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# *Salmonella* virulence factors and their role in intracellular parasitism

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## *Abstract*

*Salmonella* is an intracellular pathogen, whose virulence relies on the function of two type three secretion systems (T3SSs). The T3SSs are responsible for the delivery of effector proteins into the host cell cytoplasm in order to mediate invasion of the cell and to shape *Salmonella*'s intracellular life. In a recent review, we highlighted the newest findings on the structure, regulation and function of these complex bacterial structures.

*Salmonella*'s intracellular survival and replication depends on its niche, the *Salmonella* containing vacuole (SCV), a compartment that is derived from host plasma membrane. Several effectors shape the SCV and give rise to a tubular network, which is implicated in the SCV's stabilization and consists of three different kinds of tubules. We were able to show that the effector proteins SseF and SseG play in concert to form one kind of tubules, the recently discovered LAMP-1-negative tubules (LNTs). Their function is important to *Salmonella*, as strains having only LNTs but none of the other tubules are able to create a stable SCV, which leads to better replication and virulence *in vivo* compared to a strain that lacks in tubule formation. Starting from these LNTs as working model, we tried to understand the contribution of tubules to the formation of the SCV and their interactions with the late endosomal / lysosomal compartment (LE/lys). We deciphered the small GTPase Arl8B to play an essential role in the fusion of tubules with LE/lys. Thereby, the knockdown of Arl8B reduced *Salmonella*'s capability to replicate within host cells. We were able to show that an interaction between the effector SifA and Arl8B was responsible for our observations.

In cooperation with the Starnbach Laboratory at the Harvard Medical School, we touched another topic related to infectious disease. By exploring a newly developed method to measure protein stability *in cellulo* and its application on several infection scenarios, we discovered that certain bacteria induce an increase in protein synthesis (IPS). We then demonstrated that different recognition receptors, which can be intra- or extracellular, lead to this IPS. As our experiments were performed with epithelial cells lines, we propose that the IPS represents the effect of an immunological response of host epithelium to the identified invader. Even more, the signal responsible for the induction of IPS can be transferred to non-infected cells. Epithelial cells seem to work in tight

interaction with neighboring cells in order to orchestrate an adequate answer to the danger presented by an invading organism.

In this work, we were able to improve our understanding of several infection related topics. This ranges from the description of the basic tools for infection, the T3SSs, over functional properties of effectors inside the host cell to mechanisms for the recognition of the invading organism by the host epithelium and its appropriate signaling and response.

## *Abbreviations*

| | |
|---|---|
| BMDM | Bone marrow-derived macrophages |
| CFP | cyan fluorescent protein |
| DC | dendritic cell |
| DsRed | *Discosoma sp.* Red fluorescent protein |
| FACS | fluorescence-activated cell sorting |
| GAP | GTPase activating protein |
| GEF | guanine nucleotide exchange factor |
| GDP | guanosine diphosphate |
| GFP | green fluorescent protein |
| GPS | global protein stability |
| GTP | guanosine triphosphate |
| HOPS | homotypic fusion and vacuole protein sorting complex |
| IL | Interleukin |
| IRES | Internal ribosomal entry site |
| I.P. | intraperitoneally |
| IPS | increase in protein synthesis |
| KLC | kinesin light chain |
| LAMP1 | lysosome-associated membrane protein 1 |
| LE/lys | late endosomes / lysosomes |
| LGP | lysosomal glycoprotein |
| LNT | LAMP1-negative tubule |
| LPS | lipopolysaccharide |
| M cells | Microfold cells |
| MAPK | Mitogen-activated protein kinase |
| MLN | mesenteric lymph node |
| MTOC | microtubule-organizing center |
| M6PR | Mannose 6-phosphate receptor |
| NLR | nuclear oligomerization domain (NOD)-like receptor |
| ORF | open reading frame |

| | |
|---|---|
| PAMP | pathogen-associated molecular pattern |
| PMN | polymorphonuclear leukocyte |
| P.O. | Perorally |
| PRR | pattern recognition receptors |
| ROS | reactive oxygen species |
| SCAMP3 | secretory carrier membrane protein 3 |
| SCV | *Salmonella*-containing vacuole |
| Sif | *Salmonella*-induced filament |
| SILAC | stable isotope labeling by amino acids in cell culture |
| SIST | *Salmonella*-induced SCAMP3 tubule |
| SNX | sorting nexin |
| SPI | *Salmonella* pathogenicity island |
| spv | *Salmonella* plasmid virulence |
| T3SS | type tree secretion system |
| TLR | Toll-like receptor |
| TNT | tunneling nanotube |
| TGN | *trans*-Golgi network |
| vATPase | vacuolar adenosine triphosphatase |

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# Chapter 1
# Introduction

## *Host-pathogen interaction – at the edge of life*

Beginning with the appearance of life on earth and throughout evolution with the development of thousands of different species, their interaction with each other had to be defined. In a never-ending tug-of-war – adaptive coevolution – all kind of interactions and symbioses from mutualism to parasitism developed. The balance of these interactions depends much on the respective strength of the opponents. They are not static but can be seen as an "arm race" with adaptive changes on both sides [1].

For a successful infection, pathogens had to develop an armory adapted to all the necessary step of the infectious process, such as the survival close to the host, transmission, colonization and eventually invasion of the host and the control of inflammation [2]. The tools of this armory are called virulence factors. In the case of bacterial pathogens, they comprehend subcellular structures and secreted molecules such as adhesins, fimbriae, flagellae and secretion systems (for infection) or biofilm-related proteins, protective capsules, immunoglobulin proteases, toxins, altered lipopolysaccharides (LPS) and other mechanisms of surface variability (for the protection from the immune system) [1]

Several mechanisms may have contributed to the acquisition of such virulence related genes. The most notable mechanism is horizontal gene transfer via transduction by phages or conjugation with other bacteria. Others are mutations or extragenic elements such as transposons [1].

Being active players of this tug-of-war, bacteria outnumber human cells by ten-fold inside our body [3]. Most of them are beneficial for us by protecting us from pathogens, producing metabolites and supporting acquisition of nutrients. These commensals, together with pathogens, have also had an important contribution to the development of the immune system [4,5]. Unfortunately, some bacteria – among them *Salmonella* – can cause fatal diseases by disrupting the normal microflora surrounding us [6].

## *Salmonella – general description*

*Salmonella* are common Gram-negative, rod-shaped, non-spore-forming, motile enterobacteria that can survive in the environment but can also infect cold- and warm-blooded animals. They are intracellular pathogens with the ability of systemic spread and can also populate the intestinal lumen, causing diseases such as typhoid fever, blood infections and food borne gastroenteritis, depending on the host-pathogen-pairing. *Salmonella* accounts for three million deaths and for 300 million to 1.3 billion cases of infection per year and is an important economical factor in both developing and industrialized countries.

The first strain of *Salmonella* was isolated in 1885 by Dr. Daniel E. Salmon. Today, the genus is known to consist of the two species *S. bongori* and *S. enterica,* which have a high genomic sequence similarity (96–99%). *S. enterica* is responsible for most infections in humans and other warm-blooded animals, while *S. bongori* has usually been associated with cold blooded animals but is poorly pathogenic for humans [7,8]. To date, 2500 *Salmonella* serovars have been identified but most of them are not pathogenic [9].

The three main serovars being pathogenic to humans are *Salmonella enterica* subspecies enterica serovar Typhimurium, Enteritidis and Typhi (pronounced short as *S.* Typhimurium, *S.* Enteritidis and *S.* Typhi). *S.* Typhi (and *S.* Paratyphi) – which are exclusively pathogenic to humans – causes typhoid fever, whereas the other ones induce self-limited gastroenteritis and bacteremia. However *S.* Typhimurium infections in elder, younger and immunocompromised people lead to a typhoid fever like disease. This is especially striking for HIV infected adults. Case studies in Africa report 95% of non-typhoidal *Salmonella* infections to occur in HIV positive people [10].

*S.* Typhimurium has a broader host range than *S.* Typhi; its main reservoir are birds, especially chicken. Interestingly it induces a disease in mice that shows the symptoms of typhoid fever, making it an interesting model to study.

## *The immune system – the enemy to battle*

The mammalian immune system is a sophisticated entity with the mission to eliminate invaders from the host's body. It is constituted of two big branches that differ in speed and efficacy of response on an infection – the innate and the adaptive immune response.

The innate immune system is the first line of defense against invading organisms and responsible for the onset of an adapted immune response. It represents the steady state defense system of the host and reaches its maximal capacity immediately after exposure. It triggers a non-specific response to any pathogen, thus being less efficient in clearing the pathogen. It is particularly important at interfaces between the environment and the interior of the body, such as the skin or the intestinal tract. Its cellular part is composed of phagocytic cells, such as macrophages, DCs and neutrophils, mast cells, baso- and eosinophils, natural killer cells and a subset of T-cells, the γδ T-cells. The humoral response of the innate immune system consists of the complement system.

The adaptive immune system has a lag phase before developing its defense mechanisms, since its activation depends on antigen presentation by the innate immune system and selection of appropriate clonal effector cells. Its effector cells are cytotoxic T-cells and plasma cells, which secrete antigen specific antibodies. The strength of the adaptive immune system is it specificity to the pathogen, which allows a more piercing response than by the innate immune system. Additionally, the adaptive immune system provides memory to the encountered antigen, which allows a fast and specific response in the case of reinfection.

### The intestinal immune system

The intestinal tract displays the largest surface between the body and the external environment and is inhabited by about 100 trillion microorganisms [11,12]. The ensemble of bacteria present in the intestine is called microbiome and has several important roles during the host's life. On the one hand, it contributes to the host metabolism by providing vitamin K, folate and short-chain fatty acids, such as butyrate, a major energy source for enterocytes. It also mediates the breakdown of dietary carcinogens [13,14]. On the other hand the microbiome is indispensable for a normal development of the host's immune system. Its absence is associated with reductions in

mucosal cell turnover, vascularity, muscle wall thickness, motility, baseline cytokine production, digestive enzyme activity and with defective cell-mediated immunity [15]. The intestinal bacteria also contribute directly to the protection of the host from hostile organisms by secretion of antimicrobial peptides – bacteriocins – and colonization resistance, which is the competition for physical and nutritional niches [16].

The intestinal immune system itself plays a crucial role in the defense against pathogens but also the tolerance to beneficial intestinal microorganisms. It is exposed to an excessive amount of bacteria-derived components that it samples and to which it tries to respond adequately.

The first layer of defense in the intestine is a thick layer of the mucus, which gets secreted by goblet cells, and the glycocalyx of the enterocyte brush border [17,18]. This layer presents a physical barrier that is hard to penetrate. It is additionally soaked by IgA, which gets secreted by the plasma cells of the villous lamina propria and transferred to the mucus by the epithelial cells, and a broad set of antimicrobials that get secreted by specialized secretory cells in the crypts of the intestinal epithelium, the paneth cells [16,19].

The epithelial cells present a second physical barrier to the intestinal microflora. They are connected through an intense network of intercellular junctions: tight junctions, adherens junctions, desmosomes and gap junctions. Tight junctions, localized to the apical end of the basolateral membrane, play key roles to establish the epithelial polarity and, supported by adherens junctions, a physical barrier that prevents leakage of molecules and passing of microorganisms [20]. The integrity of this layer is of big interest for the homeostasis of the intestinal immune system; in other words to prevent unspecific inflammation [4].

The epithelial cells additionally are a substantial component of the cellular intestinal innate immune system. They express pattern recognition receptors (PRR) like Toll-like receptors (TLR) or nuclear oligomerization domain (NOD)-like receptors (NLR) to sense the intestinal microflora [21,22]. Their recognition leads to the production and apical secretion of antimicrobial effector molecules and basolateral secretion of proinflammatory cytokines, leading to the attraction of immune cells [23].

At several sites in the ileum, the intestine has specific sentinels, lymphoid follicles called peyer's patches; similar follicles exist throughout small and large intestine [24]. Peyer's patches are designed for the sampling of antigens and for the induction of a fast and appropriate immune reaction. Therefore, they do not exhibit a mucus layer and do have specialized cells to promote antigen uptake, the M cells. Underlying the M cells, DCs and macrophages sample the incoming antigens. Peyer's patches reunite parts of the innate and the adapted immune systems, having B-cell follicles interspersed with T-cell zones for the induction of an adapted immune response [24,25].

The effector sites of the intestine are the epithelium and the underlying lamina propria along the small and large intestine. Activated T-cells, plasma cells, mast cells, DCs and macrophages are present, whereat DCs, macrophages, regulatory T-cells and immunomodulatory cytokine production seem to have a mediating role between inflammation and tolerance [24].

## *Infection*

### Infectious cycle

*Salmonella* is a food-born pathogen that is ingested via contaminated water or food. Also contact with domestic animals can rarely lead to infection. (Fig. 1)

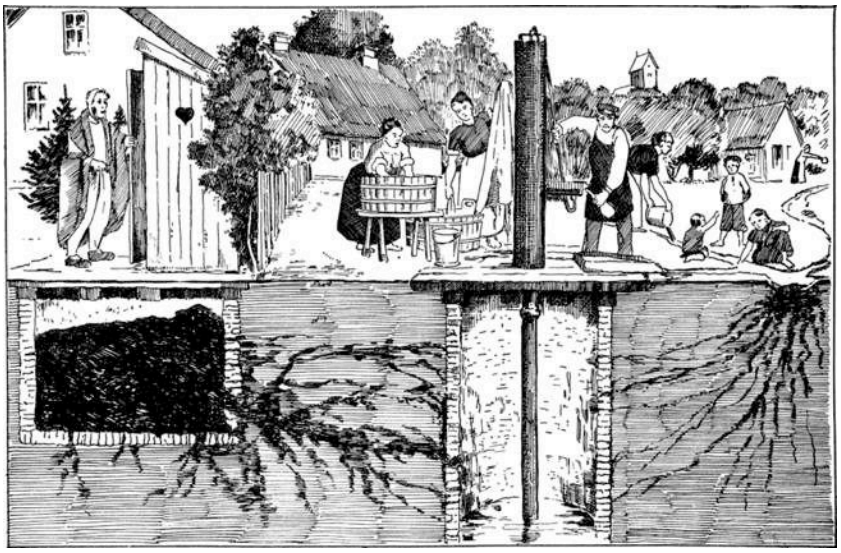

Fig. 1: Graphic representation of *Salmonella* circuit in the human environment. From Vore Sygdome; Bind II, side 116, in 1939.

*Salmonella* is in large parts killed by the low pH of the stomach and therefore has a rather high infectious dose with $>10^5$ bacteria definitively leading to an infection, but lower numbers may already cause infection [26]. Once *Salmonella* reaches the intestine, it starts its infectious cycle.

The first important step is breaching the intestinal epithelium. *Salmonella* can invade M cells, cells of Peyer's patches that are specialized on sampling of the intestinal content. M cells then further transfer *Salmonella* to the underlying immune cells. It can also be captured in the lumen by dendritic cells (DC) that send their dendrites through the intestinal epithelium [27,28]. Finally, *Salmonella* can actively invade enterocytes, a mechanism that depends on a distinct virulence system (T3SS-1, see Chapter 2).

Especially M cells seem to be important targets for the initiation of infection since it has been shown that *S.* Typhi attaches to and destroys the M cells of ileal Peyer's patches in mice [29]. In addition, it has been described that *S.* Typhimurium invades them within 30 min after introduction into murine intestinal loops, without interacting with adjacent enterocytes [30,31]. It can as well induce the transformation of follicle-associated epithelial cells into M cells after invasion in a RANKL dependent mechanism, promoting easier uptake for fellow bacteria [32].

After breaching the barrier, strains that don't have the ability to spread systemically will induce self-limiting gastroenteritis, which is characterized by an acute inflammatory response and the infiltration of neutrophils in the gastrointestinal tract [33]. During gastroenteritis, *Salmonella* re-infects and replicates within enterocytes. The infected cells will be extruded into the intestinal lumen, allowing its repopulation by *Salmonella*, but leaving the epithelial layer disrupted. The extrusion is accompanied by caspase-1 dependent inflammatory cell death and the apical release of IL-1β, IL-8 and IL-18, triggering the influx of polymorphonuclear leukocytes (PMNs) and fluid secretion into the lumen [34,35].

Strains that are able to induce a systemic infection rapidly move to the intestinal lymphoid follicles and to the mesenteric lymph nodes after crossing the epithelium (Fig. 2). *Salmonella* is taken up by macrophages and DCs and spreads via the blood to lymph nodes, then to the liver, spleen, bone marrow and gall bladder where it replicates inside the epithelium or immune cells [36]. *Salmonella*-targeted cells include M cells, epithelial

cells, macrophages, neutrophils, monocytes, dendritic cells, granulocytes, B-cells and T-cells (Fig.3) [9][37].

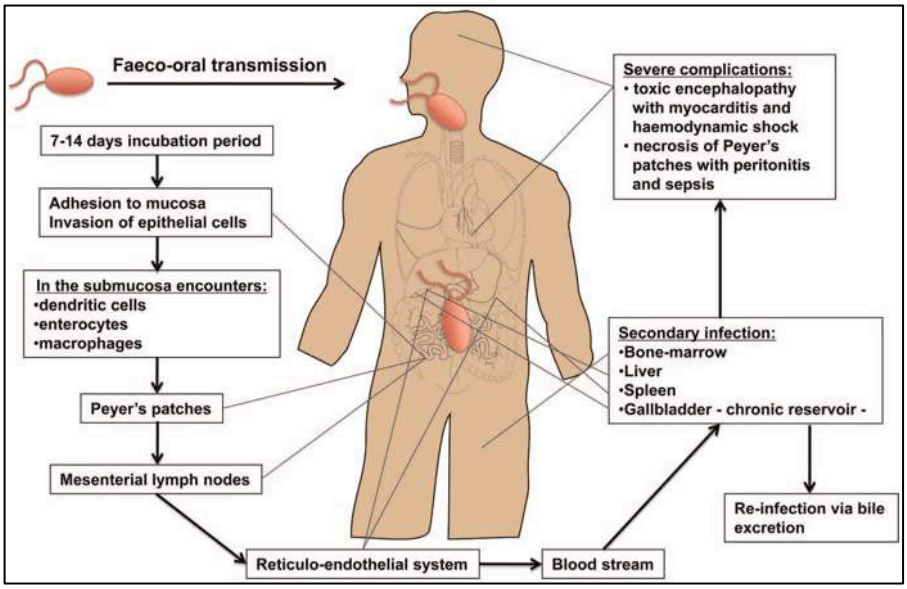


Fig. 2: Dissemination of *S*. Typhi during infection. Copied from [36].

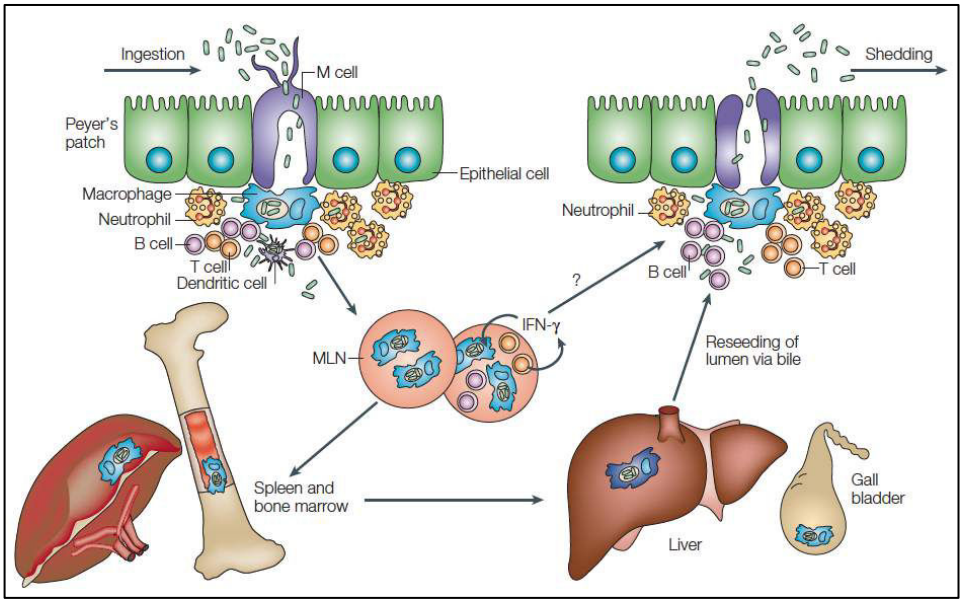


Fig. 3 *Salmonella* interactions with host cells. Intestinal epithelium, mesenteric lymph nodes (MLN), spleen, liver, gall bladder and bone marrow are target organs. *Salmonella* can reside inside epithelial cells, DCs, macrophages and PMNs, but also T-cells, B-cells and granulocytes. Copied from [38].

**Interaction with the immune system**

Throughout infection, *Salmonella* encounters distinct parts of the immune system and needs adequate tools to face them. This starts as soon as in the stomach, whose acidic pH challenges incoming bacteria. Salmonella's acidic tolerance response is mediated by the phoPQ two-component system and the alternate sigma factor RpoS [9].

In the intestine, several challenges await *Salmonella*. By the induction of inflammation (and subsequent diarrhea), it attains growth advantages above the intestinal flora, which in status quo provides colonization resistance to the host (Fig. 4) [39]. *Salmonella* induces a controlled inflammation in a T3SS-1 dependent manner, whereas several effector proteins have either pro- or anti-inflammatory roles [34].

In these inflammation conditions, *Salmonella* can exploit host derived ethanolamine as a carbon source [40]. To do so, it needs tetrathionate, which is a byproduct of the release of ROS by PMNs inside the intestinal lumen. *Salmonella* uses it as a respiratory electron acceptor, thereby allowing the anaerobic growth on ethanolamine. [41]. *Salmonella* is also resistant to lipocalin-2, a molecule that sequesters the bacterial iron chelator enterobactin. *Salmonella* does produce enterobactin, but is also able to produce a glycosylated derivate of enterobactin called salmochelin, which is not susceptible to lipocalin-2 [42].

*Salmonella* can as well partly avoid the action of defensins and other cationic antimicrobics by modifying the charge of its own LPS [43].

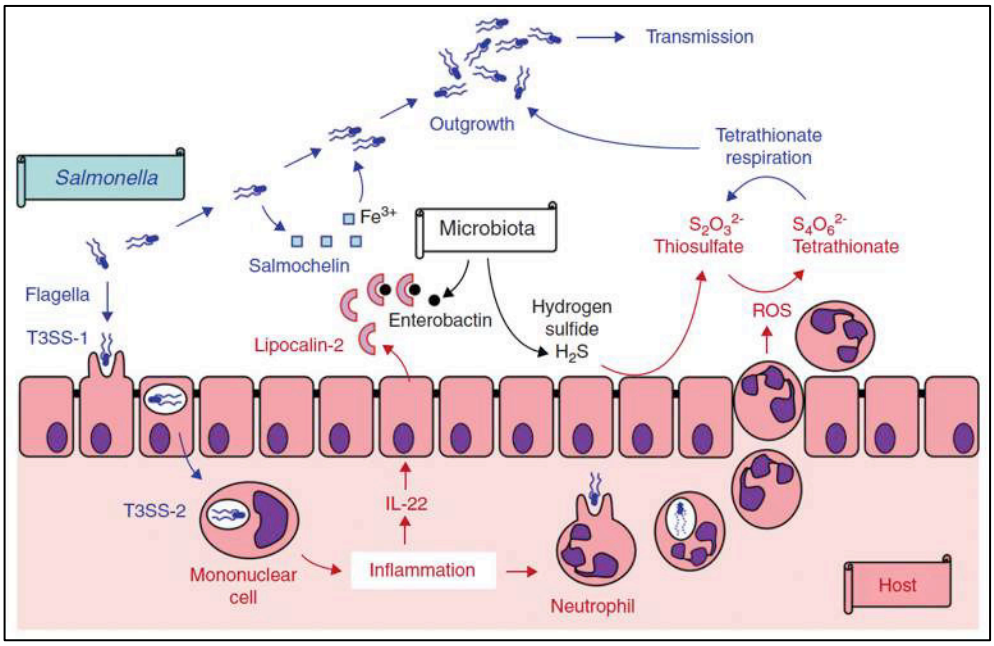


Fig. 4: Ways to overcome the microbiome. Copied from [44].

During the systemic phase of infection, *Salmonella* basically resides inside host cells. Therefore, it developed mechanisms to resist to the cell's proper defenses and to cut signaling to the immune system. An important cellular defense to incoming organisms is the phagocytic pathway, with phagolysosomes as the final step in which the pathogen gets digested. It is commonly accepted that *Salmonella* could interfere with this defense mechanism by preventing fusion with lysosomes and promoting an alternate pathway of SCV maturation [45]. However, we (compare Chapter 3) and others show that fusion with lysosomes happens and that rather a precise negative selection of (Golgi derived) anti-bacterial molecules allows *Salmonella* to survive in a compartment that is derived from the phagocytic pathway [46,47].

*Salmonella* can also face oxidative burst via periplasmic superoxide dismutases or cytoplasmic catalases/peroxidases [37,48]rather than by excluding the NADPH complex from the vacuole as it was previously proposed [49]. It interferes as well with antigen presentation through MHC-I and –II in order to block activation of an adaptive immune response [50,51]. Finally, *Salmonella* can rapidly and efficiently kill DCs in a T3SS-1 dependent manner [52].

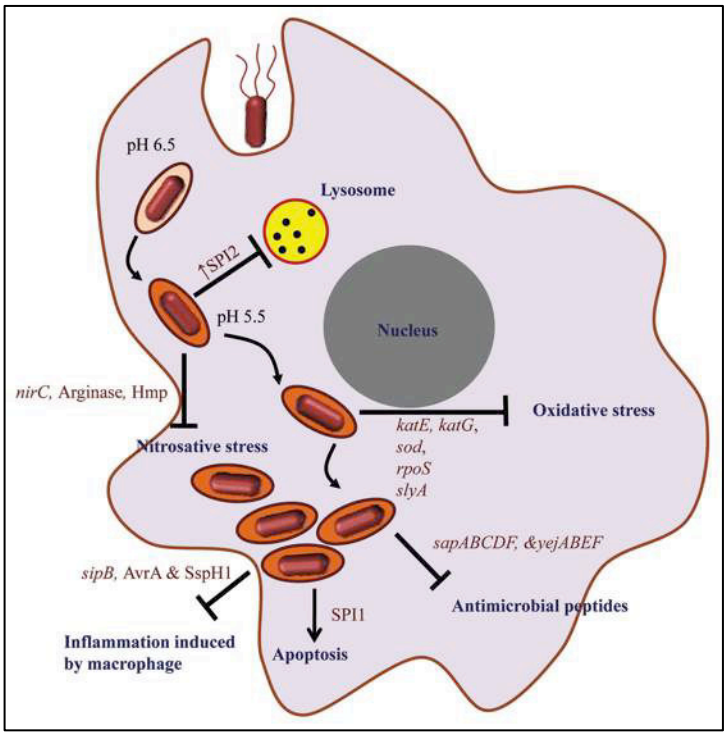


Fig. 5: Intracellular immune modulation by *Salmonella*. Copied from [9].

## *Disease and symptoms*

Depending on the host-pathogen-pairing, *Salmonella* can cause different diseases. In humans, *Salmonella* Typhi and Paratyphi are responsible for the development of typhoid fever. Other serovars like *Salmonella* Typhimurium cause a food borne gastroenteritis, salmonellosis, and rarely bacteremia.

### Salmonellosis

Salmonellosis is a form of gastroenteritis. The disease starts after a short incubation time of 12 to 72 hours. Most people infected with non-typhoidal *Salmonella* develop fever, vomiting, abdominal cramps and especially diarrhea, which can be bloody and can contain mucus. In most cases, the illness lasts four to seven days and most people recover without treatment. In some cases, though, the diarrhea may be so severe that the patient becomes dangerously dehydrated. Especially elderly, children and people with an impaired immune system are susceptible to severe infections of non-typhoidal *Salmonella* strains and can develop a typhoid fever like disease.

**Typhoid fever**

*Salmonella* Typhi and Paratyphi cause typhoid fever. It is a severe systemic disease that can last for up to one month and can cause death in 10 to 30 % of patients when untreated. After an incubation time similar to an infection with *S*. Typhimurium, the infection can be divided into four stages, which last approximately one week each. The first stage is characterized by bradycardia, headache and cough. It is followed by high fever (40°C), diarrhea or constipation, a painful abdomen and enlarged spleen and liver. Also delirium is frequent. In the third stage, severe complications such as intestinal hemorrhage, perforation of the distal ileum and encephalitis can occur. Last, a slow recovery begins with decreasing fever and the cease of the other symptoms.

One to six percent of infected people become chronic carriers. They will live without symptoms, but will shed bacteria via their feces and urine for periods of time ranging from one year to a lifetime, being highly infectious for their environment.

# Chapter 2
# T3SS - *Salmonella*'s tools for infection

The infectiousness of *Salmonella* depends on its genetic bases and a sophisticated system of interaction with the host. Based on genome sequencing, at least 4% of *S.* Typhimurium's genome is required for fatal infection of mice, which corresponds to over 200 genes [53]. The virulence-associated genes can be found throughout the whole genome including plasmids, but are mostly clustered on distinct genetic regions, called *Salmonella* pathogenicity islands (SPI). Pathogenicity islands are characterized by a set of unique features: they are large clusters (30-200 kb) with a distinct GC content, are flanked by LTR like sequences, associated with tRNA loci – presumably acting as targets for the integration of foreign DNA – and often possess (pseudo-)genes encoding for genetic mobility [54].

Twelve pathogenicity islands (SPI-1 to SPI-12) have been identified and shown to be involved in the virulence of *Salmonella*. SPI-1 and SPI-2 are the major virulence determinants of *S. enterica.* In contrast to other SPIs, they code each for a type tree secretion system (T3SS).

### *Salmonella* type three secretion systems (T3SS)

The two T3SSs are the basis for the virulence of *Salmonella*. They function as nanosyringes for the secretion of virulence factors, the effector proteins and control the infectious cycle of *Salmonella* (see below). Whereas T3SS-1 deficient strains can't actively invade cells and therefore have a lower possibility to successfully induce an infection of the host, T3SS-2 deficient strains show a strongly impaired intracellular lifestyle and replication. However, there is increasing evidence for a crosstalk of both T3SSs. Double negative strains are (quasi) avirulent and don't cause disease in the host.

The formation, regulation of expression and interplay of T3SSs is a highly complex process that deserves closer attention in order to better understand how *Salmonella* reacts on its environment – inside the intestine, inside the host cell – and is able to use its host as replicative reservoir.

In the instant review we present an overview on *Salmonella*'s T3SSs, focusing on important recent findings and trying to evoke the importance of understanding these fascinating structures.

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Current Opinion in Microbiology

# *Salmonella* T3SSs: successful mission of the secret(ion) agents

Thomas P Moest[1,2,3] and Stéphane Méresse[1,2,3]

Bacteria of the genus *Salmonella* express nanosyringe-like organelles called type three secretion systems (T3SSs). These systems promote the secretion of bacterial compounds and their translocation into host cells. Pathogenic *Salmonella* use two distinct T3SSs, with specialized functions, having the purpose to modify the biology of the host organism and to ensure a successful infection. The bacterial proteins translocated through the first T3SS (T3SS-1) facilitate the entry of *Salmonella* into host cells, whereas T3SS-2 is an important factor for shaping the intracellular replication niche. In addition both T3SSs have a strong impact on the host inflammation. For a long time the two T3SSs were thought to act separately. However, there is increasing evidence that their regulation depends not only on separate but also shared regulatory mechanisms and that their time of action during infection overlaps. Here, we review the current understanding of the structure and of the regulation of expression and activity of both T3SSs. The output image is multifaceted, as recent studies show that subpopulations of *Salmonella* present diverging patterns of expression and activity of T3SSs during important steps of infection. These diversities may advance the chances of *Salmonella* to outpace competitors and to well establish itself in its niche in the host.

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## Introduction

*Salmonella* are Gram-negative bacteria, which are divided into two species, *bongori* and *enterica*. Both species carry the so-called pathogenicity island (SPI)-1 (SPI-1), a distinct chromosomal operon that encodes a type three secretion system (T3SS-1). All pathogenic *Salmonella* belong to the species *enterica* and carry an additional pathogenicity island named SPI-2, which encodes a distinct T3SS (T3SS-2). Both T3SSs play a role with regard to interactions with the host during pathogenesis. T3SS-1 facilitates the invasion of non-phagocytic cells and contributes to the crossing of epithelia. T3SS-2 is required for bacterial replication inside many eukaryotic cell types [1,2] of the various organs reached during the development of a systemic infection [3].

Bacterial secretion systems are structures which allow the transport of various molecules (proteins, ions, DNA, etc.) across the bacterial cell wall. Saprophytes as well as symbiotic bacteria use them to influence their environment and/or to control the homeostatis of the bacterial cytoplasm. At least seven different types of bacterial secretion systems have been described (reviewed in [4]). Three of them — T3SS, T4SS and T6SS — are specific to Gram-negative symbiotic (mutualistic or parasitic) bacteria and used to translocate bacterial compounds into the cell of the interaction partner. They differ in terms of structure and mechanism of action. T3SSs are used to inject bacterial proteins, named effectors, into host cells and have therefore been described as nanosyringes [5,6•,7]. Among bacteria using T3SSs, which can be divided into seven phylogenetic groups, very few have two T3SSs and among these the greater part does not use both T3SSs for infectious matter [8,9]. The two T3SSs of *Salmonella* differ in terms of time of expression and function. However, in recent years more evidence has been observed that regulation can be interdependent and that periods of secretion overlap.

