Salmonella enterica Remodels the Host Cell Endosomal System for Efficient Intravacuolar Nutrition

Graphical Abstract

Highlights
- Salmonella converts host cell endosomes into interconnected tubular vesicles called SIFs
- SIFs contain endocytosed medium compounds available to Salmonella
- Intracellular nutrition of Salmonella depends on access to endocytosed nutrients
- Connection to SIFs promotes intracellular replication of Salmonella

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In Brief
The intracellular proliferation of the vacuolar pathogen Salmonella enterica depends on conversion of the host endosomal system into a network of interconnected tubular vesicles. Liss et al. use single-cell analyses to reveal a role for this tubular network in conveying nutrients from the host endosomal system to vacuolar bacteria.
**Salmonella enterica** Remodels the Host Cell Endosomal System for Efficient Intravacuolar Nutrition

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**SUMMARY**

*Salmonella enterica* is a facultative intracellular pathogen that survives and proliferates in the *Salmonella*-containing vacuole (SCV), yet how these vacuolar bacteria acquire nutrition remains to be determined. Intracellular *Salmonella* convert the host endosomal system into an extensive network of interconnected tubular vesicles, of which *Salmonella*-induced filaments (SIFs) are the most prominent. We found that membranes and lumen of SIFs and SCVs form a continuum, giving vacuolar *Salmonella* access to various types of endocytosed material. Membrane proteins and luminal content rapidly diffuse between SIFs and SCVs. *Salmonella* in SCVs without connection to SIFs have reduced access to endocytosed components. On a single-cell level, *Salmonella* within the SCV-SIF continuum were found to exhibit higher metabolic activity than vacuolar bacteria lacking SIFs. Our data demonstrate that formation of the SCV-SIF continuum allows *Salmonella* to bypass nutritional restriction in the intracellular environment by acquiring nutrients from the host endosomal system.

**INTRODUCTION**

Bacterial pathogens have evolved a variety of intracellular lifestyles within mammalian host cells. *Salmonella enterica* is a frequent gastrointestinal pathogen causing diseases ranging from gastroenteritis to systemic typhoid fever. After host cell invasion or phagocytic uptake, *Salmonella* remains within a specialized membrane-bound compartment that permits bacterial survival and proliferation. The *Salmonella*-containing vacuole (SCV) has certain characteristics of late endosomal vesicles; however, the maturation of the SCV to a bactericidal compartment does not occur. *Salmonella* actively manipulates infected host cells, and a key virulence factor is the type III secretion system (T3SS) encoded by *Salmonella* pathogenicity island 2 (SPI2) (LaRock et al., 2015). Mutant strains defective in SPI2-T3SS are severely attenuated in systemic virulence in the murine infection model (Hensel et al., 1995) and show reduced intracellular replication in cell-based infection models (Hensel et al., 1998).

By action of SPI2-T3SS translocated effector proteins, vacuolar *Salmonella* induces the formation of specific tubular membrane compartments growing out of the SCV, called *Salmonella*-induced filaments (SIFs). SIFs were found in various cell types and possess several endosomal membrane markers, such as LAMP1, Rab7, and v-ATPase (Brumell et al., 2001b; Drecktrah et al., 2007; Rajashekar et al., 2008). Several SPI2-T3SS effectors were reported to manipulate the host endosomal system and induce vesicle fusion events with the SCV. The SPI2-T3SS effectors SifA, SseF, SseG, PipB2, SseJ, and SopD2 were reported to be involved in SIF biogenesis (Figueiredo and Holden, 2012). Effector mutant strains deficient in sifA, sseF, sseG, and pipB2 showed most striking SIF phenotypes when analyzed by fluorescence microscopy (Rajashekar et al., 2008, 2014). The key factor involved in SIF formation is the effector SifA (Stein et al., 1996). *Salmonella* deficient in sifA exhibits a defect in SCV integrity and is released into the host cytosol. A sifA deletion strain shows a similar replication defect in macrophages and attenuation in mice as observed for SPI2-T3SS-deficient *Salmonella* (Beuzón et al., 2000; Brumell et al., 2001a; Stein et al., 1996). An sifA sseJ double-mutant strain was reported to maintain the SCV membrane but still lack SIF formation, similar to an ssaV mutant strain (Ruiz-Albert et al., 2002). Strains deficient in sseF or sseG lead to the formation of SIFs with a thinner appearance and weaker LAMP1-GFP signal.

We recently investigated the dynamic and ultrastructural features of the intracellular habitat of *Salmonella*, focusing on the induced SIF structures. The data revealed that SIFs are highly dynamic structures that contain various types of endosomal cargo (Rajashekar et al., 2008; Zhang and Hensel, 2013). The ultrastructure of SIFs is surprisingly complex, indicating a unique mechanism to remodel host cell membrane compartments through SPI2-T3SS effector proteins (Krieger et al., 2014). Proteomic studies suggested that *Salmonella*-modified membranes can incorporate vesicular membranes of diverse cellular origins (Santos et al., 2015; Vorwerk et al., 2015).

The formation of SIFs was shown to coincide with the onset of bacterial replication, though the functional contribution is not understood. Prior studies postulated several possible functions of
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SIFs (LaRock et al., 2015). One of these studies proposed that the SPI2-T3SS-dependent fusion of endosomes with Salmonella-induced compartments is required to maintain the integrity of SCVs for the growing intracellular bacterial population (Beuzón et al., 2000). We speculate that fusion events also provide nutrients enabling intracellular replication of vacuolar Salmonella. Here we investigated, on a single-cell level, the dependency of intracellular replication of Salmonella within the SCV on SPI2-T3SS-induced fusion of endosomal compartments. We provide several lines of experimental evidence that intracellular Salmonella convert the host cell endosomal system into a continuum that provides access to nutrients.

RESULTS

The SCV Is Accessible to Endosomal Cargo

To follow the fate of Salmonella within the SCV, we used live-cell imaging (LCI) of infected host cells after pulse chase (P/C) with endocytic probes (EPs). LAMP1-GFP-expressing host cells allowed the identification of SCVs and SIFs. BSA-rhodamine or BSA-rhodamine-gold nanoparticles (NPs) were used as EPs. EPs were added overnight (O/N) prior to infection or for 1 hr at 6–7 hr post-infection (p.i.) (Figure 1A) and we observed efficient uptake by the cells and distribution within the endosomal system (Figure 1B). At 8 hr p.i., Salmonella wild-type (WT) and SPI2-T3SS-deficient ssaV strains were both located in intact SCVs, while the sifA strain was predominantly without SCVs. An extensive tubular LAMP1-GFP-positive network was induced in WT-infected HeLa cells but was absent in ssaV- or sifA-infected cells (Figure 1B). Next, LAMP1-GFP-expressing RAW264.7 macrophages were interferon γ (IFNγ) activated and P/C with EPs was performed, followed by infection and LCI (Figure 1C). Activated macrophages show a higher degree of endosomal tubulation (see mock-infected cell); however, in Salmonella WT-infected cells the induction of a complex network of LAMP1-positive tubular vesicles was obvious. This phenotype was absent in macrophages infected with the ssaV strain. The SIF networks in HeLa and RAW264.7 cells were positive for endocytosed EPs, as previously described (Drecktrah et al., 2008; Rajashekar et al., 2008; Zhang and Hensel, 2013). To analyze accessibility of the SCV to endosomal content, we quantified the amount of EPs delivered to the lumen of the SCV (Figure 1D). Delivery of EPs was observed for SCVs containing WT or ssaV-deficient Salmonella. EP delivery to sifA-containing SCVs was detectable 4 hr p.i., but not at later time points, in line with the reported loss of the SCV integrity (Beuzón et al., 2000). The amounts of EPs within the SCV volume of WT or ssaV were largely identical at 8 hr p.i. if P/C was performed O/N, and slightly higher EP amounts were present in WT SCVs compared to ssaV SCVs with P/C at 6–7 hr p.i. and imaging at 8 hr p.i. Notably, the shorter the interval between EP pulsing and imaging, the stronger the difference in fluorescence intensity (FI) between WT SCVs and ssaV SCVs (Figure 1E). To probe the accessibility of Salmonella within SCVs to endosomal cargo, we performed live-cell correlational light and electron microscopy (CLEM) (Krieger et al., 2014) and used rhodamine-mediated 3,3′-diaminobenzidine (DAB) precipitation as marker for ultrastructural analyses (Figure 1F). DAB precipitates were observed in the WT SCVs and ssaV SCVs, and precipitates were located between the SCV membrane and the bacterial envelope. Deposition of DAB precipitates appeared localized in ssaV SCVs, while DAB labeling of the entire SCV and connecting SIFs was found in WT SCVs. These data demonstrate that SCVs are accessible to fusions with endosomal/lysosomal vesicles, regardless of the function of the SPI2-T3SS. However, SPI2-T3SS function mediates a faster access, probably due to highly increased frequency of vesicular fusions.

