding the intracellular pathogens investigated here, for *L. pneumophila* it is known that an effector of the Dot/Icm secretion system, RalF, acts as GEF for Arf1 recruiting to the LCV (Nagai *et al.*, 2002) and Arf1 then binds to p23 (Gommel *et al.*, 2001). These new insights ask for a closer look of the retrograde trafficking contribution for PCV biogenesis and intracellular replication that was, especially in the context of *Salmonella,* not shown before and needs to be elucidated in more detail.

Moreover, an influence on the *Salmonella* replication niche with its extensive tubular network might be possible. COPI coat polymerization induces membrane curvature for vesicle budding (Beck *et al.*, 2009). A possible impact also on the formation and stabilisation of SITs is thus worth to investigate.

Ras-related protein Rab14

The small GTPase Rab14 is located at TGN and EE. It mediates membrane trafficking in the biosynthetic/recycling pathway between the Golgi complex and endosomes (Junutula *et al.*, 2004; Proikas-Cezanne *et al.*, 2006). An impact of Rab14 on PCV biogenesis has been identified for all intracellular pathogens discussed here. However, its role in the PCV biogenesis seems to be bacterial specific. It has been demonstrated that Rab14 is recruited to the SCV (Brumell and Scidmore, 2007; Hashim *et al.*, 2000; Kuijl *et al.*, 2013; Madan *et al.*, 2012; Smith *et al.*, 2007) and SIFs (Vorwerk *et al.*, 2015) (Chapter II.I). For *Salmonella* it is assumed that binding of activated Rab14 intervenes in the phagosomal maturation process of the SCV. This is mediated by the effector SopB that activates AKT1 (or protein kinase B, PKB). The activated AKT1 phosphorylates the Rab14-GAP AS160 whereby AS160 is not able to bind to SCVs and deactivates Rab14 (Kuijl *et al.*, 2007). Another interaction partner, Nischarin, binds to GTP-bound Rab14 and PtdIns(3)*P* to control the maturation of EE to Rab9/CD63-positive LE. Kuijl *et al.* (2013) showed that Rab14 recruits Nischarin to SCVs. The authors suggest that Nischarin leads to intensified acidification of SCVs by interplay with PtdIns(3)*P*, and consecutive interaction with Rab14

and Rab9 leads to establishment of growth-enhancing conditions for *Salmonella* (Kuijl *et al.*, 2013).

The GTPase Rab14 is likewise recruited to the LCV of *L. pneumophila* (Hoffmann *et al.*, 2014; Urwyler *et al.*, 2009). However, knockdown of Rab14 enhances intracellular growth (Hoffmann *et al.*, 2014) which is in contrast to the reduced replication of *Salmonella* after Rab14 depletion (Kuijl *et al.*, 2013). Recently, the *Legionella* effector LidA has been identified as target of Rab14 (So *et al.*, 2016). LidA is known to modulate Rab1 and Rab8 activity by stabilizing active forms of these GTPases, as well as binding PtdIns(3)*P* and PtdIns(4)*P* (Finsel and Hilbi, 2015).

Rab14-mediated vesicle transport also contributes to *C. trachomatis* inclusion biogenesis and replication through the supply of sphingolipids (Capmany and Damiani, 2010). Initiating 10 h p.i., throughout the remainder of the developmental cycle, Rab14 is located at the inclusions. This is dependent on bacterial protein synthesis, but independent of Golgi integrity or microtubules. Inclusion enlargement and bacterial replication is impeded by the overexpression of dominant-negative Rab14 or siRNA knockdown resembling the effect on *Salmonella* (Capmany and Damiani, 2010). One important protein for the accumulation of Rab14 to the chlamydial inclusion has been identified: FIP2 (Rab11-family of interacting proteins 2) binds to Rab11 at the membrane and recruits Rab14 (Leiva *et al.*, 2013).

Rab14 is additionally critical for the maturation of the mycobacterial phagosome (Kyei *et al.*, 2006). Following phagocytosis, Rab14 is recruited to the *M. tuberculosis*-containing phagosome and stops the maturation to phagolysosomes. The maturation block is released by overexpression of dominant-negative Rab14 mutants or siRNA knockdown and phagosomes with live mycobacteria end up in phagolysosomes. Rab14 facilitates fusion of phagosomes and EE but not LE. Accumulation of Rab14 at the mycobacterial phagosome allows maintaining EE characteristics and impeding recruitment of LE/lysosomal components (Kyei *et al.*, 2006).