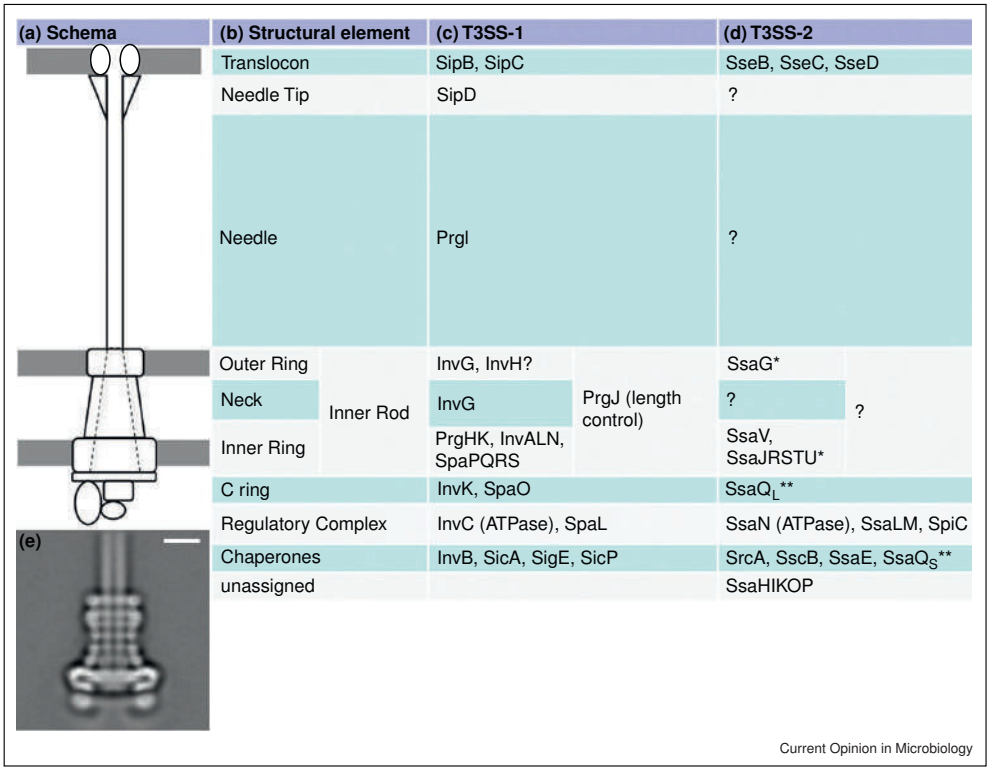
T3SSs derive from flagella and still share regulatory mechanisms with them [10–12]. In addition, although T3SSs of various genera of pathogens have discrete repertoires of effectors, they have kept functional homologies as shown by the ability to secrete and translocate effectors from a bacterial genus by the T3SS of another [13].

## Structure and function of T3SSs

T3SSs are macromolecular organelles composed of a basal body that spans the cell membranes and a needle-like complex that extends from the outer membrane, through which effectors are secreted (Figure 1). The basal body consists of an inner ring, a neck that spans the periplasmic zone and an outer ring. In order to be functional, a T3SS requires additional elements: a cytosolic regulatory complex that is linked to the basal body, an inner rod that constitutes the base of the needle, and a translocon, a protein complex forming a pore in the host membrane which is associated to the needle tip [14].

As it is expressed by *Salmonella* grown in regular medium and more abundant than T3SS-2, most of the structural data have been obtained with T3SS-1. Substructures of T3SS-1 are homomultimeric or heteromultimeric protein

**Figure 1**



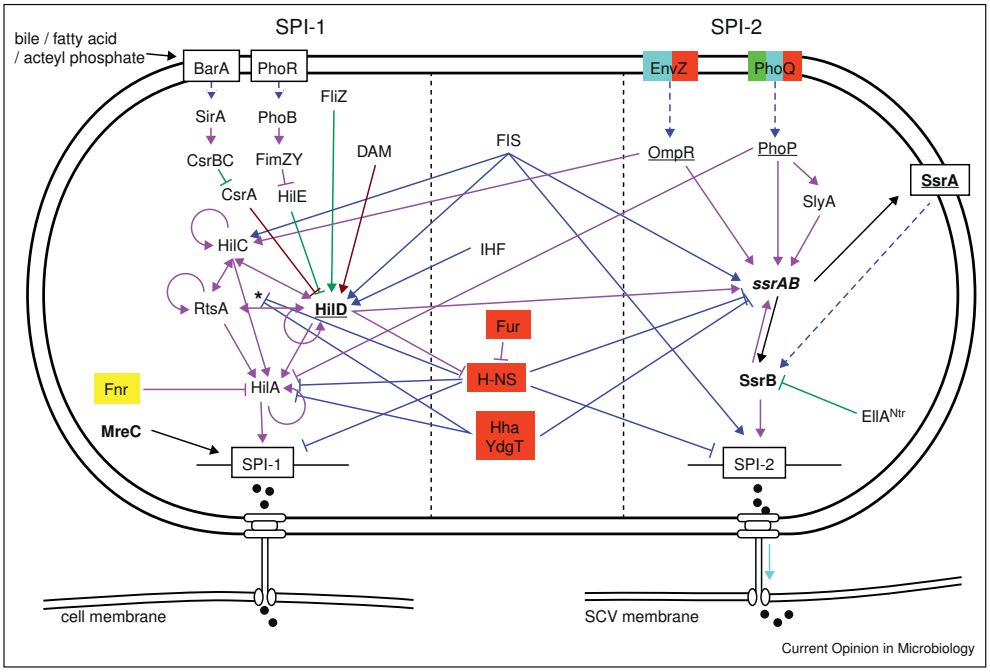

Structure and composition of *Salmonella* T3SSs. **(a)** Schematic representation of the structure of T3SSs. (b) Structural elements of T3SSs. **(c) and (d)** Proteins involved in composition of T3SS-1 and T3SS-2. *Estimations due to similarities to other T3SSs. **$SsaQ_{LS}$ (long, short) tandem translation leads to expression of two proteins, where $SsaQ_S$ acts as a chaperone for the structural component $SsaQ_L$ [61]. **(e)** Picture of a class-average from cyro-electron microscopy of a *Salmonella* T3SS-1. Scale bar: 10 nm.
Photo Credit: Thomas C. Marlovits, Research Institute of Molecular Pathology (IMP), Vienna, Austria.

complexes (Figure 1). The inner ring consists of 24 subunits of both PrgK and PrgH. The outer ring and the neck are made of 15 copies of InvG [15•,16]. These proteins show a wedge-shaped structure with two α-helices folding against one β-sheet. This motif is responsible for the formation of the inner and outer ring [17].

The needle is composed of replicates of the sole protein PrgI, which consists of two parallel α-helices. α1–α1 and α2–α2 interactions favor the homo-polymerization and the formation of a right-handed helical assembly similar to that of the flagellar filament [18]. It is growing at the distal end by spontaneous assembly of PrgI, which is probably mediated by a α-helix-to-β-strand conversion on the C-terminus [19]. The central lumen of the needle through which effectors are secreted has a diameter of ~25 Å [18]. The needle is connected to the inner rod, which is made of PrgJ. This substructure traverses the basal body and controls the length of the needle [20]. The tip of the needle is formed by five molecules of SipD interacting with PrgI via conformational changes in α-helices of both proteins [21,22••].

The translocation of effector proteins is a multistep process. Effectors are delivered from the cytosol towards the T3SS and recognized by a regulatory complex at the basal body. The secretion itself is promoted by an ATPase [23]. Finally, the effectors are translocated through the translocon. Sequences corresponding to secretion signals are usually located at the N-terminal part of the protein and are natively disordered, an important characteristic for the recognition by the T3SS apparatus [24]. Some cytosolic effectors interact with chaperones, which prevents them from folding and also facilitates targeting to T3SSs. The standard translocation process occurs from the bacterial to the host cytosol, but there is evidence that effectors localized on the bacterial surface can also be translocated. Such transport, which has

**Figure 2**




Regulation of *Salmonella* T3SSs. (Left) Regulation of SPI-1. (Middle part) DNA structuring elements with impact on both pathogenicity islands. (Right) Regulation of SPI-2. Key regulators are in bold and main players in cross-regulation are underlined. The nature of the impact on regulation is indicated with a color code. Environmental factors: osmolarity (red), pH (light blue), oxygen (yellow) and antimicrobial peptides (light green). Protein synthesis: promoter binding/transcription (pink), DNA structuring/transcription (dark blue), posttranscriptional regulation on mRNA (brown) and translational regulation (orange). Protein interactions: direct binding (dark green), phosphorylation (dark blue dashed). *Regulation mechanisms on the triumvirate of HilD, HilC and RtsA.

been shown for the *Yersinia pseudotuberculosis* effector YopH, also occurs in *trans* by *Salmonella* T3SS-1 [25].

## Regulation of T3SSs expression and function

*Salmonella* is an enteric pathogen that, as a function of the strain–host couple, triggers a local or systemic infection. In the latter case, *Salmonella* successively needs T3SS-1 and T3SS-2 to first invade the intestinal epithelium and subsequently survive in tissue phagocytes. The adaptation to different environmental conditions requires a rapid and accurate regulation of the expression of these virulence genes. T3SSs activities are controlled by a complex system of activating and inhibiting signals acting with different strength on different levels — ranging from transcription and translation to protein–protein interactions and secretion. A broad range of internal and external factors influence these regulatory networks [26•,27] (Figure 2).

## Regulation of T3SS-1

T3SS-1 expression is stimulated by the intestinal environment [28] and is needed to cross the intestinal barrier. T3SS-1 regulation (reviewed in [29]) follows a switch-on–off mechanism with a coupled positive feedback loop and a threshold of activation [30]. The central players of this regulation are HilA, its activators HilD, HilC, RtsA and its inhibitor HilE. The strong expression of HilD is responsible for the on-off pattern and the absence of dynamic upregulation, which has been shown at the single-cell level. HilC and RtsA have a lower level of expression. On their own, they are not able to activate HilA but rather act as amplifiers of the HilD-mediated transcription [26•,31]. Many more proteins are involved in the regulation of T3SS-1 and some new players have recently been identified: Fur [32], DAM [33], FliZ [31]. The bacterial cytoskeleton is also crucial for the regulation of T3SS-1 [34••] (Figure 2).

## Regulation of T3SS-2

In contrast to the switch-on–off mechanism of T3SS-1, the T3SS-2 regulation is rather continuous and depends on three two-component regulatory systems: SsrA/SsrB, PhoP/PhoQ and EnvZ/OmpR. SsrA/SsrB is the central regulator of T3SS-2 functions (reviewed in [27]) because SsrB directly activates the transcription of T3SS-2

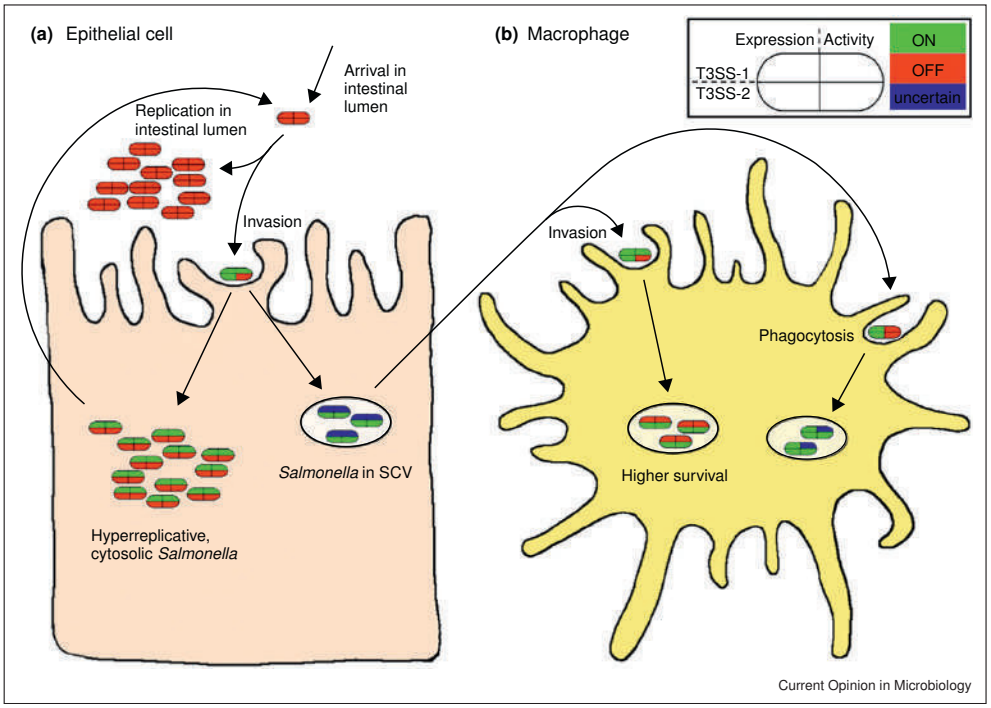
substrates (SifA, SifB, SseJ, etc.) and also promotes the expression of the T3SS-2 apparatus itself by displacing the DNA-binding protein H-NS from the promoter region of SPI-2 [35]. In addition, SsrB activates its own transcription. This is negatively controlled by $EIIA^{Ntr}$, which directly interacts with SsrB, thereby preventing it from binding its target promoters and avoiding undesirable effects due to overexpression of SPI-2 genes [36] (Figure 2). Remarkably, the T3SS-2 function is also regulated at the level of secretion by an unidentified 'pH sensor' that reacts to the neutral pH of the eukaryotic cytoplasm. T3SS-2 assembles under conditions of low pH in the vacuolar environment and secretes translocon proteins but negligible levels of effectors. A regulatory complex of three proteins (SsaL, SsaM and SpiC) (Figure 1), which is located in the bacterial cytosol and likely in contact with the basal body, controls this selective secretion. The formation of the translocon allows the sensing of the neutral pH of the eukaryotic cytoplasm. This causes dissociation of the SsaL–SsaM–SpiC complex thus enabling secretion of effector proteins [37••].

## Transition from SPI-1 to SPI-2 uses both differential regulation and crosstalk

The process of transition of the two SPIs expression depends on two means. On the one hand, the different environmental conditions in the host turn on or shut down each SPI separately (described above). On the other hand, some regulatory mechanisms are at the cutting edge of transition and influence the expression of both SPIs. These fine tuning mechanisms are discussed here.

DNA topology and structuring proteins are important players in transcriptional processes [27]. Not only the structuring of the DNA itself (Figure 2) but also specific events targeting structuring proteins can influence transition from SPI-1 to SPI-2. An example is the discovery that the SPI-1 *invA* gene is repressed by DNA relaxation, while *ssrA* expression is induced [38]. DNA topology is modified in response to environmental conditions such as oxygen pressure or cell-specific vacuolar environment. DNA becomes relaxed when *Salmonella* resides in macrophages but not in epithelial cells [38]. Relaxed DNA

**Figure 3**




Patterns of expression and activity of T3SSs during infection (compare to Box 1). Ingested *Salmonella* passes different host niches and encounters various environmental conditions and cell types. These different situations will promote or repress the expression of T3SSs and their activities. Once in the gut lumen only a fraction of *Salmonella* will express T3SS-1 and invade epithelial cells. Intra-epithelial bacteria might be vacuolar or cytoplasmic and have dissimilar T3SS patterns. In macrophagic cells, the initial fate and T3SS pattern are functions of the entry mode (invasion versus phagocytosis).

recruits OmpR but the relative contributions of OmpR and DNA topology to promoter activity seem to be gene-specific and have diverging consequences on the expression of SPI-1 and SPI-2 genes [39].

HilD is the main player of SPI-1 expression. It is under certain conditions [40] and in concert with OmpR able to crosstalk and to mediate a first step in the transition from SPI-1 to SPI-2. This happens before invasion, and before any cell contact [41,42]. HilD binds to the regulatory region of *ssrAB*, thereby counteracting H-NS mediated repression and promoting OmpR binding [40]. This favors the expression of SPI-2 in the intestine (Figure 3), which is a priming mechanism for the future fate of *Salmonella* inside the host cell.

After entering into the cell, *Salmonella* resides inside a membrane-bound compartment named *Salmonella*-containing vacuole (SCV). At this time, SPI-1 is to be down-regulated and SPI-2 expression to be increased by environmental factors. One important feature of the SCV is the shortage of magnesium that leads to H-NS polymerization, stiffening the DNA and repressing transcription of both SPIs [43]. However, the effect on SPI-2 is specifically overcome by PhoQ/PhoP, which is known to induce SPI-2 under $Mg^{2+}$ shortage. PhoQ/PhoP also acts as a crosstalk repressor of HilA thereby further shutting down SPI-1 expression, ending the transition and heralding the SPI-2 phase.

Yet, there are some examples where both T3SSs are coexpressed and where T3SS-1 even plays a role in intracellular replication. This is the case in hemophagocytic macrophages, a type of activated phagocyte, which is present in various infectious and inflammatory circumstances [44]. This may explain why SPI-1 is needed for bacterial survival in the mouse model of chronic *Salmonella* infections [45]. Moreover, SPI-1 genes are upregulated following phagocytic uptake by resting macrophages. Even though the locus is not required for intracellular survival or replication in these cells [46], it may finely tune the host biology and play a subtle role *in vivo*. Thus, the two T3SSs rather seem interdependent in terms of regulation and function.

## Copy number and distribution of *Salmonella* T3SSs

T3SSs of *Salmonella* differ in both number per bacterium and distribution. T3SS-1 exists in six to eight copies [47] and tends to be located along the axis of the bacterium, its distribution being similar to those of flagella. T3SS-2 exists in one or two copies and is located at the poles of the bacterial cell [34••,48]. T3SSs are also regulated differently by the cytoskeleton. A knockout of MreC leads to a dramatic downregulation of T3SS-1 and flagella expression while T3SS-2 appears to remain functional [34••].

## Activities of *Salmonella* T3SSs

The two *Salmonella* T3SSs have a functional dichotomy in infection. T3SS-1 is mainly responsible for invasion, a process mediated by re-arrangement and polymerization of actin, leading to membrane ruffling and engulfment of the bacteria (Figure 3). It is also responsible for intestinal inflammation [49]. After irreversible docking to the cell membrane via the T3SS-1 needle tip protein SipD and the translocases SipB, SipC [47,50], the whole T3SS-1 effector repertoire is translocated into the cytoplasm. However, *Salmonella* strains without translocase SipB or SipC are capable of secreting effector proteins into the extracellular environment. These strains have been used to show that effector secretion starts before invasion and that this pool of extracellular effectors contributes to the activation of proinflammatory signal transduction pathways [51].

T3SS-2 is necessary for shaping the SCV, the intracellular niche of replication (reviewed in [52]) (Figure 3). T3SS-2 effector secretion occurs 2–4 hours after invasion [53]. This time delay is required for the maturation of the SCV, docking of the T3SS-2 to the SCV and pH sensing [37••]. Secretion is primed by the expression of T3SS-2 effectors already in the intestine [40–42,54].

In addition to the capacity to invade and to replicate in host cells, the expression of T3SSs has also important consequences for host defence. T3SS-1 is a potent activator of caspase-1 [55] which elicits a strong intestinal inflammation. This helps *Salmonella* to colonize host tissues by competing with the natural microbiota (compare to Box 1). However, this inflammation is counteracted by other T3SS-1 and T3SS-2 effectors, which maintain an adequate balance between colonization and killing by the host defence.

**Box 1 Bistable gene expression**

Each regulation is limited in its precision and in biology chance always plays a certain role. An important recent perception is that many processes during *Salmonella* infection happen on the level of single bacteria [57]. Diversity is known to exist in populations of bacteria. For example, in the intestine, only a fraction (about 15%) of the *Salmonella* expresses T3SS-1 [58] (Figure 3). Even more strikingly, after invasion of the epithelium, there is a small population of bacteria, which does not reside inside a vacuole but in the cytoplasm of epithelial cells (Figure 3). They express T3SS-1 and not T3SS-2, show a high replication rate and are partly mobile [59••] (Figure 3). How are these diversities possible? Genetic regulation is dependent on a phenomenon called phenotypic noise or bistable gene expression [60]. This is a random switch-on or switch-off that can depend on environmental signals or intracellular stoichiometry. It may be supposed that this is the reason why different *Salmonella* populations can be found in the intestine and epithelial cells. Perhaps both diversified populations present a desired and regulated mechanism to serve as a reservoir of intestinal re-infection and out-competition of other bacteria.

## Conclusions

The traditional concept that the two T3SSs have separate roles in infection and are independent of each other is increasingly revoked. Recent results rather point towards an interdependent regulation and function. The regulatory network of the two entities shows cross-regulations in both directions. As an effect, T3SS-2 effectors are already expressed in the intestine, priming bacteria for intracellular colonization. In addition, T3SS-1 effectors may play a role even after invasion, during the T3SS-2 phase. Apart from their main function, that is, the secretion and translocation of effectors, T3SSs are involved in other processes during infection. T3SS-1 (and probably also T3SS-2) provides a tool for irreversible attachment to the host cell membrane. The T3SSs are likewise recognized by the immune system, leading to inflammation, and might even be used as vaccination targets [56].

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# Chapter 3
# *Salmonella*'s effector proteins

**What is an effector, how does it act?**

Certain pathogens rely on virulence factors in order to invade the host's cells, survive and replicate within these, acquire nutrients, manipulate these cells and escape from the immune system. Many different components of a pathogen are implicated in these tasks. A most successful way of manipulation is the secretion of effector proteins by the bacterium and their translocation into host cells.

The universality of intracellular bacterial pathogens is able to manipulate many prominent host functions. Examples are the targeting of processes from transcription to post-translational modifications and the modification of endocytic and secretory pathways [55-58]. Surprisingly, recent findings show an impact of effectors on epigenetics, for instance mediated by LLO from Listeria monocytogenes, PFO from Clostridium perfringens and PLY from Streptococcus pneumonia, effectors that induce dephosphorylation of Ser10 on H3 [59].

***Salmonella*'s effector proteins**

To date, more than 40 different T3SS-1 and -2 protein effectors with many different functions have been identified. The effectors are mostly attributed to one T3SS, but some of them are able to get secreted by both secretion systems (Fig. 3.1).

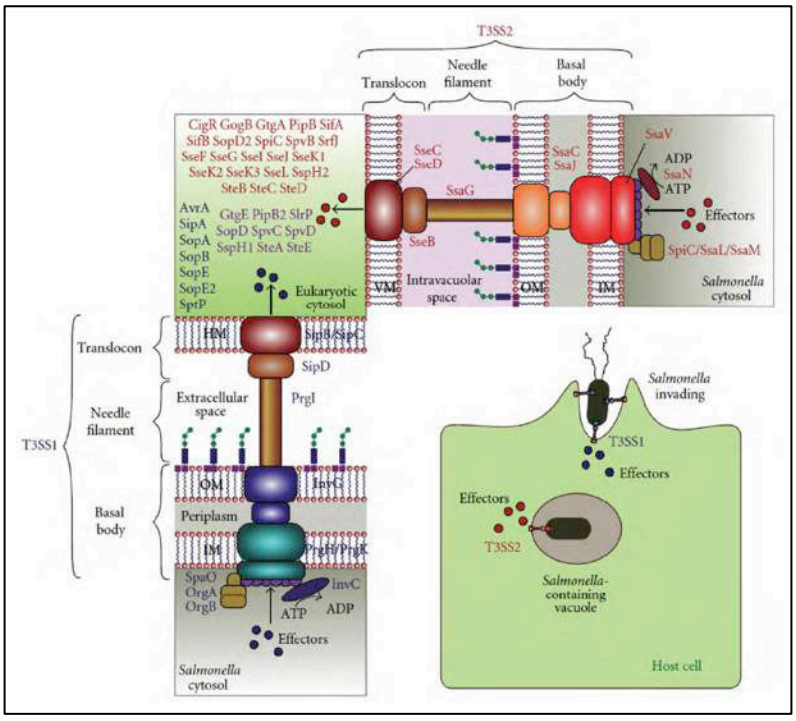

Fig. 3.1: Schematic structures of *Salmonella* T3SS-1 and T3SS-2 with associated effector proteins. HM: host membrane; VM: vacuolar membrane; OM: outer membrane; IM: inner membrane. T3SS-1 components and effectors are in blue. T3SS2 components and effectors are in red. Effectors translocated by both systems are in purple. Adapted from [60].

**Functions of the T3SS-1 effectors**

Invasion

Effectors that are attributed to the T3SS-1 promote the invasion of non-phagocytic cells and regulate the inflammation of the intestine during the gastrointestinal phase of *Salmonella*. The effectors get injected into the cell after docking of *Salmonella* to the cell membrane. Subsequently, the entry of *Salmonella* into the cell is triggered via the induction of membrane ruffles through rearrangements of the actin cytoskeleton. An accorded action of several effectors is necessary to do so. The effectors SipA and SipC stabilize and nucleate actin, but are themselves not sufficient to induce membrane ruffling. They are supported by the indirect actin rearrangement through the effectors SopE, SopE2 and SopB, which act in concert on the small GTPases Cdc42, Rac1 and RhoG. These host proteins are responsible for the rearrangement of the actin cytoskeleton. SopE and SopE2, which exert a GEF activity toward Cdc42 and Rac1, get ubiquitinated after injection into the cell and get quickly degraded within less than 30 min after invasion. Their effect is reversed by the effector protein SptP, which acts as a GAP on the same small GTPases, leading to the recovery of the normal architecture of the cytoskeleton after the internalization of the bacteria in a primary vacuole [61,62].

Immune modulation

Most of the T3SS-1 effectors also contribute to inflammation. A side effect of the action of SopE and SopE2 on Cdc42 and Rac1 is the activation of the transcription factors AP-1 and NK-κB, leading to the production of IL-8. IL-8 itself is a potent attractant for neutrophils and promotes their transmigration into the intestinal lumen. Also SipA and SopA promote this transmigration of neutrophils. SipB can induce inflammation by activating caspase-1, which leads to the production and release of IL-1 β

and IL-18 [62]. SopA mimics an E3 ubiquitin ligase and acts together with host E2 ubiquitin ligases that are involved in inflammation [63]. The concerted action of T3SS-1 effectors on the actin cytoskeleton has been reported to destabilize the tight junctions of the intestinal epithelium, promoting the trans-epithelial crossing of neutrophils and the efflux of liquids that contribute to diarrhea. To keep things in balance, the three effectors SptP, AvrA and SpvC (a T3SS-2 effector) can inhibit the host's Erk, Jnk and NK-κB signaling [62].

**Transition time – from the uptake to the intracellular niche**

Bacterial pathogens have created different ways to persist and replicate in their hosts. One important factor is the creation or adaption of their replicative niche, which for *Salmonella* consists in a membrane-bound compartment called *Salmonella* containing vacuole (SCV).

After invasion of the cell, the primary SCV is composed of plasma membrane. On its way towards its final destination, at a juxtanuclear position close to the MTOC and the Golgi apparatus, its composition and characteristics change. The SCV interacts with the endosomal compartment, namely early and late endosomes, and acquires surface markers and properties of the respective compartments. Along the way, it transiently turns positive for markers of early endosomes such as endosomal antigen 1 (EEA1), transferrin receptor, Rab5 and Rab11. After, markers such as Rab7, LAMP1 and vATPase are associated to the SCV (Fig. 3.2) [64,65]. Although the SCV turns positive for certain LE/lys markers, others such as Cathepsin D and the mannose-6-phosphate receptor (M6PR), a protein that is involved in the transport of hydrolases to late endosomes, stay excluded [66,67].

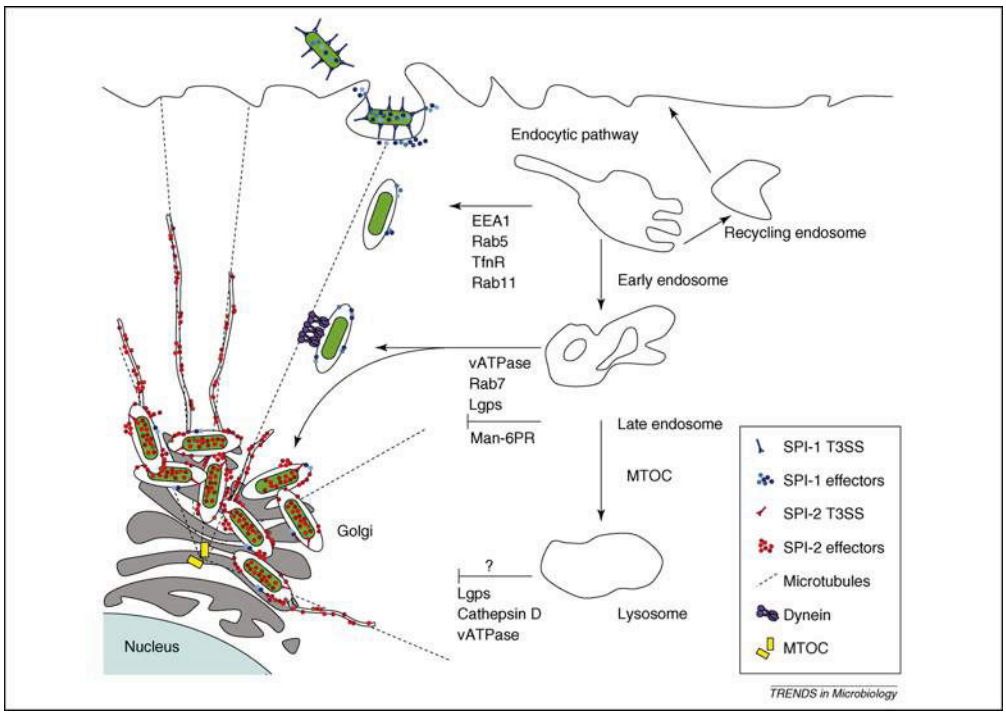


Fig. 3.2: Maturation of the SCV and interaction with the endosomal compartment. Copied from [65]

Together with the change of membrane-imbedded proteins, the membrane itself changes. Already during invasion the phosphoinositides PI(3,4)P2 and PI(3,4,5)P3, which are ubiquitously present in the plasma membrane, get dephosphorylated to PI(3)P. This is mediated through the recruitment of Rab5 and VPS34 onto the SCV by SopB and promotes membrane flexibility and therefore fission of the SCV [68]. Cholesterol plays an important role in the invasion of cells by *Salmonella*. It gets relocated and accumulated at the entry site, and *Salmonella* preferentially targets mitotic cells for invasion due to their high concentration of plasma membrane cholesterol [69,70]. Later, up to 30% of the cells total cholesterol get recruited onto the SCV [69,71]. However, it remains unclear why cholesterol gets recruited. It could be necessary for the formation of lipid rafts, thereby regulating signaling events or fusion of the SCV with other compartments. Also, cholesterol is a determinant of membrane fluidity [72]. Finally lysosomes carry 6% of the cells total amount of cholesterol [73]. This leaves much space for speculation about a fusion of lysosomes with the SCV or the tubular network that arises from it, mechanisms that will be discussed later on.

**Functions of the T3SS-2 effectors**

The preparation of the intracellular phase starts as soon as in the intestine. Here, the expression of T3SS-2 effectors is upregulated through complex regulatory networks. Hence, *Salmonella* enters the cell with a fully loaded armory. However, the secretion of these effectors is delayed until two to four hours after invasion, when the SCV already reached its final position in the cell. This delay is due to the trafficking and maturation of the SCV and the docking of the T3SS-2 to the SCV. This docking allows the sensing of the low pH of the SCV, which is the required signal for the secretion of the effectors [74].

About 30 effectors were identified to be secreted by the T3SS-2. They are responsible for the localization of the SCV and its membrane dynamics, modify the actin skeleton and microtubules and interfere with immune signaling. However, the function of many effectors is still not known (see Table 3.1 and Fig. 3.3).