Membranes and Luminal Content of SIFs and SCVs Are Rapidly Interchanging

Our observations suggest an extensive and connected network of endosomal compartments in Salmonella-infected cells. To analyze interchange within this network, we performed fluorescence loss in photobleaching (FLIP) experiments (Figures 2A–2C). If EPs inside distal parts of SIF networks were repeatedly photobleached, FI in the entire network and connected SCVs decreased dramatically as a function of bleaching. Only EP-containing vesicles not connected to the SIF network maintained signals, and FI of non-bleached SIF networks in neighboring cells was not affected. From these results, we conclude that lumina of the SIF network and SCVs are connected and interchanging.

SIFs rapidly extend, branch, or collapse (Drecktrah et al., 2008; Rajashekar et al., 2008). To analyze the kinetics of...
distribution of membranes and luminal content of SIFs and SCVs, fluorescence recovery after photobleaching (FRAP) experiments were performed. To trace membranes of SIFs and SCVs, host cells permanently expressing LAMP1-GFP as SIF membrane marker were used. For labeling of the SCV and SIF luminal content, P/C with EP Dextran-Alexa Fluor 488 (D488) was performed. Sections of SIFs distal to the SCVs were photo-bleached and recovery of LAMP-GFP or EP signals was determined (Figures 2D–2G). FRAP after P/C with EPs indicated a very rapid exchange of the luminal content of SIFs (Figure 2E). Bleached membrane markers of SIFs also recovered rapidly (Figure 2D), and recovery was independent from the position of the bleached area within the SIF network. About 40%–50% of the initial FI was recovered (Figures 2F and 2G). The kinetics of recovery of SIF membranes and of luminal content at various time points p.i. were comparable within the dataset (Figures 2D and 2E); thus, they were not affected by the different dynamics of SIFs early or late in infection as previously reported (Drecktrah et al., 2008; Rajashekar et al., 2008). This indicates that integral membrane proteins within SIF membranes as well as the luminal content of SIFs can rapidly interchange. We hypothesized that SCVs connected to SIFs would be subject to higher degrees of interchange compared to SCVs without connection to SIFs. The recovery of SIF membranes and luminal content was again analyzed in FRAP experiments (Figure 3). Quantification of FRAP of SIFs containing WT bacteria with or without connection to SIFs was performed for cells expressing LAMP1-GFP or after P/C with EP D488. In addition, SCVs were analyzed containing ssaV or sifA ssaJ mutant strains, both unable to induce SIFs but maintaining an intact SCV (Ruiz-Albert et al., 2002). Rapid recovery of luminal EPs in the SCV was only observed for SIFs containing WT bacteria and if connected to SIFs (Figure 3A). FRAP was about 35%, while recovery of EPs in SCVs without SIF connection was less than 5% of initial FI (Figure 3B). Finally, FRAP of membrane marker LAMP1-GFP in SIF membranes was compared for WT and ssaV-containing SCVs. FRAP was slower than for luminal EPs, but again dependent on the function of SPI2-T3SS (Figures 3C and 3D).

Taken together, these data demonstrate that membranes and luminal content of the SIF network are dynamically interchanging with the SCV. This SPI2-T3SS-dependent interchange allows the fast access of Salmonella to endocytosed material.

Intracellular Salmonella Converts the Host Cell Endosomal System to an Extensive Interconnected Network

Our previous studies (Zhang and Hensel, 2013) showed that SIFs are accessible to various types of EPs regardless of the time point of P/C, and data reported here demonstrate that SIF formation leads to rapid interchange of membrane and luminal material with the SCV. We hypothesized that an extensive endosomal compartment is formed with a continuum of SIF and SCV membranes as well as luminal content. The conversion of the normal host cell endosomal system by intracellular Salmonella was analyzed in a volume-based approach (Figure 4A) by quantification of EP-positive objects and the relative increase in surface area of Salmonella-modified membranes (SMMs). Over the time of intracellular activity of Salmonella, we observed a decreased number of endosomal compartments, as previously described (Rajashekar et al., 2008). This was accompanied by a steady increase in the surface areas of Salmonella-induced compartments (Figure 4A). As resolution of light microscopy does not allow unequivocal detection of a vesicular continuum, we applied the previously established live-cell CLEM approach (Krieger et al., 2014) to link the live-cell analyses of dynamics of SIFs and SCVs to ultrastructural delineation of the membrane organization (Figure 4B). Live-cell images of LAMP1- and EP-positive compartments were registered (Figure 4Ba) and correlated to stitched sets of transmission EM (TEM) images (Figure 4Bb), allowing a precise localization of the SCV and tubular compartments (Figure 4Bc). Higher magnifications confirmed the double-membrane organization of SIFs we previously reported (Krieger et al., 2014) and revealed a continuum of SIFs as well as SIF and SCV membranes at various positions investigated (Figures 4Bd–4F). Based on these observations, we modeled the dimensions of SCV/SIF endosomal continuum, using diameters of SIFs and SCVs as determined by TEM and the overall length of the SIF network as determined by light microscopy (Figures 4C and 4D). For double-membrane SIFs induced by WT Salmonella, based on previous results (Krieger et al., 2014) we considered only the outer SIF lumen positive for EPs as continuous with the SCV lumen (see Figure S3 for details). For comparison, dimensions of SCVs without connection to the SIF network were used. On average, 71- and 131-fold increases in luminal volume and membrane surface area,
respectively, were determined for SCVs connected to an SIF network. We also analyzed cells infected with the sseF mutant strain, which induces a network of SIFs smaller in diameter and only composed of single-membrane tubules (Krieger et al., 2014; Rajashekar et al., 2014), thus, only a single SIF lumen. Here, only 34- and 37-fold increases of luminal volume and membrane surface area, respectively, were determined in comparison to WT SCVs without SIFs.

Taken together, these results demonstrate that WT Salmonella has access to a large volume of luminal content due to the SPI2-T3SS-dependent exchange within an extensive vacuolar continuum. Compared to WT, the sseF strain has access to a lower SIF volume due to the induction of narrower single-membrane SIFs.

Probing Access of Internalized External Compounds to SIFs and SCVs

Our P/C experiments with EPs suggest that internalization of external material and distribution within SIF network and SCVs is rather non-selective. The internalized compounds may be beneficial for Salmonella if acting as a nutrient, but may also be detrimental if conferring antimicrobial activity. We tested both scenarios.

Salmonella can utilize various C-sources and rapidly adjust metabolic pathways to the available C-source. However, in contrast to E. coli, S. enterica typically lacks the ability to utilize lactose. Also, RAW264.7 macrophages do not metabolize lactose as C-source (Figure S4A). We generated a Salmonella strain able to grow on lactose as sole C-source by cloning and transferring the lac operon of E. coli to Salmonella. Using lac+ Salmonella, we tested if lacZ-encoded β-galactosidase (β-Gal) activity of intracellular bacteria can be induced by extracellular application of lactose after infection (Figures 5A and 5B). Intracellular WT Salmonella showed an increased β-Gal activity compared to ssaV or sifA strains if lactose was externally added 1 hr p.i. Glucose-free cell culture medium was used from 1 hr before infection to restrict availability of C-sources. The results revealed lacZ induction in Salmonella dependent on SPI2-T3SS and SifA function (Figure S4B). While the deletion of neither ssaV nor sifA had a measurable impact on β-Gal activity in vitro, differences were highly significant during growth in RAW264.7 macrophages (Figure 5B). Addition of glucose or lactose also increased intracellular proliferation of WT Salmonella, but to a far lesser extent the proliferation of ssaV or sifA strains (Figure S4C). In HeLa cells, high β-Gal activities per bacterial cell were observed for lac+ strains, but induction was independent from SPI2-T3SS function (Figure S4D).