These multifarious findings elucidate the versatile mechanisms how intracellular bacteria utilize a host protein for their own intention. This small GTPase is associated with the PCVs to regulate its two objectives known until now. On the one hand Rab14 contributes to the maturation block to phagolysosomes in the *Mycobacterium* specific maturation step by maintaining EE/LE characteristics and allows due to this intracellular survival (Kuijl *et al.*, 2013; Kuijl *et al.*, 2007; Kyei *et al.*, 2006). On the other hand recruitment of Rab14 serves to tap the secretory system for nutrition (Capmany and Damiani, 2010). Regarding Rab14 it will be of special interest to clarify the exact mechanisms how the different bacteria manipulate Rab14 functions, either directly by bacterial proteins or indirectly through other host interaction partners.

Comparing the pathogens' impacts on host cell transport processes and organelle characteristics will improve our understanding of how bacteria avoid their degradation and facilitate the development of general strategies against intracellular bacteria.

III.4 Conclusion and Outlook

This work focused on the identification of the protein composition of *Salmonella*-modified membranes to get a deeper understanding of origin and biogenesis processes of these filigree membrane structures arising from the activity of intracellular *Salmonella*. For proteomic analysis of the SMM proteins a specific purification method was developed which combined differential fractionation with immunomagnetic separation (IMS) of the membrane-integral SPI2-T3SS effector SseF (Chapter II.1). This approach was successfully applied to *Salmonella*-infected cells of the epithelial cell line HeLa (Chapter II.1) and mouse macrophage-like cell line RAW264.7 (Chapter II.2) and should be easily adaptable to other cell lines and primary cells such as macrophages or DCs. However, utilizing another *Salmonella*-effector for affinity purification of SMM or changes in the MS-analytics have to be considered carefully and need further investigation (Chapter II.3).

Analysis of the proteomic results (Chapter II.1/2) reveals the contribution of proteins of the cytoskeleton (actin, myosin and associated proteins) and proteins regulating the endocytic and secretory vesicle trafficking. The influence of MS-identified Rab GTPases, e.g. Rab5c and Rab14, for SITs at late phase of infection should be elucidated in more detail. Beside co-localisation studies that showed association with SIFs (Chapter II.1), siRNA knockdowns and/or CRISPR/Cas9-mediated genome editing would be suitable tools to clarify the impact on SIT biogenesis. First experiments demonstrated an influence of Rab14 for SIF formation (A. Kehl, personal communication). Additionally and previously not considered as involved in SMM biogenesis, ER/Golgi trafficking proteins (e.g. COPI/II) might be also important for SMM (Chapter II.1/2). Here it would be interesting to understand if the identified proteins of different cellular origin are distributed equally in the SMM or if foci of local accumulation or microdomains exist. It might be that proteins of the ER/Golgi trafficking are concentrated in SIFs which are close to the ER/Golgi, for instance. Applying methods developed for quantitative analysis of endosomal tubulation and microdomain organization mediated by the WASH complex (Derivery and Gautreau, 2015; Derivery *et al.*, 2012; Derivery *et al.*, 2009) would be an interesting tool for further SIT investigations. The identification of microdomains would help to understand how vesicles of different origin fuse with SITs and what happens after fusion.

The major task for future work will be filtering more interesting candidates out of the large number of identified proteins. To do this, subsequent validation methods are needed. A promising approach has recently been developed to identify the infection-dependent interactomes of effectors in *L. pneumophila* (So *et al.*, 2016). Translocated effector complexes were isolated from infected cells by tandem-affinity purification (TAP) using a hexahistidine (His_6) and BirA-specific biotinylation tag and subsequently analysed by MS. A pathogen expressing the effector of interest is fused to a His_6-tag and a 15 amino acid residue BirA biotinylation site (Bio) and infects host cells that stably express BirA (biotin ligase of *Escherichia coli*). This purification method allows discrimination of translocated effector from the remaining intrabacterial pool and strong reduction of the false-positive rate since only the secreted His_6-tagged and biotinylated effectors and their interactomes are purified. These small tags are inert to detergents and denaturating chemicals which enable the application of diverse lysis and purification conditions, e.g. formaldehyde crosslinking prior cell lysis. Especially, the usage of small tags can be an advantage of this approach. *Salmonella* effectors fused with BirA were not sufficiently translocated to detect biotinylation in infected HeLa cells (unpublished data) thus these small tags should have no effect on translocation efficiency. Moreover, stably BirA-transfected cells provide an adequate biotinylation rate and background biotinylation is negligible because of the tandem affinity purification. The comparison of the proteins identified by two different approaches would result in more compelling protein target lists for further investigation. A further more versatile tag is APEX2 which can be used in electron microscopy and proteomics (Hung *et al.*, 2016; Lam *et al.*, 2015). Furthermore, the HaloTag is a promising and diverse applicable tag (England *et al.*, 2015).