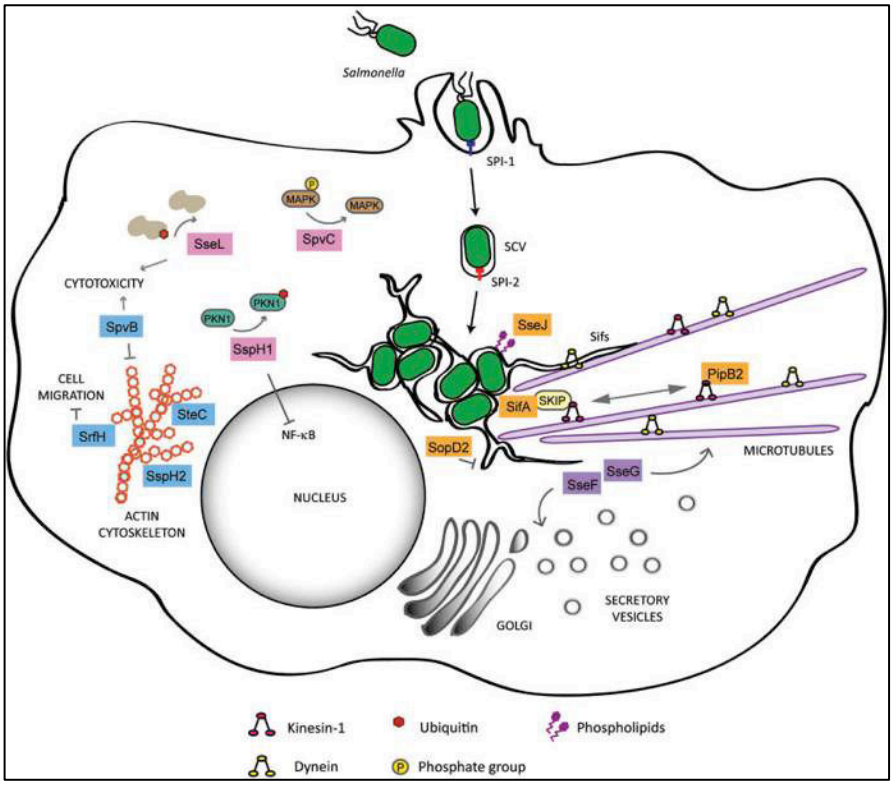


Fig. 3.3: Functions of the T3SS-2 effectors. They are involved in the maintenance of the SCV (orange boxes) and its localization in proximity of the Golgi apparatus (purple boxes). They can target and modulate immune signaling pathways (pink boxes) and interfere with the cytoskeleton (blue boxes). Copied from [75].

| Effector | Function(s) | Biochemical activity | Host target |
|---|---|---|---|
| **SCV localization** | | | |
| SseF | SCV positioning at peri-Golgi region, microtubule bundling, Sif formation | ? | ? |
| SseG | | | |
| **Vacuole membrane dynamics** | | | |
| SifA | Sif formation, maintenance of vacuolar membrane, enhanced kinesin-dependent anterograde movement along microtubules | ? | SKIP, RhoA |
| PipB2 | Recruitment of kinesin-1 to the SCV, Sif extension | ? | Kinesin-1 |
| SopD2 | Sif formation, vacuole integrity | ? | ? |
| SseJ | Esterification of cholesterol in infected cells, vacuole integrity | Acyltransferase | Phospholipids, cholesterol |
| **Host immune signalling** | | | |
| SpvC | Dephosphorylation of MAP kinases | Phosphothreonine lyase | MAPKs |
| SseL | Macrophage delayed cytotoxicity, downmodulation of NF-κB-dependent cytokine production, altered lipid metabolism in infected cells | Deubiquitinase | IκB, OSBP |
| SspH1 | Inhibition of NF-κB-dependent gene transcription | E3 ubiquitin ligase | PKN1 |
| **Host cytoskeleton** | | | |
| SteC | Formation of SCV-associated F-actin meshwork | Kinase | ? |
| SspH2 | Recruited to SCV-associated F-actin meshwork | E3 ubiquitin ligase | Profilin |
| SrfH/SseI | Recruited to SCV-associated F-actin meshwork, migration of infected phagocytes | ? | TRIP6, IQGAP1 |
| SpvB | Inhibition of actin polymerization, macrophage cytotoxicity, P-body disassembly in infected cells | Actin ribosyltransferase | ? |
| **Unknown function** | | | |
| SlrP | ? | E3 ubiquitin ligase | Thioredoxin, ERdj3 |
| GtgE | Cleavage of Rab29 accumulated on SCV | Protease | ? |
| PipB | ? | ? | ? |
| SifB | ? | ? | ? |
| SseK1 | ? | ? | ? |
| SseK2 | ? | ? | ? |
| SseK3 | ? | ? | ? |
| GogB | ? | ? | ? |
| SteA | ? | ? | ? |
| SteB | ? | ? | ? |
| SteD | ? | ? | ? |
| SteE | ? | ? | ? |
| SpvD | ? | ? | ? |
| GtgA | ? | ? | ? |
| CigR | ? | ? | ? |

Table 3.1: Overview about T3SS-2 effectors and their functions during infection. Copied from [75].

Positioning of the SCV – Implications of motor proteins

After invasion of the host cell, the trafficking of the SCV towards its juxtanuclear position depends on dynein [76]. This first movement along microtubules initiates a long history of interactions between T3SS-2 effectors and the microtubular network. Microtubules have actually been in the focus of *Salmonella*-related research during the last years, as they are implicated in many vital processes of *Salmonella*'s intracellular life [77].

In mammalian cells, cargo transport is carried out along cytoskeletal structures. Microtubules are one of them and allow movement mediated by dyneins and kinesins.

They are long polar structures with two distinct ends, plus and minus. They spread from the MTOC, where the minus end is located, to the cell periphery, location of the plus end [78]. Their structure is asymmetric, which is important for the orientation of motor proteins. That way, dyneins are able to move towards their minus end and (almost all) kinesins towards their plus end.

Kinesins and dyneins have essential functions in *Salmonella*'s intracellular life. After the bacteria reach their juxtanuclear niche in a dynein-dependent manner, the SCV stably resides there. The dynein-mediated movement is reverted by the effector protein SifA, which prohibits the binding of Rab7 – Rab7 is responsible for the minus end directed movement of LE/lys – to its effector protein RILP [76]. A knockout of SifA stops this effect as it stops the removal of kinesin-1 from the SCV through the formation of SIFs; the ensuing, uncontrolled opposing motor activity might contribute to the loss of a stable SCV [79-81]. Additionally, the SCVs of Δ*sifA*, Δ*sseF* and Δ*sseG* strains don't show the normal juxtanuclear positioning close to the Golgi apparatus, representing another indication for an undesired motor function. It is likely that during infection with a Δ*sifA* strain this is mediated by the accumulation of the plus end directed motor kinesin-1 on the SCV. For knockout strains of the effectors SseF and SseG, the delocalization of the SCV might happen because of their proposed interaction with dynein, which allows positioning of the SCV close to the MTOC and the Golgi apparatus [82,83]. This would mean that after a first uncoupling of dynein from the SCV via the interaction of SifA with Rab7, other effectors take over. Perhaps they provide a more controlled function of dynein, which corresponds better to the needs of *Salmonella*. Additionally, the SCVs of Δ*sseF* and Δ*sseG* strains show higher motility, speaking for the importance of motor regulation by T3SS-2 effectors [84].

Motor proteins are also strongly involved in the formation of *Salmonella*'s tubular network, which impacts the development, dynamics and stability of the SCV, in order to promote a good intracellular replication.

Vacuole membrane dynamics and the tubular network – The replicative niche

The establishment and maintenance of the SCV as niche is crucial for the intracellular survival and replication of *Salmonella*. To establish the niche and to create new SCVs for replicating bacteria, a constant supply of membrane is needed. This is closely linked to the appearance of a dynamic and complex network of tubular structures, which arises from the SCV and is one particular attribute of *Salmonella*'s intracellular lifestyle. The establishment of the network depends on the interplay of the T3SS-2 effectors SifA, SopD2, SseJ, SteA, PipB2, SpvB, SseF and SseG (Table 3.2) [85-91].

| Effector | Intracellular localisation | Function(s) | Target(s)[a] |
|---|---|---|---|
| SopB/SigD | Plasma membrane, SCV | Phosphoinositide phosphatase | PI(4,5)P2, Rab5 |
| PipB | SCV, SIFs | n.d. | n.d. |
| PipB2 | SCV, SIFs, SISTs, LNTs | SIF and LNT extension, recruitment of kinesin-1 to SCV | Kinesin-1 |
| SifA | SCV, SIFs, SISTs, LNTs | SIF, SIST, LNT formation, SCV integrity, SCV positioning, redirection of post-Golgi trafficking, putative GEF | SKIP, Rab7, GDP-RhoA |
| SifB | SCV, SIFs | n.d. | n.d. |
| SopD2 | SCV, SIFs, SISTs, LNTs, late endosomes | SIF and SIST formation, inhibition of LNT formation | n.d. |
| SpvB | n.d. | ADP-ribosyltransferase, actin depolymerisation, induction of apoptosis, negative modulation of SIF formation | Actin |
| SseF | SCV, SIFs, SISTs | SIF and SIST formation, microtubule bundling, SCV positioning, redirection of post-Golgi trafficking, possible recruitment of dynein | n.d. |
| SseG | SCV, SIFs | SIF and SIST formation, microtubule bundling, SCV positioning, redirection of post-Golgi trafficking, possible recruitment of dynein | n.d. |
| SseJ | SCV, SIFs, SISTs, LNTs | Deacylase, phospholipase, glycerophospholipid-cholesterol acyltransferase (GCAT), SCV dynamics, SIF formation | GTP-RhoA |
| SseL | SCV, SIFs | Deubiquitinase, macrophage cytotoxicity | Ubiquitin |
| SteA | TGN, SCV, SIFs | n.d. | n.d. |
| SteC | SCV, SIFs | Kinase, F-actin remodelling in proximity to SCVs | n.d. |

Table 3.2: T3SS-2 effectors which are present on the SCV or *Salmonella*'s tubular network. Adapted from [92].

The generation of the tubular network starts four to six hours after infection and gives rise to three kind of tubules – *Salmonella*-induced filaments (SIFs), *Salmonella*-induced SCAMP3 tubules (SISTs) and LAMP-1 negative tubules (LNTs) (Fig. 3.4 and Table 3.3) – which all depend on a functional microtubular network [93-96].

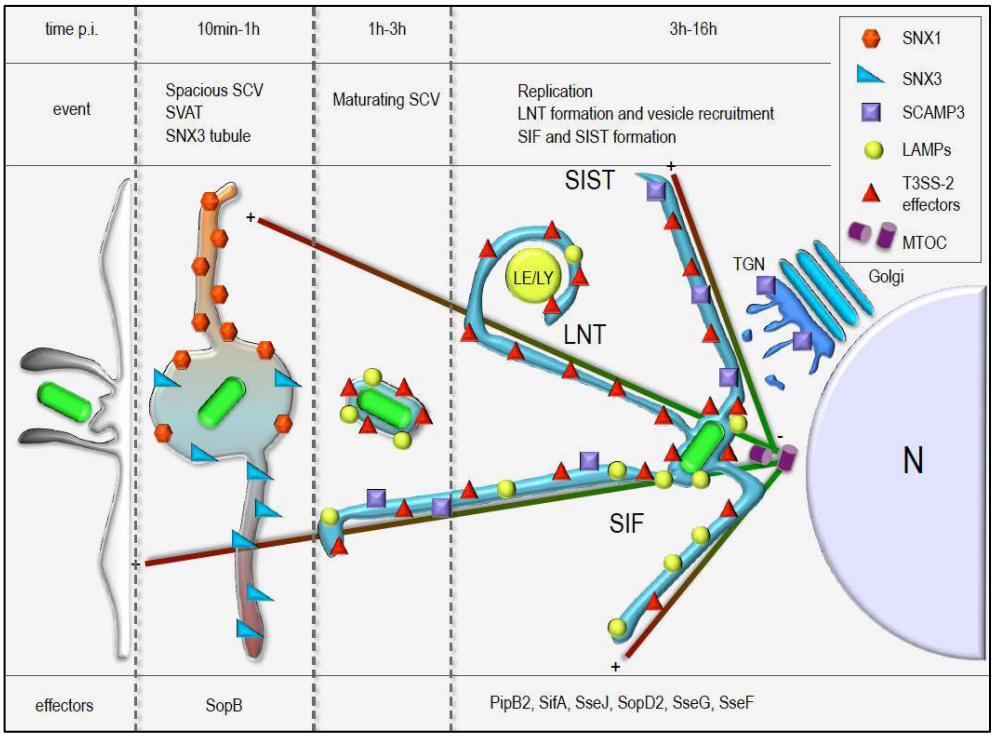


Fig. 3.4: *Salmonella*'s tubular network. Copied from [92].

| Marker (compartment)* | SIFs | SISTs | LNTs |
|---|---|---|---|
| LAMP1 (LE/Lys) | + | – | – |
| Scamp3 | +/– | + | – |
| T3SS-2 effector | + | + | + |
| EEA1 | – | n.d. | – |
| Rab5 (EE) | – | n.d. | – |
| Rab7 (LE) | + | n.d. | – |
| Rab9 (LE) | – | n.d. | – |
| vATPase (LE/Lys) | + | – | + |
| Yip3 (LE) | – | n.d. | – |
| Syntaxin 7 (LE) | n.d. | n.d. | – |
| LBPA (LE) | + | n.d. | – |
| CD63 (LE/Lys) | + | – | – |
| Mannose 6-Phosphate Receptor (LE, TGN) | – | n.d. | – |
| VPS26 (TGN) | – | n.d. | – |
| Syntaxin 6 (TGN) | – | n.d. | – |
| P230 (TGN) | – | n.d. | – |
| GM 130 (*cis* Golgi) | – | n.d. | – |
| Calreticulin (ER) | – | n.d. | – |
| Calnexin (ER) | – | n.d. | – |
| Dynein ($P150^{glued}$) | – | n.d. | – |
| Cholesterol | + | n.d. | + |
| **Susceptibility** | | | |
| Nocodazole | + | + | + |
| Cytochalasin D | – | n.d. | – |
| Brefeldin A | – | + | – |
| SKIP RNAi | + | n.d. | – |

Table 3.3: Features of the three types of tubules, SIFs, LNTs and SISTs. EE, early endosome; ER, endoplasmatic reticulum; LE, late endosome; Lys, lysosome; TGN, *trans*-Golgi network. Copied from [96].

SIFs are tubular structures that arise from the SCV and are positive for several T3SS-2 effectors and host proteins derived from the LE/lys compartment (Fig. 3.4). The formation of SIFs is linked to the presence of the effector SifA and a Δ*sifA* strain is deprived of the formation of tubules and can't maintain a stable SCV [85,93,94]. The interaction of SifA with the host protein SKIP leads to budding of vesicles from the SCV. Via the interaction of SKIP with Kinesin-1 this complex promotes the formation of SIFs along microtubules[79,97,98]. SIFs are highly dynamic structures. Especially during the first hours of formation (4h to 6h post infection), they show bidirectional movement,

elongation, retraction and branching. At later time points they become less dynamic and don't either react on the disruption of microtubules [99].

Apart from SifA, other effectors are implicated in the formation of SIFs; these are PipB2, SopD2, SteA, SseF and SseG. PipB2 recruits Kinesin-1 to the SCV, providing it to the SifA-SKIP complex [80]. Δ*pipB2* strains therefore show shorter SIFs [89]. The impact of SopD2, SteA, SseF and SseG is less well understood. Knock-out of SopD2, SseF or SseG leads to a decreased number of SIFs and apparition of SIF-like structures, which are called pseudo-SIFs and characterized by a reduced presence of LAMP-1 [86,100]. As for SopD2, it might contribute to the formation of SIFs by its interaction with LE/lys [101]. The contribution of SseF and SseG, which form a functional complex, may be from the massive bundling of microtubules that they induce [82,102,103]. It is as well possible that their interaction with LE/lys contributes to the formation of SIFs [91,102]. Also the knock-out of SteA leads to a decrease of SIFs, but the mechanism has not yet been described [88]. Contrarily to these effectors, SseJ and SpvC seem to antagonize the formation of SIFs. A Δ*sifA* Δ*sseJ* strain exhibits a more stable SCV than a Δ*sifA* strain [104]. SseJ is a glycophospholipid-cholesterol acyltransferase, thus it is esterifying cholesterol [105]. Through this modification cholesterol becomes more hydrophobic, which might change and disrupt the structure of the SCV. As a possible result, Δ*sifA* strains have been shown to possess an unstable SCV. Yet, it is not clear whether the destabilizing action of SseJ or the deregulation of motor proteins is the deciding factor for the destabilization of the SCV. Besides, SseJ may play a role in the initiation of SIFs (via its function in the "destabilization" of the SCV in the Δ*sifA* mutant strain). This hypothesis is supported by eukaryotic expression of SifA together with SseJ, which leads to tubulation of endosomes [106].

SIFs interact intensively with the LE/lys compartment. They can fuse with lysosomes and are also accessible to incoming material from the endocytic pathway [46,107]. The association of LE/lys with the SCV is an active process, as interactions of the SCV with the LE/lys compartment were described to be more frequent than for instance those of the phagosome of *Escherichia coli* [46]. Their fusion with the SCV is even essential for *Salmonella*. For instance, the recruitment of vATPase and the linked acidification of the

SCV induce the secretion of T3SS-2 effectors [108], which then facilitate the replication and survival of *Salmonella* inside its niche.

Although the fusion with LE/lys is required, this compartment could be harmful to *Salmonella* due to its degrading nature. But certain harmful substances such as the hydrolase Cathepsin D are known to be excluded from the SCV [47,67]. To explain these absences, it has been proposed that *Salmonella* partly blocks trafficking of harmful substances from the Golgi to the LE/lys compartment.

Unlike SIFs, LNTs are negative for host proteins, although they sometimes show punctuated and weak staining for LAMP-1, but are positive for several T3SS-2 effectors (see Fig. 3.4 and Table 3.2). They were discovered in infections with a Δ*sifA* Δ*sopD2* strain [96]. A Δ*sifA* strain is defective for tubule formation. This indicates that SopD2 acts as a negative regulator of LNT formation. The LNTs of a Δ*sifA* Δ*sopD2* strain are sufficient to ensure a stable vacuole and bacterial replication. Blocking LNT formation through the action of SopD2 may be one of the reasons for the destabilization of the SCV. LNTs can also be found in infections with wild-type *Salmonella*, hence LNT induction in presence of SifA seems to be dominant over their repression by SopD2. However, they are mainly found in early stages of tubule development and only exist in small numbers. Other than SIFs, LNTs show only restricted interaction with the LE/lys compartment. LNTs sometimes seem to wrap around them, but no efficient fusion has been shown yet [96]. SseF, SseG and SteA are present on LNTs and SIFs and their knockout leads to less SIFs and reduced recruitment of LAMP-1 on SIFs. Rather than being independent tubules LNTs may therefore be precursors and integral parts of SIFs.

Unlike SIFs and LNTs, SISTs obtain their membrane substrate from the secretory pathway, although the precise mechanism of recruitment is unknown. They can thereby contribute to the membrane recruitment to the SCV. Otherwise, they are negative for markers that originate from the endosomal compartment [95].

Therefore all three kinds of tubules seem to be implicated in the recruitment of membrane, although from different sources. However, it can't be excluded that they have other functions during *Salmonella*'s intracellular development.

**Paper introduction: *Salmonella* induced host tubules are functional organelles**

*Salmonella* is an intracellular pathogen and resides in a membrane-bound compartment, the *Salmonella* containing vacuole (SCV). The SCV derives from membrane of the endosomal compartment an from Golgi vesicles and its stability is crucial for *Salmonella*'s intracellular survival and replication [46,95,109]. A particular feature of *Salmonella* is the development of a network of tubular structures. It arises from the SCV, is important for the SCV's stability and consists of three different types of tubules [92]. Among the tree kind of tubules, only SIFs are well described and the mechanisms of their formation well understood [79,80,93,94,97]. The two other types, SISTs and LNTs, were discovered recently and little is known about the T3SS-2 effectors and host proteins being involved in their formation [95,96]. Here, we try to decipher the formation of LNTs and their contribution in the membrane stability of the SCV and the depending intracellular replication inside the host cell.

LNTs can be found in infections with wild-type *Salmonella*. They seem to extensively interact with late endosomes / lysosomes (LE/lys), thereby providing a stable SCV to the bacteria and allowing higher intracellular replication [96]. While a Δ*sifA* strain does not give rise to any tubules, LNTs can be found in a Δ*sifA*Δ*sopD2* strain [94,96]. This implies that SopD2 has a negative regulatory effect on the formation of LNTs, which is overcome by the action of SifA. However, other effectors must be implicated in their formation, as due to their appearance in the Δ*sifA*Δ*sopD2* strain. We took advantage of this strain and showed that the two effectors SseF and SseG act in concert to build the LNTs.

We were then looking for host factors that are involved in their formation and interaction with LE/lys. Arl8B is a small GTPase that is responsible for the movement of lysosomes and for their fusion with late endosomes. We showed that over-expressed Arl8B gets recruited to LNTs. A knockdown of Arl8B does not drastically impact the formation of tubules, although they are shorter and less abundant. However, over-expression of Arl8B leads to the recruitment of LAMP-1 onto LNTs. We concluded that the predominately active Arl8B promotes the fusion of LNTs with LE/lys. The acquired membrane allows the stabilization of the SCV and also contributes indirectly to the formation of the LNTs, as also tubules need a certain amount of membrane to arise.

# *Salmonella*-induced host tubules are functional organelles

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## Abstract

*Salmonella* is a bacterial pathogen that invades host cells in order to survive and replicated within its host. *Salmonella*-infected cells are characterized by the appearance of membranous tubular structures that extend from its replicative niche, a membrane-bound compartment called *Salmonella* containing vacuole (SCV). Different kinds of tubules with varying host protein contents have been identified and this diversity is thought to reflect the capacity of these tubules to interact with different host compartments. Membrane tubules that are essentially devoid of host proteins, named LAMP1-negative tubules (LNT), have been observed at early time of host infection suggesting they are precursors of other kinds of tubules. As LNTs have been observed to wrap around LAMP1-positive vesicles, we propose that these tubules promote the recruitment of lysosomal glycoproteins onto the bacterial vacuole and therefore facilitate the formation of a replicative niche. In this study, we performed a biochemical and functional characterization of LNTs. We show that the effector proteins SseF and SseG are required for the formation of LNTs and demonstrate that the presence of LNTs is associated with an enrichment of LAMP1 on the bacterial vacuole and a better replicative capacity of the bacteria inside cells and an increased virulence. Altogether these data support the idea that LNTs are functional organelles that support the establishment of the replicative niche by favoring the recruitment of host proteins and membrane and their transport towards the bacterial vacuole.

## Introduction

*Salmonella* Typhimurium is an intracellular pathogen that causes a typhoid-like infection in mice. The bacterium spreads systematically and grows in epithelial cells and cells of the immune system [1,2]. *Salmonella*'s virulence relies on its capacity to establish an intracellular replicative niche, a membrane-bound compartment named *Salmonella*-containing vacuole (SCV) that is shaped by the activity of effector proteins. The latter are translocated from the bacterial cytosol into the infected host cell by the pathogenicity islands 1- and 2-encoded type three secretion systems (T3SS-1 and T3SS-2) [3,4].

The SCV is characterized by a high enrichment of lysosomal glycoproteins (LGPs) such as LAMP1 and the presence of membrane tubules that emerge from the SCV and extend towards the cell periphery [5]. The first observed tubules in *Salmonella*-infected cells were named SIFs (*Salmonella*-induced filaments) and are like SCVs highly enriched in LGPs [6]. Later on, the presence of SCAMP3-positive tubules was shown. These tubules are either LAMP1-positive (SIFs) or LAMP1–negative (SISTs, *Salmonella*-induced SCAMP3 tubules) [7]. More recently, we described the existence of LAMP1-negative tubules (LNT) that are essentially devoid of host proteins [8].

Approximately thirty T3SS-2 effectors have been identified and a number of these proteins accumulates on SCVs and tubules after their translocation into the host cell. They are involved in the shaping, constitution, stability and membrane dynamics of the SCVs and tubules. In 1996, a large screening in *Salmonella*-infected HeLa cells identified the effector SifA as essential for the formation of SIFs [9]. A subsequent screening added SseF and SseG to the list of effectors required for the SIF phenotype [10]. However, it was shown that effector labeled tubules with a discontinuous distribution of LAMP1 are produced in absence of SseF/G [11]. These so-called pseudo-SIFs, which could be intermediates of SIF formation, have also been described in absence of SopD2 [12]. Finally PipB2, which directly binds the molecular motor kinesin-1, participates to the elongation of *Salmonella*-induced tubules [8,13].

*Salmonella*-induced tubules are dynamic structures with bidirectional movement along microtubules and sustained interactions with endocytic compartments [14]. However, whether *Salmonella*-induced tubules are functional organelles remains a matter

of debate. Indeed, these tubules are difficult to observe in non-epithelial cells and to our knowledge they have not been observed *in vivo*. These facts have instilled a doubt regarding the physiological role of tubules. Yet, mutant *Salmonella* strains that induce no or altered tubules have decreased virulence in mouse models, while conversely a regain of virulence has been observed in strains in which the formation of tubules is restored [6,8].

In this study, we performed a molecular and functional characterization of LNTs, which are possible precursors of other kinds of *Salmonella*-induced tubules. LNTs are also the only kind of tubules that can be present in cells infected with a strain with deletion of SifA, under condition of a supplementary knockout of the effector SopD2. A Δ*sifAsopD2* strain therefore presents a convenient model to study LNTs and the interactions of *Salmonella*-induced tubules with the late endosomal compartment. We found that the formation of LNTs requires the translocation of the T3SS-2 effectors SseF/G. The disappearance of these structures in absence of SseF or SseG is associated with an impaired enrichment of LGPs on the SCVs and a lower capability of *Salmonella* to replicate inside cells. This study shows that *Salmonella*-induced tubules are functional organelles that are necessary for the establishment of a compartment that supports bacterial replication.

## Results

### SseF/G are required for the formation of LNTs.

To investigate if any single T3SS-2 effector was required for the formation of LNTs, we tested the impact of their individual deletion in a Δ*sifA*Δ*sopD2* strain (hereafter called ΔΔ), chromosomally expressing a 2HA-tagged version of PipB2. Most mutants did not affect the formation of LNTs in HeLa cells (see list of tested effectors in the legend of Figure 1). However, the deletion of *sseF* or *sseG* dramatically decreased the percentage of cells presenting LNTs (Figure 1A & 1B). The formation of these tubular structures was rescued by complementing these mutants with plasmids encoding the respective effector protein (Figure 1A & 1B) but we did not observed a complementation of the Δ*sseF* strain by overexpression of SseG or *vice versa*. A similar SseF/G-dependent phenotype was also observed in bone marrow-derived mouse macrophages (not shown). Other than wild-type or ΔΔ bacteria, which are enclosed in distinct individual vacuole, Δ*sifA*, ΔΔ Δ*sseF* or ΔΔ Δ*sseG* mutants often persist in clustered, instable vacuoles and form balloon-like structures made of several SCVs (see Figure 1A). These results indicate that SseF/G are necessary for the formation of LNTs and that the absence of either effector impacts the shape and the distribution of SCVs.

As *Salmonella* effectors are translocated into the host cell cytosol, ectopic expression of effectors by the host cells can in some cases complement the respective bacterial mutants [15]. Expression of GFP-tagged SseG supported the formation of LNTs in HeLa cells infected with a ΔΔ Δ*sseG* mutant (Figure 1C). However no complementation of the ΔΔ Δ*sseF* or ΔΔ Δ*sseF*/*G* strains was observed by the expression of GFP-SseF and/or GFP-SseG. This suggests that ectopically expressed SseF is not or poorly functional and that SseG is not sufficient to induce the formation of LNTs in absence of SseF. This confirms previous results indicating that SseF and SseG form a functional protein complex [16].

### Functions of SseF/G in a Δ*sifA*Δ*sopD2* strain

In contrast to a Δ*sifA* mutant, a ΔΔ mutant resides in a stable, LAMP1-positive SCV that supports the bacterial replication. We previously suggested that LNTs promote interactions with late endosomal compartments, thus favoring the recruitment of LGPs

onto tubules and their transport towards the SCV [5,8]. To examine this hypothesis we analyzed whether the deletion of *sseF/G* in a ΔΔ strain had consequences on the presence of LGPs on SCVs and on the intracellular replication of *Salmonella*.

We first examined whether deletion of *sseF/G* in a ΔΔ strain had consequences on the presence of LAMP1 on SCVs. HeLa cells were infected with various strains and the presence of LAMP1 SCVs was scored and illustrated by confocal microscopy 16 hours post-invasion (Figures 2A & 2B). We observed that the percentage of LAMP1-positive vacuoles was not significantly different between a Δ*sifA* mutant and ΔΔ strains deleted of either *sseF* or *sseG* and significantly reduced as compared to a ΔΔ mutant strain. This phenotype was complemented with plasmids for the expression of SseF/G in the corresponding strain (Figures 2B). Similar effects were observed in RAW264.7 and bone marrow-derived mouse macrophages (data not shown). These data indicate that SseF and SseG are required for the recruitment of LGPs and confirm the possible role of LNTs in this function.

To investigate the consequences of *sseF/G* deletions on intracellular replication, RAW 264.7 mouse macrophages were infected with wild-type or mutant bacteria and the fold increase of intracellular bacteria between 2 and 16 h after phagocytic uptake was determined (Figure 2C). As expected, a Δ*sifA* mutant presented a strong replication defect while a ΔΔ mutant replicated well, although not at wild-type levels [8,17]. We observed that deletion of *sseF* in a ΔΔ mutant limited bacterial replication to a level with insignificant difference to a Δ*sifA* strain and that this phenotype was reverted by complemention with SseF (Figure 2C). We concluded that the functions mediated by SseF in the formation of LNTs and the recruitment of LAMP1 support the replication of a ΔΔ strain.

## Molecular requirements for the formation and function of LNTs

The results presented above support the idea that LNTs promote interactions with the late endosomal compartment and the recruitment and transport of LAMP1 towards the SCV. However, due to the absence of SifA, this process is limited or incomplete and the LGPs content of SCVs and tubules low. As LNTs are observed in the concomitant absence of SifA and SopD2, and since SifA has two domains, we asked whether the

expression of one or the other domain in a ΔΔ strain would support the recruitment of LAMP1 by *Salmonella*-induced tubules. We constructed strains deleted of *sopD2* and expressing the N-terminal domain of SifA [*sifA(1-136)*] or a full-length point mutated form of SifA in which only the C-terminal domain is functional ($sifA^{L130D}$) [18]. In HeLa cells infected with these strains we observed that the C- but not the N-terminal domain of SifA favors the recruitment of LAMP1 onto *Salmonella*-induced tubules (Figure 3A). Though these tubules are morphologically similar to LNTs (thinner and shorter than SIFs), their high LAMP1 content indicates that the C-terminal domain of SifA is functional and supports the membrane exchange with late endosomal compartments.

The Arf-like G protein Arl8b is localized on lysosomes, where it is linked to kinesin-1 via SKIP and therefore responsible for their movement. It is as well responsible for fusion events of lysosomes with other cellular compartments leading to cargo delivery [19-21]. Arl8B is present on SCVs and on associated tubules in *Salmonella*-infected cells [22]. Therefore, we investigated the presence of this GTPase specifically on LNTs. We observed that over-expressed Arl8b is present on ΔΔ SCVs and on associated tubules (Figure 3B). Over-expression of Arl8b significantly increased the presence of LAMP1 on tubules (Figure 3B and 3C), while this effect was not observed with a dominant negative form of the GTPase ($Arl8b^{T24N}$). The percentage of ΔΔ-infected cells exhibiting LNTs was not significantly different in control and HeLa cells with an shRNA mediated knock-down of Arl8b (Figure 3D). These results indicate that Arl8b contributes to the process of LGP recruitment onto *Salmonella*-induced tubules but is not required for their formation. We also investigated the presence of this GTPase on vacuoles enclosing other *Salmonella* mutant strains. Over-expressed Arl8b was not detected on Δ*sifA*, ΔΔ Δ*sseF* or ΔΔ Δ*sseG* SCVs indicating that Arl8b is not recruited onto the vacuole by default (data not shown). This result suggests that the presence of Arl8b results from the interaction of LNTs with late endosomal compartments on which this GTPase localizes. In support of this hypothesis we found no significant decrease for the presence of LAMP1 on tubules in Arl8B knock-down cells (Figure 3C).

The minus-end microtubule molecular motor dynein has been shown to accumulate on intracellular *Salmonella* colonies in a manner dependent of SseF or SseG [23]. Therefore, to investigate whether dynein is involved in the formation of LNTs, we

constructed a HeLa cell line with a stable knock-down of dynactin $p150^{Glued}$. This protein is encoded by the gene DCTN1 and required for the activity of cytoplasmic dynein in eukaryotes [24]. We observed the presence of PipB2-positive tubules in $p150^{Glued}$ knock-down cells infected with wild-type or ΔΔ strains (Figure 3E). LNTs, though shorter, were still elongating from the SCVs that tended to accumulate at the cell periphery (Figure 4). This result indicates that the SseF/G function required for the formation of LNTs is not mediated by dynein.

## Discussion

*Salmonella*-infected cells are characterized by the appearance of host membrane tubules that develop from the bacterial vacuole and extend along microtubules [5]. The diverse compositions of tubules likely reflect their capacity to interact with different host compartments. Several T3SS-2 effectors are required for their formation but SifA seems to play a central role as no tubules have been observed during infections with a Δ*sifA* strain. Nevertheless, SifA is not absolutely required for tubule formation. An additional knockout of *sopD2* in the ΔsifA strain leads to the reappearance of LNTs during infection [8]. Our study allowed the identification of two important players in the formation and function of LNTs. We found that the effector proteins SseF and SseG are necessary for their formation and that ΔΔ Δ*sseF/G* mutants are very similar to a Δ*sifA* mutant regarding the reduced LAMP1 content of SCVs and the diminished capacity of these mutants to replicate inside the host cell.