We next investigated if extracellular C-sources directly affect replication of intracellular Salmonella (Figures 5C and 5D). Prior to infection, RAW264.7 cells were cultured in medium lacking glucose, then infected, and incubation from 1 to 8 hr p.i. was performed in presence or absence of glucose. Intracellular replication of all strains was highly reduced (~2-fold) in the absence of glucose. Presence of glucose in the culture medium restored 5.5-fold intracellular replication of the WT strain, but not of the ssaV strain (2.1-fold). Addition of glucose resulted only in a small increase (2.7-fold) of replication of the sifA sseJ mutant strain, while an intermediate replication rate was determined for the sseF strain (3.8-fold). These data show that formation of the SIF network enables acquisition of external C-sources for nutrition of intracellular Salmonella.
We also speculated that the access of intracellular *Salmonella* to external medium would have detrimental effects if antimicrobial substances are present in the medium. Gentamicin is an antibiotic that cannot penetrate eukaryotic cell membranes and is commonly used to kill extracellular bacteria. Gentamicin protection assays are usually experimentally used to quantify bacterial invasion and intracellular replication. Here, we investigated the effect of increased gentamicin concentrations on intracellular *Salmonella*. While gentamicin protection assays are routinely performed with medium containing 10 μg/mL gentamicin, we increased gentamicin concentration up to 200 μg/mL and determined the effect on intracellular replication of WT and SIF-lacking *Salmonella* (Figure 6). We performed cell viability assays and excluded detrimental effects of high antibiotic concentration on the host cell viability (Figure S5A). In line with previous reports, with 10 μg/mL gentamicin intracellular proliferation of WT *Salmonella* was 5.1- and 12.0-fold higher compared to the ssaV strain, and 1.3- and 8.8-fold higher compared to the sifA sseJ strain in HeLa and RAW264.7 infection models, respectively. If gentamicin was increased to 100 μg/mL, intracellular proliferation of WT, ssaV, and sifA sseJ was almost identical, while assays performed with 200 μg/mL revealed improved intracellular survival and proliferation of the ssaV and sifA sseJ strains. Increased gentamicin concentrations did also affect intracellular proliferation of the sseF-deficient strain (Figure S5B). Since gentamicin is a primary bactericidal antibiotic, these results indicate that the SIF/SCV continuum induced by WT *Salmonella* increases the exposure of bacteria in the SCV to antibiotics that are internalized by endocytosis rather than due to formation of persisters of the ssaV strain. To exclude antibiotic-specific effects, we conducted such protection assays with polymyxin B and cefotaxime as further antibiotics that do not penetrate the mammalian plasma membrane. We obtained results similar to gentamicin with increased concentrations of both antibiotics (Figures S5C and S5D).

These findings are in line with the hypothesis that *Salmonella* in SCVs with SIF connection, i.e., WT and sseF strains, are exposed to internalized antibiotics. The ssaV or sifA sseJ strains do not induce this continuum but remain largely secluded from endocytosed portions of medium and thus from the high amounts of antibiotics applied here.

**Connection of SCVs to SIFs Is Required for Efficient Intracellular Metabolism**

To directly analyze the physiological consequence of SIF formation for *Salmonella* within the SCV, we performed photoconversion experiments of intracellular *Salmonella*. Analyses of mKikumeGR with photoconversion from green to red fluorescence have previously been used to analyze the metabolic state of the parasite *Leishmania major* in response to innate immune defense (Müller et al., 2013). Here we interrogated the metabolic state of vacuolar *Salmonella* on a single-cell level. We anticipated that within metabolically active bacteria, photoconverted red fluorescent mKikumeR is continuously diluted due to growth and division of bacteria, concomitantly with re-synthesis of green fluorescent mKikumeG. In contrast, in *Salmonella* with low metabolic activity and slow intracellular replication, no dilution of mKikumeR and replacement by mKikumeG should take place (Figure 7A). If bacteria were grown in vitro on agarose pads, replacement of mKikumeR by mKikumeG was dependent on bacterial growth and independent from function of the SPI2-T3SS (Figure S6).

*Salmonella* strains synthesizing mKikumeG under control of a constitutive ribosomal promoter (Habuchi et al., 2008) were used for infection experiments with HeLa and RAW264.7 cells. At 3 hr p.i. (HeLa) and 6 hr p.i. (RAW264.7), *Salmonella* within the SCV with or without connection to SIFs were identified and mKikumeG was photoconverted to mKikumeR. Subsequent time-lapse microscopy revealed that WT bacteria within SCVs connected to an SIF network showed dilution of mKikumeR and re-synthesis of mKikumeG, indicating metabolic activity while WT in SCVs without SIF connection showed strongly decreased metabolic activity. Bacteria treated with chloramphenicol (Figure 7) showed very low replacement of mKikumeR by mKikumeG. This was observed for both infected HeLa and RAW264.7 host cells. The ssaV strain also showed decreased metabolic activity with a stronger attenuation in RAW264.7 macrophages. Interestingly, the sifA sseJ strain, unable to form SIFs but having all other SPI2-T3SS effectors, showed higher metabolic activity when compared with ssaV mutant, indicating functional benefits of other SPI2-T3SS effectors.

These data demonstrate that *Salmonella* in SCVs connected to SIFs are metabolically active, while *Salmonella* in SCVs without SIFs show highly reduced metabolic activity.

**DISCUSSION**

Despite being confined to a membrane-bound compartment within host cells, bacterial pathogens such as *Salmonella* are capable of rapid intracellular proliferation. This feature is dependent on the proper spatiotemporal activation of virulence factors that modify host cell functions. However, how these manipulations are linked to intracellular proliferation is mostly unknown. A central requirement for intracellular proliferation within the SCV is the availability of nutrients and absence of antimicrobial functions.

Dependent on SPI2-T3SS function and a subset of effector proteins, intracellular *Salmonella* converts the host cell endosomal system into an extensive interconnected tubular network growing from the SCV. This also results in a massive increase of membrane surface and luminal volume that is in interchange with the SCV. If SCVs are connected to the tubular network, membrane components and, more importantly, the luminal content can freely interchange between SIFs and SCVs. Our data suggest that virtually all endocytosed material is entering the tubular network, and by this is available for access by *Salmonella* within the SCV. We demonstrate that this interchange is required for the efficient intracellular replication of vacuolar *Salmonella*, while lack of interchange leads to reduced intracellular proliferation.

A unique feature of SIFs is the double-membrane structure, resulting in an inner SIF lumen that is most likely derived from host cytosol and not interchanging with *Salmonella* in the SCV. The outer lumen, however, is generated from various types of endosomal cargo and is in continuity with the SCV lumen.

We recently showed that the replication defect of amino acid auxotrophic *Salmonella* strains in host cells can be
HeLa cells expressing LAMP1-GFP were infected with Salmonella expressing GFP (A) or mCherry (B) and pulse chased with rhodamine-labeled NPs (A) or BSA (B).
complemented by addition of external amino acids if bacteria are able to induce SIF formation (Popp et al., 2015). Here we demonstrate that SIF formation allows access to C-sources for growth of intracellular Salmonella. We and others have previously reported that SIFs contain markers that are indicative for various types of endosomal compartments. The proteome of Salmonella-modified membranes showed organelle signatures indicating that intracellular Salmonella recruit diverse types of host organelles for fusion to the SCV/SIF continuum (Vorwerk et al., 2015). This diversity of fusion events will also result in a diversity of luminal content and nutrients delivered to the SCV and available to Salmonella. Interaction of the SCV with ER membranes has recently been detected in a study combining quantitative proteomics and CLEM of individual bacteria in the initial phase of intracellular life. The interaction with ER-derived COPII vesicles was considered to be decisive for maintaining the SCV or SCV/SIF continuum (Santos et al., 2015).

