The COPII complex and lysosomal VAMP7 determine intracellular \textit{Salmonella} localization and growth

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\textbf{Summary}

\textit{Salmonella} invades epithelial cells and survives within a membrane-bound compartment, the \textit{Salmonella}-containing vacuole (SCV). We isolated and determined the host protein composition of the SCV at 30 min and 3 h of infection to identify and characterize novel regulators of intracellular bacterial localization and growth. Quantitation of the SCV protein content revealed 392 host proteins specifically enriched at SCVs, out of which 173 associated exclusively with early SCVs, 124 with maturing SCV and 95 proteins during both time-points. Vacuole interactions with endoplasmic reticulum-derived coat protein complex II vesicles modulate early steps of SCV maturation, promoting SCV rupture and bacterial hyper-replication within the host cytosol. On the other hand, SCV interactions with VAMP7-positive lysosome-like vesicles promote \textit{Salmonella}-induced filament formation and bacterial growth within the late SCV. Our results reveal that the dynamic communication between the SCV and distinct host organelles affects both intracellular \textit{Salmonella} localization and growth at successive steps of host cell invasion.

\textbf{Introduction}

\textit{Salmonella enterica} serovar Typhimurium (\textit{Salmonella}) is a Gram-negative enteric pathogen that can cause acute gastroenteritis in humans after ingestion of contaminated food or water. Salmonellosis is one of the most common sources of food-borne disease in humans and is a major public health and economic burden worldwide (Majowicz et al., 2010; Agbor and McCormick, 2011). A key aspect of \textit{Salmonella} virulence is its ability to invade and survive within non-phagocytic intestinal epithelial cells, processes driven by two type III secretion systems (T3SS1 and T3SS2) that together inject more than 30 effector proteins into the host cell (Haraga et al., 2008; Figueira and Holden, 2012).

Host cell invasion is mostly mediated by T3SS1-injected effectors, triggering fast and massive rearrangements of the actin cytoskeleton (Zhou et al., 2001; Scott et al., 2005; Patel and Galán, 2006) followed by the formation of plasma membrane (PM) ruffles and \textit{Salmonella} engulfment into the \textit{Salmonella}-containing vacuole (SCV). The SCV is a unique, modified membrane-bound compartment that enables bacterial survival and replication. The early SCV [<30 min post-invasion (p.i.)] has been shown to share some similarities with early endosomes, namely an association with phosphatidylinositol 3-phosphate, Rab5, Vps34, early endosomal antigen-1 (EEA-1) and sorting nexins -1 and -3 (Hernandez et al., 2004; Bujny et al., 2008; Mallo et al., 2008; Steele-Mortimer, 2008; Braun et al., 2010). The maturing SCV [between 30 min and 5 h p.i.] undergoes extensive membrane remodelling and acquires late endosome/lysosomal markers, such as Rab7, lysosomal-associated membrane-associated protein-1 (Lamp-1) and vacuolar ATPase (vATPase) (reviewed in Steele-Mortimer, 2008; Schroeder et al., 2011). This is accompanied by movement of the SCV along microtubules (MTs) to a juxtanuclear position adjacent to the MT-organizing centre (Harrison et al., 2004). Maintaining the SCV in the perinuclear region is thought to be important for promoting bacterial replication, which is initiated 3–4 h p.i. (Ramsden et al., 2007a,b; Bakowski et al., 2008). The last stages of SCV maturation (>5 h p.i.), mostly mediated by T3SS2 effectors, are characterized by concomitant intravacuolar bacterial replication and formation of Lamp-1-enriched membrane tubules, named \textit{Salmonella}-induced filaments (SIFs), that extend from the SCV along MTs (Drecktrah et al., 2008). SIFs are highly dynamic structures and can spread throughout the entire
cell to form a complex network. They are also enriched in vATPase, Rab7 and cholesterol (Brumell et al., 2001). In addition to SIFs, other tubular networks emanate from the late SCV, such as Salmonella-induced SCAMP3 tubules (Mota et al., 2009) and Lamp-1-negative tubules (Schroeder et al., 2010). However, the biological role of all these Salmonella-induced tubules remains largely unknown (Schroeder et al., 2011). Membrane damage during the early or maturing stage has also been described for some SCVs, which gives bacteria access to the host cell cytosol. This can allow for bacterial detection and degradation by autophagy mechanisms (Jo et al., 2013). Nevertheless, recent data show that in approximately 9% of infected epithelial cells, these cytoplasmic bacteria can replicate at much faster rates than those within an SCV (doubling time of ~20 min); this is termed hyper-replication (Knodler et al., 2010; Malik-Kale et al., 2012). Thus, intracellular Salmonella growth can be different depending on its localization within the host cell.