In addition, determination of *Salmonella* proteins as components of the SMM was not satisfying (unpublished data) or discrimination between secreted or intrabacterial proteins was not possible (Chapter II.3). A second recently developed approach would allow handling one major problem of identification of secreted *Salmonella* proteins against the overwhelming protein content of the host by a special enrichment. A fascinating approach has been published for the selective enrichment of mycobacterial proteins from infected macrophages (Chande *et al.*, 2015). By using a *M. tuberculosis*-selective protein labelling method based on genetic incorporation of azidonorleucine (ANL) through the expression of a mutant methionyl-tRNA synthase (MetRS). The incorporation of ANL into native bacterial proteins allows pulling out secreted proteins with low abundance from the lysate of infected cells. The workflow would be as follows: treatment of *Salmonella*-infected cells with ANL, lysis of the host, clearance of the lysate by centrifugation, click-reaction for linking of azide-functionalized bacterial proteins with alkyne immobilized on resin, stringent washing, on-resin digestion of enriched bacterial proteins, and finally MS

analysis. If the expression of this mutant MetRS had no influence on *Salmonella* growth, infection rate or intracellular growth, this approach would be a suitable tool for the identification of secreted proteins. Time course experiments, independent of the host, in a quantitative manner would allow deep insights into the *Salmonella* secretome and its influences on the host cell.

To sum up, in this work a new method for proteomic analyses of SMM has been developed that facilitates the identification of numerous proteins associated with SMM with systematic proteome-wide analyses. They are an important starting point for further investigation to understand their contributions to biogenesis of the SCVs and its connected tubular structures. The group of host proteins common to PCVs and unique proteins allow defining common and pathogen specific signatures that can be used as marker for identification or as targets for vaccines and antibiotics.

IV Summary/Zusammenfassung

IV.1 Summary

The foodborne, facultative intracellular pathogen *Salmonella enterica* serovar Typhimurium has the capability to remodel the host endosomal system and establish a network consisting of tubular membranous structures connected with the *Salmonella*-containing vacuole (SCV). Five different kinds of *Salmonella*-induced tubules (SITs) are distinguishable: *Salmonella*-induced filaments (SIFs), sorting nexin 3 (SNX3), *Salmonella*-induced SCAMP3 (SIST), LAMP1-negative (LNT), and spacious vacuole-associated tubules. However, despite its ability to form these *Salmonella*-modified membranes (SMM), the cellular origin and composition of SMM are mostly unknown.

Thus we developed a novel approach facilitating the isolation of SMM. Therefore, we combined differential fractionation and affinity-based enrichment using an epitope-tagged SPI2-T3SS effector SseF with liquid chromatography-tandem mass spectrometry (LC-MS/MS). This enabled us to probe the composition of SMM during infection of the epithelial cell line HeLa and mouse macrophage RAW264.7 cells. The identified SMM proteome consists of 247 host proteins in HeLa cells and 262 proteins in macrophages, respectively. The protein compositions revealed the importance of cytoskeletal and host trafficking proteins for SMM and indicated a redirection of host trafficking towards SMM. In addition our results suggested a contribution of the traffic between ER and Golgi apparatus, e.g. COPI and COPII vesicles, that has not been assumed as origin for SMM before. A selection of proteins involved in trafficking and cytoskeleton formation were validated by confocal light microscopy.

Furthermore we analysed the effect of IFNγ activation on the SMM proteome in RAW264.7 macrophages and revealed a strong impact on the protein composition. The identification of IFNγ-inducible proteins and a higher percentage of ER proteins implied that *Salmonella* is able to adjust its SMM according to the cell status.