Analyses of intracellular nutrition of Salmonella in a mouse typhoid fever model by proteomics, microbial genetics, competitive infections, and computational approaches indicated that Salmonella has access to a set of at least 31 different nutrients (Steeb et al., 2013). Salmonella colonization was mainly dependent on utilization of glycerol, fatty acids, N-acetylgalactosamine (GlcNAc), gluconate, glucose, lactate, and arginine (Steeb et al., 2013). Several nutrients were exploited simultaneously by Salmonella, in contrast with most microbes, which utilize only a single preferred nutrient when exposed to nutrient mixtures (Poncet et al., 2009). One explanation was the scarce availability of individual nutrients, thus demanding that the intracellular Salmonella population uses several nutrient sources at the same time (Steeb et al., 2013). As intracellular ensembles were analyzed, phenotypic heterogeneity of individual intracellular Salmonella was not addressed. In RAW264.7 macrophages, nutrient limitation in the SCV could be relieved by adding external glucose or mannitol to the cell culture medium.

(A) Quantification of endosomal compartments by LCI at various time points p.i. with Salmonella WT as indicated. 3D volume reconstruction was performed, and number of rhodamine-positive objects and relative increase in surface area of Salmonella-modified membranes (SMMs, yellow) were quantified for representative cells.

(B) CLEM analyses of SIF and SCV continuity in a Salmonella-infected HeLa cell. LCI was performed and cell fixed and processed for TEM. Several ultra-thin sections were stitched to generate a cell section with complete SIF network. SIFs were pseudo-colored orange, and magnified details (partially pseudo-colored) show the double-membrane organization of SIFs. Inner and outer SIF membranes were indicated with open and filled arrowheads, respectively. Scale bars, 10 μm (a) and 250 nm (d–f).

(C) Determination of the luminal volume and overall endosomal membrane area of the SCV/SIF continuum. The dimensions of bacteria and of SCV and SIF network were measured by means of TEM micrographs and 3D fluorescence images. Volumes and membrane areas were calculated by simplification of the bacterial body, the SCVs, and SIFs to spheres and cylinders as described in Figure S3. For defining the volume of SCVs, the volume of Salmonella within SCVs was subtracted. For the outer volume of WT double-membrane SIFs, the volume of the inner tubule was subtracted. For comparison of SIF networks, cells with similar SIF length of 350 μm were used, either harboring WT or sseF strains.

(D) Overall membrane areas and volumes of the SCV/SIF continuum were determined for cells harboring WT Salmonella with or without connection to SIFs, or cells infected with the sseF strain. Values for SCVs without SIFs were set to 1 and x-fold increase of SCV/SIF continuum was calculated.

Statistical analysis was performed as for Figure 2 comparing SCVs without SIFs to SCVs with SIFs. SDs were calculated from error propagation of the measured length and width.
followed by incubation with medium containing gentamicin at 10

determined 2 and 16 hr p.i. and intracellular replication is the CFU ratio of 16 to 2 hr. Depicted are means and SD of four biological replicates, each performed in

triclicate. Statistical analysis was performed as for Figure 2.

4 hr p.i. (Steeb et al., 2013). Complementation of amino acid auxotrophies by extracellular amino acids (Popp et al., 2015),

expression by external lactose, and dependency of growth on extracellular glucose are in line with this

findings. Interestingly, most nutrients used by intracellular Salmo-

ella are usually products of macromolecule hydrolysis within the host cell. Glycerol and fatty acids are derived from lipids, GlicNac is part of glycans/glycoproteins, and most amino acids are generated by proteolysis (Steeb et al., 2013). Based on our

findings, we propose that vacuolar Salmonella retrieve host

utrients by means of endosomal remodeling, fusion with endo-

somes/lysosomes containing degraded macromolecules, and/ or degrading macromolecular cargo present in transport vesicles

using bacterial hydrolytic enzymes. Glucose and lactate are usually delivered from the blood (Abu Kwaik and Bumann, 2015), and vacuolar Salmonella could have gained access to these host-derived nutrients also via the endosomal pathway.

Various vacuolar pathogens have evolved different strategies for acquisition of host-derived nutrients. However, the mechan-

isms of transport are not completely understood (Abu Kwaik and Bumann, 2015). The massive endosomal remodeling caused by Salmonella is one of the strategies of intracellular pathogens to adapt to life in mammalian host cells. However, it is very possible that other pathogens are also connected to distinct types of mem-

brane networks that have to be revealed by identifying suitable host cell markers. Legionella pneumophilia fuses with ER-derived secretory vesicles, associates with mitochondria, and later inter-

acts with ER membranes (Isaac and Isberg, 2014; Prashar and Terebiznik, 2015). Besides recruiting various host organelles to the Legionella-containing vacuole, Legionella exploits the protein degradation system of the host cell for using amino acids as a nutrient source (Price et al., 2014). Thus, Legionella probably deploys several nutritional strategies and endosomal tubulation is not necessary. Other vacuolar pathogens are known to recruit and interact with host organelles or organelle-derived vesicles for nutrient acquisition. Chlamydia trachomatis recruits exocytic vesicles, MVB, and mitochondria as well as ER-derived lipid droplets (Elwell and Engel, 2012; Saka and Valdivia, 2010).

Figure 6. SPI2-T3SS-Dependent Endosomal Remodeling Increases Exposure of Intracellular Salmonella to Endocyted Antibiotics

Intracellular replication of Salmonella was determined by the gentamicin protection assay. HeLa cells (A) or RAW264.7 macrophages (B) were infected with Salmonella WT, ssAV, or sifA ssAV strains at an MOI of 1. After infection, medium with 100 μg x mL⁻¹ gentamicin was used to kill extracellular bacteria for 1 hr, followed by incubation with medium containing gentamicin at 10 μg x mL⁻¹ (standard condition), or at 100 or 200 μg x mL⁻¹. Intracellular CFU counts were determined 2 and 16 hr p.i. and intracellular replication is the CFU ratio of 16 to 2 hr. Depicted are means and SD of four biological replicates, each performed in

triclicate. Statistical analysis was performed as for Figure 2.

Mycobacterium tuberculosis mainly interacts with early endo-

somes (Stanley and Cox, 2013). However, the formation of tubular networks for any of these pathogens has yet not been reported.

Recently, formation of tubovesicular compartments (TVCs) was described for liver cells infected with the malaria parasite Plasmodium spp. (Grützke et al., 2014). These dynamic exten-
sions of the Plasmodium-containing vacuole (PCV) were decorated with proteins secreted by the parasite, and co-localization with several organelle markers for early and late endosomes as well as autophagosomes was observed. Further work has to

reveal if TVCs contribute to Plasmodium nutrition in the PCV or

mediate the connection between the PCV with the extracellular space as previously discussed (Baumeister et al., 2010).

Our observations can also resolve the paradox of fusion of the SCV with late endosomes and lysosomes (McGourty et al., 2012). These vesicles normally deliver antimicrobial and proteolytic activi-
ties to the maturing endosomes. In case of presence of a large interconnected network, such antimicrobial activities would be rapidly diluted by mixing with the content of the network, probably resulting in a decrease below a critical local activity. Other cellular mechanisms for avoidance of antimicrobial activities by intracellular Salmonella have been proposed, but the dilution by mixing with luminal content of SIFs could provide a simple phys-

iological explanation. In addition to supply of vesicular membran-

es for the growing intracellular population and the provision of nutrients, SIF formation would provide a further protective effect by reduction of exposure to bactericidal host activities.