Proteomics has been used to reveal the protein composition of phagosomes (Desjardins et al., 1994; Gagnon et al., 2002; Stuart et al., 2007; Rogers and Foster, 2008). Other proteomics studies have reported the protein composition of bacteria-containing vacuoles, such as Legionella pneumophila (Shchuk et al., 2009; Urywyler et al., 2009; Hoffmann et al., 2014) or Mycobacterium bovis BCG (Lee et al., 2010), and have given important insights into the mechanisms of intracellular bacterial survival. In this study, we applied a quantitative proteomics approach to identify novel host factors associated with the SCV at different stages of its maturation. We reproducibly isolated SCVs and determined their protein composition. Using functional and correlational ultrastructural approaches, we then characterized and showed that two specific SCV–protein interactions affect intracellular Salmonella growth. We demonstrate that early interactions between the SCV, the endoplasmic reticulum (ER) and the coat protein complex II (COPII) complex promote cytoplasmic Salmonella localization and hyper-replication. At late stages, bacterial growth is regulated by interactions between intact SCVs and VAMP7-positive lysosome-like vesicles, which also determine SIF formation.

**Results**

**Purification of SCVs from infected epithelial host cells**

We developed a fractionation methodology to obtain a subcellular fraction highly enriched in intact SCVs that could be used to determine the SCV proteome. Vacuoles were isolated at two time-points of Salmonella infection representing two stages of SCV maturation: 30 min, corresponding to the early SCV; and 3 h, corresponding to the maturing SCV. Later stages of SCV maturation were not isolated, as bacterial replication and Salmonella-induced tubule formation interfere with our purification procedures. In order to obtain intact SCVs with sufficient purity, we performed careful cell homogenization and centrifugal separation in density gradients. SCV integrity was quantified in the post-nuclear supernatant (PNS) after cell homogenization, by a novel ELISA-based assay (Fig. 1; see Experimental procedures for details). In this assay, non-vacuolarized Salmonella adhere to an immobilized antibody and are then quantified by a secondary, biotinylated antibody, whereas vacuolarized bacteria do not adhere and are thus not counted (Fig. 1A, left panel). Total bacteria in each sample were also determined using this method by first subjecting samples to osmotic shock in order to rupture the SCVs and free all vacuolarized bacteria. In order to evaluate the robustness of our assay and to quantify the number of non-vacuolarized Salmonella in the PNS, a standard curve with known amounts of bacteria was generated and statistically validated (Fig. S1A and B). In the non-infected control PNS, to which a known amount of bacteria was added, the percentage of non-vacuolarized and total, osmotically shocked, Salmonella was the same (Fig. 1A, right panel). At the two time-points of infection, non-vacuolarized Salmonella accounted for only 16% of the total number of bacteria in the PNS, indicating that the vast majority of SCVs are intact after cell homogenization.

**Fig. 1.** Isolation of highly enriched and intact SCVs from infected host cells. A. Salmonella-infected HeLa cells were mechanically homogenized in an isotonic buffer and the SCV integrity in the PNS was tested by ELISA. Non-infected PNS supplemented with a known amount of added bacteria was used as control. A small aliquot of sample was subjected to a sandwich ELISA, either at isotonic conditions (non-treated) or after osmotic shock. Data are represented as the mean ± SEM from three independent and representative experiments. B. The different PNSs (non-infected control, 30 min and 3 h infection) were fractionated by ultracentrifugation in a density gradient and the number of Salmonella in each fraction (F1 to F12) was determined by counting CFUs (black bars). Simultaneously, the density of each fraction was the same (Fig. 1A, right panel). C. Aliquots of fractions F6 to F8 were subjected to the ELISA method described in (A). D. All fractions F1 to F12 and the PNS were tested using markers for the following compartments: early endosomes (EEA-1 and Rab5), late endosomes and lysosomes (Lamp-1), Golgi (GM130), peroxisomes (catalase), mitochondria (TOM22) and ER (calreticulin). The organelle distribution in the density gradients was the same in the non-infected control (left panel), 30 min (right panel) and 3 h (Fig. S2). Salmonella distribution, detected with a specific anti-Salmonella LPS antibody, was the same in the 30 min (right panel) and 3 h (Fig. S2) fractionations. All P-values were determined using the Student's t-test.

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We performed five independent infection experiments, followed by ultracentrifugation, with highly reproducible linear density gradients (Fig. S1C). This separated SCVs from remaining subcellular organelles. To determine the SCV position within the gradient, we measured the number of colony forming units (CFUs) of total *Salmonella* in each fraction. As a control, we added *Salmonella* to a non-infected PNS before fractionation and found that the majority of non-vacuolized bacteria (Fig. 1B, left panel) accumulated in fractions F7 and F8 (1.11 and 1.12 g cm\(^{-3}\) respectively). In contrast, the fractionation of the infected PNS, both at 30 min and 3 h (Fig. 1B, middle and right panels), led to a 500-fold enrichment of total bacteria in fraction F6 (1.10 g cm\(^{-3}\)). We next evaluated SCV integrity in fractions F6 to F8 by ELISA. The number of non-vacuolized bacteria in fractions F6 and F7 was significantly lower than the total *Salmonella* as determined by osmotic shock (Fig. 1C, middle and right panels). This indicates that the bacteria in these two fractions were mostly within intact SCVs. In contrast, fraction F8 contained solely non-vacuolized *Salmonella*, as no increase was seen after osmotic shock. Thus, in fractions F6 and F7, we successfully isolated intact SCVs and separated them from non-vacuolized bacteria.