In wild-type infected cells, SIFs were shown to associate and fuse with membranes of the endocytic pathway, suggesting that they recruit membrane and associated proteins from these compartments [14]. LNTs seem to play a similar role since their formation comes along with LAMP1 enrichment and increased stability of ΔΔ SCVs as compared to a *sifA* mutant strain [8]. LNTs have also been observed in cells infected with wild-type *Salmonella*, at the onset of T3SS-2-positive tubules formation. Indeed, most tubules seen at 4 h of infection are LNTs [8]. Therefore, we proposed that LNTs are precursors of other *Salmonella*-induced tubules. We also previously observed that LNTs wrap around late endosomal compartments. Thus, these tubules could mediate the recruitment of LGPs by an interaction with host compartments. The fact that ΔΔ SCVs are enriched in LGPs as compared to Δ*sifA* SCVs supports this hypothesis. Since LNTs are essentially LAMP1-negative, while SCVs connected to these tubules tend to get enriched in LAMP1, we believe that tubules induce the capture and then favor the transport of host membrane and associated proteins towards the SCV. The inhibition of LNT formation by deletion of *sseF/G* deeply correlates with the formation of LAMP1-negative or LAMP1-low SCVs that are from this point of view hardly distinguishable from Δ*sifA* SCVs.

These data strongly support our hypothesis regarding the function of LNTs in the recruitment of LGPs and their transport to the SCV. However, it is clear that LNTs are

not fully functional tubules as they are essentially deprived of host proteins [8]. We suggest that LNTs have unproductive encounter with late endosomal compartments due to the fact that they have a limited capacity to exchange membrane with the host compartments. Indeed, we noticed that the LAMP1 content of tubules is dramatically increased for a *sifA*$^{L130D}$Δ*sopD2* strain. This indicates that maturation of tubules through the recruitment of LGPs onto them via an efficient fusion with late endosomal/lysosomal compartments requires the function of the C-terminal domain of SifA. This domain of SifA has a GEF fold and may sustain the active, GTP-bound form of a lysosomal GTPase that is necessary for the fusion between *Salmonella*-induced tubules and host compartments [25].

Several phenotypes have been associated with the expression and translocation of SseF/G but none can be easily linked to the supporting role of these effectors in the formation of LNTs. Both SseF and SseG are membrane associated and SseF has been shown to possess two trans-membrane domains and to behave as an integral membrane protein [26]. Translocated SseF/G are both associated with the SCV membrane and SCV-associated tubules and are involved in the formation of these membranous structures [11]. While mature SIFs are hardly observed in cells infected with Δ*sseF/G* strains, one observes effector positive tubules presenting a discontinuous labeling for LGPs, named pseudo-SIFs [11]. In addition these effectors have been reported to interfere with microtubules, though it remains uncertain whether this association reflects a direct interaction or a more likely presence of these effectors on host vesicles associated with the microtubules cytoskeleton [27]. In line with these results, SseF and SseG were shown to be required for the formation of bacterial micro-colonies in close proximity to the Golgi apparatus of epithelial cells [23,28]. This localization requires the activity of the minus end-directed motor dynein and micro-colonies of *sseF* or *sseG* mutant strains show a marked decrease in their ability to recruit dynein [23].

Little is known about the molecular requirements for the formation of tubules. Previously we showed that it requests an intact microtubules network whereas PipB2 and SKIP, which both directly bind kinesin-1, are dispensable [8]. In this study we observed SIFs and LNTs in p150$^{glued}$ knock-down HeLa cells indicating that their formation does not require functional dynein either, although this interaction has been proposed in the

context of SCV positioning. Therefore, the molecular mechanism by which LNTs elongate on microtubules, which very probably involves the pulling force provided by a molecular motor, remains to be discovered.

The late endosomal membrane fusion machineries are absent on LNTs. For example, the small GTPases Rab7 and Rab9 are both found on late endosomal compartments but have not been detected on LNTs [8]. The small GTPase Arl8B however localizes on lysosomes and is responsible for the recruitment of fusion machinery onto them [21]. We found that over-expressed Arl8b localizes on LNTs and supports the recruitment of LAMP1 to SCVs but we could not detect it on Δ*sifA* or ΔΔ Δ*sseF/G* SCVs. This indicates that the presence of Arl8b on LNTs results from sustained interactions of tubules with lysosomal compartments in the presence of high level of this GTPase rather than from a default recruitment on the SCV. Since Arl8b is present on tubules that form in wild-type *Salmonella*-infected cells and leads to the recruitment of LAMP1 onto LNTs, one can consider that Arl8b recruitment onto SCV and tubules depends on SifA, most probably through interaction with its C-term domain. Further investigation will be required to fully understand the role of SseF/G in the formation of LNTs and whether Arl8b and the C-term domain of SifA have functional interactions that are necessary for the development of fully functional *Salmonella*-induced tubules.

In this study we contribute new information about the complexity of the formation and regulation of the SCV and *Salmonella*-induced tubules. We also show the importance of the interaction of these tubules with late endosomal compartments for the establishment and maintenance of the SCV and propose for the first time an interaction of an effector with a host protein to promote this interaction.

## Figure Legends

**Figure 1**. *SseF and SseG are required for the formations of LNTs.* (**A** and **B**) LNTs do not emerge from SCVs enclosing ΔΔ Δ*sseF* or ΔΔ Δ*sseG* mutant bacteria. HeLa cells were infected for 16 h with various ΔΔ strains expressing GFP, PipB2-2HA and deleted of one of the following gene: *gogB*, *pipB*, *sifB*, *slrP*, *sopD*, *spvB*, *spvC*, *spvD*, *spvR*, *srfJ*, *sseF*, *sseG*, *sseI*, *sseJ*, *ssek1*, *ssek2*, *sseK3*, *sseL sspH1*, *sspH2*, *steA*, *steB*, *steC*, *steD*, *steE*. Deletion of *pipB2* was tested in a strain chromosomally expressing SseJ-2HA. Infected cells were immunostained for HA (PipB2- or SseJ-2HA) as a SCV and LNT membrane marker and imaged for GFP (green) and HA (red) using a confocal microscope. Panel **A** presents cells infected with Δ*sifA*, ΔΔ, ΔΔ Δ*sseF* or ΔΔ Δ*sseG* strains expressing GFP (pFPV25.1) and with ΔΔ Δ*sseF* or ΔΔ Δ*sseG* complemented with plasmids for the expression of SseF or SseG, respectively, and expressing GFP (pGFPLow $Tet^R$). Magnified insets showing single labeling for GFP (left) or HA (right) are presented below each image. Bar, 20 µm or 10 µm for the magnified insets. (**B**) The formation of LNTs in cells infected with a selection of mutant strains presented in panel **A** was scored. (**C**) Ectopic expression of GFP-SseG restores LNT formation in HeLa cells infected with the corresponding mutant. HeLa cells were infected with CFP expressing ΔΔ Δ*sseF* or ΔΔ Δ*sseG* mutant strains and then transfected with a plasmid for the expression of GFP-SseF or -SseG, respectively. Infected and transfected cells were scored for the presence of LNTs 16 h post-infection. (**B** and **C**) Values are means ± SD of three independent experiments. Ordinary one-way ANOVA and Tukey's post-tests were used to determine whether two values were significantly different. *P*-values: ns, not significant; ***, $P<0.001$.

**Figure 2**. *LNTs support the recruitment of LAMP1 onto SCVs and the intra-celullar replication of Salmonella*. (**A** and **B**) SseF/G are necessary for the recruitment of LAMP1 onto the ΔΔ SCVs. HeLa cells were infected with various mutant strains expressing CFP or GFP and PipB2-2HA. 16 h post-infection, cells were fixed and immunostained for HA and LAMP1. (**A**) Cells were imaged for CFP (blue), HA (red) and LAMP1 (green) using a confocal microscope. Magnified insets showing single labeling for CFP (left), LAMP1 (middle) or HA (right) are presented below each image. Bar, 10

µm or 5 µm for the magnified insets. (**B**) Infected cells were scored for the presence of LAMP1 on PipB2-positive tubules. (**C**) SseF supports the intracellular replication of a ΔΔ strain. RAW264.7 mouse macrophages were infected with wild-type *Salmonella* or different mutant strains and lysed at 2 or 16 h post-infection for the enumeration of intracellular bacteria. The values shown represent the fold increase calculated as a ratio of the intracellular bacteria between 16 and 2 h and normalized to that of the wild-type strain. (**B** and **C**) Values are means ± SD of three independent experiments. (**B** and **C**) Values are means ± SD of three independent experiments. Ordinary One-way ANOVA and Tukey's post-tests were used to determine whether two values were significantly different. *P*-values: ns, not significant; ***, $P<0.001$.

**Figure 3**. *Molecular characterization of LNTs*. (**A**) The C-term domain of SifA supports the recruitment of LAMP1 onto *Salmonella*-induced tubules. HeLa cells were infected with the following *Salmonella* strains: wild-type, ΔΔ with or without expression of SifA from a plasmid and Δ*sopD2* chromosomally expressing the N-terminal domain of SifA [SifA(1-136)] or a point mutated form of SifA in which only the C-term domain is functional ($SifA^{L130D}$) . These strains also expressed GFP and chromosomal PipB2-2HA. Cells were fixed 16 h post-infection, immunostained for HA and LAMP1. Infected cells were scored for the presence of LAMP1 on PipB2-positive tubules. (**B**) Arl8b is recruited on *Salmonella*-induced tubules. HeLa cells were infected with a ΔΔ mutant strain expressing PipB2-2HA and further transfected with plasmids for the expression of GFP (left panel) or Arl8b-GFP (right panel). 16 h post-infection, cells were fixed, immunostained for HA and LAMP1 and imaged for GFP (Top row), HA (red) and LAMP1 (green) using a confocal microscope. *Salmonella*-induced tubules are essentially LAMP1-negative (arrows in cells expressing GFP) while Arl8b-positive tubules present a discontinuous LAMP1 labeling (arrowheads). Magnified insets showing single labeling for LAMP1 (left) or HA (right) are presented below each image. Bar, 10 µm or 5 µm for the magnified insets. (**C**). Arl8b supports the recruitment of LAMP1 onto *Salmonella*-induced tubules. HeLa cells were infected with wild-type or ΔΔ strains of *Salmonella* expressing mTFP and PipB2-2HA and further transfected or not (N.T.) with plasmids for the expression of GFP, Arl8b-GFP or Arl8bT24N-GFP. HeLa cells with a stable knock-

down of Arl8b (Arl8bKD) were infected with a ΔΔ strain. Cells were fixed 16 h post-infection and immunostained for HA and LAMP1. Infected and transfected cells were scored for the presence of LAMP1 on PipB2-positive tubules. (**D**) Inhibition of Arl8b expression does not significantly impair the formation of LNTs. HeLa cells stably expressing shRNAs against GFP (control) or Arl8b were infected with a ΔΔ strain expressing CFP and PipB2-2HA and scored for the formation of LNTs 16 h post-infection. (**E**) Dynein activity is not required for the formation of *Salmonella*-induced tubules. Control or $p150^{Glued}$ knock-down HeLa cells were infected with wild-type or ΔΔ strains expressing CFP and PipB2-2HA and scored for the formation of *Salmonella*-induced tubules 16 h post-infection. (**A**, **C** and **D**) Values are means ± SD of three independent experiments. Unpaired two-tailed t test (**D**) or ordinary one-way ANOVA and Tukey's post-test (**A**, **C**) were used to determine whether two values were significantly different. *P*-values: ns, not significant; **, $P<0.01$; ***, $P<0.001$.

## Materials & Methods

### Statistical Analyses

Statistical analyses were performed with Prism 6 software (GraphPad).

### Ethic statement

Animal experimentation was conducted in strict accordance with good animal practice as defined by the French animal welfare bodies (Law 87–848 dated 19 October 1987 modified by Decree 2001-464 and Decree 2001-131 relative to European Convention, EEC Directive 86/609). All animal work was approved by the Direction Départementale des Services Vétérinaires des Bouches du Rhône (authorization number 13.118 to S.M.).

### Bacterial strains and growth conditions.

Bacterial strains used in this study are listed in Table 1. Strains were cultured in LB broth (Difco), LB broth plus 5 g/L NaCl or minimal medium (M9, glycerol 0.2%, $MgSO_4$ 1 mM, $CaCl_2$ 200 mM, thiamine 1 mg/ml, casamino acids 1 mg/ml. Ampicillin (50 µg/ml), kanamycin (50 µg/ml), tetracycline (10 µg/ml) and chloramphenicol (50 µg/ml) were added when required.

### Construction of plasmids

Plasmids pGG2-CFP and pGG2-mTFP were constructed by using the In-Fusion® HD cloning system. CFP and mTFP fragments were amplified by using oligos O-701 (AAGGAGATATACATATGGTGAGCAAGGGCGAGGAG) and O-702 (CTACCGCATTAAGCTTTTACTTGTACAGCTCGTCCATGCC). The pGG2 backbone was digested with NdeI and HindIII. The ligation reaction was performed according to the manufacturers protocol.

### Construction of mutant strains

Strains carrying several mutations were created by transduction using the phage P22 HT105 int.

### Eukaryotic cells and culture conditions

RAW 264.7, HeLa and primary bone marrow-derived macrophages were grown in DMEM (GibcoBRL) supplemented with 10% foetal calf serum (FCS; GibcoBRL), 2 mM nonessential amino acids, and glutamine (GibcoBRL) at 37°C in 5% CO2.

### Lentiviral shRNA mediated silencing of Arl8b

HeLa or RAW 264.7 cells were plated in 6-well plates. 24 hours later the growing medium was changed for the viral supernatant of 293T cells previously transfected with a Mission shRNA (Sigma-Aldrich) and Δ8.9, VSVG, TAT plasmids for the production of lentiviral transduction particles and to which polybrene (8 µg/ml) was added. Puromycin (5 µg/ml) was added after 24 h to select transductants. shRNAs target sequences were AGGTAACGTCACAATAAAGAT (human Arl8b, TRCN0000291597), TGCTATTCAGGATAGAGAAAT (mouse Arl8b, TRCN0000296944), GCCCATCTACAGGATGTGAAT (human DCTN1/P150$^{Glued}$, TRCN0000299621). Efficiency of the knock-down was proven by Western blotting using polyclonal rabbit anti-Arl8B (Abcam, ab105792).

### Bacterial infection and replication assays

Bone marrow-derived macrophages, HeLa and RAW 264.7 macrophage were grown, infected and treated as previously described [8].

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## Figures

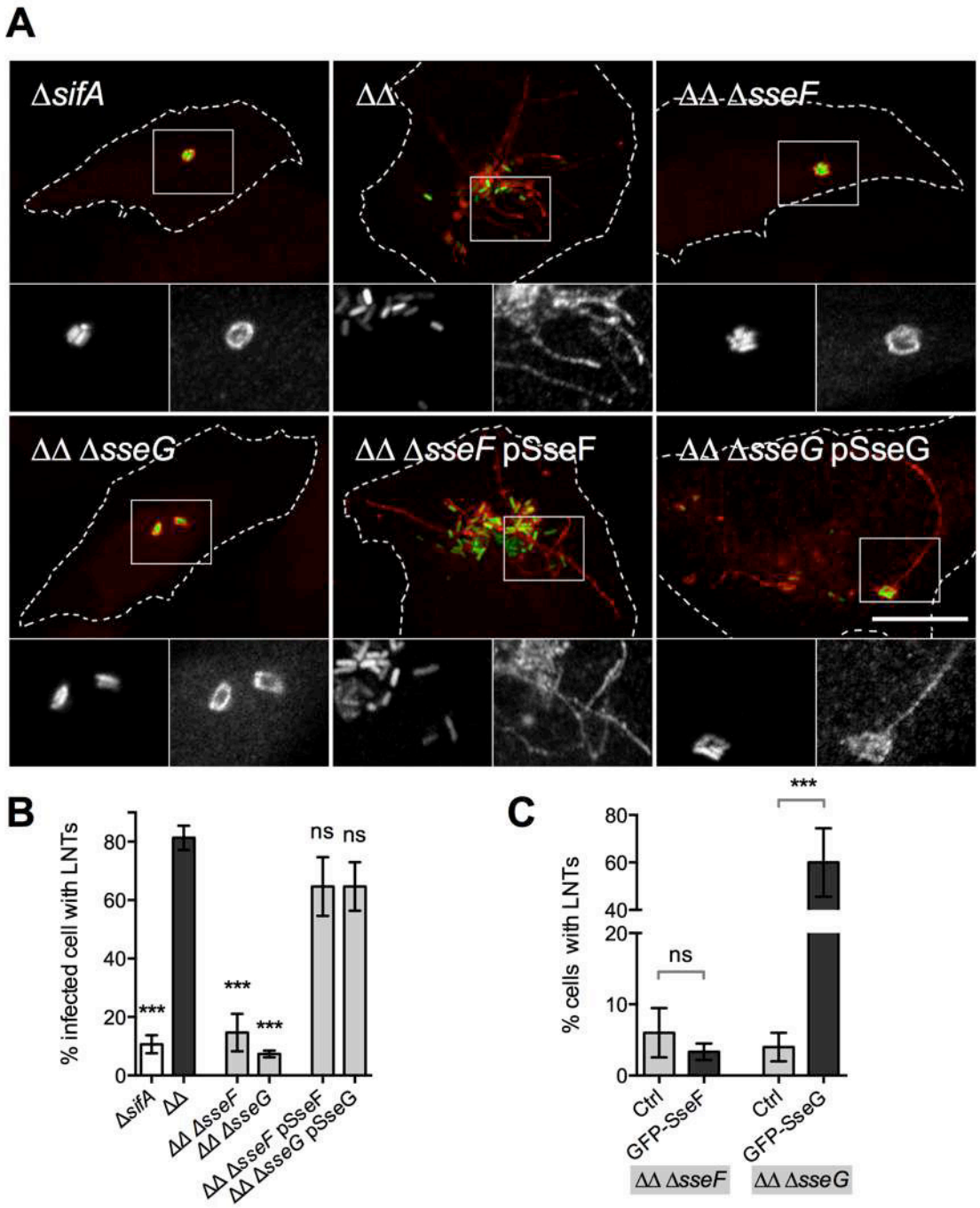


Figure 1

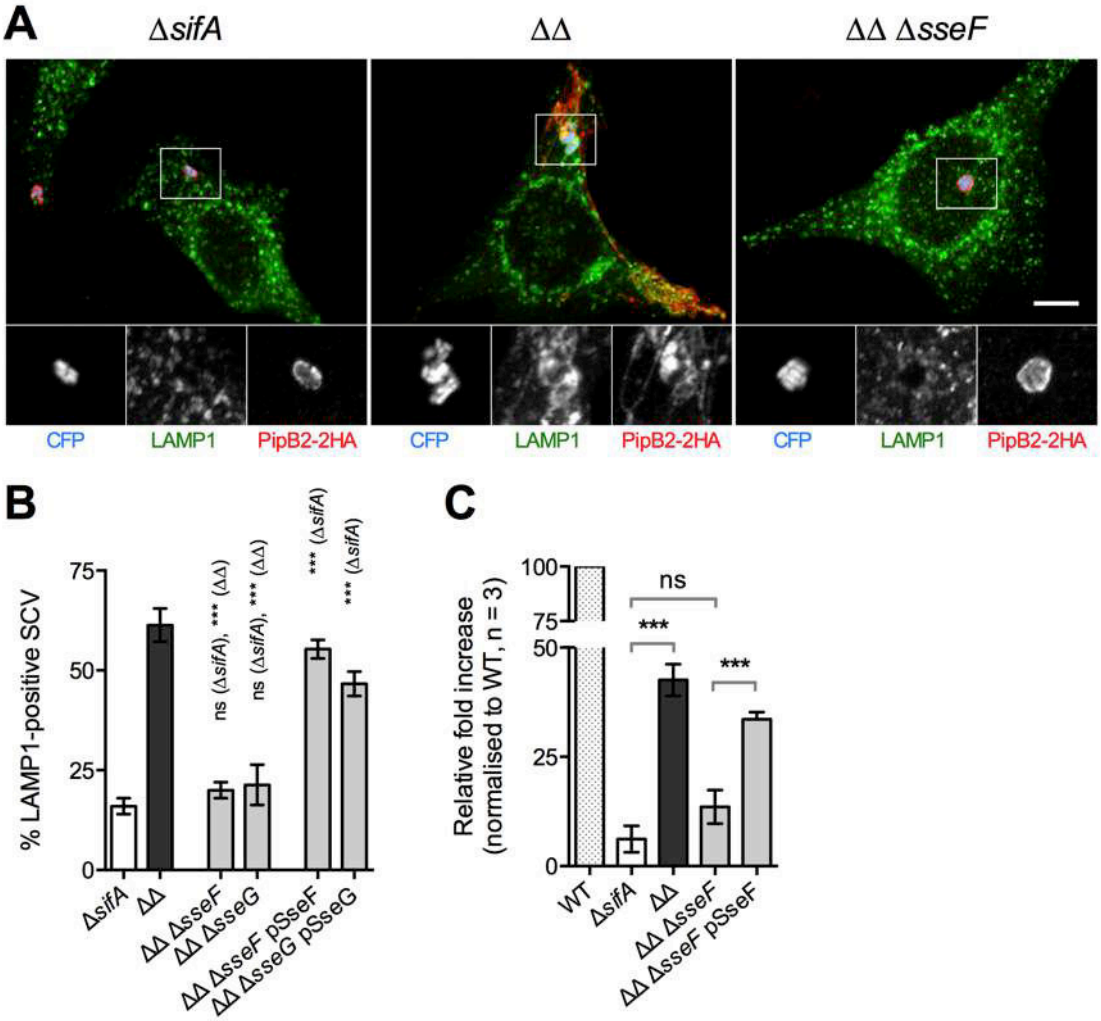
A
ΔsifA
ΔΔ
ΔΔ ΔsseF
CFP
LAMP1
PipB2-2HA
B
% LAMP1-positive SCV
75
50
25
0
ns (ΔsifA), *** (ΔΔ)
ns (ΔsifA), *** (ΔΔ)
*** (ΔsifA)
*** (ΔsifA)
ΔsifA
ΔΔ
ΔΔ ΔsseF
ΔΔ ΔsseG
ΔΔ ΔsseF pSseF
ΔΔ ΔsseG pSseG
C
Relative fold increase
(normalised to WT, n = 3)
100
75
50
25
0
ns
***
***
WT
ΔsifA
ΔΔ
ΔΔ ΔsseF
ΔΔ ΔsseF pSseF

Figure 2

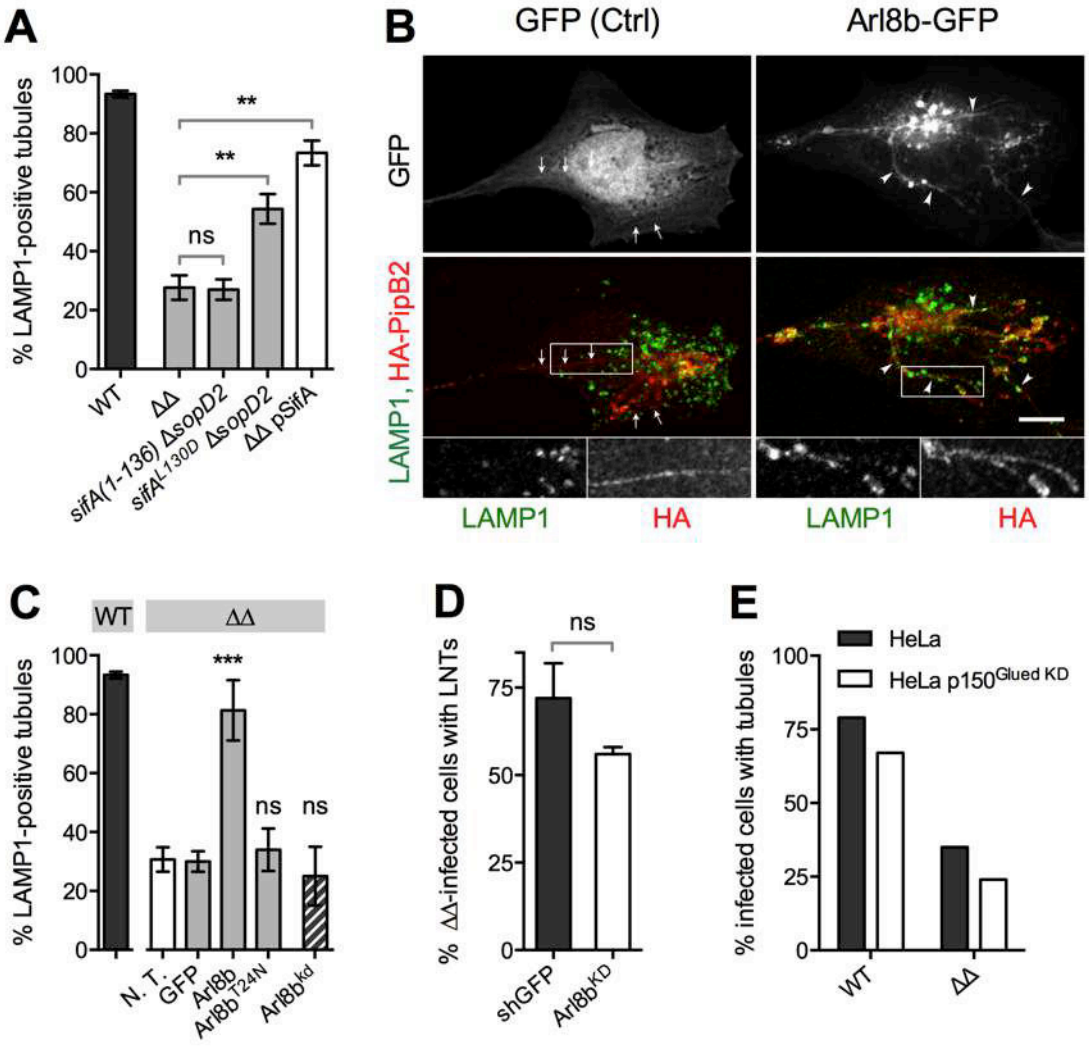
A
% LAMP1-positive tubules
100
80
60
40
20
0
**
**
ns
WT
ΔΔ
sifA(1-136) ΔsopD2
sifAL130D ΔsopD2
ΔΔ pSifA
B
GFP (Ctrl)
Arl8b-GFP
GFP
LAMP1, HA-PipB2
LAMP1
HA
LAMP1
HA
C
WT
ΔΔ
% LAMP1-positive tubules
100
80
60
40
20
0
***
ns
ns
N. T.
GFP
Arl8b
Arl8bT24N
Arl8bkd
D
% ΔΔ-infected cells with LNTs
75
50
25
0
ns
shGFP
Arl8bKD
E
% infected cells with tubules
100
75
50
25
0
HeLa
HeLa p150Glued KD
WT
ΔΔ

Figure 3

***Table 1***. *Salmonella* strains and plasmids

| Name | Description | Reference |
|---|---|---|
| Strains | | |
| 12023 | Wild-type S. Typhimurium (*Salmonella* enterica subsp. enterica serovar Typhimurium) strain 12023 | Laboratory stock |
| AAG020 | 12023 pipB2-2HAchr::FRT | [8] |
| AAG022sc4 | 12023 *pipB2-2HAchr*::FRT, Δ*sifA*::FRT | [8] |
| AAG025scs | 12023 *pipB2-2HAchr*::FRT, Δ*sifA*::FRT, Δ*sopD2*::FRT | [8] |
| TM014 | 12023 *pipB2-2HAchr*, Δ*sifA*::FRT, Δs*opD2*::FRT, Δ*sseF*::km$^R$ | This study |
| TM048 | 12023 *pipB2-2HAchr*::FRT, Δ*sifA*::FRT, Δ*sopD2*::FRT, Δ*sseF*::km$^R$, pSseF-M45 | This study |
| TM003 | 12023 *pipB2-2HAchr*::FRT, Δ*sifA*::FRT, Δ*sopD2*::FRT, Δ*sseG*::km$^R$ | This study |
| TM049 | 12023 *pipB2-2HAchr*::FRT, Δ*sifA*::FRT, Δ*sopD2*::FRT, Δ*sseG*::km$^R$, pSseG-M45 | This study |
| Plasmids | | |
| pFPV25.1 (V208) | pFPV25 derivative for expression of GFP under control of the *rpsM* promoter | [29] |
| pGG2 (V271) | pFPV25 derivative for expression of DsRed under control of the *rpsM* promoter | [30] |
| pGG2-CFP (C0974) | pFPV25 derivative for expression of CFP under control of the *rpsM* promoter | This study |
| pGG2-mTFP (C0975) | pFPV25 derivative for expression of TFP under control of the *rpsM* promoter | This study |
| pArl8B-GFP (C0984) | For ectopic expression of Arl8B-GFP | [19] |
| pGPFLow Tet$^R$ (V243) | For low expression of GFP in *Salmonella* | Laboratory stock |
| psSseF-M45 (C0977) | pWSK29 derivative for expression of SseF-M45 under control of the *sseA* promoter | [31] |
| pSseG-M45 (C0979) | pWSK29 derivative for expression of SseG-M45 under control of the *sseA* promoter | [31] |

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**Paper introduction: SifA and its dual function in *Salmonella* pathogenesis**

The survival and replication of *Salmonella* inside the host cell depends on its niche, the SCV. Once the SCV is established at its final, juxtanuclear position close to the Golgi apparatus, the formation of filamentous structures, the SIFs, starts [93]. Their formation depends on the action of the T3SS-2 effector SifA [94]. A Δ*sifA* strain can't induce the formation of tubules and loses the integrity of the SCV. This leads to the release of bacteria to the host cytosol, together with a stop of replication in macrophages [85,104]. The role of SifA in the formation of SIFs is linked to its interaction with the host protein SKIP, which binds Kinesin-1 and is therefore responsible for the centrifugal movement of the complex [79,97].

SifA is a protein with a bi-modular structure and it is its N-terminal domain that is responsible for the interaction with SKIP (Fig. 3.6). Mutation of a single amino acid residue of SifA at the interface with SKIP ($Leu^{130}$) is sufficient to abolish the interaction [98].

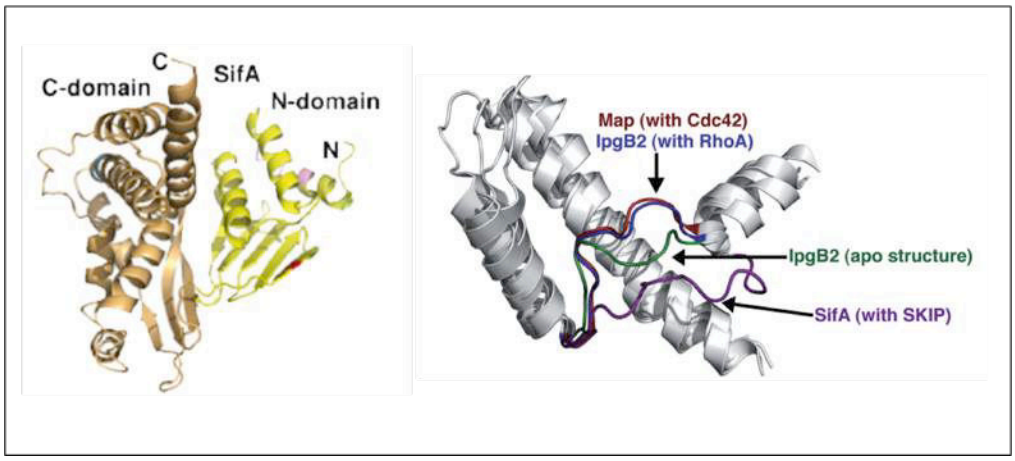


Fig 3.5: Bi-modular structure of the SifA and overlay of the C-terminal part with other bacterial GEFs. Adapted from [98,110].

SifA's C-terminal part however has a structure that is similar to bacterial guanine exchange factors (GEFs), suggesting that it does act as a GEF (Fig. 3.6) [106]. SifA can interact with GDP-bound RhoA in pull-down experiments. If it was really a GEF for RhoA, this could present a biochemical link between the functions of SifA and SseJ and their interplay in membrane dynamics, because SseJ gets activated by active GTP-bound RhoA [105]. However, structural modeling does not favor a productive interaction of

SifA with RhoA and the proposed GEF activity of SifA on RhoA could not be proven biochemically. The putative host target of the C-terminal GEF function remains a subject of interest [111,112].

In modular proteins, which are often toxins, subunits are prone to influence each other. One subunit could either prevent promiscuous activity of the other one, allow a more precise regulation of the proteins function or even stand for a synergistic effect [56,113]. Considering these possible interdependences and control mechanisms within bi-modular proteins, it can be suggested that the target of SifA's C-terminal part will be discovered in the spacio-functional vicinity of SifA's functions, such as the modulation of membrane dynamics [114].

Arl8 is a small GTPase that is located on lysosomes and exists in two isoforms that share 91% sequence homology [115]. Whereas Arl8A is restricted to certain tissues, Arl8B is abundantly expressed in every cell type [116,117]. Arl8B prevails in its GTP-bound active form and controls several aspects of the lysosomal function [115]. First, it binds SKIP thereby being responsible for the movement of lysosomes towards the cell periphery [118]. Overexpression of Arl8B increases the motility of lysosomes and leads to their redistribution towards the cell periphery [115]. Secondly, Arl8B is responsible for the recruitment of the homotypic fusion and vacuole protein sorting (HOPS) complex onto lysosomes via binding its VPS41 subunit (Fig. 3.7) [119]. The HOPS complex is a multi-protein complex that leads to tethering of late endosomes and lysosomes and their subsequent fusion, therefore facilitating the last fusion step of a functional endocytic / phagocytic pathway.