Our observations give rise to many new questions. After several

hours of intracellular growth, a stable SIF network is formed and

the number of normal host cell endosomes (LAMP1-positive compartments) is highly reduced (Rajashekar et al., 2008). Is this the end of nutritional supply that terminates bacterial proliferation? What is the role of other tubular membrane compartments observed in Salmonella-infected cells (Schroeder et al., 2011)? Are the other networks part of the SCV/SIF continuum or separate supply chains for further sources of nutrients? Does the SCV/SIF continuum result in dilution to sublethal concentrations of antimicrobial compounds delivered by phagocytic cells to phagosomes?
containing *Salmonella*? Does formation of the SCV/SIF continuum alter the acidic pH in the lumen of the SCV? Does formation of the SCV/SIF continuum correlate with the divergent growth rates of *Salmonella* observed in tissues of infected organs (Claudi et al., 2014; Sheppard et al., 2003)? Further analyses of host-pathogen interactions on the single-cell level will likely contribute answers to these questions.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**

Infection experiments were performed with *Salmonella enterica* serovar Typhimurium NCTC 12023 as WT strain and isogenic mutant strains deficient in *ssaV*, *sseA*, *sseF*, or *ssaV* *sseJ* (Table S1). For LCI, bacterial strains harbored pFPV25.1 or pFPV-mCherry for constitutive expression of GFP or mCherry, respectively. Plasmid mKikGR-Bug for constitutive expression of mKikumeGR was kindly provided by Andreas Muller. The construction of plasmid p3595 for expression of the E. coli lac operon is described in the Supplemental Information. Bacterial strains were routinely cultured in LB (D647) and subsequently infected with *Salmonella* WT, *ssaV*, or *ssaV* *sseJ* strains at MOI of 75 and 25 for HeLa and RAW264.7 cells, respectively. All strains constitutively expressed mKikumeGR. Infected cells were imaged directly before and after a 405 nm laser pulse for photoconversion from KikumeG to KikumeR, and green and red FI were measured over the indicated time frames. As control, chloramphenicol (Cm) was added to block protein biosynthesis. At least 50 events per condition were analyzed, except 30 events for Cm controls. The D647 FI was recorded to select bacteria within intact SCVs and connection to SIFs for WT *Salmonella*. The ratio of green and red fluorescence of vacuolar bacteria was calculated and normalized values are displayed referring to the value before conversion.

(A) KikumeGR for probing metabolic activity. In proliferating, metabolically active bacteria, dilution of KikumeR, and new synthesis of KikumeG are anticipated. Non-proliferating, metabolically inactive bacteria are expected to maintain KikumeR.

(B) Infection of HeLa cells. Representative stills of the time series of WT-infected cells are shown in Figure S7.

(C) The slope of the green/red ratio was calculated as percent (%) × hr⁻¹ for infected HeLa cells.

(D) Infection of IFNγ-activated RAW264.7 cells.

(E) The slope of the green/red ratio was calculated as percent (%) × hr⁻¹ for infected RAW264.7 cells.

Statistical analysis was performed as for Figure 2.

**Host Cell Infection**

For infection of HeLa or HeLa LAMP1-GFP cells, *Salmonella* strains were grown in LB O/N, diluted 1:31 in fresh LB, and subcultured for 3.5 hr. The infection of HeLa cells was performed with indicated MOI for 25 min. RAW264.7 or RAW264.7 LAMP1-GFP cells were infected with O/N cultures of *Salmonella* strains for 25 min. Subsequently, all cells were washed thrice with phosphate-buffered saline (PBS) and incubated for 1 hr with medium containing 100 μg/mL 1-gentamicin (Applichem) to kill non-invaded bacteria. Medium was replaced by medium containing 10 μg/mL 1-gentamicin for the rest of the experiment.

Quantification of intracellular replication was performed as described in the Supplemental Information.

**Live-Cell Imaging**

Imaging medium was used for LCI consisting of minimal essential medium (MEM) with Earle’s salts, without NaHCO₃, without L-glutamine and phenol red (Biochrom), and supplemented with 30 mM HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid) (Sigma-Aldrich) (pH 7.4). For P/C and
CLEM experiments, LCI was performed using the confocal laser-scanning microscope (CLSM) Leica SP5 (Leica) equipped with an incubation chamber maintaining 37°C and humidity during LCI, polychromic mirror TD 488/543/633 for GFP/FPF/FRFP, and objectives as follows: HC PL FL 10×, NA 0.3; HC PL APO CS 20×, NA 0.7; HCX PL APO CS 40×, NA 1.25-0.75; HCX PL APO CS 100×, NA 1.4-0.7. LAS AF software (Leica) was used for setting adjustment, image acquisition, and image processing. For FLIP, FRAP, and KikumeGR experiments, LCI was performed using the Cell Observer Spinning Disk microscope (SDM, Zeiss) equipped with a Yokogawa Spinning Disc Unit CSU-X1a 5000, Evolve EMCCD cameras (Photometrics), DirectFRAP slider, and an α-Plan-Apochromat 63×, NA 1.4 objective. During LCI, 37°C and absolute humidity were maintained. Images were acquired and processed using ZEN software (Zeiss) and the following filter combinations: GFP with BP 525/50, RFP with BP 593/46, and FRFP with BP 690/50.

Correlative Light and Electron Microscopy and DAB Photooxidation
CLEM and rhodamine-based DAB photooxidation of infected HeLa cells stably transfected with LAMP1-GFP and after P/C with BSA-rhodamine were basically performed as described before (Krieger et al., 2014). A detailed protocol is provided in the Supplemental Information.

Quantification of EPs within SCVs and Number and Surface Area of Endosomal Compartments and Quantification of Volume and Membrane Area of SCV and SIF Network
See the Supplemental Information for detailed description.

Quantification of Lactose, Infection for β-Gal Assays, and Determination of β-Gal Activity
See the Supplemental Information for detailed description.

FLIP, FRAP, and mKikumeGR Photoconversion Experiments
FLIP analyses were used to determine the continuity of SCV/SIF network and FRAP analyses were used to determine the interconnection of membrane and luminal content of SCVs and SFs. The photoconvertible fluorescent mKikumeGR was used to measure the metabolic activity of *Salmonella* as previously described for *Leishmania major* (Müller et al., 2013). Details are given in the Supplemental Information.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, seven figures, one table, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2017.02.005.

AUTHOR CONTRIBUTIONS

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REFERENCES


Supplemental Information

*Salmonella enterica* Remodels the Host Cell Endosomal System for Efficient Intravacuolar Nutrition

Viktoria Liss, A. Leoni Swart, Alexander Kehl, Natascha Hermanns, Yuying Zhang, Deepak Chikkaballi, Nathalie Böhles, Jörg Deiwick, and Michael Hensel
Suppl. Figures and Legends
Fig. S 1. **Interchange of SIF and SCV membranes determined by FRAP analyses.** Fig. S 1A related to Fig. 2D, Fig. S 1B related to Fig 3C.

A) Examples of FRAP experiments for SIF membranes shown in Fig. 2D. HeLa cells expressing LAMP1-GFP were infected with *Salmonella* WT expressing mCherry with an MOI of 25. At various time points p.i., FRAP analyses were performed using spinning disk microscopy (SDM). Small parts of single SIF at different positions within the SIF network were imaged just before and after a 488 nm laser pulse for photobleaching of GFP and recovery of green fluorescence signal was measured over time. Relative fluorescence intensities (RFI) are displayed referring to the before-value.

B) Examples of FRAP experiments for SCV membranes shown in Fig 3C. HeLa cells stably transfected with LAMP1-GFP were infected with *Salmonella* WT or *ssaV* mutant strains expressing mCherry with an MOI of 25. At 7-9 hr p.i. FRAP analyses were performed by imaging single SCV just before and after a 488 nm laser pulse for photobleaching of GFP and recovery of green fluorescence signal was measured over time. RFI are displayed as for A).

Scale bars, 10 (overview) and 5 µm (detail).
Fig. S 2. **Interchange of SIF and SCV lumen determined by FRAP analyses.** Fig. S 2A related to Fig. 2E, Fig. S 2B related to Fig 3A.

A) Examples of FRAP experiments of SIF lumen shown in Fig. 2E. HeLa cells were infected with *Salmonella* WT strain expressing mCherry with an MOI of 25 and pulse-chased with Dextran-Alexa Fluor 488 (D488) for 3 hr before microscopy. At various time points p.i., FRAP analyses were performed using SDM. Small parts of single SIF at different positions within the SIF network were imaged just before and after a 488 nm laser pulse for photobleaching of D488 and recovery of green fluorescence signal was measured over time. RFI are displayed referring to the before-value.

B) Examples of FRAP experiments of SIF lumen shown in Fig. 3A. HeLa cells were infected with *Salmonella* WT strain, *ssaV* mutant or *sifA sseJ* double mutant expressing mCherry with an MOI of 25 and pulse-chased with D488 at 3-7 hr p.i. At 7-9 hr p.i. FRAP analyses were performed. Therefore single SCV were imaged just before and after a 488 nm laser pulse for photobleaching of D488 and recovery of green fluorescence signal was measured over time. RFI are displayed as for A).