Comparison of the SMM proteome with host compartments of other intracellular pathogens indicated communalities that might be important for the biogenesis of pathogen-containing vacuoles despite of their individual replication niches.

Furthermore, the methods were adopted for a non-membrane integral *Salmonella* effector SseJ. Proteome analyses were conducted in collaboration with Professor Becher (Department of Microbial Proteomics, University of Greifswald) using the sensitive Velos

Orbitrap system. This data set revealed not only the impact of the bait protein during the SMM probing but also differences between MS platforms. Nevertheless, interesting host and *Salmonella* proteins were identified using this approach, which are starting points for further research.

Altogether, this work provides new insights into the origin of *Salmonella*-modified membranes and serves as a rich source for new theories that will help to understand the biogenesis and function of the *Salmonella*-containing vacuole and its connected tubular membrane network.

IV.2 Zusammenfassung

Salmonella enterica serovar Typhimurium ist ein durch Nahrungsmittel übertragbares, fakultativ intrazelluläres Pathogen. Es besitzt die Fähigkeit das Endosomalsystem des Wirtes umzuformen und ein Netzwerk aus tubulären Membranstrukturen zu etablieren, die mit einer Vakuole, die die Salmonelle beinhaltet ("*Salmonella*-containing vacuole", SCV), verbunden ist. Fünf verschiedene Arten von Membrantubuli („*Samonella*-induced tubules, SITs) werden wie folgt unterschieden: Salmonellen-induzierte Filamente („*Salmonella*-induced filaments", SIFs), Sorting Nexin 3 (SNX3), Salmonellen-induzierte SCAMP3 („*Salmonella*-induced SCAMP3", SIST), LAMP1-negative (LNT), und Tubuli, die mit der frühen Vakuole („spacious vacuole-associated tubules", SVAT) assoziiert sind. Jedoch abgesehen von ihrer Fähigkeit Salmonellen-modifizierte Membranen (SMM) zu bilden, ist der Ursprung und die Zusammensetzung der SMM überwiegend unbekannt.

Aus diesem Grund wurde ein neuer, faszinierender Ansatz zur Isolation der SMM entwickelt. Hierfür wurde differentielle Fraktionierung mit affinitätsbasierender Anreicherung kombiniert, die sich den Epitop markierten SPI2-T3SS Effektor SseF zu Nutze macht. Die Analyse wird mittels Flüssigkeitschromatographie mit Tandem-Massenspektrometrie (LC-MS/MS) durchgeführt. Es ist uns gelungen die Zusammensetzung der SMM in infizierten Epithelzellen (HeLa) und Mausmakrophagen ähnlichen RAW264.7 Zellen zu bestimmen. Das SMM-Proteom besteht bei HeLa Zellen aus 247 und bei Makrophagen aus 262 Wirtsproteinen. Die Zusammensetzung zeigt die Bedeutung des Zytoskelettes und der Proteine, die im Vesikeltransport involviert sind, auf. Dies weist auf eine Umleitung des Vesikeltransportes zu Gunsten der SMM hin. Zusätzlich legen unsere Ergebnisse nahe, dass der Transport zwischen dem ER und dem Golgi-Apparat, z. B. COPI und COPII, an der SMM Biogenese beteiligt ist, das bisher nicht vermutet wurde. Eine Auswahl an Proteinen, die am Vesikeltransport oder der Zytoskelettbildung beteiligt sind, wurde mittels konfokaler Lichtmikroskopie validiert.

Des Weiteren wurde der Effekt von IFNγ-Aktivierung auf das SMM-Proteom in RAW264.7 Makrophagen analysiert und ein großer Einfluss auf die Zusammenstellung festgestellt. Die Identifizierung von INF-induzierten Proteinen und ein hoher Prozentsatz an ER Proteinen implizieren die Fähigkeit von Salmonellen ihre SMM-Zusammensetzung entsprechend des Zellstatus anzupassen.

Der Vergleich der SMM-Proteome mit den Proteomen weiterer intrazellulärer Pathogene deutet auf Gemeinsamkeiten hin, welche wichtig für die Biogenese von Pathogen-beinhaltenden Vakuolen sein könnten trotz der Unterschied ihrer replikativen Nischen.