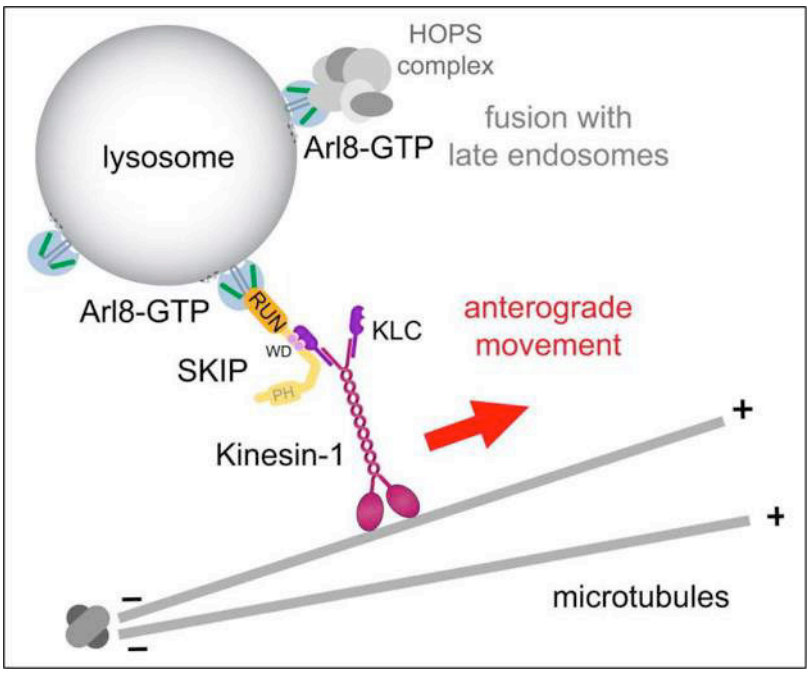


Fig. 3.6: The two functions of Arl8B. It is responsible for the anterograde movement of lysosomes in a SKIP and Kinesin-1 dependent manner and for to fusion of lysosomes with late endosomes by inducing the recruitment of the HOPS complex onto lysosomes. Copied from [118].

During infection with *Salmonella*, over-expressed Arl8B was shown to be recruited to *Salmonella*'s tubular network, on both LAMP-1$^+$ and LAMP-1$^-$ tubules [120]. It was also proposed that Arl8B has an impact on the development of tubules, which was assessed by scoring LAMP-1$^+$ tubules [120]. However, LAMP-1 recruitment does not represent the formation of tubules – for instance LNTs are negative for LAMP-1 per definition – but rather their interaction or fusion with the LE/lys compartment. On the one hand, SIFs – staining positive for LAMP-1 – are able of such a fusion with late endosomes / lysosomes (LE/lys) [46]. On the other hand, the contact of LNTs with LE/lys is less intense and fusion might occur only rarely. Expression of SifA in a Δ*sifA* Δ*sopD2* strain, which *per se* only induces LNTs, leads to the reappearance of LAMP-1$^+$ tubules, the SIFs [96]. One could conclude that SifA therefore has a role in the fusion of tubules with LE/lys and that this role is mediated by an interaction of its C-terminal domain with Arl8B.

As described, the interaction of SifA with SKIP has been thoroughly confirmed *in vitro*, but no data support the importance of this interaction *in vivo*. Also, a functional link between SifA and SseJ via a GEF activity of SifA's C-terminus could not be proven. The SKIP$^{-/-}$ mouse model allows the investigation of these points. By infecting wild-type and SKIP$^{-/-}$ mice with wild-type *Salmonella*, we were able to show that SKIP$^{-/-}$ mice survive longer upon a challenge with S*almonella*. This indicates that the SifA-SKIP axis is important for the virulence of *Salmonella*. Besides, a Δ*sifA* mutant still has attenuated virulence compared to wild-type bacteria in the SKIP$^{-/-}$ mouse. Therefore we conclude that the C-terminal part of SifA does have a distinct, virulence relevant function. However, its function is not linked to the activity of SseJ. A Δ*sifA* Δ*sseJ* strain is less virulent than a Δ*sifA* or a Δ*sseJ* strain; therefore it is unlikely that these two effectors share a common pathway within the host cell. This result encouraged us to search for another interaction partner of SifA's C-terminal domain.

Arl8B seemed a promising target. It binds to SKIP in uninfected cells, gets recruited on the tubular network upon infection and recruits LAMP-1 to LNTs. It is therefore in the spacio-functional vicinity of SifA. We showed that either a mutant form of SifA, which has only a functional C-terminus, or Arl8B are able to increase the recruitment of LAMP-1 onto the SCV an associated tubules and give biochemical proof for the interaction of the C-terminal part of SifA with Arl8B. We propose that this interaction is responsible for the fusion of Sifs with the LE/lys compartment.

# The *Salmonella* effector protein SifA plays a dual role in virulence

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Running title:

Keywords: *Salmonella*, Type three secretion system, SifA, Plekhm2, SKIP, virulence

## Abstract

The virulence of the intracellular pathogen Salmonella Typhimurium relies on the expression of bacterial effector proteins that are translocated into infected host cells by type three secretion systems. Upon entry in eukaryotic host cells, this bacterium resides in a membrane-bound compartment named the Salmonella- containing vacuole (SCV). The Salmonella effector protein SifA is secreted and translocated into host-cells where it localizes onto the SCV and SCV-associated membrane tubules. SifA is made of two distinct domains. The SifA N-term domain interacts with the host protein SKIP. This interaction is required for the stability of the SCV membrane and the removal of the molecular motor kinesin-1 from the vacuole. The SifA C-term has a fold that is similar to other bacterial effector proteins having a guanine nucleotide exchange factor (GEF) activity. Indeed, SifA preferentially binds a GDP-bound form of the RhoA GTPase but does not stimulate GDP dissociation. Therefore it remains unknown whether the SifA C-term contributes to the function of SifA in Salmonella virulence and, if it does, whether it has a GEF activity. We used a model of SKIP knockout mice to show that SKIP mediates susceptibility to Salmonellosis and to establish that SifA contributes to Salmonella virulence even independently of its interaction with SKIP. The SifA C-term domain supports this contribution and binds the Arl-like GTPase Arl8b. Finally this study shows that both the SifA C-term and Arl8b support the recruitment of lysosomal glycoproteins onto Salmonella-induced tubules, suggesting that SifA might favor the GDP to GTP exchange and sustain the activity of Arl8b.

## Introduction

The Gram-negative bacterium *Salmonella* is an intracellular pathogen whose virulence relies on the capacity to survive and replicate inside cells of the infected host. The intracellular life requires the expression of a type three secretion system-2 (T3SS-2), which is expressed by the intracellular bacterium in response to the vacuolar environment [1-3]. The T3SS-2 mediates the translocation of a set of bacterial effector proteins, which collectively support the intra-vacuolar replication, across the vacuolar membrane (for review see [4,5]).

The T3SS-2 effector protein SifA [6,7] plays a significant role in *Salmonella* virulence and several phenotypes are linked to the translocation of SifA. SifA is required to maintain the integrity of the *Salmonella*-containing vacuole (SCV) [8,9] and in epithelial cells promotes the formation of tubular membranous structures connected to SCVs, which have been named *Salmonella*-induced filaments (Sifs) [10-12]. In absence of SifA, the molecular motor kinesin-1, which is directly recruited by the T3SS-2 effector PipB2 [13,14], accumulates on the SCV. This accumulation on the SCV is also visible for PipB2 and other membrane bound T3SS-2 effectors such as SifA or SseJ. We have suggested that these accumulations result from a slow formation of SCV-derived vesicles [2,15].
Several functional peptide stretches and domains of SifA have been identified. The N-terminal residues direct the T3SS-2 mediated secretion/translocation of this effector [2,4,16]. The last C-terminal residues form a CAAX motif, which is found in eukaryotic Rab GTPases but also in many bacterial effectors [17,18]. Following translocation, the CAAX motif of SifA is isoprenylated and S-acylated by the eukaryotic enzymatic machinery [10,12,19], which allows the membrane anchoring of the effector [20,21]. The resolution of the crystal structure of SifA showed the presence of two distinct domains [10,12,22] that are separated by a potential caspase-3 cleavage site [23,24]. Thus, the two domains of SifA might act independently of each other upon cleavage. However, the functionality of the proteolytic cleavage has not been demonstrated.

The SifA N-term domain (residues 1 to 136) interacts with the eukaryotic protein SKIP [2,4,13]. SKIP interacts with kinesin-1 [1-3] and this interaction is thought to activate this anterograde molecular motor [4,5]. SKIP interacts also with the GTP- bound form of the lysosomal Arf-like G protein Arl8b [6,7]. Thereby Arl8b/SKIP provides a

link between the microtubule-engaged kinesin-1 and lysosomal membranes. In the context of infected cell, Arl8b is present on SCV and on SCV- associated tubules [8,9].
The SifA C-term domain has a fold similar to the *Salmonella* effector SopE, which is a guanine nucleotide exchange factor (GEF). SifA interacts with the GDP bound form of the RhoA GTPase [10-12] but does not stimulate the nucleotide exchange for this GTPase [13,14]. Thus, the contribution of the SifA C-term to the virulence via RhoA remains undefined.

We took advantage of a SKIP knockout mouse model in combination with *Salmonella* strains expressing various point mutated or truncated version of SifA to assess the involvement of the SifA C-term domain in *Salmonella* virulence. The present study establishes that SKIP mediates the function of SifA in virulence in the mouse model but also that the C-term domain of SifA has a SKIP-independent function in virulence. It shows that the SifA C-term specifically binds Arl8b. Both Arl8b and the SifA C-term support the recruitment of lysosomal glycoprotein onto the SCV through the *Salmonella*-induced tubules. Altogether these results indicate that SifA might support the nucleotide exchange and sustain activity of Arl8b.

## Results

### *Characterization of SKIP$^{-/-}$ mice.*

We used an anti-SKIP antibody [2,15] to confirm the absence of the protein in *SKIP*$^{-/-}$ mice. By Western blotting, the antibody recognized in HeLa cells a protein with an apparent molecular mass of ≈ 150 kDa. This protein was not detected in peritoneal macrophages prepared from *SKIP*$^{-/-}$ mice while it was present in C57BL/6 macrophages (Figure 1A).

*SKIP*$^{-/-}$ mice are healthy, fertile and appear to behave normally. *SKIP*$^{-/-}$ and C57BL/6 hematology profiles obtained from cohorts of sex- and age-matched mice were indistinguishable (data not shown). At the subcellular level we found identical distributions of early and late endosomal compartments and of the Golgi apparatus in bone marrow macrophages or embryonic fibroblastic cells derived from these mouse strains (data not shown).

The consequences of the lack of SKIP expression have been previously described in cultured cells using a siRNA-mediated knock-down [2,4,16]. In *Salmonella* infected HeLa cells this results, among other phenotypes, in the accumulation of kinesin-1 and T3SS-2 effectors on SCVs. We checked these phenotypes using mouse embryonic fibroblasts (MEF) prepared from C57BL/6 and *SKIP*$^{-/-}$ mice. MEFs were infected with a wild-type or Δ*sifA* strain of *S.* Typhimurium and immunostained. A microscopic analysis showed that kinesin-1 accumulated both on wild-type and Δ*sifA* SCVs in the absence of SKIP (Figure 1B). In C57BL/6 MEFs, we detected 32 ± 6 % Δ*sifA* SCVs decorated by the anti-kinesin antibody as compared to 10 ± 2 % for wild-type SCVs. By contrast, the percentages of kinesin-positive SCVs were superior to 30 % and not significantly different in *SKIP*$^{-/-}$ MEFs infected by one or the other strain (Figure 1C). Using strains that express chromosomally tagged T3SS-2 effectors (PipB2-2HA or SseJ-2HA), we also observed the accumulation of effectors on SCVs in the absence of SKIP (not shown). We concluded that, as far as the accumulation of kinesin-1 and effectors are concerned, the consequences of the absence of SKIP are similar in *SKIP*$^{-/-}$-derived cells and in HeLa cells.

### *SKIP$^{-/-}$ mice are less sensitive to Salmonella infection than congenic C57BL/6.*

Next, we investigated whether SKIP mediates susceptibility to Salmonellosis. We perorally (P.O.) inoculated C57BL/6 or *SKIP*$^{-/-}$ mice with *S*. Typhimurium and monitored their survival. *SKIP*$^{-/-}$ mice succumbed to wild-type *Salmonella* infection significantly later than C57BL/6 mice. C57BL/6 and *SKIP*$^{-/-}$ mice had all succumbed by day 8 and 10, and had a median survival time of 7.5 and 9 days, respectively (Figure 2A). We obtained very similar results for wild-type and Δ*sifA* strains of *Salmonella* in C57BL/6 mice (Figure 2B), with a median survival time of 7 and 9 days, respectively. These data indicate that the absence of SifA or SKIP results in longer survival of mice after a challenge with *Salmonella*.

We examined how the lack of SKIP could decrease the susceptibility of mice to a *Salmonella* challenge. At day five post-inoculation, we found lower bacterial loads in organs of *SKIP*$^{-/-}$ as compared to C57BL/6 mice (Figure 2C). The ratio of bacterial burden between C57BL/6 and *SKIP*$^{-/-}$ mice was 23, 32 and 25 in the spleen, the liver and mesenteric lymph nodes, respectively. Although statistical analysis revealed no significance, it suggests that the absence of SKIP limits *Salmonella* replication. We thought to verify this point using bone marrow-derived macrophages prepared from the two mouse strains. Cells were infected with a wild-type or Δ*sifA* strain and the fold increase of intracellular bacteria between 2 and 16 h after infection was determined. As expected, we observed a dramatic replication defect for the Δ*sifA* mutant with respect to the wild-type strain in C57BL/6 macrophages (Figure 2D). In contrast there was not significant difference for the replication of these bacterial strains in *SKIP*$^{-/-}$ macrophages and the replication of the wild-type strain was significantly reduced in absence of SKIP. We concluded that SKIP is an important mediator of the role that SifA plays in the intracellular replication of *Salmonella*. All together, our data indicate that SKIP, by interacting with SifA, mediates susceptibility to Salmonellosis.

### *Characterization of a SKIP-independent function of SifA.*

Having shown that SKIP is an important mediator of SifA's function, we determined if the role of SifA in virulence is exclusively mediated by its interaction with SKIP. For

this purpose, we analyzed the virulence attenuation of a Δ*sifA* strain in C57BL/6 and *SKIP*$^{-/-}$ mice (Figure 3A & 3D). Groups of mice were inoculated intraperitoneally (I.P.) or P.O. with different two strain combinations (1:1 mix) and bacteria were recovered from mouse spleens after two (I.P.) or five (P.O.) days to determine the competitive index (CI) [17,18]. We found that a Δ*sifA* mutant is still significantly attenuated as compared to wild-type *Salmonella* in *SKIP*$^{-/-}$ mice inoculated I.P. or P.O. (CI of 0.83±0.14 and 0.23 ± 0.11, respectively) (Figure 4A). As control, we tested the virulence attenuation of a Δ*sseG* mutant. The Δ*sseG* mutant presented an attenuation of virulence, which did not differ significantly between mice expressing or not SKIP (Figure 4B). Taken together, these data indicate that SifA mediates a SKIP- independent function in virulence.

To confirm this, we performed a mixed inoculation of wild-type *Salmonella* and a strain chromosomally expressing SifA$^{L130D}$, a point mutant form of SifA that does not interact with SKIP [10,12,19]. This mix was used to estimate the contribution of SKIP to the virulence mediated by SifA (Figure 3B). The CIs in C57BL/6 mice inoculated I.P. or P.O. were both ≈ 0.5 (Figure 4C). In *SKIP*$^{-/-}$ mice (Figure 3G) we obtained CI values that were almost equal to or not significantly different from 1 (Figure 4C), therefore confirming that the CI values obtained in C57BL/6 mice strictly reflect the contribution of the SKIP-SifA interaction to *Salmonella*'s virulence.

Finally, we infected C57BL/6 mice with a mix of *sifA*$^{L130D}$ and Δ*sifA* strains (Figure 3E). We observed a CI of 0.62 ± 0.22 (Figure 4D). This value is slightly higher than the one observed for the CI of wild-type versus Δ*sifA* in *SKIP*$^{-/-}$ mice (Figure 3D and Figure 4A) even though these two mixed infections are both expected to reveal the SKIP-independent role of SifA in virulence. Yet, this result confirms that SifA plays a SKIP-independent function in virulence.

### *The SKIP-independent function of SifA is associated with the C-term domain.*

To determine which of the N- or C-term domain of SifA is responsible for the SKIP-independent function during infection, we constructed *Salmonella* strains that chromosomally express either SifA deleted of the C-term domain [*sifA(1-136)*], SifA containing an internal double haemagglutinin tag at the boundary between the N- an C-

term domains (*sifA-2HA*) [20,21] or a HA tagged form deleted of the C-term domain [*sifA(1-136)-2HA*]. SifA(1-136)-2HA was used in an *in vitro* assay to verify that the removal of the C-terminal domain of SifA did not impair the secretion (Figure S1). Then, these strains were tested in mixed infections. We compared the virulence of *sifA(1-136)* and Δ*sifA* mutant strains (Figure 3F & 4E) and obtained a CI of 0.55 ± 0.36. This value is similar to the one found for the SKIP-dependent contribution of SifA to virulence (Figure 3B & 4C). We also carried this mixed infection out in *SKIP*$^{-/-}$ mice (Figure 3H). We obtained a CI not significantly different from 1 (1.03 ± 0.42, Figure 4E) indicating that the SifA N-term is not functional in absence of SKIP and that consequently the function of this domain is exclusively mediated by SKIP. Therefore, we concluded that the SifA C-term is solely mediating the SKIP- independent virulence effect of SifA.

*SifA and SseJ contribute independently to Salmonella virulence.*

The T3SS-2 effector SseJ has a lipase activity that increases the esterification of cholesterol in host cell membranes [10,12,22]. SseJ is activated by binding the GTP-bound form of the eukaryotic RhoA GTPase [23,24] while SifA preferentially binds GDP-bound RhoA. Therefore, though a GEF activity of SifA toward RhoA could not been demonstrated [2,4,13], it has been proposed that SifA participates to the activation of SseJ by favoring the recruitment of RhoA to the SCV membrane and possibly activating it [25]. Thus, we tested whether the SKIP-independent role of SifA in virulence could be linked to this proposed activity. We compared the virulence attenuation of strains deleted of *sseJ*, *sifA* or both genes in I.P inoculated mice. We found that a Δ*sifA*Δ*sseJ* strain is far more attenuated that each individual mutant as compared to wild-type *Salmonella* (Figure 4G). This indicates that the two genes are unlikely to share a signaling pathway. To confirm this point, we defined the CI of Δ*sifA* versus Δ*sifA*Δ*sseJ* and obtained a value (0.54 ± 0.01) that was not significantly different from the CI value of wild-type versus Δ*sseJ* (0.47 ± 0.16, Figure 4G). It shows that *sifA* does not influence the contribution of *sseJ* to virulence. We concluded that the two genes contribute independently to *Salmonella*'s virulence and that consequently the SKIP-independent function of SifA in virulence is unrelated to the RhoA-SseJ signaling pathway.

### *The SifA C-term is important for the recruitment of LAMP1 onto tubules/vacuoles*

Next we investigated the role of the C-term domain in the function of SifA. Compared to wild-type SCV membranes, the ones of a Δ*sifA* strain are characterized by a very low level of lysosomal glycoproteins (LGPs) [9]. Thus, we tested if the SifA C-term could be important for the recruitment of LGPs onto membrane tubules extending from the SCV. To facilitate this investigation, we used a Δ*sifA*ΔsopD2 strain that is able to form T3SS-2 effector-positive tubules, which are essentially negative for LAMP1 as they undertake only non-productive interactions with late endosomal compartments [26]. Figure 5A shows that, as compared to wild-type *Salmonella*, only a small fraction of tubules seen in Δ*sifA*ΔsopD2-infected cells was LAMP1 positive (93.3 ± 1.2% versus 30.7 ± 4.2%). Then, we used a Δ*sifA*ΔsopD2 strain and analyzed the consequences of the plasmidic expression of SifA or SifA$^{L130D}$ on the LAMP1 content of tubules. While SifA induced a striking complementation (73.3 ± 4.2%), SifA$^{L130D}$ moderately but significantly increased the proportion of LAMP1-positive tubules (49.3 ± 5%), thus suggesting that the SifA C-term is involved in the recruitment of LGPs to *Salmonella*-induced tubules.

### *The Arl8b hypothesis*

As previously mentioned, the SifA C-term has a GEF fold. It preferentially binds GDP-bound RhoA but has no GEF activity towards this protein. Therefore we tested other GTPases that may interact with this effector. SKIP has recently been shown to interact with the Arf-like G protein Arl8b [6], which is present on SCVs and associated tubules in *Salmonella*-infected cells [8]. We investigated the involvement of Arl8b in the SifA C-term function. First we analyzed the consequences of an increased expression of Arl8b on the presence of LAMP1 on tubules in cells infected by a Δ*sifA*ΔsopD2 strain. Compared to GFP, expression of Arl8b significantly increased the percentage of LAMP1-positive tubules (Figure 5A) while this effect was not observed with a dominant negative form of this GTPase (Arl8b$^{T24N}$). These results point at a possible contribution of Arl8b in the process of LGP recruitment onto *Salmonella*-induced tubules and prompted us to further investigate the relation between SifA and Arl8b.

*SifA and Arl8b interact*

We tested the interaction between Arl8b and SifA by transfecting HeLa cells with plasmids for the expression of Arl8b-GFP and Myc-tagged variants of SifA. Proteins bound to GFP-Trap® beads were analyzed by anti-Myc Western blotting. We found that SifA specifically binds to Ar8b (Figure 5B, left panel). Unexpectedly both domains of SifA interact with Arl8 (Figure 5B, right panel). Disruption of the SKIP binding domain in full SifA ($SifA^{L130D}$) or the SifA N-term [$SifA^{L130D}$(1-140)] did not alter the interaction with Arl8b, indicating a SKIP-independent interaction. As compared with the SifA N-term, the SifA C-term seems to interact less efficiently with Arl8b and removal of its membrane-anchoring motif [SifA(1-330)] abrogates the interaction. To determine if the last residues of SifA are directly involved in binding Arl8b or if the interaction requires SifA to be membrane-bound, we expressed the proteins in the presence of different pharmacological inhibitors for protein lipidation and then performed the co-immunoprecipitation. GGTI-298 and cerulenin are inhibitors of the geranylgeranyltransferase 1 and the fatty acid synthetase, respectively. HeLa cell treatment with these drugs increased the cytosolic pool of SifA (Figure S2) and inhibited the interaction between the SifA C-term domain and Arl8b (Figure 5C). A farnesyltransferase inhibitor (FTI-227) used as control had a moderate effect on this interaction. It shows that the membrane anchoring of SifA favors the interaction with Arl8b.

Then we analyzed the consequences of the deletion of the membrane-anchoring motif of SifA on its contribution to virulence. The mean CI in C57BL/6 mice infected with a mix of strains expressing SifA(1-330) or SifA(1-136) was not significantly different from 1 (0.93 ± 0.72, Figures 3I & 4F). We concluded that the SifA C-term does not play a significant role in virulence in absence of its membrane-anchoring domain. Altogether these data demonstrate that Arl8b interact with both domains of SifA. Moreover, SifA C-term needs to be membrane-anchored for its function in virulence and its interaction with Arl8b. The association of these results might indicate that the strong virulence defect of a strains expressing SifA(1-330) is due to its lack of interaction with Arl8b.

## Discussion

### *The SKIP$^{-/-}$ mouse model*

We obtained *SKIP$^{-/-}$* mice from the Sanger Institute and maintained a colony of homozygote *SKIP$^{-/-}$* mice. These mice are viable, have a good prolificacy and do not present signs of disease or debility. Thus, SKIP is dispensable for the life of C57BL/6 mice indicating that in laboratory stabling conditions the function of this protein is not required and/or that another protein is capable of complementing SKIP's functions. At the cellular level we did not observed the Golgi scattering or the clustering of the LAMP1-positive compartments that we previously described upon siRNA-mediated knock- down of SKIP expression [2,4]. Thus the function of SKIP regarding the positioning and the organization of these organelles has been complemented and these results rather support our second hypothesis.

We found that *SKIP$^{-/-}$* mice are more resistant to a *Salmonella* challenge than congenic C57BL/6 mice. Remarkably, we observed very similar profiles for the survival curves of wild-type *Salmonella* in *SKIP$^{-/-}$* mice and for a Δ*sifA* strain in C57BL/6. We concluded that SKIP is one mediator for the function of SifA in virulence.

### *The two domains of SifA are functionally linked*

Our data indicate that in *SKIP$^{-/-}$* mice a Δ*sifA* mutant is still attenuated as compared to the wild-type strain of *Salmonella*. The use of strains expressing various mutant forms of SifA in CI experiments allowed us to conclude that SifA exerts a SKIP-independent function that is borne by the C-term domain. Therefore, the main conclusion of this work is that SifA has two domains and two functions. However, a comprehensive analysis of the CI results emphasizes other interesting information regarding the functional and physical interactions between the two domains.

A putative caspase-3 cleavage site separates the N- and C-term domains of SifA [24]. Yet it is not known whether this cleavage site is functional and if the two domains of SifA are split apart after translocation. We estimated the SKIP-dependent role of the SifA N-term domain in the context of a membrane anchored (Figure 3B, WT *versus sifA$^{L130D}$*) or cytosolic (Figure 3F, *sifA[1-136] versus* Δ*sifA*) domain. In both cases we obtained CI values of about 0.5 indicating that the N-term domain is equally functional in either

context. Therefore these data do not exclude the possibility that translocated SifA is cleaved.

The role in virulence of the SifA C-term domain was analyzed in the context of a fully functional molecule (Figure 3C, WT versus *sifA[1-136]*) or in absence of SKIP-dependent function (Figure 3E, *sifA*$^{L130D}$ *versus* Δ*sifA,* and Figure 3D, WT *versus* Δ*sifA* in *SKIP*$^{-/-}$ mice). Of note, the CI results were considerably divergent and showed that the contribution of the SifA C-term is far more important in the presence of a functional N-term domain. These data strongly suggest that the two domains are functionally linked and participate to the same signaling cascade. In conclusion, the two domains of SifA are functionally but not necessarily physically linked.

### *SifA is not a GEF for RhoA*

Having shown that the SifA C-term is functional we investigated the role of this domain in virulence. The T3SS-2 effector SseJ, which possesses a lipase activity, binds to and is activated by GTP-RhoA [23] while SifA interacts preferentially with GDP-RhoA. Considering the GEF-fold structure of SifA C-term, it has been proposed that SifA acts as a GEF for RhoA and therefore participates to SseJ's function in virulence [25]. We analyzed the interaction of SifA and SseJ in systemic infection of mice and found that they contribute independently to *Salmonella* virulence. Thus, SifA is not capable of activating SseJ by favoring the GDP to GTP exchange of RhoA. This result supports previous biochemical analysis that did not detect a GEF activity of SifA toward this GTPase [10,13].

### *The role of the SifA C-term in Salmonella virulence*

In infected HeLa or RAW 264.7 cells, the *sifA*$^{L130D}$ and Δ*sifA* strains are phenotypically comparable as far as the formation of Sifs and the modulation of kinesin-1 recruitment are concerned [12]. However, we observed for the strain expressing SifA$^{L130D}$, which possesses a functional SifA C-term domain, a modest but significant increase in the capacity to replicate in RAW264.7 mouse macrophages. Therefore we investigated the capacity of this mutant to preserve a SCV supporting the bacterial replication and more particularly its capacity to recruit LGPs, which is a hallmark for a

canonical wild-type SCV. To facilitate this analysis we used a Δ*sifA*Δ*sopD2* strain that is able to induce the formation of LAMP1- negative tubules. The plasmidic expression of SifA$^{L130D}$ significantly increased the percentage of LAMP1-positive tubules emerging from the SCVs, though less efficiently than the wild-type form of SifA. Nevertheless, this indicates that the SifA C- term is likely to play a role in the recruitment of LGPs by SCV-associated tubules.

As Arl8b interacts with SKIP and is present on SCVs and Sifs [8] we tested the capability of this GTPase to influence the formation of LGP-positive tubules. We found that the over-expression of Arl8b noticeably increased the proportion of LAMP1-positive tubules in cells infected by the Δ*sifA*Δ*sopD2* strain. This leads us to the assumption that the function of the SifA C-term and of Arl8b are linked. This theory was confirmed by showing the specific interaction between the two proteins. We found that Arl8b interacts with the C-term domain and – independently of SKIP – with the N-term domain of SifA.

We are currently uncertain if the recruitment of LAMP1 induced by the SifA C- terminus requires its interaction with Arl8b. To investigate this crucial point, future researches will study the phenotype induced by *Salmonella* in the absence of either SifA C-term or Arl8b. We observed a lower bacterial replication in Arl8b knock-down cells (Add.Fig 1). However, variation of bacterial growth can be observed between cell clones and it will be necessary to confirm this results by comparing the replication of two strains within the same cell clone. We plan to analyze the intracellular replication of the *sifA*$^{L130D}$ and Δ*sifA* strains in Raw 264.7 macrophages expressing or not Arl8b. HeLa cells cannot be used in this kind of experiment since a Δ*sifA* mutant replicates well in the cytosol of these cells. Arl8B knock-down Raw 264.7 macrophages are currently in the process of production and we anticipate it will tell us whether the expected phenotype, i.e. a better replication of the strain expressing a functional SifA C-term domain, is linked or not the expression of Arl8b.

### *The membrane anchoring of SifA favors its interaction with Arl8b*

The membrane localization of SifA is important for its function in virulence as a strain expressing a mutant form deleted of the carboxy terminal domain (SifA[1-330]) is highly attenuated in the mouse model [21]. Interestingly, we found that the SifA C-term deleted of its carboxyl terminal motif hardly interacts with Arl8b. Inhibitors of host cell prenylation and S-acylation [19,21] which prevent the membrane anchoring of SifA also decrease its interaction with Arl8b. These results demonstrate an association between the

function of SifA in virulence and its capacity to interact with Arl8b. However, it remains unknown if the function of SifA is limited to the binding/recruitment of Arl8b or if it induces the exchange of GDP for GTP and the activation of this GTPase. Further investigation will test this possibility.

In addition to Arl8b, mammalian cells can express Arl8a. The human proteins are 91% identical, localize both to lysosomes and the over-expression of either protein results in a microtubule-dependent redistribution of lysosomes towards the cell periphery [27]. In a yeast two-hybrid test both Arl8a and Arl8b interact with SKIP [6]. Thus, it is very likely that Arl8a and Arl8b may act redundantly and that Arl8a might compensate the knock down of Arl8b. However, HeLa cells express predominately Arl8b, as a siRNA mediated knock-down of Arl8b is sufficient to decrease the specific signal of an antibody that recognizes both proteins [6]. We will have to investigate whether the same is true in Raw 264.7 macrophages though Arl8a does not seem capable of complementing the loss of Arl8b expression for the phenotype specifically observed in *Salmonella*-infected cells [8].

A complex of host and bacterial proteins regulates the membrane exchanges between host compartments and SCVs and SCV-associated tubules. We previously showed that the *Salmonella* effector SifA recruits the kinesin-1-binding protein SKIP [2], which in non-infectious situation is recruited onto the lysosomal membrane by the Arl8b GTPase [6]. Here we have shown that in infected cells, Arl8b also interacts with SifA. It remains to understand whether this interaction leads to activation of Arl8 by promoting GDP for GTP exchange.

In this study we show that the T3SS-2 effector SifA plays an essential role in virulence in mice and that several of its domains contribute to this role. SifA N-term binds the host protein SKIP and SifA N-term and C-term binds the small GTPase Arl8B, whereby the interactions of SifA N-term with the two host proteins do not depend on each other. Also the membrane anchoring of SifA, which is mediated by a short carboxy terminal sequence, is crucial for its interaction with Arl8B. As also Ar8B and SKIP interact in non-infection conditions, a firm complex seems to form through action of SifA. This complex is important for *Salmonella*'s intracellular life, as it induces the formation of tubules (Sifs) and allows the interaction / fusion with the late endocytic compartment.

## Figure Legends

**Figure 1**. *Phenotyping of SKIP$^{-/-}$ mice.* (**A**) *SKIP$^{-/-}$* mice do not express SKIP. Triton X-100 extracts from peritoneal macrophages (PM) prepared from C57BL/6 or congenic *SKIP$^{-/-}$* mice and HeLa cells were examined for the presence of SKIP by Western immunoblotting with antibodies against the pleckstrin homology domain of SKIP. An anti-actin blot was used as control. (**B** & **C**) Kinesin-1 accumulates on SCVs in absence of SifA or in absence of SKIP. MEFs prepared from C57BL/6 or congenic *SKIP$^{-/-}$* mice were infected with GFP-expressing wild-type or Δ*sifA* strains of *Salmonella* (green). Cells were fixed 16 h post-infection and immunostained for kinesin HC (red). Nuclei were stained with DAPI (light blue). (**B**) Scale bar, 20 μm. (**C**) The percentage of kinesin-positive SCVs was scored. Results shown are the means ± SD of three independent experiments.