Scale bars, 10 (overview) and 5 \( \mu \)m (detail).
Fig. S 3. **Quantification of volumes and membrane surface areas of SCV and SIF. Related to Fig. 4.**

For the calculations of SCV/SIF volume and membrane area the dimensions of bacteria, SCV and SIF were determined based on TEM micrographs using ImageJ. The SIF length was measured based on 3D fluorescence images using Imaris Filament Tracer tool in AutoPath mode. For simplification, the bacterial body and SCV were fragmented into geometrical forms of sphere and cylinder. SIF were also simplified to cylinders. Using formulae for sphere and cylinder the volumes and membrane areas were calculated. For determination of SCV volumes, the volumes of bacteria within SCV were subtracted. For defining the outer volume of WT double membrane SIF the volume of the inner tubule was subtracted. For comparison of SIF networks induced by WT or
sseF-deficient strains, a similar SIF length of 350 µm was used. The length of 350 µm was determined as a mean SIF length after measuring several SIF networks in 3D reconstructions (data not shown).
Fig. S 4. Controls for β-Gal assays with *Salmonella enterica*. Related to Fig. 5B.

A) Lactose is not metabolized by RAW264.7 macrophages. RAW264.7 macrophages were grown in DMEM supplemented with either glucose or lactose as sole carbon source for 45 hr. Concentrations of lactose and glucose were measured by an enzymatic assay.

B) Lactose as external C-source during macrophage infection led to *lacZ* induction in a SPI2-T3SS-dependent manner. Carbohydrate-starved RAW264.7 macrophages were infected with *Salmonella* WT, *ssaV* or *sifA* strains each harboring a plasmid for expression of the *lac* operon. The WT strain without the plasmid was included as control (mock). After growth for 8 hr with medium containing either 25 mM lactose or glucose β-Gal activities were measured using Flourescein di(β-D-galactopyranoside (FDG) as substrate and correlated to numbers of intracellular *Salmonella*. Insert:
correlation of β-Gal activity of induced bacteria in presence and absence of 3 x 10^6 macrophages indicating a strong correlation and no significant activity in macrophages.

C) Effect of glucose or lactose addition on intracellular replication in RAW264.7 cells. Various strains without or with (+ lac) a plasmid for expression of the lac operon were used to infect RAW264.7 cells. Cell culture medium was supplemented with lactose or glucose as indicated. Standard gentamicin protection assays were performed and the x-fold intracellular replication was determined by calculating CFU 8 hr p.i./CFU 1 hr p.i.

D) β-Gal activities of Salmonella in HeLa cells were determined as for B). For comparison, the activity of WT + lac in minimal medium containing lactose (in vitro) was determined.

Statistical analysis was performed by Student’s t-test and significances are indicated as follows: n.s., not significant; *, p < 0.05; **, p < 0.0005.
Fig. S 5. Effect of various antibiotics on intracellular *Salmonella enterica*. Related to Fig. 6.
A) Elevated antibiotic concentrations are not detrimental to epithelial cells. Cell viability of HeLa cells was determined using an MTT assay. Cells were exposed to experimental conditions according to previous replication assays with infection by *Salmonella* WT. After infection and antibiotic application as indicated, cells were submitted to MTT for 4 hr and metabolic activity was measured at 595 nm. Untreated cells as negative control were set as 100% and phosphate–buffered saline (PBS)-treated cells served as positive control. Depicted are means and SD of three biological replicates (n = 3) each performed in triplicate.

B) Effect of increased gentamicin concentrations on intracellular replication of WT and sseF-deficient strains. The experiment was performed as described for Fig. 6.

C) Effect of increased concentrations of polymyxin B (PMB) on intracellular replication of *Salmonella* in HeLa cells or RAW264.7 macrophages. Intracellular replication of *Salmonella* was determined using a variation of a gentamicin protection assay. HeLa cells or RAW264.7 macrophages were infected with WT (green bars) or SPI2-T3SS-deficient *ssaV* (red bars) at an MOI of 1. After infection, cells were incubated for 1 hr in medium containing 150 µg x ml⁻¹ PMB to kill extracellular bacteria. Subsequently, cells were incubated in medium containing 75, 250 or 500 µg x ml⁻¹ PMB for HeLa cells, or 15, 25 or 50 µg x ml⁻¹ PMB for RAW264.7 cells as indicated.

D) Effect of increased concentrations of cefotaxime (Cefo) on intracellular replication of *Salmonella* in HeLa cells or RAW264.7 macrophages. The experiment was performed as for B) but cells were incubated for 1 hr in medium containing 350 µg x ml⁻¹ Cefo to kill extracellular bacteria, followed by medium containing 35, 350, or 700 µg x ml⁻¹ Cefo for HeLa cells, or 100, 125, or 150 µg x ml⁻¹ Cefo for RAW264.7 cells as indicated.

Cell lysis and enumeration of antibiotics-protected intracellular bacteria were done 2 hr and 16 hr p.i. The x-fold intracellular replication is the ratio of CFU 16 hr/2 hr.

Depicted are means and SD of assays performed in triplicates representative of two biological replicates. Statistical analysis was done using Student’s *t*-test and significances are indicated as: n.s., not significant; *, p <0.05; **, p <0.01; ***, p <0.001.
Fig. S 6. Metabolic activity of *Salmonella* growing on agarose pads determined by mKikumeGR. Related to Fig. 7.

*Salmonella* WT, *ssaV* and *sifA sseJ* strains harboring a plasmid for expression of photoconvertible fluorophore mKikumeGR were subcultured for 3.5 hr in LB media and transferred to the surface of agarose pads consisting of various media as described before (Young et al., 2012). Loaded agarose pads were subjected to time-lapse LCI by SDM. Green and red fluorescence of bacteria was imaged just before and after a 405 nm laser pulse for mKikumeGR conversion from green to red for 5 hr.

A) The ratio of green and red fluorescence was calculated and normalized values are displayed in % referred to the initial value.

B) The slope of the green/red ratio was calculated in % x hr⁻¹.

All *Salmonella* strains growing on PCN minimal medium show a comparable high metabolic activity, whereas the metabolic state of bacteria grown on PBS is restricted. For statistical analysis Student’s *t*-test was performed and significances are indicated as follows: n.s., not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001. For the calculation at least three biological replicates with n > 11 were used.
Fig. S 7. **Metabolic activity of intracellular Salmonella determined by mKikumeGR.** Related to Fig. 7.

HeLa cells were pulsed-chased O/N with the EP Dextran-Alexa Fluor 647 (D647) for labeling of endosomal compartments and subsequently infected with *Salmonella* WT, or *ssaV* or *sifA sseJ* strains (not shown) harboring a plasmid for expression of photoconvertible mKikumeGR at an MOI of 75. Infected cells were imaged just prior and after a 405 nm laser pulse for mKikumeGR photoconversion from green and red and both fluorescence signals were measured from 3 to 8 hr p.i. The D647 signal (blue) was imaged to locate *Salmonella* within SCV and the connection to SIF. Scale bar, 20 µm.
**Suppl. Movies**

Movie 1: **Fluorescence loss in photobleaching (FLIP) of SIF lumen. Related for Fig. 2ABC.**

FLIP experiments were performed for SIF lumen at 7-9 hr p.i. Photobleaching was performed for a small circular ROI (yellow dashed circle) with the 488 nm laser for 400 ms (FLIP) and 100 % laser power. A series of a Z-stack followed by a bleach pulse and 20 s delay was repeated 20 times. D488 fluorescence intensity for a part of a SIF network was measured by means of MIP projections within ROI1 close to bleaching ROI and within the SIF network of a neighboring cell (control cell, ROI2). Green background fluorescence outside of a cell (background, ROI3) was subtracted from ROI1 and ROI2. The FI is indicated as percentage of the initial signal at t=0.

**Suppl. Tables**

Table S1. *Salmonella enterica* serovar Typhimurium strains used in this study. Related to Experimental Procedures.