Zusätzlich wurde die Methode für den Salmonellen-Effektor SseJ, der nicht membranintegral ist, angepasst. Die Proteomanalysen wurden in Zusammenarbeit mit Prof. Becher (Abteilung für mikrobielle Proteomik, Universität Greifswald) durchgeführt, die ein sensitives Velos Orbitrap System zur massenspektrometrischen Analyse benutzen. Diese Ergebnisse zeigen auf, dass es Einflüsse des verwendeten Epitop-markierten Effektors für Affinitätsreinigung und der unterschiedlich verwendeten MS-Plattformen gibt. Dennoch ist es uns gelungen mit diesem Ansatz interessante Wirts- und Salmonellenproteine zu identifizieren, die ein spannender Startpunkt für weitere Forschung sein können.

Zusammengefasst stellt diese Arbeit neue Einblicke über den Ursprung der Salmonellen modifizierten Membranen bereit und dient als reiche Quelle für neue Theorien, die helfen werden die Biogenese und Funktion der Salmonellen beinhaltenden Vakuole und der verbundenen tubulären Membranstrukturen zu erklären.

V References

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VI Appendix

Table S III-3: Comparison of overlapping (orthologue) genes of PCVs of *S.* Typhimurium (HeLa and RAW264.7) (Vorwerk *et al.*, 2015; Chapter II-1; Chapter II-2), *L. pneumophila* (Hoffmann *et al.*, 2014; Urwyler *et al.*, 2010), *C. trachomatis* (Aeberhard *et al.*, 2015), *M. bovis* (Lee *et al.*, 2010) and the combined overlap

SCV HeLa/RAW264.7	SCV Hela/RAW264.7; LCV	SCV HeLa/ RAW264.7; *C. trachomatis* inclusion	SCV HeLa/RAW264.7; *M. bovis* phagosome	complete overlap
ACTN1			ACTN1	
ALDH18A1				
ALDOA		ALDOA	ALDOA	
ANXA1	ANXA1	ANXA1	ANXA1	ANXA1
AP2B1			AP2B1	
ARPC4	ARPC4	ARPC4	ARPC4	ARPC4
ATP5C1	ATP5C1		ATP5C1	
ATP5F1	ATP5F1		ATP5F1	
ATP5H	ATP5H			
ATP5O	ATP5O		ATP5O	
CAPZB	CAPZB	CAPZB	CAPZB	CAPZB
CCT3	CCT3	CCT3	CCT3	CCT3
CCT5	CCT5	CCT5	CCT5	CCT5
CCT6A	CCT6A	CCT6A	CCT6A	CCT6A
COPA	COPA	COPA		
COPG1	COPG1	COPG1	COPG1	COPG1
DDX39A				
EEF1B2	EEF1B2			
EEF1G	EEF1G	EEF1G		
EHD4	EHD4			
G3BP2				
HNRNPD				
HYOU1	HYOU1	HYOU1	HYOU1	HYOU1
LARS				
LDHA		LDHA	LDHA	
MRPL19	MRPL19			
MRPL48	MRPL48			
MTHFD1L	MTHFD1L			
MYH9	MYH9		MYH9	
NAPA	NAPA			
NDUFS1	NDUFS1			
NDUFS3	NDUFS3			
RAB14	RAB14	RAB14	RAB14	RAB14
RAB5C	RAB5C		RAB5C	
RAP1B		RAP1B	RAP1B	
RARS	RARS			
RPL32				
RPN2	RPN2	RPN2		

SCV HeLa/RAW264.7	**SCV Hela/RAW264.7; LCV**	**SCV HeLa/ RAW264.7; *C. trachomatis* inclusion**	**SCV HeLa/RAW264.7; *M. bovis* phagosome**	**complete overlap**
SEC61B				
SLC25A1	SLC25A1			
SNRPA				
SRPR	SRPR			
TMED10	TMED10	TMED10	TMED10	TMED10
TRAP1	TRAP1		TRAP1	
VAPA	VAPA	VAPA		
VARS		VARS		