**Figure 2**. *SKIP$^{-/-}$ mice are more resistant to Salmonellosis than congenic C57BL/6 mice*. (**A**-**C**) Mice were inoculated perorally with $10^5$ CFU of *S.* Typhimurium. (**A**) Survival of C57BL/6 or *SKIP$^{-/-}$* mice to a wild-type strain challenge. (**B**) Survival of C57BL/6 to a wild-type or Δ*sifA* mutant strain challenge. (**C**) Bacterial loads in spleen, liver and MLNs of C57BL/6 (light circle) and *SKIP$^{-/-}$* (dark square) mice 5 days P.O. (5 mice per group). (**D**) Bone marrow macrophages prepared from C57BL/6 or *SKIP$^{-/-}$* mice were infected with a wild-type or Δ*sifA* mutant strain and lysed at 2 and 16 h post-infection for enumeration of intracellular bacteria. The values shown represent the fold increase calculated as a ratio of the intracellular bacteria between 16 and 2 h and normalized to the value of the wild-type strain. Values are means ± SD of three independent experiments.

**Figure 3**. Schematic representation of the SifA status in mixed infection performed with different *Salmonella* strains and in C57BL/6 (light, upper right part) or SKIP$^{-/-}$ (shadowed lower left part) mice. The two-domain protein SifA is symbolized by a cylinder (N-term domain, in blue) connected to a parallelepiped (C-term in green). The C-term CAAX motif is lipidated (square bracket, in red). A *sifA*(1-330) strain expresses a non-lipidated from of SifA. A Δ*sifA* strain does not express the SifA protein (colorless/dotted line). SifA$^{L130D}$ corresponds to a SifA variant carrying a mutation (L130D, in orange) that

prevents the interaction with SKIP (colorless N- term). The strain *sifA*(1-136) expresses only the SifA N-term. In SKIP$^{-/-}$ mice, the N- term domain is not functional (in grey), as far as the interaction with SKIP is concerned. The diverse mixed infections tested are marked with a letter and used to analyzed the: (**A**) Contribution of SifA; (**B** & **G**) SKIP-dependent contribution of SifA; (**C**) Contribution of the SifA C-term; (**D** & **E**) SKIP-independent contribution of SifA; (**F**) Contribution of SifA N-term; (**H**) SKIP-independent contribution of SifA N-term; (**I**) Contribution of SifA C-term deleted of its membrane anchoring motif - to *Salmonella* virulence.

**Figure 4**. *The SifA C-term is accountable to the SKIP-independent function of SifA in virulence*. C57BL/6 or *SKIP*$^{-/-}$ mice were inoculated intraperitoneally (I.P.) or perorally (P.O.) with $10^5$ CFU of various combinations of two *Salmonella* strains as indicated. Mice were sacrificed two (I.P.) or five days (P.O.) post-inoculation and spleens were harvested for bacterial counts and determination of competitive indexes (CI). Each symbol represents one mouse. Mean CI ± SD values are represented by both vertical and horizontal error bars and are also indicated on the top of each mouse group. (**A**) A Δ*sifA* strain is still significantly attenuated in *SKIP*$^{-/-}$ mice. (**B**) A Δ*sseG* mutant strain presents the same attenuation of virulence in C57BL/6 and *SKIP*$^{-/-}$ mice. (**C**) Evaluation of the SKIP contribution to the virulence mediated by SifA. (**D**) SifA plays a SKIP-independent function in virulence. (**E**) The SKIP-independent function of SifA in virulence is linked to the SifA C-term. (**F**) The membrane-anchoring motif is required for the function of SifA C-term in virulence. (**G**) SifA and SseJ contribute independently to *Salmonella* virulence.

**Figure 5**. *SifA C-term and Arl8b interact and contribute to the recruitment of LAMP1 onto Salmonella-induced tubules.* (**A**) SifA C-term and Arl8b modulate the presence of LAMP1 on *Salmonella*-associated tubules. HeLa cells were infected with a wild-type (WT) or Δ*sifA*Δ*sopD2 Salmonella* strains. The Δ*sifA*Δ*sopD2* strains were carrying or not (ctrl) a plasmid for the expression of SifA-2HA or SifA$^{L130D}$-2HA (left panel). HeLa were over-expressing GFP (ctrl), Arl8b-GFP, Arl8b$^{T24N}$-GFP or bared a knock-down of Arl8b (right panel). (**B**) Arl8b interacts with both domains of SifA. HeLa cells were transfected

with plasmid for the ectopic expression of GFP, Arl8b-GFP, Myc-SifB and different forms of mutated and/or truncated Myc-SifA. Note that $SifA^{L130D}$(1-140) migrated in SDS-PAGE with an apparent molecular mass superior to that of SifA(1-140) (**C**) HeLa cells were pre-treated or not for 8 to 10 h with different enzymatic inhibitors and transfected with plasmids for the expression of Arl8b-GFP and Myc-SifA(137-336). (**B & C**) Myc-tagged proteins bound to GFP-Trap® beads were analyzed by Western blotting.

**Figure S1**. *C-terminally truncated forms of SifA are secreted*. Strains expressing full length SifA-2HA or forms deleted from the C-terminal domain [SifA(1-136)-2HA] or the carboxy terminal hexapeptide [SifA(1-330)-2HA] were tested for the *in vitro* secretion of effectors. A strain expressing SifA-2HA but being unable to secrete T3SS-2 effectors (Δ*ssaV*, *sifA-2HA*) was used as negative control.

**Figure S2**. *Treatment with GGTI and cerulenin increases the cytosolic pool of SifA*. HeLa cells were pretreated or not for 8 to 10 h with 2.5 µM GGTI-298 and 2.5 µM cerulenin or with 10 µM FTI-277 and transfected overnight with plasmids for the expression of Arl8b-GFP and myc-SifA. Fixed cells were immunostained for SifA and LAMP1 and imaged using a confocal microscope. Scale bar, 20 µm.

**Additional Figure 1.** *Testing the role of Arl8b in Salmonella infection*. (A) Expression of Arl8b. Control shRNA HeLa cells and Arl8b shRNA HeLa cells were examined for the expression of Arl8b by Western immunoblotting with a polyclonal antibody against Arl8b. An anti-actin antibody was used as control. (B) Control shRNA and Arl8b shRNA HeLa cells were infected with a wild-type strain and lysed at 2 and 16 h post-infection for enumeration of intracellular bacteria. The values shown represent the fold increase calculated as a ratio of the intracellular bacteria between 16 h and 2 h. Values are means ± SD of three independent experiments.

## Materials & Methods

### *Statistical Analyses*

Statistical analyses were performed with Prism 5 software (GraphPad) with one-way ANOVA and Tukey post-test or two-tailed unpaired Student's t test. *P*-values: ns, not significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.0005$.

### *Ethic statement*

Animal experimentation was conducted in strict accordance with good animal practice as defined by the French animal welfare bodies (Law 87–848 dated 19 October 1987 modified by Decree 2001-464 and Decree 2001-131 relative to European Convention, EEC Directive 86/609). All animal work was approved by the Direction Départementale des Services Vétérinaires des Bouches du Rhône (authorization number 13.118 to S.M.).

### *Mouse Strains*

SKIP$^{-/-}$ mice (B6N;B6J-Tyr$^{c\text{-}Brd}$ Plekhm2$^{tm1a(EUCOMM)Wtsi/Wtsi)}$ were obtained from the Wellcome Trust Sanger Institute. A mouse colony was maintained by incrossing homozygotes, which have been genotyped as described by the producer.

### *Bacterial strains and growth conditions*

Bacterial strains used in this study are listed in Table 1. Strains were cultured in LB broth (Difco) or minimal medium (M9, glycerol 0.2%, MgSO4 1 mM, CaCl2 200 mM, thiamine 1 mg/ml, casamino acids 1 mg/ml. Ampicillin (50 µg/ml), kanamycin (50 µg/ml), tetracycline (10 µg/ml) and chloramphenicol (50 µg/ml) were added when required.

### *Construction of mutant strains*

Non-polar gene-deletion mutants were generated by the lambda Red recombinase system [28], using gene-specific primer pairs to amplify pKD4 kanamycin or pKD3 chloramphenicol resistance genes as shown in Table 2. All mutagenesis was performed in the 12023 wild-type strain. When necessary *Salmonella* mutants were transformed with the pCP20 plasmid to excise the antibiotic cassette.

### *Eukaryotic cells and culture conditions*

RAW 264.7, HeLa, primary bone marrow-derived macrophages and embryonic fibroblasts were grown in DMEM (GibcoBRL) supplemented with 10% foetal calf serum (FCS; GibcoBRL), 2 mM nonessential amino acids, and glutamine (GibcoBRL) at 37°C in 5% CO2.

### *Bacterial infection and replication assays*

Bone marrow-derived macrophages, HeLa and RAW 264.7 macrophage were grown, infected and treated as previously described [26].
When indicated lipidation inhibitors (GGTI-298, Cerulenin, FTi-277) were add onto recently splitted cells 8-10 hours prior cell transfection.

### *Western Blotting analysis*

Tagged proteins were detected by Western Blotting using mouse monoclonal anti-Myc (Clone 9E10), or anti HA (Covance, clone 16B12). A previously described rabbit polyclonal serum was used to detect SKIP [2]. Secondary antibodies were goat anti-mouse or anti-rabbit IgG HRP conjugate (Sigma–Aldrich).

### *Competitive index*

C57BL/6 or congenic *SKIP*$^{-/-}$ mice (six to eight weeks old) were inoculated intraperitoneally or perorally with equal amounts of two bacterial strains for a total of $10^5$ bacteria per mouse. The spleens were harvested two (I.P.) or five (P.O.) days after inoculation and homogenized. Bacteria were recovered and enumerated after plating a dilution series onto LB agar with the appropriate antibiotics. Competitive indexes (CI) were determined for each mouse [17]. The CI is defined as the ratio between the mutant and wild-type strains within the output (bacteria recovered from the mouse after infection) divided by their ratios within the input (initial inoculum).

*Preparation of peritoneal macrophages and mouse embryonic fibroblasts*

For peritoneal macrophages, mice (C57BL/6 or *SKIP*$^{-/-}$) were injected intraperitoneally with a thioglycollate solution for a volume of 1 ml per mouse. Four days post injection, the thioglycollate-pretreated mice were sacrificed and macrophages were harvested from the peritoneal cavity by washing with 5 ml of cold PBS. Cells were collected from the washing solution by centrifugation, washed again twice with cold PBS, resuspended in DMEM based growing medium and seeded at a density of $10^5$ cells per $cm^2$ in 6- or 24-well plates. Cells were used for infection after one day of culture.

For the preparation of mouse embryonic fibroblasts C57BL/6 or *SKIP*$^{-/-}$ mice were sacrificed at 13-14 days gestation. The uterine horns were collected and washed three times with 10 ml PBS. Then, visceral tissues were separated from embryos. Embryos were washed again for three times with PBS and then finely minced with a curved dissecting scissors. A volume of 2 ml of trypsin was added and incubated for 5 min, during which the tissue was minced consistently. 5 ml trypsin were added and the cells were pipetted vigorously up and down. The cells were placed into incubator for 20-30 min and again pipetted vigorously up and down. Cells were diluted in DMEM-derived growing medium and seeded in 75 $cm^2$ flasks and incubated at 37°C in a tissue culture incubator until the flasks are at least 90% confluent. Then, the cells were splitted using trypsin and seeded in 6- or 24-well plates for infection.

## Acknowledgments

We acknowledge the EUCOMM Consortium for *SKIP*$^{-/-}$ mice and Sean Munro for providing a plasmid for expression of Arl8b-GFP. TM was a recipient of a DOC-fellowship of the Austrian Academy of Sciences at the CIML.

## Figures

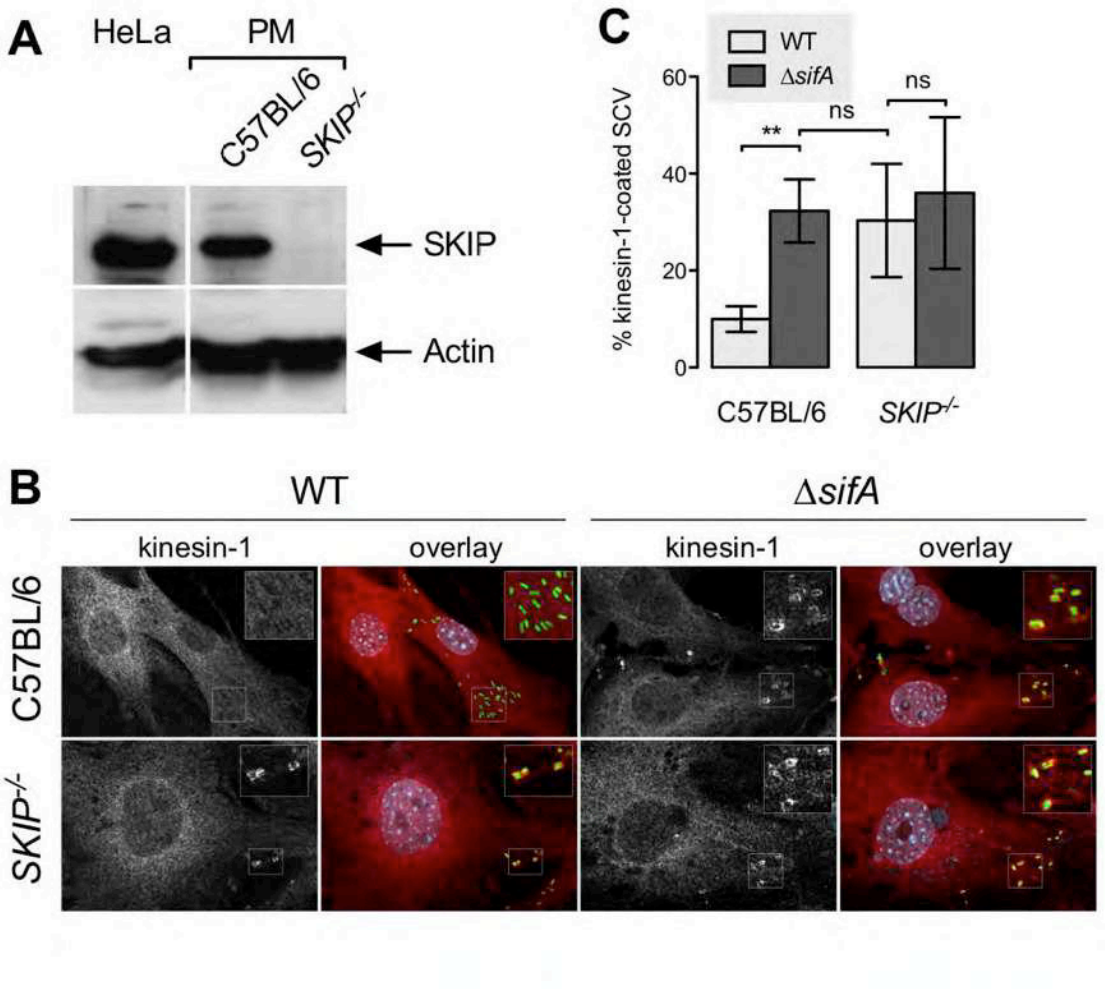



Figure 1

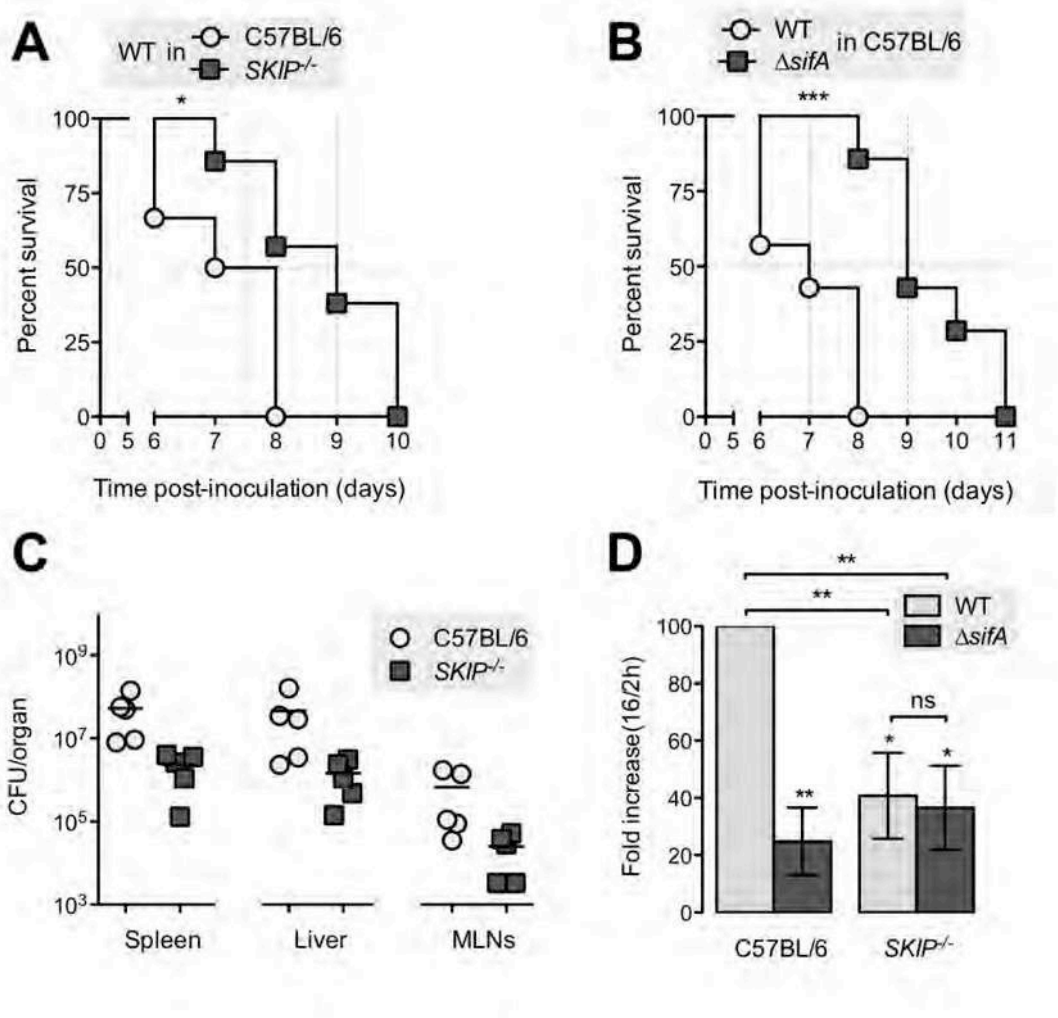

A
WT in
C57BL/6
SKIP-/-
Percent survival
Time post-inoculation (days)
B
WT
ΔsifA
in C57BL/6
Percent survival
Time post-inoculation (days)
C
C57BL/6
SKIP-/-
CFU/organ
Spleen
Liver
MLNs
D
WT
ΔsifA
Fold increase(16/2h)
ns
C57BL/6
SKIP-/-

Figure 2

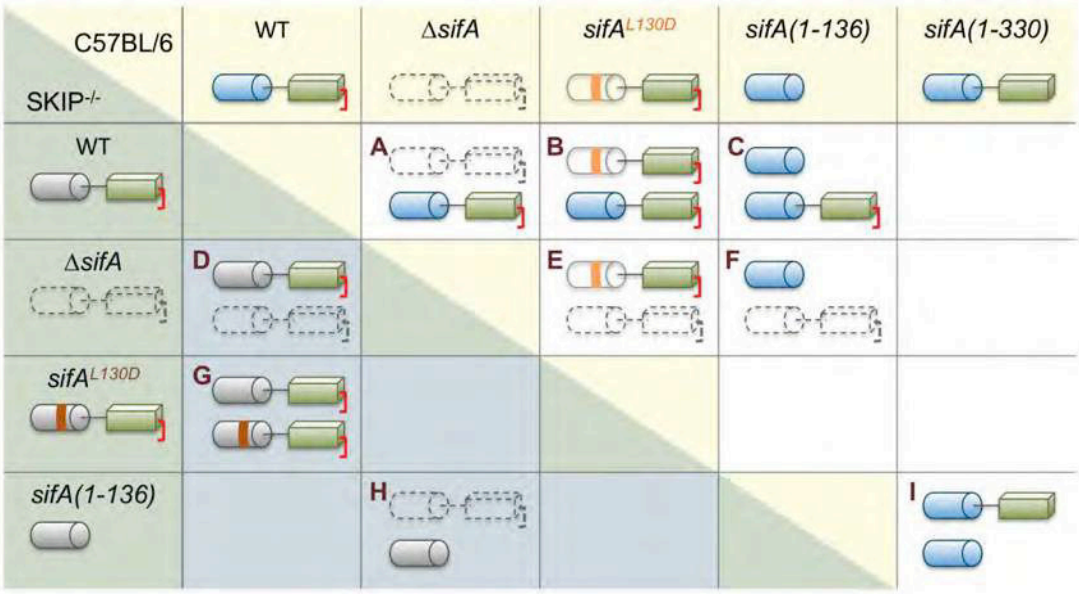

C57BL/6
SKIP-/-
WT
ΔsifA
sifAL130D
sifA(1-136)
sifA(1-330)
A
B
C
D
E
F
G
H
I

Figure 3

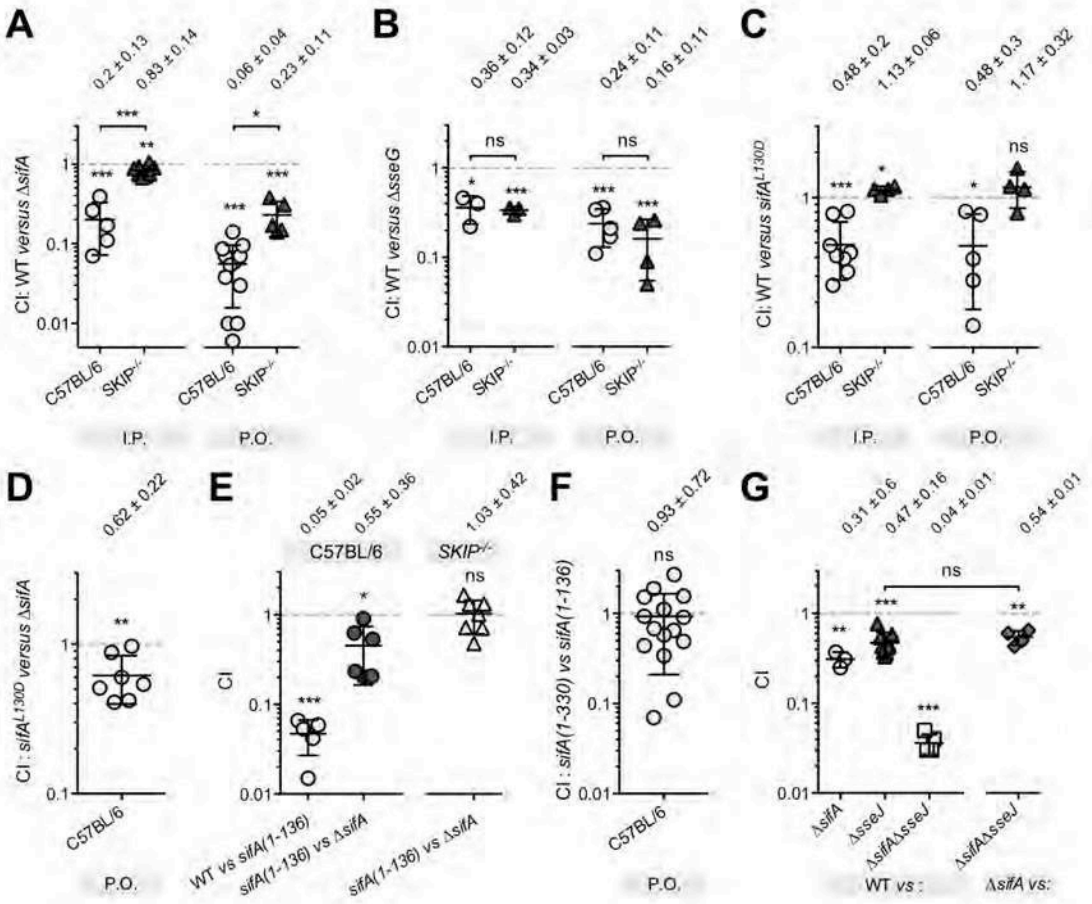

A
0.2 ± 0.13
0.83 ± 0.14
0.06 ± 0.04
0.23 ± 0.11
CI: WT versus ΔsifA
C57BL/6
SKIP-/-
I.P.
P.O.
B
0.36 ± 0.12
0.34 ± 0.03
0.24 ± 0.11
0.16 ± 0.11
CI: WT versus ΔsseG
ns
C
0.48 ± 0.2
1.13 ± 0.06
0.48 ± 0.3
1.17 ± 0.32
CI: WT versus sifAL130D
D
0.62 ± 0.22
CI : sifAL130D versus ΔsifA
E
0.05 ± 0.02
0.55 ± 0.36
1.03 ± 0.42
C57BL/6
SKIP-/-
CI
WT vs sifA(1-136)
sifA(1-136) vs ΔsifA
sifA(1-136) vs ΔsifA
F
0.93 ± 0.72
CI : sifA(1-330) vs sifA(1-136)
G
0.31 ± 0.6
0.47 ± 0.16
0.04 ± 0.01
0.54 ± 0.01
ΔsifA
ΔsseJ
ΔsifAΔsseJ
ΔsifAΔsseJ
WT vs :
ΔsifA vs:

Figure 4

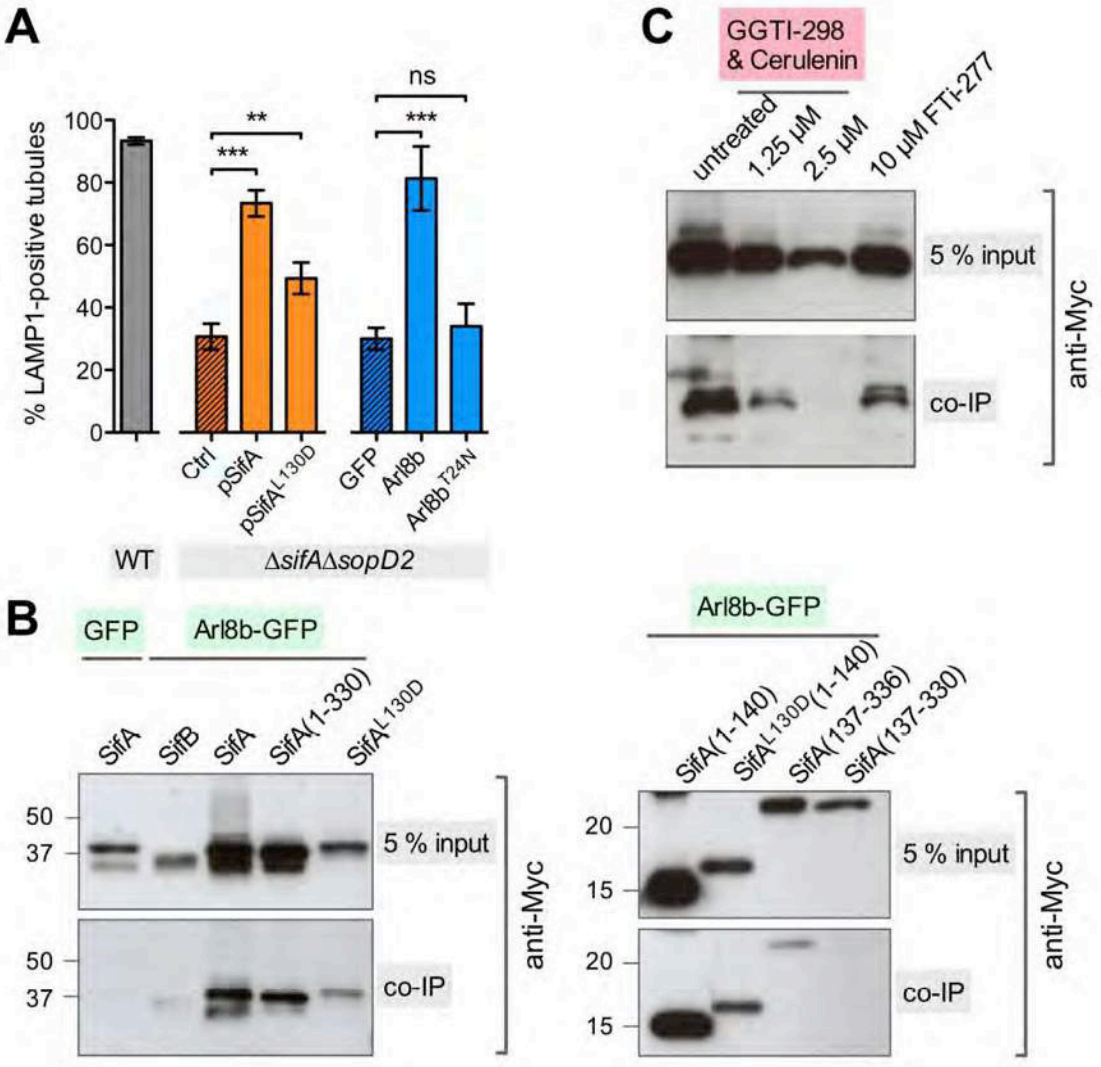

A
% LAMP1-positive tubules
100
80
60
40
20
0
**
***
ns
***
Ctrl
pSifA
pSifA L130D
GFP
Arl8b
Arl8b T24N
WT
ΔsifAΔsopD2
B
GFP
Arl8b-GFP
SifA
SifB
SifA
SifA(1-330)
SifA L130D
50
37
5 % input
co-IP
anti-Myc
C
GGTI-298
& Cerulenin
untreated
1.25 μM
2.5 μM
10 μM FTi-277
5 % input
co-IP
anti-Myc
Arl8b-GFP
SifA(1-140)
SifA L130D (1-140)
SifA(137-336)
SifA(137-330)
20
15
5 % input
co-IP
anti-Myc

Figure 5

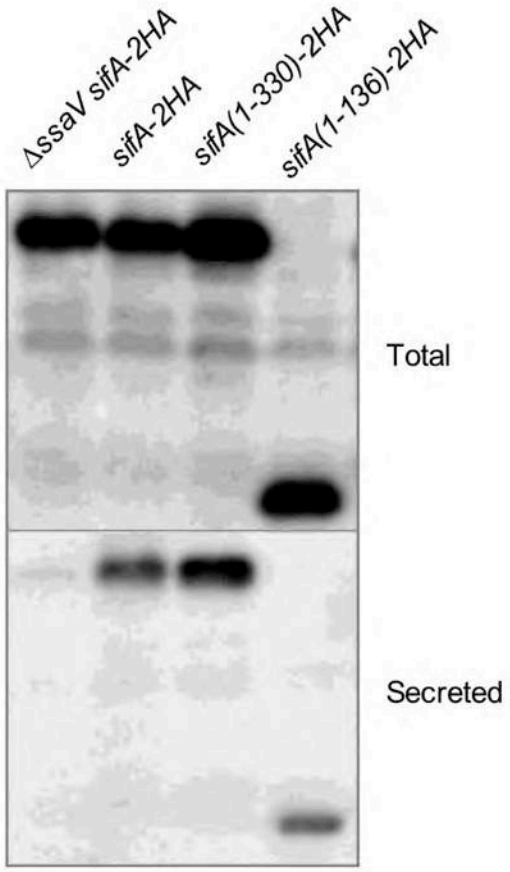

ΔssaV sifA-2HA
sifA-2HA
sifA(1-330)-2HA
sifA(1-136)-2HA
Total
Secreted

Figure S1

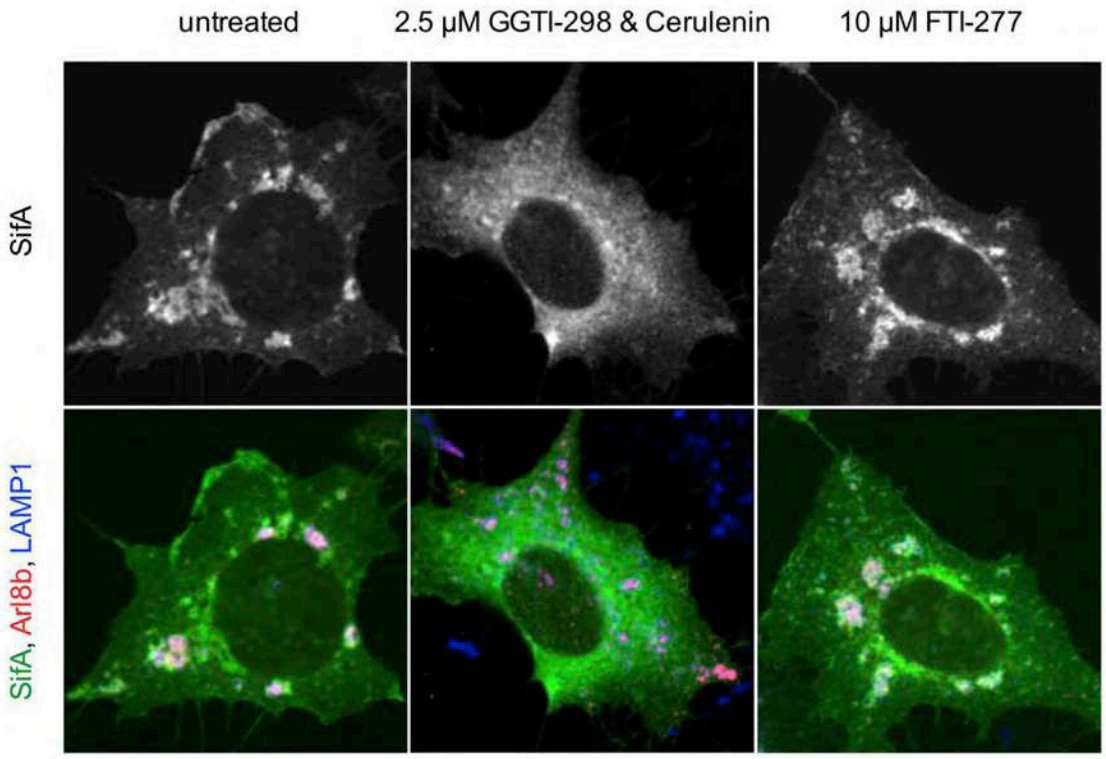
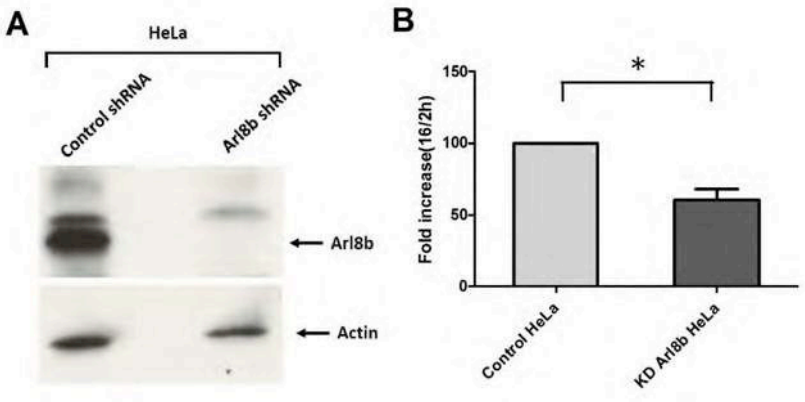

untreated
2.5 μM GGTI-298 & Cerulenin
10 μM FTI-277
SifA
SifA, Arl8b, LAMP1

Figure S2




Additional Figure 1

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# Chapter 4
# Revelations by the global protein stability profiling

## Quantitative approaches for the description of host-pathogen interactions

Quantitative approaches to describe host-pathogen interactions are a powerful tool and complement qualitative approaches, which describe segregate processes during infection. Quantitative approaches allow the systematic description of cohesion of higher entities. They have multiplied during recent years and can be based on interactions on different molecular or biological level, targeting the genome, transcriptome, proteome or lipidome [121-125]. But so far, posttranslational modifications and protein stability, two major playgrounds for intracellular pathogens, were spared out [126,127]. Their quantitative measurement was hindered by the proteomes chemical heterogeneity, its large dynamic range of abundance and the inability of specific recognition [128,129]. This issue was addressed by the development of the global protein stability profiling (GPS) [130].