<table>
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<tr>
<th>Designation</th>
<th>relevant genotype</th>
<th>source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC12023</td>
<td>WT</td>
<td>NCTC, lab stock</td>
</tr>
<tr>
<td>P2D6</td>
<td>ssaV::mTn5</td>
<td>(Shea et al., 1996)</td>
</tr>
<tr>
<td>MvP497</td>
<td>∆sifA::aph</td>
<td>(Chakravortty et al., 2002)</td>
</tr>
<tr>
<td>HH107</td>
<td>∆sseF::aphT</td>
<td>(Hensel et al., 1998)</td>
</tr>
<tr>
<td>MvP450</td>
<td>∆sseJ::FRT ∆sifA::aph</td>
<td>(Rajashekar et al., 2008)</td>
</tr>
</tbody>
</table>
Suppl. Experimental Procedures

Construction of lac plasmid

For generation of a low copy plasmid encoding the entire lac operon, the latter was amplified using *E. coli* K12 as template in two fragments using the primers LacZ-SacI-For (AACGAGCTCCTGCACTGGATGGTG), LacA-XbaI-Rev (GGCTCTAGAATTTAATTACGATTCAAC), LacI-KpnI-For (CGGGGTACCGCGGCATGCATTTACGTTGACACC) and LacZ-SacI-Rev (CAGGAGCTCGTTATCGCTATGACGG). The two fragments were introduced into pWSK29. The plasmid obtained (p3595) conferred to *S. Typhimurium* a lactose-positive phenotype on MacConkey lactose agar and growth in MOPS minimal medium with lactose as sole C-source.

Intracellular replication assay

Different conditions and antibiotics were tested varying the standard gentamicin protection assay. 2 x 10⁵ HeLa cells or 4 x 10⁵ RAW264.7 cells in 24-well plates were infected in triplicates with cultures diluted 1:31 from O/N cultures and grown for additional 3.5 hr or O/N cultures, respectively, of *S. Typhimurium* WT or *ssaV* with an MOI of 1. Infection was synchronized by centrifugation at 500 x g for 5 min and allowed to proceed for 25 min at 37°C, 5 % CO₂. Cells were washed thrice with PBS and incubated in medium containing 100 µg x ml⁻¹ gentamicin for 1 hr to eliminate extracellular bacteria. Then medium containing either 10 µg x ml⁻¹ gentamicin as standard or the indicated concentrations as variation was applied for the remainder of the experiment. Cell lysis was performed 2 hr and 16 hr p.i. if not otherwise indicated by washing thrice with PBS and incubation for 10 min with PBS containing 0.1 % Triton X-100. Colony-forming units (CFU) were enumerated by plating serial dilutions of the lysates onto Mueller-Hinton agar plates. Replication is defined as CFU ratio of 16 hr to 2 hr, or as indicated. Experiments with carbohydrate variation and polymyxin B and cefotaxime were done accordingly with time points, conditions and concentrations as indicated.

Quantification of lactose

Lactose concentration was quantified according to the enzymatic assay of Roche (R-Biopharm, Darmstadt, Germany) with slight modifications. The assay was adopted for a 96-well format that was analyzed by a microplate reader (Chameleon V, Hidex, Turku, Finland) using a 340 nm filter.
Infection for β-galactosidase measurement

RAW264.7 macrophages were seeded (8 x 10^5 cells) in 6-wells with DMEM 1 d before infection and medium was exchanged to DMEM without glucose 1 hr prior infection with an MOI of 10. Infection was synchronized by centrifugation at 500 x g for 5 min before incubation for 30 min. Infected cells were washed with PBS and incubated in DMEM without glucose but with 100 µg x ml^-1 gentamicin for 1 hr. For additional 8 hr infected cells were incubated in DMEM with either 25 mM glucose or lactose and 10 µg x ml^-1 gentamicin. For lysis cells were kept at 4°C, washed with PBS twice and lysed with 0.75 ml 0.1 % Triton X-100. This was repeated to collect residual cells from 6 well plates. Aliquots of 1 hr or 8 hr lysates were used for determination of colony-forming units (CFU). The residual lysates were centrifuged at 22,000 x g and pellets were stored at -80°C until determination of β-galactosidase activity.

β-galactosidase assay

Fluorescein di(β-D-galactopyranoside) (FDG) measurement was performed as described (Garcia-del Portillo et al., 1992) with following modifications: bacterial pellets were lysed in 110 µl lacZ-buffer (Maloy et al., 1996) containing 0.003 % SDS with 5.5 µl chloroform at 28°C for 20 min. After chloroform had settled 100 µl were mixed with 10 µl of 10 mM FDG and analyzed in U-bottom black plates (Dynex, Chantilly, VA, USA) using a microplate reader (Chameleon V, Hidex, Turku, Finland) for 1 hr of fluorescence development at 22°C (485 nm ex., 535/20 nm em.). Results were calculated with fluorescein as standard to classical enzymatic units (µmol FDG x min^-1) and related to the amount of bacteria determined. Analysis of in vitro induction of β-galactosidase was performed with ortho-nitrophenyl-β-D-galactopyranoside (ONPG) of bacteria grown O/N in PCN medium (Neidhardt et al., 1974) supplemented with 25 mM glucose or lactose as described before (Deiwick et al., 1999).

Cell viability assay

Modifying a standard MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) assay measuring metabolic activity 2 x 10^4 HeLa cells in 96 well plates were first submitted to the indicated experimental conditions. Then 20 µl of 5 mg x ml^-1 MTT were applied and cells were incubated for additional 4 hr at 37°C, 5 % CO2 in the dark. Medium was replaced by 100 µl DMSO and incubated for 10 min with agitation in the dark to solve metabolically built formazan crystals. Absorbance was measured at 595 nm with a microplate reader (Chameleon V, Hidex, Turku,
Finland). Cell viability was blank-subtracted and calculated as percentage compared to a negative control (i.e. untreated cells) set as 100 %. Cells incubated with PBS served as positive control.

**Quantification of EP within SCV and number and surface area of endosomal compartments**

HeLa cells stably expressing LAMP1-GFP were infected with various *Salmonella* strains expressing GFP with an MOI of 100. P/C with fluorescent EP, here BSA-Rhodamine NP, was performed O/N prior infection, or for 1 hr at 6-7 hr p.i. LCI by CLSM was performed at 4 hr and 8 hr p.i. and the relative amount of EP within the SCV was calculated with the software Imaris (Bitplane, Zürich, Switzerland). In short, by using the Surface tool and measuring GFP signals, the 3D structure of bacteria within Z-stacks was recognized as previously described (Zhang et al., 2015). For this, surface (S1) and the volume (Vs1) were calculated. Subsequently, a second shell (S2) was created around the bacteria with a thickness of 0.374 µm simulating the SCV lumen. The volume of SCV enclosing *Salmonella* was determined by distance between SCV membrane and bacterial LPS layer by means of fluorescence intensity peak measurements with Imaris in a line plot after immuno-labeling (n = 20). Finally, the Rhodamine signal of EP was measured for the volume of S1 and S2 (FIEPS1, FIEPS2), and the relative amount of EP within SCV was calculated as follows: (FIEPS1 + FIEPS2)/Vs1. For each strain and condition at least 48 cells were analyzed.