VII List of Abbreviations

2DE	2D electrophoresis
aa	amino acids
ADP	adenosine diphosphate
aph	aminoglycoside phosphotransferase (Km^R)
ATP	adenosine triphosphate
BCG	Bacille Calmette-Guérin
BCV	*Brucella*-containing vacuole
BSA	bovine serum albumin
CCV	*Coxiella*-containing vacuole
CLSM	confocal laser-scanning microscopy
COFRADIC	combined fractional diagonal chromatography
COP	coat protein complex
Da	Dalton
DC	dendritic cells
DIGE	difference gel electrophoresis
DMEM	Dulbecco's modified eagle medium
EDTA	ethylene diamine tetraacetic acid
EE	early endosome
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
ESI	electrospray ionization
FACS	fluorescence associated cell sorting
FTICR	Fourier transform ion cyclotron resonance
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	G-nucleotide exchange factor
GEMN	Golgi-endoplasmic reticulum-microtubule organizing centre-nucleus
GFP	green florescent protein
GMP	guanosine monophosphate
GO	gene ontology
GTP	guanosine triphosphate
h	hour(s)
HeLa cells	cervical cancer cells from Henrietta Lacks
HPLC	high performance LC
HRP	horseradish peroxidase

HSP	heat shock proteins
ICAT	isotope-coded affinity tag
iFCS	inactivated fetal calf serum
ILV	intraluminal vesicles
IMS	immunomagnetic separation
IP	immunoprecipitation
iRiKO	inducible Rictor-knockout
iTRAQ	isobaric tags for relative and absolute quantitation
LAMP	lysosome-associated membrane protein
LB	Luria-Bertani broth
LC	liquid chromatography
LCV	*Legionella*-containing vacuole
LCV	large cell variant
LE	late endosome
LMP	lysosomal membrane protein
LNT	LAMP1-negative tubule
LPS	lipopolysaccharid
Lys	lysosome
MALDI	matrix-assisted laser desorption/ionization
min	minutes
mm	millimetres
MOI	multiplicity of infection
MS	mass spectrometry
MS/MS	tandem mass spectrometry
mTn*5*	minitransposon Tn*5*
MTOC	microtubule-organizing centre
MudPit	multidimensional protein identification technology
MVB	multivesicular body
N/A	not available
NPF	nucleation promoting factor
p.i.	post infection
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS/T	phosphate buffered saline supplemented with 0,1% Tween20
PCR	polymerase chain reaction
PCV	pathogen-containing vacuole
PFA	paraformaldehyde
PMN	polymorphonuclear leukocytes
PtdIns	phosphatidylinositol
PtdIns(3)*P*	phosphatidylinositol-3-phosphate

PtdIns(4,5)P_2	phosphatidylinositol-4,5-bisphosphate
PTM	post-translation modification
RAW264.7	murine macrophage-like cell line
RE	recycling endosome
REP	Rab escort protein
RNAi	RNA interference
RPLC	reversed-phase liquid chromatographic
rpm	rounds per minute
RT	room temperature
SCV	*Salmonella*-containing vacuole
SCV	small cell variant
SDS	sodium dodecyl sulphate
sec	second(s)
SIF	*Salmonella*-induced filament
SILAC	stable-isotope labelling with amino acids in cell culture
SIST	*Salmonella*-induced SCAMP3 tubule
SIT	*Salmonella*-induced tubule
SMM	*Salmonella*-modified membranes
SNX3	sorting nexin 3
SPI	*Salmonella* pathogenicity island
spp.	species (plural, various)
SR	super resolution
subsp.	subspecies
SV	secretory vesicle
sv.	serovar
SVAT	spacious vacuole-associated tubules
T3SS	type III secretion system
TBST	Tris-buffered saline supplemented with 0.1% Tween20
TCS	two-component system
TGN	*trans*-Golgi network
TMT	tandem mass tags
TOF	time-of-flight
VAP	vacuole-associated actin polymerizations
vATPase	vacuolar ATPase
WT	wild type

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XI List of Publications

Proteomes of host cell membranes modified by intracellular activities of *Salmonella enterica*

Vorwerk, S., Krieger, V., Deiwick, J., Hensel, M., Hansmeier, N.

Molecular & Cellular Proteomics. 2015 Jan;14(1):81-92. doi: 10.1074/mcp.M114.041145. Epub 2014 Oct 27. http://www.mcponline.org/content/14/1/81.long

How *Salmonella* modifies its killer – proteomic analysis of *Salmonella*-modified membranes in macrophages

Vorwerk, S., Hensel, M., Hansmeier, N.

in preparation

The SPI2-T3SS effector SseJ based *Salmonella* modified membrane proteome

Vorwerk, S., Barlag B., Böhles N., Becher, D., Hensel M., Hansmeier N.

in preparation

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XIII Erklärung über die Eigenständigkeit der erbrachten wissenschaftlichen Leistung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.

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Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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