## The global protein stability profiling

### General description

The GPS was designed to evaluate posttranslational changes and protein turnover of the human proteome under various physiological and disease conditions [130]. It is based on a lentiviral expression plasmid – the GPS vector – that allows bicistronic expression of the components of the GPS system. The bicistronic mRNA codes for the expression of DsRed and – via an internal ribosomal entry site (IRES) – of GFP, the latter one being tagged to any protein of the human proteome (Fig. 4.1). DsRed is thus independently expressed, but it is always prevalent in the same ratio as the GFP-tagged protein of interest. Therefore it can serve as an internal expression control, evening out the cell-based variability of expression of the GFP-tagged protein of interest. The turnover of GFP and its signal strength in each cell depend on the stability and turnover of the tagged protein. The comparison of signal strengths of the independent DsRed and the variable GFP hence allows a measurement of the tagged proteins stability.

Lentiviral transduction of a proteome wide library of GPS vectors into 293T cells leads to the creation of the primary tool of the GPS system, the GPS expression library. Within this library, presumably all proteins of the GPS library should be equally expressed. By manipulating the GPS expression library – through genetic manipulation of the cells, administration of chemical compounds or infection – certain proteins may be

targeted and their stability changes. Flow cytometry (FACS) based sorting into different bins depending on the GFP/DsRed ratio and subsequent analysis via genomic isolation and microarrays on the GPS inserts of the respective populations will allow to distinguish proteins that suffered stability relevant modifications from untargeted proteins (Fig. 4.1). Screening the entire proteome for modifications allows discrimination of proteins or entire pathways that are changed by the treatment.

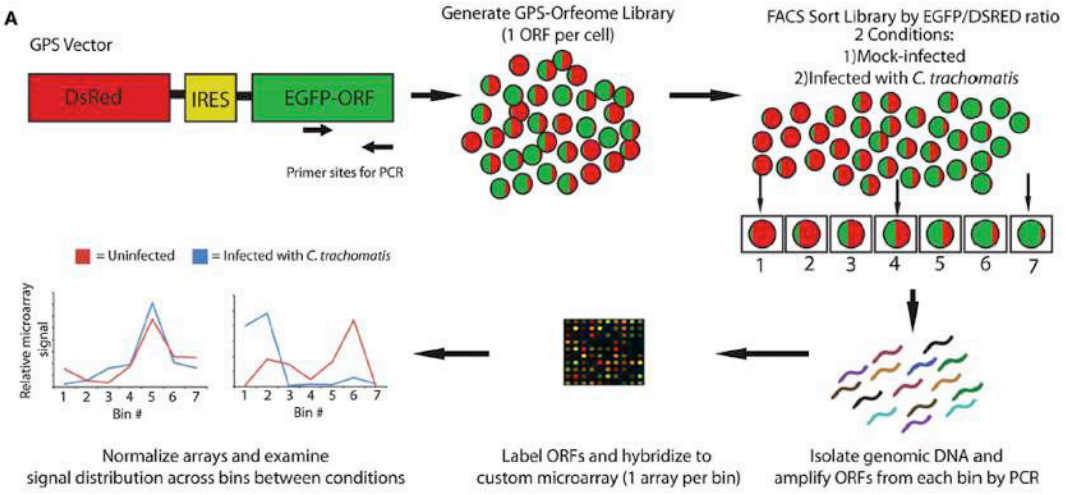


Fig 4.1: Visualization of the steps of the GPS profiling [131].

Distinction from other high-throughput methods

When investigating the effects of infections – although also other manipulations may be aimed for – on the proteome, the GPS distinguishes itself from other high-throughput screening assays in important details that stand for the interest of its application in all biological fields. Genome and transcriptome based approaches give appropriate overviews on the initial steps of reaction on the infection, which lead to transcription of pathogen induced genes or host defense related mechanisms. But modifications that happen after transcription but before the final adaption of the protein – like translational regulation, direct degradation of the newly formed protein due to unfolded protein response (UPR) in the endoplasmatic reticulum or specific proteolysis – are spared out although they relate directly to the functionality of the cell's proteome [132-135].

Stable isotope labeling by amino acids in cell culture (SILAC) in combination with mass spectrometry allows the quantitative detection of changes in the protein synthesis, but posttranslational changes can't be assessed precisely. It may, under certain conditions, be adapted to follow up posttranslational changes, but to obtain information on the entire proteome seems rather improbable [136].

As the GPS was developed to measure all kind of posttranslational changes that somehow affect protein stability, it can fill this knowledge gap that can't be addressed by other approaches and can complement findings or answer open questions that concern – here – the intracellular lifestyle of pathogens and the reaction of the host cell on the invader.

The choice of the GPS

*Salmonella* is a well-studied pathogen and many functional particularities of its intracellular lifestyle have been described. However, only for few of its secreted effector proteins host cell interaction partner and biochemical activity are known [75].

Our group is much interested in the tubular network that forms upon infection of the host cell. We were able to describe that SseF and SseG are the effector proteins that are responsible for the formation of LNTs and the connected effects on the stability of the SCV and virulence (Chapter 3). Others report implications of SseF and SseG in the positioning of the SCV, SIF formation, interaction with the Golgi apparatus and microtubules bundling (see Introduction). Our group also described the negative regulatory effects that SopD2 has on the formation of LNTs, especially in the absence of SifA and its implication in membrane instability in a Δ*sifA* mutant [96]. However, the host interaction partners of SseF, SseG and SopD2 are not described yet [75]. Recent papers address filling these gaps by biochemical methods and even propose interaction partners, but partly suffer from important methodical inaccuracies and none of the proposed interaction partners has been proven yet. In a SILAC based paper two proteins of the desmosome complex, junction plakoglobin and desmoplakin, were proposed as interaction partners for SseF and SseG and Caprin1, a cell cycle related protein, for SseG [137]. SseF and SseG were here expressed ectopically in the experimental cell line, whereby they localize in the Golgi apparatus [102,138]. Consequently, they do not reach

their biologically relevant position, which is in on LNTs and Sifs upon infection (compare Chapter 3 and [107]). Therefore they can't interact with neatly assembled desmosomes at their right subcellular localization. Apart from that, their involvement in tubules formation does not go along with the hypothesis that they might bind to desmosomal proteins, which are exclusively located at the cell membrane and expressed at very low levels in the 293T cell, which were the experimental cell line of this SILAC based study. Apart from that, an interaction of SseG with Caprin1 would indicate an impact of *Salmonella* on the cell cycle, which contradicts recent findings of our laboratory (unpublished data). More interestingly, an association of the cellular motor protein dynein with SseF and SseG has been proposed but never been proven [83].

Considering SopD2, no interaction partners have been proposed so far. Several attempts by our laboratory to identify a possible host target have not been successful, which is also true for SseF and SseG.

Proteins are the functional units of biological processes and their posttranslational modifications – from activation to flagging for degradation – indispensable for fine-tuning the adaption to the constantly changing situations. This fact is also recognized by pathogens, which modify proteins – the host interaction partners – corresponding to their needs [126,127]. The GPS is the first tool that allows a systemic analysis of these posttranslational changes. It can therefore serve to reveal unidentified host interaction partners of intracellular pathogens, here *Salmonella*. It can equally complement other high-throughput analyses to give insight into the complex mechanisms of host defense. We were keen to try it on different *Salmonella* infection scenarios to identify these effects on the proteome and to get insight into mechanisms of cellular stress and immune response upon infection. Besides, we also hoped to lay hands on the host targets of SseF, SseG and SopD2 and improve our understanding of the formation and regulation of *Salmonella*'s tubular network.

# Critics on the Global Protein Stability Profiling and what we can learn from it in the context of intracellular bacterial pathogens

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Keywords: *Salmonella*, infection, effector protein, global protein stability

## Abstract

The Global Protein Stability Profiling (GPS) is a sophisticated method that allows an *in cellulo* characterization of the stability of proteins. It was successfully applied to follow up posttranslational changes induced by the ubiquitin machinery and allowed to discover new host interaction partners for *Chlamydia trachomatis*. It is therefore of high interest to apply this method to infection scenarios with other intracellular bacterial pathogens with the purpose of identify host proteins that get targeted by these pathogens and to better understand the biology of infections. Here, we describe an unexpected effect of infections on the GPS. *Salmonella* Typhimurium, *Shigella flexneri* or non-infectious *Escherichia coli* corrupt the GPS itself by increasing protein synthesis, rendering its application impossible. We were able to show the effect to be about a host response, which can be induced by different means. We observed that cell signaling via direct cell contact induces this increased protein synthesis in uninfected cells. By using two different epithelial cell lines, we point out the importance of cell communication during infection at the outmost barriers of the human body.

The GPS is a powerful system but has, as each system, its limitations. Certain infection scenarios seem to perturb is function by the induction of a host response on the infection. Therefore, the GPS has to be used with care and eventual effects on the host cells have to be evaluated carefully.

## Introduction

In recent years, there has been an increase in global approaches to screen for eukaryotic host factors that are critical during intracellular bacterial infections [1-4]. While many of these approaches can identify large scale changes of host cell processes such as transcription, most are unable to examine host-pathogen interactions that occur post-translationally, even though these are considerably targeted by secreted bacterial effectors [5,6].

Recently, the global protein stability screening platform (GPS) has been described as a method that allows to characterize how the stability of proteins changes on a global scale in response to defined perturbations [7]. The GPS system has been used to investigate substrates of the mammalian ubiquitin system and recently has been used to understand how infection with the intracellular pathogen *Chlamydia trachomatis* alters the host proteome [8,9].

The GPS platform is based on the ratio of signals from two fluorescent proteins within a cell, DsRed and GFP. Both proteins are expressed and translated from a single mRNA via an internal ribosomal entry site (IRES). DsRed is expressed independently, while GFP is fused to a host protein. Because the levels of GFP are dependent on the stability of this fused host protein, the GFP to DsRed ratio under basal conditions represents the inherent stability of the host protein. Following perturbations, such as infection by a pathogen, the host protein may be modified directly or indirectly. These changes in stability can then be uncovered by changes in the GFP:DsRed ratio using flow cytometry.

Here we set out to use the GPS platform to dissect interactions between the bacterial pathogen *Salmonella* Typhimurium and human epithelial cells. *S.* Typhimurium is an intracellular pathogen whose virulence depends on entry into host cells, establishing an intracellular replicative niche – the *Salmonella* containing vacuole (SCV) – and avoiding immune clearance [10-15]. These processes rely on two independent and distinct type three secretion systems (T3SS), T3SS-1 and T3SS-2. T3SSs are nanosyringe-like structures that mediate the translocation of bacterial proteins, called effectors, into the host cell cytoplasm where they can manipulate the host to promote bacterial growth and survival [16]. Whereas T3SS-1 and its related effectors are mainly involved in the invasion of non-phagocytic cells, T3SS-2 and its effectors are responsible for the

maintenance of the SCV and allowing intracellular replication of *Salmonella* [16]. A large repertoire of effector proteins has been described to play an important role in *Salmonella* virulence and knocking out one or multiple effectors can lead to distinct defects in virulence within the host (reviewed in [17,18]).

While many host processes that are targeted during *Salmonella* infection have been described, others remain to be discovered and the function of several secreted effectors to be elucidated [18]. Several groups have described direct post-translational modifications of host proteins that are driven by secreted effectors during infections with *S.* Typhimurium [19-22]. We hypothesized that many uncharacterized interactions still remain, and are ripe for characterization using the GPS platform.

We set out to use the GPS system to compare the stability of host proteins in uninfected cells to cells infected with either wild-type *S.* Typhimurium or a strain deficient for the secretion of T3SS-2 effectors. We sought to identify new interactions of *Salmonella* and the mammalian host. Unexpectedly, we observed that *S.* Typhimurium, along with other gram-negative pathogens, led to a perturbation of host translation by inducing an increase in protein synthesis (IPS).

## Results

### *Salmonella* infection broadly perturbs fluorescent protein expression in human cells lines

We initially intended to use the GPS screening platform to identify host proteins that are altered directly following infection with *Salmonella* Typhimurium. The GPS platform is well suited to do so. It allows comparison of the fluorescence intensity of a free, stable, DsRed with a host protein-fused GFP, whose levels depend on the inherent stability of the fused protein, under varying experimental conditions. In order to adapt the GPS platform to understand host-pathogen interactions during *S.* Typhimurium infection we first created stable human 293T GPS reporter cell lines expressing GFP-fusions of RhoA or Profilin-1, which are known interaction partners for the *S.* Typhimurium T3SS-2 effectors SifA and SseJ (RhoA) or SspH2 (Profilin-1). Upon infection with wild-type *S.* Typhimurium we found increased stability of both host proteins as expected (Fig. 1A). However, when we examined a Δ*ssaV* mutant strain, which is deficient for translocation of T3SS-2 effector proteins to the host cell cytoplasm due to a non-functional secretion system, we still found an increase in the stability of RhoA and Profilin-1 following infection (Fig. 1A). To understand whether this increase in protein stability was T3SS-2 independent, but related to real increases in stability during *Salmonella* infection, we examined different GPS reporter cell lines expressing GFP with distinct half-lives of 1 hour , 4 hours (thereafter called 293T RG4) and 24 hours that was not fused to any host protein. We infected these cell lines for 8 hours and observed shifts in the GFP:DsRed ratio similar to RhoA and Profilin-1 cell lines for cell lines expressing 1 hour and 4 hour half-life GFP but not 24 hour half-life GFP (Fig. 1B).

Further examination of the individual GFP and DsRed fluorescence intensities showed that the shift in the GFP:DsRed ratio mainly comes from an increase in the signal intensity of GFP. However, we also observed a slight increase in the signal intensity of DsRed. When we compared fluorescence intensities 8 hours and 14 hours following infection, we found that the DsRed signal increases strongly at later time points post infection, which reduces the shift in the GFP:DsRed ratio (Fig. 1C). Similar to 8 hours, we did not observe a shift in the GFP:DsRed ratio in cells expressing the 24 hour half-life GFP 14 hours following *Salmonella* infection (Fig. 1B). However, we did note an

increase in individual fluorescence intensities of both GFP and DsRed in these infected cells. This suggests that there is no change in the GFP:DsRed ratio in these cells because both proteins accumulate at the same rate (compare also Fig. 5). Altogether, we found that infection with *Salmonella* leads to increased DsRed and GFP signal intensities independently of host proteins fused to GFP. We conclude that infection with *Salmonella* activates a host response that drives an increase in the accumulation of fluorescent proteins irrespective of protein fusions.

**The observed increase in fluorescence is due to an increase of protein synthesis (IPS) within the host cell**

We found increased fluorescence intensity for both DsRed and GFP upon infection of GPS reporter cell lines with *S.* Typhimurium, independently of an eventually fused host protein. This suggested that direct post-translational modifications were unlikely. Therefore, we hypothesized that infection of host cells with *Salmonella* may lead to an infection-mediated increase in host transcription or translation. In support of this concept DsRed has been shown to mature more slowly than GFP, which is in line with its delayed accumulation we observed above [23-25]. In order to test our hypothesis, we infected cells, blocked their translation by adding cycloheximide to the culture medium and examined the cells 8 hours following infection. No significant change was observed in the individual fluorescence intensities of GFP or DsRed and the GFP:DsRed ratio remained unchanged following cycloheximide treatment (Fig. 2). These results suggest that an increase in protein production occurs following infection with *Salmonella*, which in turn leads to an accumulation of fluorescent proteins.

Pathogens can influence the proliferation or modify the cell cycle of their host cells [26,27] and we wondered if the IPS could result from such alteration. To test this hypothesis, we measured cell proliferation of infected cells for three days. We used an attenuated Δ*ssaV* strain that triggers a signal increase of the FPs (Figure 1A) but does not kill the cells in this time frame. We did not observe any difference in proliferation of infected and non-infected cells (Fig. S1) indicating that IPS does not result of an infection-mediated proliferation signal [28].

**Infection by two distinct intracellular pathogens induces an increase in protein accumulation independently of the intracellular niche**

We next sought to clarify whether only infections with *S.* Typhimurium can induce alterations in host translation or if this is phenomenon is induced by other bacterial infections as well.

We first sought to understand at what step during infection the increase in protein translation was induced. We incubated 239T RG4 cells with heat killed wild-type *S.* Typhimurium or an invasion deficient (Δ*prgH*) strain. In contrast to live *S.* Typhimurium, heat killed or non-invasive bacteria did not induce changes in fluorescent protein accumulation (Fig. 3) indicating that an invasion of the cell by live *S.* Typhimurium in necessary. We next tested if the increase in host translation was restricted to *S.* Typhimurium infection or if other intracellular pathogens could induce this response. We compared *S.* Typhimurium, *Shigella flexneri*, *Brucella abortus* and *Chlamydia trachomatis* and observed that only *S.* Typhimurium and *S. flexneri* induced the increased production of fluorescent proteins (Fig. 3). Since *S.* Typhimurium resides in a vacuole and *S. flexneri* enters the cytoplasm rapidly following infection, we concluded that intracellular pathogens trigger this increase in fluorescent protein accumulation independently of the replicative niche.

Interestingly we also observed an IPS upon administration of the non-infectious *Escherichia coli* on our control cell lines (Fig. 3). We concluded from these results that there are probably various bacterial components that can be sensed extracellularly or in different intracellular compartments and induce IPS.

**Direct cell contact is necessary for increased protein synthesis in non-infected cells**

A very curious observation is that all cells in infected wells, whether we could detect intracellular bacteria or not, showed an increase in protein synthesis. We hypothesized two distinct models in which this could occur. In the first, all cells might have initially been infected, but the pathogen either lost its fluorescent signal or was eliminated from the cells. In the second model, infection of a subset of cells with *Salmonella* would lead to the secretion of cytokines or other signaling molecules that activate an increase in protein synthesis in all cells. To test these two models, we conducted an experiment

where we implemented transwell-like structures into wells with infected cells one hour after infection. These cells were not directly in contact with *S.* Typhimurium infected cells, but shared the culture medium with the cells that were exposed to infection. Interestingly, these cells showed no change in the amount of fluorescent proteins suggesting that secretion of a soluble factor does not drive this response (Fig. 4A). We next tested the possibility that direct contact of uninfected cells with infected cells may be responsible for our observation in fluorescent protein accumulation. In order to investigate this, we stained uninfected cells with a cell tracker dye, mixed them in culture with infected cells and let mixed infection progress overnight (Fig. 4B). We found by flow cytometry that almost all labeled cells remained uninfected after the overnight incubation (Fig 4B, 1.49 % of infected traceable cells), ruling out the possibility of a secondary infection. We observed that uninfected labeled cells added to unlabeled infected cells showed an increase in fluorescent protein levels compared to uninfected labeled cells added to a mock well (Fig. 4C). Moreover the GFP:DsRed ratio of infected cells, uninfected cells from the initial infection and cell tracker labeled uninfected cells that were mixed with these cells did not show any significant differences, yet were distinct from mock infected cells (Fig. 4D). Together these data suggest that direct contact of uninfected cells with *Salmonella* infected cells can lead to the increased accumulation of fluorescent proteins.

**The increased protein synthesis is not cell line dependent**

293T cells are highly responsive cells. To make sure that the effects we saw were not specific to infected 293T cells, we transferred the GPS system into HeLa cells. We were able to confirm the observed phenomenon in HeLa cells, ruling out a 293T-dependent artifact (Fig. 5).

## Discussion

*Salmonella* manipulates its host cells in many different and complex ways [17,18]. Despite the previous use of high-throughput assays targeting protein-protein interactions and due to the lack of high-throughput methods targeting post-translational modifications, many interaction partners for *Salmonella* effector proteins are still unknown [29]. We wanted to fill some of these gaps by applying the GPS profiling to *S.* Typhimurium infections. However, we found that the GPS system itself is sensitive to the presence of *S.* Typhimurium, rendering its application impossible. Therefore, we investigated how and why a pathogen could influence the GPS system. By administration of different bacteria onto the 293T RG4 control cell line we demonstrated that infections with *S.* Typhimurium and *S. flexneri* lead to an increase in the fluorescence of GFP and DsRed, the two fluorophores used in the GPS system. When blocking translation during an infection with *S.* Typhimurium we were able to inhibit this fluorescence increase and thus attributed it to an increase in protein synthesis, which might be due to a transcriptional or a translational boost. Infections with other, slow growing pathogens like *C. trachomatis* or *B. abortus* did not result in an IPS, but an infection with a replication defective *S.* Typhimurium Δ*ssaV* strain had an effect. This excludes the possibility that the IPS is only initiated by high numbers of intracellular bacteria. Curiously, the administration of non-infectious *Escherichia coli* into the culture medium led to IPS as well, whereas neither heat-killed nor non-invasive live *S.* Typhimurium did so. This implies that intra- and extracellularly recognized bacteria can cause the effect and that the recognition mechanism differs between bacteria.

Comparing data at 8 hours post-infection with those obtained at 14 hours post-infection, we found that the difference in the ratio of GFP and DsRed diminished with time, probably due to a slower maturation rate of DsRed. GFP and DsRed are distant homologs but differ in many aspects. Whereas GFP is a monomeric protein with a weak tendency to dimerize, DsRed is obligatorily tetrameric [24,30]. Their chromophores are very similar and share certain steps of formation, but additional covalent modifications are necessary for the formation of the final chromophore of DsRed [30]. This results in different formation dynamics. GFP formation follows a single exponential curve with a formation time $t_{0.5}$ of 30 min to 1 hour, while DsRed maturation can best be described by

a three step model with a $t_{0.5}$ of about 10 hours [23-25]. These biochemical properties explain why the increase in protein formation observed in the GPS system can be observed in the GFP:DsRed ratio, but is getting weaker after a long infection time.

Upon exposure, a targeted cell reacts in many ways, which strongly depends on the pathogen. In most cases, infected cells try to kill or stop the replication of the pathogen and to alarm surrounding cells and the immune system to fight infection. The cell has a broad set of tools to sense the pathogen, the pattern recognition receptors (PRR), which are located at the cell surface, within specialized compartments for the degradation of the pathogens and in the cytosol. They can detect various components of the pathogens, so called pathogen-associated molecular patterns (PAMPs). These are LPS, peptidoglycans, T3SS, DNA, RNA and others [31]. However 293T cells are mostly devoid of TLRs and their use as a model didn't allow us to distinguish canonical receptors to be involved in IPS. Still 293T seem to be able to sense bacteria at different interfaces and by different receptors and pathways as non-infectious *E. coli* but not a non-invasive *S.* Typhimurium strain induced IPS extracellularly and only *S. flexneri* and *S.* Typhimurium but not *C. trachomatis* and *B. abortus* were recognized in their different intracellular niches, thereby leading to IPS.

When infecting tissue culture cell lines, infection ratios differ depending on the pathogen and the conditions used. For instance, ratios of 30 to 50% were normal in our experiments for an infection with wild-type *S.* Typhimurium. Curiously, we found that not only infected cells but all cells that were present in the well of infection showed an IPS. We reasoned that signaling between the cells could be responsible for this effect. The reactive repertoire of infected cells is indeed prominent. Signaling molecules as cytokines get secreted, cell contact via gap junctions and ion channels can alert adjacent cells, tunneling nanotubes and epithelial bridges lead to exchange between cells and the cell can even enter apoptosis to deprive the pathogen from metabolic and anabolic bricks in its replicative and protective niche [32-36]. We were able to proof that the IPS is not obtained by paracrine signaling, as shown by transwell-like insertions of uninfected cells. By mixing and incubating infected and not infected cells together we showed that direct contact is necessary for the transduction of the signal and the IPS. Signaling via gap junctions and oxygen radical formation has already been described to induce the secretion

of cytokines by neighboring epithelial cells upon infection with *S. flexneri* or *Listeria monocytogenes* [32,33]. As shown by the infection of HeLa GPS control cell lines, the IPS in response to *Salmonella* infection is not exclusive to 293T cells and does not represent a cell line specific artifact. Thus, these results suggest a general mechanism of host response that is linked to IPS.

Altogether we show here that the GPS profiling is not applicable to certain bacterial infections. Unidentified mechanisms, which might vary between the administered microorganisms, trigger a cell contact dependent signaling and a subsequent increase of protein synthesis. As GFP and DsRed have different maturation times, the IPS produced shift in the GFP:DsRed ratio is unrelated to posttranslational effects that are the target of a GPS based analysis. This leads the use of the GPS system *ad erratum*. The GPS must therefore be used with care, excluding all eventual perturbation before applying it on scenarios that might have an impact on its functionality. The observed cell contact dependent IPS is of high interest, as it extends knowledge on the ways that cells can react on a threat by bacteria. It suggests that there is a broader reaction of cells on a thread than expected in the past.

## Figure Legends

Figure 1: *S. Typhimurium increases the fluorescence within the GPS system.*

Different 293T GPS cell lines were infected with wild-type *S.* Typhimurium, the Δ*ssaV* mutant strain or mock infected for 14 h. Cells were then fixed and analyzed by flow cytometry. The GFP:DsRed ratio, GFP or DsRed were plotted to visualize the stability shifts and the linked changes of fluorescence. A) RhoA-GPS and Profilin-1-GPS cell lines were infected and analyzed as described. A stability shift is observed for wild-type *S.* Typhimurium and the Δ*ssaV* mutant strain. B) Negative control cell lines 293T RG1, RG4 and RG 24 were infected and analyzed as described. RG1 and RG4 cells show a similar stability shift as positive control cell lines. The effect on RG24 cells cannot be seen in the GFP:DsRed ratio, as the increases in signal of GFP and DsRed correspond (not shown). C) 293T RG4 cells were infected and analyzed as described. GFP and DsRed signals are plotted and statistically analyzed to visualize the effect of the infection on each protein. The signal increase of GFP is significant after 8 h and 14 h; the increase of DsRed has a longer lag phase and is only significant after 14h. The statistical analysis was performed out of three independent experiments. P-values: *** $P<0.001$.

Figure 2: *The increased fluorescence of GFP and DsRed is linked to an increased protein synthesis.*

293T RG4 cells were infected with wild-type *S.* Typhimurium or mock infected. Immediately after infection, 100 µg/mL cycloheximide were added to the cells or not and the cells incubated for 8 h. Cells were then fixed and analyzed by flow cytometry. A) Comparison of the plots of GFP:DsRed ratio and GFP or DsRed alone revealed that the inhibition of translation impedes the signal increase of GFP and DsRed. B) Statistical evaluation of the fluorescence of GFP and DsRed shows a significant deregulation of both in control samples. This deregulation can be blocked by the addition of cycloheximide. The statistical analysis was performed out of three independent experiments. P-values: *** $P<0.001$.

Figure 3: *The impact of different infection scenarios on the GPS.*

293T RG4 cells were infected with wild-type *S.* Typhimurium, *S. flexneri*, *B. abortus* or *C. trachomatis*. Also, heat-killed *S.* Typhimurium, the non-invasive *S.* Typhimurium Δ*prgH* mutant and *E. coli* were administered onto the cells. Cells were then fixed and analyzed by flow cytometry. Comparing the GFP:DsRed ratio plots, we found induction of IPS for infections with live, but not heat-killed wild-type *S.* Typhimurium or the non-invasive Δ*prgH* mutant. *S. flexneri* infection did, but *B. abortus* and *C. trachomatis* infections did not influence the GPS system. Administered *E. coli* induced IPS.

Figure 4: *Cell contact dependent signaling leads to increased protein synthesis.*

A) 293T RG4 cells were infected with wild-type *S.* Typhimurium or mock infected. 1 h after infection, a transwell-like insert was plunged into the well of infected cells and infection was allowed to go on for additional 13 h. Cells were then fixed and analyzed by flow cytometry. Cells from the transwell-like insert behave like mock cells. B-D) 293T RG4 cells were infected with wild-type *S.* Typhimurium or mock infected (mock and traceable cells). 1 h after infection, traceable cells were stained with CellTracker $^{TM}$, mock and infected cells were mock stained. All cells were then trypsinized and traceable cells mixed with mock and with infected cells for additional 13 h. Cells were then fixed and analyzed by flow cytometry. B) Traceable cells (CellTracker $^{TM+}$) are distinguishable from uninfected ($^{-/-}$) and infected cells (CFP$^{+}$) and do essentially not get infected. C) Traceable cells added to wild-type infections show an IPS as compared to traceable cells added to mock infections. D) Within the well of wild-type infection, uninfected, traceable and infected cells behave the same way.

Figure 5: *The increased protein synthesis is not limited to 293T cell lines.*

HeLa cell lines RG1, RG4 and RG24 were infected with wild-type *S.* Typhimurium or mock infected for 14 h. Cells were then fixed and analyzed by flow cytometry. The plotted results show a similar pattern of IPS as the corresponding 293T cell lines. A shift in the GFP:DsRed ratio can be observed for RG1 and - subtly nuanced - for RG4 cells. The inlet in the plot for RG4 cells visualizes the shift of GFP, which is mostly masked in

the GFP:DsRed ratio by the shift of DsRed. The shifts of GFP and DsRed are - as in 293T cells - not visible in the GFP:DsRed ratio of the RG24 cells.

Figure S1: *The IPS does not influence the proliferation of the host cells.*

Cells were stained with CellTracker$^{TM}$, infected with a *S.* Typhimurium Δ*ssaV* mutant strain or mock infected, then incubated for three days. Cells were then fixed and analyzed by flow cytometrie. The loss of signal strength of the CellTracker$^{TM}$ staining indicates the speed of proliferation. The initial signal strength before the start of the experiment is shown in grey. No difference between the Δ*ssaV* strain and the mock infection – thus no impact on proliferation – was observed.

## Material and methods

### Statistical analyses

Statistical analyses were performed with Prism 6 software (GraphPad) by two-way ANOVA with Bonferroni correction: ns, not significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

### Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 2. *Salmonella* Typhimurium and *Escherichia* coli strains were cultured in LB broth (Difco) or LB broth plus 5 g/L NaCl. Ampicillin (50 µg/mL) and kanamycin (50 µg/mL) and tetracycline (10 µg/mL) were added when required. *Shigella* flexnernii and *Brucella* abortus strains were cultured in TSB (Sigma). Ampicillin (100 µg/mL) was added when required.