**Quantification of volume and membrane area of SCV and SIF network**

For the calculations of SCV/SIF volumes and membrane surface area HeLa cells infected with *Salmonella* WT or sseF strain for 7-9 hr with MOI of 75 were used. The dimensions of intracellular bacteria (length [n=76] and diameter [n=85]), SCV (distance between bacterial surface and SCV membrane [n=164]) and SIF (diameter of WT outer and inner SIF tubule [n=30 SIF measured at 5 positions each], diameter of sseF SIF tubule [n=6 SIF measured at 5 positions each]) were determined by means of TEM micrographs of infected HeLa cells with the software ImageJ (http://rsbweb.nih.gov/ij/). SIF lengths were measured by means of 3D fluorescence using Imaris (Bitplane, Zürich, Switzerland) with the AutoPath mode of the Filament Tracer tool. For simplification the bacterial body and SCV were fragmented into geometrical forms as a sphere and a cylinder. The SIF were also simplified to a cylinder. With the formulae for sphere and cylinder the volumes and membrane area could be calculated. For the volume of SCV the bacterial volume inside was subtracted. For defining the outer volume of WT double membrane SIF the volume of the inner tubule was subtracted. For comparison between WT SIF network and sseF SIF network a similar SIF length of 350 µm was used. For details see Fig. S 3.
Correlative light and electron microscopy and DAB photooxidation

HeLa cells (1 x 10^5) stably transfected with LAMP1-GFP were seeded in a Petri dish with a gridded coverslip (MatTek, Ashland, MA) two days prior to microscopy. On the third day cells were infected with Salmonella strains expressing GFP with an MOI of 75 and pulse-chased with 400 µg x ml⁻¹ BSA-Rhodamine as EP at 4-7 hr p.i. At 8 hr p.i. cells were observed by LCI and fixed as fast as possible directly on microscope stage with pre-warmed 2.5 % glutaraldehyde (Electron Microscopy Sciences) in buffer (0.2 M HEPES, pH 7.4, 5 mM CaCl₂) for 1 hr at 37°C. After fixation cells were rinsed several times in buffer containing 50 mM glycine and 20 mM potassium cyanide to reduce unspecific DAB (3,3'-diaminobenzidine tetrahydrochloride, Sigma) staining, followed by rinses in buffer. For the DAB photooxidation fixed cells were covered with freshly-prepared ice-cold 1 mg x ml⁻¹ DAB in 0.2 M HEPES buffer and the sample was viewed again by CLSM. The illumination of samples for DAB photooxidation occurred with an OSRAM HXR-R120W/45C VIS lamp (used power 70 mW) with green light (filter excitation wavelength 515-560 nm) until a brown DAB polymer was visible by eye, mostly after 8-12 min. After DAB oxidation the DAB solution was removed and the cells were washed several times in HEPES buffer. Post-fixation was performed with 2 % osmium tetroxide (Electron Microscopy Sciences) in buffer containing 1.5 % potassium ferricyanide (Sigma) and 0.1 % ruthenium red (Applichem) for 1 hr at 4°C in darkness. After several washing steps the cells were dehydrated in a cold graded ethanol series and finally one rinse in anhydrous ethanol and two rinses in anhydrous acetone at RT. The gridded coverslip was removed from the Petri dish and cells were flat-embedded in EPON812 (Serva). Serial 70 nm sections were cut with an ultramicrotome (Leica EM UC6) and collected on formvar-coated EM copper grids. After staining with uranyl acetate and lead citrate, cells were observed with TEM (Zeiss EFTEM 902 A), operated at 80 kV and equipped with a 2K wide-angle slow-scan CCD camera (TRS, Moerenwies, Germany). Images were taken with the software ImageSP (TRS image SysProg, Moerenwies, Germany). For image analysis, software packages LAS AF (Leica. Wetzlar, Germany), ImageJ (http://rsbweb.nih.gov/ij/) and Imaris (Bitplane, Zürich, Switzerland) were used. Stitching and overlay of CLSM and TEM images was done using Photoshop 5.5 (Adobe) according to Keene et al. (2008).

FLIP and FRAP experiments

For analyses of SCV/SIF membrane by FRAP LAMP1-GFP-expressing HeLa cells were infected with Salmonella strains expressing mCherry. For analyses of SCV/SIF lumen by FRAP or FLIP,
HeLa cells were pulse-chased with 200 μg x ml⁻¹ Dextran-Alexa Fluor 488 (D488, Invitrogen) after infection with *Salmonella*. FRAP and FLIP analyses were performed with SDM with DirectFRAP slider. The photobleaching was performed for a small circular ROI with the 488 nm laser for 300 ms (FRAP) or 400 ms (FLIP) and 100 % laser power. For FRAP analyses, one single Z-plane was imaged before (20 frames) and after (80-180 frames) photobleaching and recovery of 488 nm fluorescence signals was measured over time. For SIF studies small parts of single SIF were bleached at various time periods after infection (3-5 hr, 7-9 hr and 14-16 hr) and at various positions within the SIF network. For SCV studies 7-9 hr p.i. single SCV containing max. 5 bacteria and connected to a SIF network were chosen for imaging and the bleaching ROI captured only the SCV. After microscopic acquisition green fluorescence intensity of a ROI was measured over time with ZEN by manually setting the ROI. The mean fluorescence intensity within the ROI was used to calculate bleaching and recovery of green fluorescence. Therefore, green background fluorescence outside of a cell was measured over time and subtracted from appropriate values. Than the value before photobleaching was set to 1 and the first value after photobleaching was subtracted from all values for normalization. Normalized mean values were graphically displayed referring to the before-value. Additionally, the final mobile fraction of LAMP1-GFP or D488 was calculated and displayed in %. For evaluation one ROI was considered as one event. Due to variation in image size for improvement of resolution the acquisition time of single events varies by few seconds. For FLIP analyses, a series of a Z-stack followed by a bleach pulse and 20 s delay was repeated 20 times. The bleaching ROI was set in the middle of a SIF network of an infected cell. D488 fluorescence intensity for a part of a SIF network was measured by means of MIP projection within a ROI1 close to bleaching ROI and within the SIF network of a neighboring cell (ROI2). Green background fluorescence outside of a cell (ROI3) was subtracted from ROI1 and ROI2. The fluorescence intensities were normalized by setting the initial fluorescence to 1.

*mKikumeGR photoconversion* in vitro

For in vitro experiments bacterial growth on agarose pads was imaged as described (Young et al., 2012). Briefly, *Salmonella* strains expressing mKikumeGR were grown O/N in LB media, subcultured in LB media for 3.5 hr and transferred to the surface of pre-made agarose pads consisting of various media. For preparation of agarose pads, 600 μl of prewarmed PCN minimal medium or PBS with 2 % low melting point agarose were pipetted on a 22 mm² cover glass slide and immediately covered with a second cover glass slide to create an agarose sandwich. After 1 hr
at room temperature (RT) the top cover glass was removed and the solidified agarose pad was cut in four pieces with a sterile scalpel. For loading of the agarose pads with bacteria 2 µl of a 3.5 hr subculture diluted to OD$_{600}$ 0.2 were pipetted on the agarose pad surface and allowed to set for 10 min. Afterwards single agarose pads were transferred upside-down to single wells of an 8-well chamber slide (ibidi, Martinsried, Germany) and the borders were sealed with liquid agarose. The 8-well chamber slide was transferred to the SDM and subjected to time-lapse LCI before and after mKikumeGR photoconversion. For bacteria on agarose pads the whole field of view was measured as one ROI.

**Measurement of metabolic activity of bacteria using mKikumeGR**

HeLa (2 $\times$ 10$^4$) or RAW264.7 (4 $\times$ 10$^4$) cells were seeded in 8-well chamber slides (ibidi, Martinsried, Germany). In order to visualize endosomal structures such as SCV and SIF P/C with EP Dextran-Alexa Fluor (D647, Invitrogen) was performed. P/C was performed O/N prior to infection with 100 or 33 µg x ml$^{-1}$ D647 for HeLa cells or RAW macrophages, respectively. Simultaneous to P/C, RAW macrophages were activated with 7.5 ng x ml$^{-1}$ IFN$\gamma$ O/N. On day three, cells were infected with various *Salmonella* strains expressing mKikumeGR. Before microscopy, cells were provided with imaging medium, the 8-well chamber slide was subjected to time-lapse LCI before and after mKikumeGR photoconversion, re-synthesis and dilution of converted molecules using SDM. Z-stacks were acquired immediately prior conversion, and after a 60 s pulse with a 405 nm laser (at 100 % power) of the whole field of view the green and red fluorescence signals of bacteria were measured over time by Z-stacks. Fluorescence intensities were measured using Imaris with the Surface tool which allows recognition of 3D structures within Z-stacks and measurement of fluorescence for these volumes. Single bacteria or growing micro-colonies within a cell were marked as ROI and green and red fluorescence was tracked over time for this ROI. The mean values of fluorescence intensities of a ROI were used to calculate ratios of green and red fluorescence of bacteria. The value before photoconversion of mKikumeGR was set to 100 % and the first value after photoconversion was subtracted from all values for normalization. Normalized values were displayed in % referred to the before-value. Additionally, slopes of green/red ratios were calculated in % x hr$^{-1}$. One ROI was considered as one event in cells evaluated.
Suppl. References