### Eukaryotic cells and culture conditions

Cell lines used in this study are listed in Table 1. HeLa and 293T were grown in DMEM (GibcoBRL) supplemented with 10% fetal calf serum (FCS; GibcoBRL), 2 mM nonessential amino acids, glutamine and sodium-pyruvate (GibcoBRL) at 37°C in 5% CO2. For experiments on translation inhibition and staining with Cell Tracer™, cells were seeded on poly-L-lysine coated wells.

### Construction of cell lines

Retroviruses were packaged in 293T cells transfected with the plasmid of interest as well as plasmids expressing the viral proteins VSVG and gag-pol. Lentiviruses were packaged in 293T cells transfected with the plasmid of interest plus plasmids expressing Δ8.91, VSVG and TAT. Viral infections were supplemented with hexadimethrine bromide at a concentration of 8 µg/ml. 293T RhoA- and PFN-1-GPS cell lines were put under puromycin selection, using a concentration of 1 µg/mL.

**Bacterial infection assays**

HeLa and 293T cells were seeded in 6-well plates at a surface ratio of 1/6 at 24 h prior infection.

*Salmonella* Typhimurium was incubated overnight in LB broth at 37˚C with shaking, diluted 1:30 in fresh LB broth (plus 5 g/L NaCl), and incubated in the same conditions for 3.5 h. The cultures were diluted in Earle's buffered salt solution and then added to the cells at a multiplicity of infection of 100:1. The infection was allowed to proceed for 12 min at 37˚C. For experiments with heat killed *S.* Typhimurium, 250µL of *S.* Typhimurium of a 2h pre-culture were incubated for 12 min at 65˚C. Bacteria were spun down 30 sec 13.000 rpm RT and re-suspended in 2 mL DMEMc, then added onto the cells. The plates were then spun down at 1000 g for 10 min at 21˚C and then incubated for 30 min at 37˚C. Experiments with *Brucella abortus* and *Chlamydia trachomatis* were performed as previously described [9,37].

Infections with *Shigella flexneri* and *Escherichia coli* DH5α followed the procedures for heat killed *S.* Typhimurium with some exceptions. S. *flexneri* was grown in TSB, final experimental concentration was $OD_{600}$ 0.3. *E. coli* final experimental concentration was $OD_{600}$ 0.05.

Infected cells were then washed three times with PBS and incubated in DMEMc + 100 µg/mL gentamicin for 1 h, then gentamicin concentration was decreased to 10 µg/mL for the remainder of the experiment.

**Analysis of the effect of infections on translation**

Cycloheximide was administered onto cells at 100 µg/mL right after *S.* Typhimurium infection and was kept throughout the remaining 8 h of the experiment.

**Analysis of cell communication**

To measure an eventual communication by soluble cues, transwell-like structures were inserted into the well that contained infected cells one hour after infection and were kept there for the remainder of the experiment.

For measuring of communication upon direct cell contact, mock-infected cells were stained with CellTrace™ Far Red DDAO-SE following the provider's instructions. These

cells and the experimental cells were trypsinized, mixed together and incubated overnight at 37˚C 5% $CO_2$.

**Staining and FACS analysis**

For detection with FACS, *S.* Typhimurium was stained with a rabbit anti-LPS antibody when needed (heat-killed strain), *S. flexnerii* with rabbit anti-Shigella V LPS antibody, *B. abortus* with cow anti-*Brucella* smooth antibody. Secondary antibodies were goat anti-rabbit APC (Invitrogen), goat anti-rabbit PacificBlue (Invitrogen).

Table 1. *Cell lines, bacterial* strains and plasmids

| Name | Description | Reference |
|---|---|---|
| *Cell lines* | | |
| 293T RhoA-GPS | 293T cells expressing RhoA within the GPS system | This study |
| 293T PFN1-GPS | 293T cells expressing Profilin1 within the GPS system | This study |
| 293T RG1 | 293T cells expressing the negative control GPS variant, modified GFP with a half-life time of 1h | Provided by Stephen Elledge |
| 293T RG4 | 293T cells expressing the negative control GPS variant, modified GFP with a half-life time of 4h | Provided by Stephen Elledge |
| 293T RG24 | 293T cells expressing the negative control GPS variant, modified GFP with a half-life time of 24h | Provided by Stephen Elledge |
| HeLa RG1 | HeLa cells expressing the negative control GPS variant, modified GFP with a half-life time of 1h | This study |
| HeLa RG4 | HeLa cells expressing the negative control GPS variant, modified GFP with a half-life time of 4h | This study |
| HeLa RG24 | HeLa cells expressing the negative control GPS variant, modified GFP with a half-life time of 24h | This study |
| *Bacteria strains* | | |
| 12023 | *S*. Typhimurium (*Salmonella enterica* subsp. *enterica* serovar Typhimurium) wild-type strain 12023 | Laboratory stock |
| TM055 | *S*. Typhimurium, wild-type, expressing CFP | This study |
| TM056 | *S*. Typhimurium, Δ*ssaV*, expressing CFP | This study |
| HH124 | *S*. Typhimurium, prgH020::Tn5lacZY, expressing CFP | [38] |
| TM051 | *Shigella* flexneri wild-type strain M90T | Provided by Guy Tran Van Nhieu |
| | *Escherichia* coli wild-type strain DH5α | Laboratory stock |
| | *Brucella* abortus wild-type strain 2308 smooth virulent | Laboratory stock |

| | | |
|---|---|---|
| | *Chlamydia* trachomatis wild-type strain | Laboratory stock |
| *Plasmids* | | |
| pCMV-lGPS (C1080) | Empty GPS expression vector for the production of lentiviruses, provides puromycin resistance | Provided by Stephen Elledge |
| GPS-RG1 (C1081) | GPS expression vector for the production of retroviral particles, modified GFP with a half-life time of 1h | Provided byStephen Elledge |
| GPS-RG4 (C1082) | GPS expression vector for the production of retroviral particles, modified GFP with a half-life time of 4h | Provided by Stephen Elledge |
| GPS-RG24 (C1083) | GPS expression vector for the production of retroviral particles, modified GFP with a half-life time of 24h | Provided by Stephen Elledge |
| pCMV GPS-RhoA (C1084) | GPS expression vector for the production of lentiviruses, RhoA in the pCMV-lGPS vector | This study |
| pCMV GPS-PFN1 (not in library) | GPS expression vector for the production of lentiviruses, Profilin-1 in the pCMV-lGPS vector | This study |
| pCMV Δ8.91 (C1075) | Supporting expression vector for the production of lentiviral particles | Laboratory stock |
| pCMV VSVG (C1076) | Supporting expression vector for the production of lentiviral and retroviral particles | Laboratory stock |
| pCMV TAT (C1077) | Supporting expression vector for the production of lentiviral particles | Laboratory stock |
| gag-pol (C1078) | Supporting expression vector for the production of retroviral particles | Laboratory stock |

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## Figures

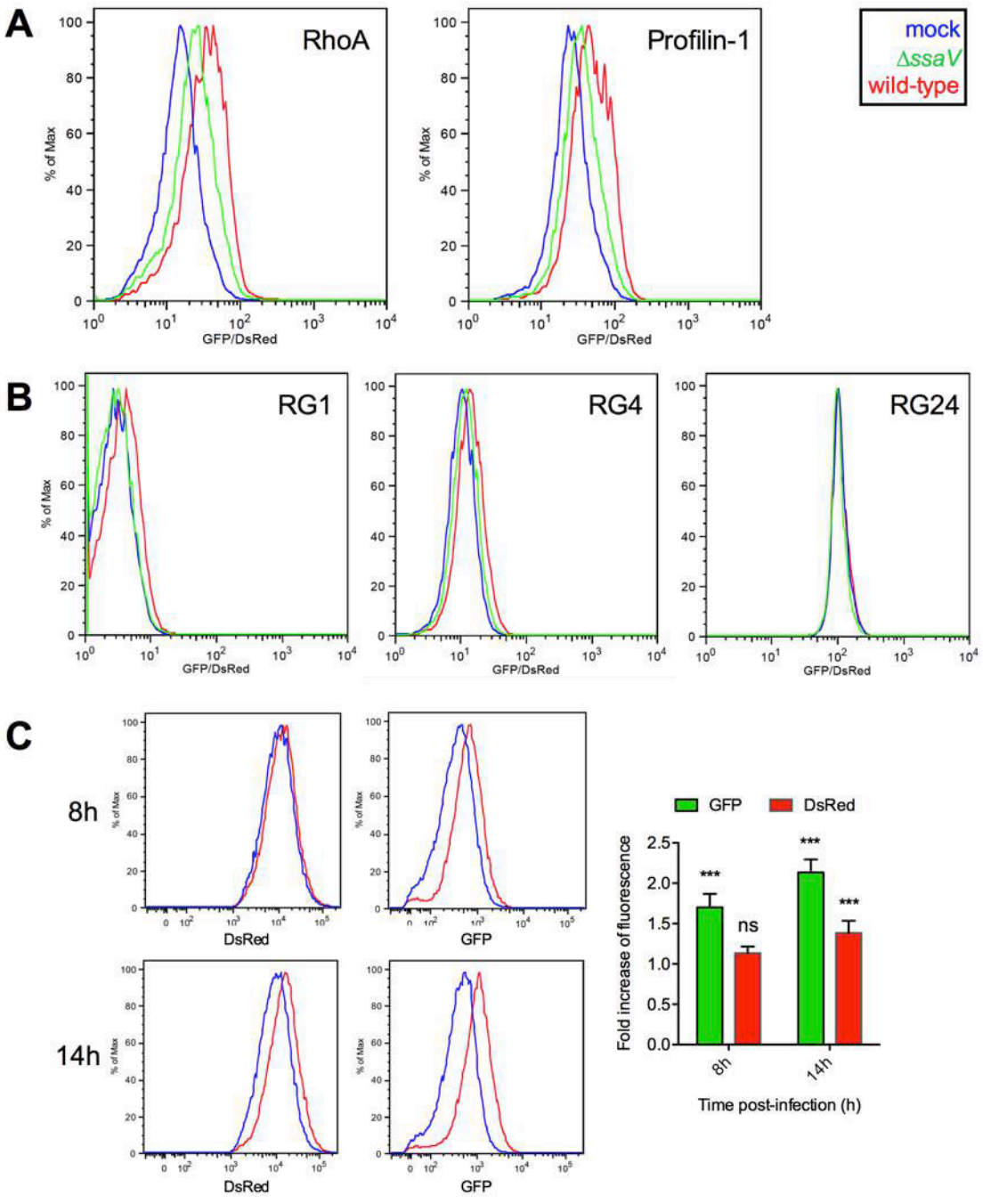
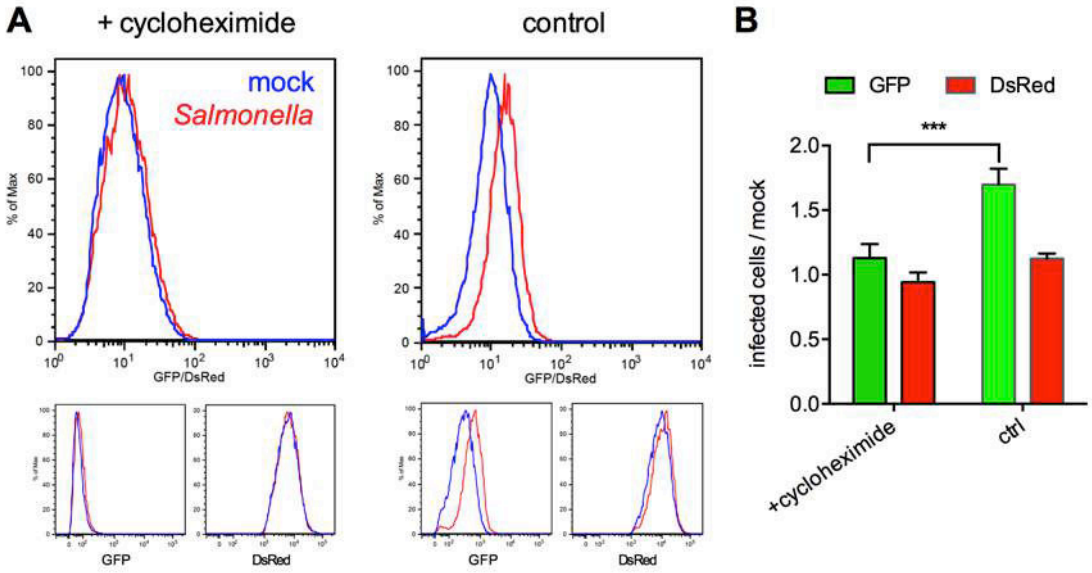



Figure 1


A
+ cycloheximide
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Salmonella
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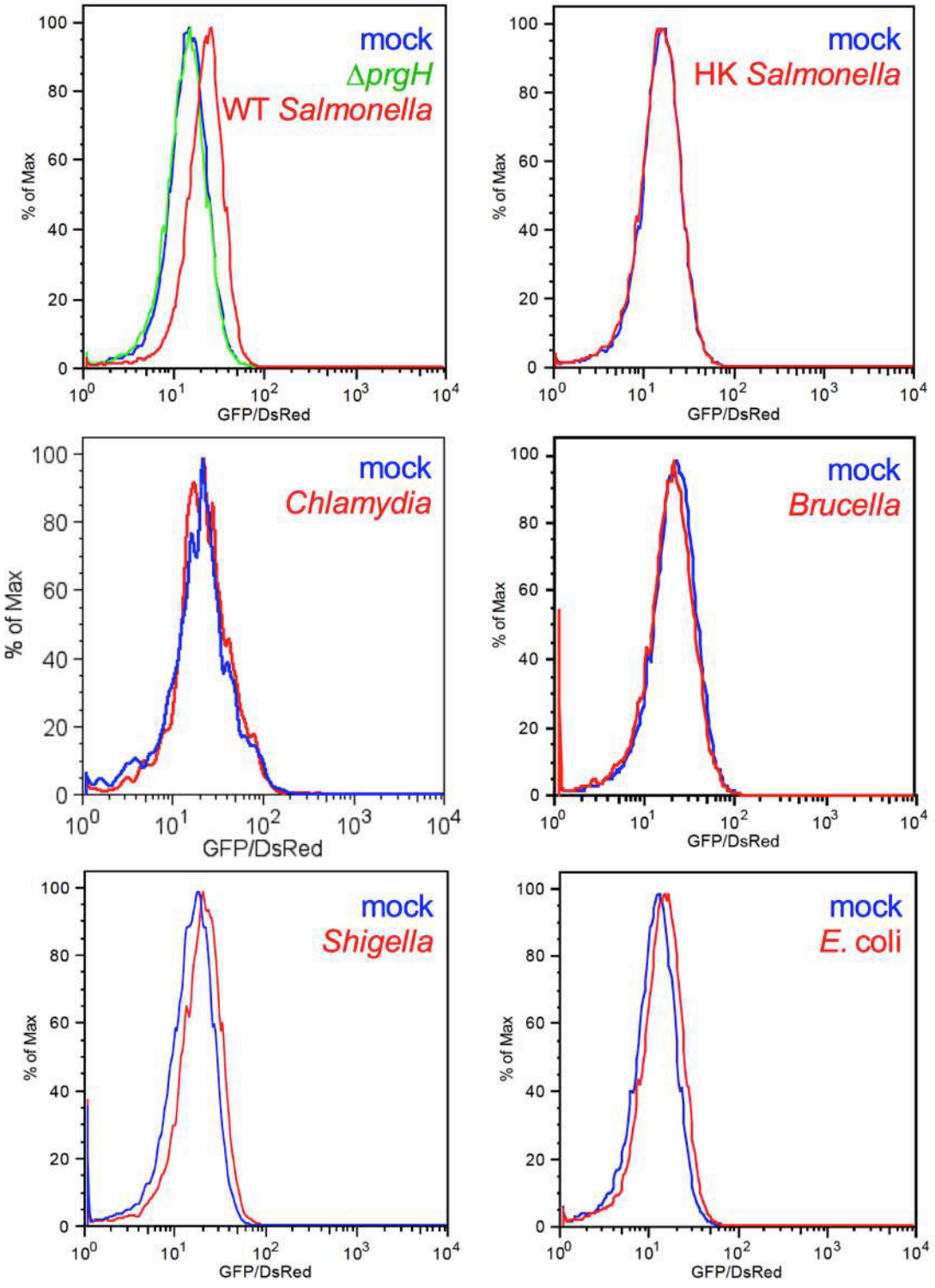
Figure 2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WT Salmonella
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E. coli
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GFP/DsRed

Figure 3

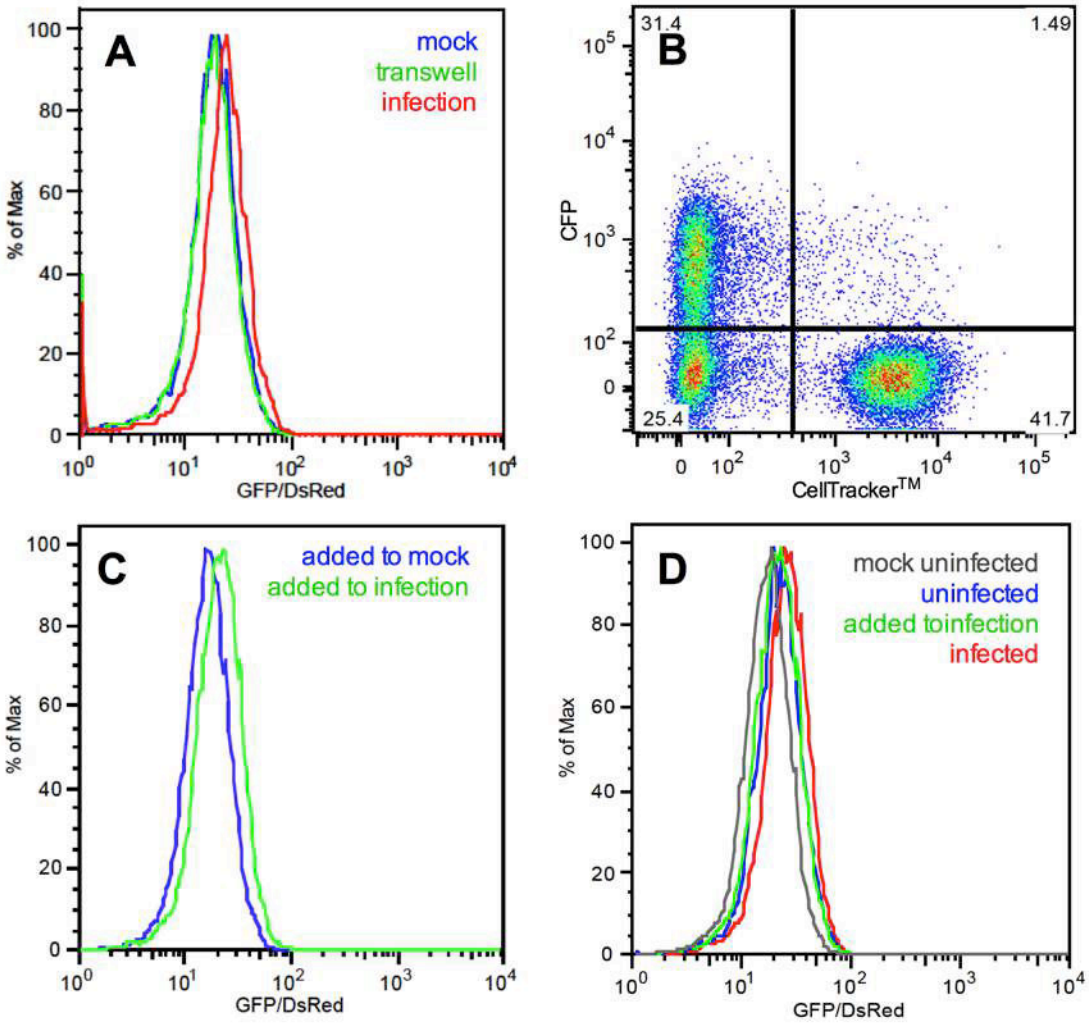
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Figure 4

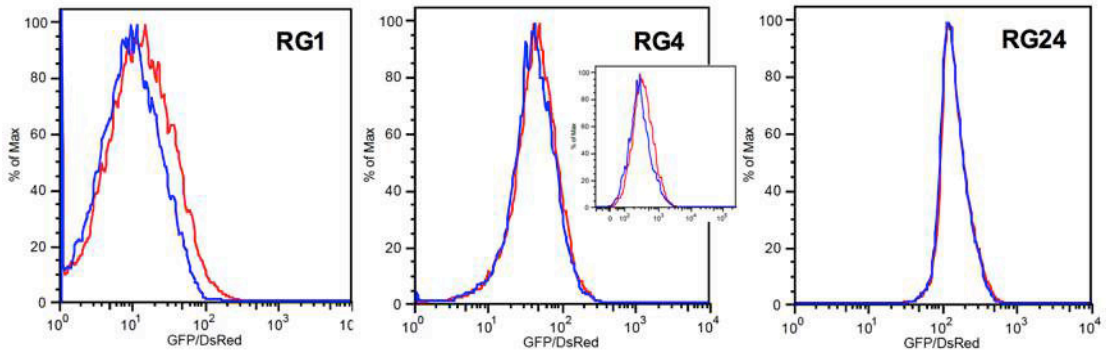


Figure 5

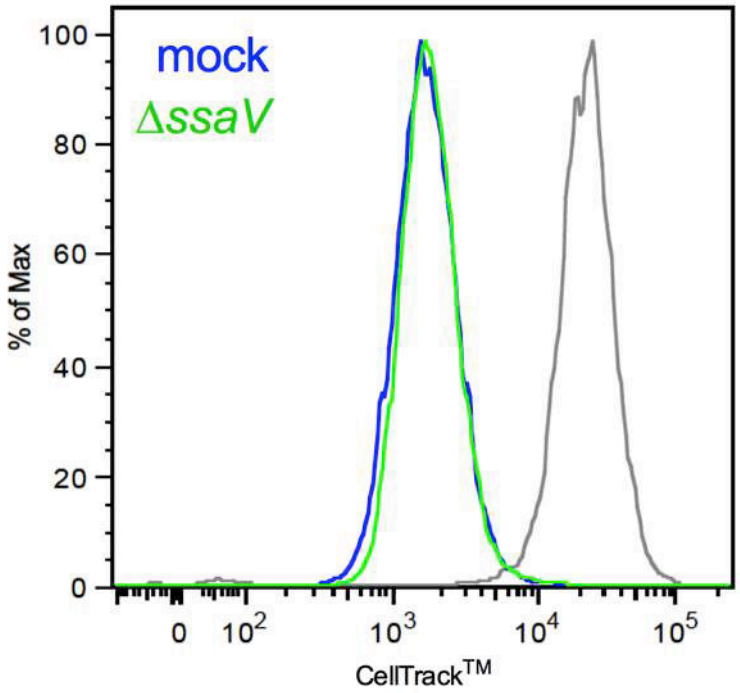


Figure S1

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### *Additional reflections about the GPS*

In this study, we show that the GPS is – in cell lines derived from epithelium – susceptible to the administration of certain bacteria. An increase in the synthesis of new protein is the consequence of the recognition of these bacteria. We propose that different mechanisms contribute to the recognition of extracellularly administered bacteria and the different intracellular pathogens. However, the host cells react the same way; the levels of GFP and DsRed rise. As for intracellular pathogens, the signal gets equally transferred to cells that are in direct contact with the infected cell. These perceptions are extremely interesting, especially when considering recent findings on the communication of epithelial cells upon infection [139,140].

Cell communication is the basis for coordinated cellular activity and hence implicated in processes such as development and tissue homeostasis and also reactive processes as wound repair and infection. It embraces ways to communicate with neighboring cells (juxtacrine signaling) but also signals that can reach distant cells (endocrine signaling). Depending on the cell type and the distance between the signaling partners, the communication tool changes. Communication can be established via the connection of the cytoplasm of neighboring cells via gap junctions, by synaptic signaling, redox signaling and the secretion of second messengers that can stay attached to the cells or can be set free [141-145]. Besides, many cell types as immune cells or neurons present a specialized, very curious way of direct communication through tunneling nanotubes (TNTs). These structures have a length of up to 100 µm and serve for signaling or the exchange of subcellular structures, but can be induced or hijacked by pathogens for intracellular spread [146]. Epithelial cells exhibit a similar, but longer and more stable subcellular communication structure, called epithelial bridges, which can be used for transfer of cellular components or cell migration [147,148].

The intestinal epithelium plays an important role in the defense against intestinal pathogens, tolerance to and regulation of the commensal microbiota. Together with a thick mucus layer that is loaded with anti-bacterial components, it presents a physical, almost impassable barrier and its integrity is crucial for the homeostasis of the intestinal immune system (see Introduction). The intestinal epithelial cells deploy tight contact between neighboring cells, rapid cell turn-over, shedding of infected cells into the

intestinal lumen and digestion of the invading bacteria as their primary arms [149]. They also induce the first wave of immune response to a pathogen by secreting signaling molecules that attract immune cells. In order to fulfill their functions, a close communication among them and with their environment is necessary. Recently signaling via gap junctions and redox signaling upon infection was shown to contribute strongly to alert the innate immune system [139,140]. We propose that the mechanisms of communication between epithelial cells are fanned out much more and can be induced by many different signals. It also seems that the reaction does not only contain the secretion of some few cytokines, but is broader positioned.

We don't know yet which bacteria trigger which mechanisms and how these signals are processed and transferred. But we think that communication between epithelial cells, as the first layer of defense at the host's outmost frontiers, will be more valorized within the next years as an important reaction of the host on the infection.

# Chapter 5
# Conclusions

Even though there are vaccines and antibiotic treatments, *Salmonella* is still a major cause of severe diseases and death, especially in countries with bad sanitary conditions and less developed health care systems.

*Salmonella*'s infectiousness and hazardousness comes from its capacity to survive within host cells, whereupon it also targets cells of the immune system. A fact that counts heavy is that especially macrophages are one of its main niches during systemic infection.

During my thesis I worked on several aspects of the infectivity of *Salmonella*. One main focus was to understand how *Salmonella* develops and maintains its intracellular niche, the SCV, and the tubular network that arises from it, and by what means it interacts with late endosomes and lysosomes. As a systemic infection stands and falls with *Salmonella*'s capacity to develop its niche, this is one of the most challenging and thrilling aspects of the field.

The development, integrity and dynamics of the SCV membrane are a very complex issue. A big set of effector proteins is involved in these processes, with – what it seems to us – often contradictory functions and effects.

SseF and SseG are an example for the difficulties that someone can encounter trying to understand the dynamics of the SCV. They are two effector proteins that are known to act together. They are responsible for the positioning of the SCV next to the Golgi apparatus. Two theories have been proposed, a Golgi tethering model, where they interact with Golgi proteins and therefore attach the SCV to the Golgi apparatus, and a motor protein based model, where an interaction with dynein is responsible for the minus end movement and juxtanuclear positioning of the SCV. There are good arguments for both models and the answer is up to date unsolved. The identification of their interaction partners would certainly shed light to that issue and boost our understanding of tubule formation and membrane dynamics. SseFG were also described to be – somehow – implicated in the formation of SIFs and / or in their interaction with LE/lys, as a knockout of each of them leads to the disruption of LAMP-1 localization with SIFs. Here, we show that SseFG are responsible for the formation of LNTs. In a Δ*sifA* Δ*sopD2* strain, only the LNTs are present out of the three types of late tubules that *Salmonella* can induce upon infection. However, these LNTs are sufficient to provide a stable SCV and an additional knockout of SseFG, which leads to the disappearance of LNTs, reverts the beneficial

effect. This deteriorates the intracellular survival and replication in epithelial and macrophage like cell lines. A Δ*sifA* Δ*sopD2* Δ*sseFG* strain actually behaves exactly like a Δ*sifA* strain, which does not show any tubules either. These two strains, but also *sseF* and *sseG* strains, additionally lose their juxtanuclear position and get scatter throughout the cell. Considering that the effect of SifA on the formation of SIFs and the positioning of the SCV are mediated by its interaction with the SKIP-kinesin complex, one might suggest that also SseFG somehow target motor proteins. Our findings would then support the motor protein based model for SseFG, which proposes that the positioning of the SCV next to the Golgi apparatus depends on the interaction of SseFG with dynein and a fine-tuning of motor protein activities on the SCV.

The fact that LNTs are able to provide a stable SCV is puzzling. Their interaction with LE/lys is reduced, which is represented by a strongly decreased presence of LAMP-1 along them. However, during infection with a Δ*sifA* Δ*sopD2* strain enough membrane – supposedly of LE/lys – is recruited to stabilize the SCV and permits the growth of tubules. Most SCVs in a Δ*sifA* Δ*sopD2* infection are even positive for LAMP-1, although the tubular network is mainly not. We think that somehow host factors, such as the small GTPase Arl8B, which is in status quo predominantly activated, get weakly recruited to the LNTs and are sufficient for a low level fusion of LNTs with the LE/lys compartment. Subsequently, LAMP1 would be predominantly transported along tubules to the SCV. As LNTs are considered to be precursors of SIFs, the fact that Δ*sseFG* strains show SIFs with a reduced presence of LAMP-1 makes a lot of sense. The full development of *Salmonella*'s tubular network, the assuring of a stable SCV and the acquisition of enough new membrane to promote *Salmonella*'s replication needs the combined action of SseF, SseG and SifA, together with other effectors like PipB2, SteA and SopD2. The difference between a tubular network consisting only of LNTs and one consisting of all three kinds of tubules is therefore in the combined strength of action of many effectors in the raise of this network.

One particular player therein may be the C-terminal part of SifA. For a long time, its role was subject of speculation. Structural studies proposed it to be a GEF and after pull-down experiments people fancied it to interact with the small GTPase RhoA, an interaction that has never be proven functionally. In a recent paper, the host protein

Arl8B was described to get recruited on *Salmonella*'s tubular network. We tried to investigate this point in more detail and were able to show that during infection with the Δ*sifA* Δ*sopD2* strain an overexpressed Arl8B recruits to LNTs. The recruited Ar8B then turns out to promote LAMP-1 positive "LNTs". Arl8B is a small GTPase that is located on lysosomes and is – among other things – responsible for the fusion of late endosomes with lysosomes. The obvious thing was to test whether there was and interaction of SifA with Arl8B. We were able to proof this interaction, whereby we attempt to explain the difference between LNTs and SIFs in the recruitment of LAMP-1, representing the fusion of tubules with the LE/lys compartment.

As described, the interaction partners of SseF and SseG, effectors in which we got interested due to their role in the formation of LNTs, are not known up to now. The two proteins are tricky to work with, due to the presence of respectively two and three trans-membrane domains. As a lack of adequate methods for such hydrophobic proteins, which tend to precipitate in the aqueous milieu that is used for many biochemical methods or are hard to produce in tools such as *E. coli*, we tempted to apply a newly described method on this subject. The global protein stability profiling (GPS) allows the analysis of host cell targets of effectors during infection *in cellulo* via comparison of the change in the stabilities of a linked reporter (GFP) and an internal control (DsRed). By applying this method, we thought to circumvent the problem of the hydrophilic properties of SseFG.

Unfortunately, an artifact that appeared during the application of the GPS on infections did not allow us to elucidate what host proteins might be the target for SseFG. We observed a shift in the "stability" of our reporters (GFP and DsRed), which was independent of the function of T3SS-2 effectors. This artifact allowed us to discover a fascinating phenomenon. We were able to link this stability shift to an increase in protein synthesis (IPS) of GFP and DsRed, which we could inhibit by blocking protein translation at the ribosomes. This was intriguing and we tried to understand more about it. The IPS is not only induced by *Salmonella*, but also by other bacteria, such as the intracellular pathogen *Shigella flexneri* or the non-infectious *Escherichia coli*. Thus, it must be induced by different means at different localizations, as *Salmonella* resides in the SCV, *S. flexneri* in the cytoplasm of the host cell and *E. coli* does not even enter the cell.

Most interestingly, the signal that is induced by intracellular pathogens can be transferred from infected to non-infected cells, which are in direct contact with each other. The cell lines used in this study are derived from two different kinds of epithelium. Epithelial cells are known to interact tightly in order to maintain a functional barrier against invading organisms and to respond to external impacts. Pathogens that enter the host's body in the intestine often breach this barrier; this rupture of the equilibrium must then have consequences. Two recent papers describe specific signaling mechanisms of the epithelium from infected to uninfected neighbor cells; redox signaling after infection with *Listeria monocytogenes* and gap junction mediated signaling after infection with *S. flexneri, S.* Typhimurium or *L. monocytogenes*. We think, that the epithelial signaling is more sophisticated than that. Our findings are a first hint in this direction and our model, which is easy to apply, could be used to better understand receptors, signaling and cellular responses that are involved in this very first step of an immune response.

Altogether, this work contributes some interesting findings to understanding the biology of infections with *Salmonella*. We were able to describe some aspects of the function of its T3SS-2 effectors SseF, SseG and SifA – their role in tubule formation and in the membrane dynamics of the SCV. Besides, we showed an immune mechanism of epithelial cell, where cell communication leads to an increase in protein synthesis as response to the recognition of non-self molecules.

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