We assessed the separation efficiency of different subcellular organelles by Western blot (Fig. 1D). As expected, the Golgi apparatus and early endosomes showed a low density (between 1.03 and 1.09 g cm\(^{-3}\)), whereas peroxisomes had a high density (between 1.12 and 1.15 g cm\(^{-3}\)) and did not overlap with fractions F6 and F7. Late endosomes/lysosomes accumulated mostly in F5 (1.09 g cm\(^{-3}\)) but also showed partial overlap with the SCV-enriched fraction F6. The ER displayed a broad distribution within the gradient, spanning from F5 to F8 (1.09 to 1.12 g cm\(^{-3}\)) and overlapped with the SCV fractions. This distribution can be explained by the complex structure of the ER and its heterogeneous physical properties. Importantly, the tested conditions led to the accumulation of mitochondria only in fractions F7 and F8 (1.11–1.12 g cm\(^{-3}\)), not overlapping with the SCV fraction, F6. For all tested antibodies, equal results were obtained for the 30 min (Fig. 1D, right panel) and 3 h fractionation (Fig. S2). We successfully isolated intact and highly enriched SCVs in fraction F6, which we then used to determine the protein composition of the SCV.

The dynamic quantitative host protein composition of the SCV

To determine the repertoire of host proteins enriched at the SCV at the two time-points, we used a label-free quantitative mass spectrometry (MS)-based proteomics approach. We compared the relative protein abundance in the SCV fraction (30 min or 3 h) with a non-infected control fraction, prepared in parallel. MS data obtained from five independent experiments were analysed by MaxQuant, both for protein identification and quantification (Cox and Mann, 2008; Luber et al., 2010). As protein abundance from contaminating organelles should not differ between the tested conditions (Rao et al., 2009), factors that are enriched in the SCV can be identified through their positive fold-change ratio compared with the control.

Analysis of the entire MS data set identified 2522 host proteins, 392 of which (~15%) showed a statistically significant fold-change increase at one or both of the two time-points of infection (Fig. 2A and Fig. S3A and B and Tables S1 and S2). In detail, 173 proteins were enriched solely at the 30 min SCV (red), 124 uniquely at the 3 h SCV (green) and 95 at both time-points (orange) (Fig. 2A). These three specific subsets of host factors (only 30 min SCV; only 3 h SCV; 30 min + 3 h SCV; complete list is in Table S3) reflect how SCV protein composition is altered during its maturation. An overview of some selected proteins dynamically associated with the SCV can be found in Table S4.

The identified proteins were grouped according to their putative subcellular localization or biological function by gene ontology analysis. We detected a significant enrichment of ER-, Golgi- and vesicle-derived proteins, all of which decreased with time. Also, lysosome-derived proteins were exclusively increased in the 30 min SCV (Fig. 2B). We observed significant enrichment of proteins involved in ER to Golgi vesicle-mediated transport at the 30 min SCV or at both time-points (Fig. S3C). Together, these data highlight that SCV protein composition dynamically varies with time and that specific biological processes are implicated at each step of SCV maturation.

Several host proteins identified by our quantitative approach were previously described to be associated with the SCV (Rathman et al., 1997; Steele-Mortimer et al., 1999; Harrison et al., 2004; Boucrot et al., 2005; Smith et al., 2007; Thurston et al., 2012) or involved in *Salmonella* infection (Cripps and Casanova, 2003; Hänisch et al., 2010; Jolly et al., 2014), confirming the potential of our work (Fig. 2C and Table S4). Importantly, we also identified several novel host proteins associated with the SCV (Tables S3 and S4). Among them, we were particularly interested in ER-derived proteins, all the factors from the coat protein complex II (COPII) machinery and lysosome-derived proteins (Fig. 2C).

**Early SCV interactions with ER-derived COPII complex promote vacuolar rupture and cytoplasmic *Salmonella* growth through hyper-replication**

A key finding in our quantitative proteomic analysis was that approximately 20% of the proteins enriched in early SCVs were derived from the ER (Fig. 2B and Table S3 for
Fig. 2. The SCV protein composition dynamically changes during vacuole maturation. Protein data from fraction F6 of each condition (control, 30 min and 3 h) was analysed with label-free quantitation algorithms (MaxQuant software). Relative host protein abundances were compared between the control and any two infection time-points. Proteins enriched at a specific time-point were considered as SCV constituents. A positive fold-change was considered when the abundance ratio 30 min/control or 3 h/control was $>1.3$ (log$_2$ fold-change $>0.387$) with a $P$-value $<0.05$.

A. Thirty minute SCV-enriched proteins were compared with 3 h SCV-enriched proteins and a Venn diagram was built.

B. The host proteins enriched in the SCV were grouped according to their subcellular localization. For each term, the analysis was performed for the proteins enriched uniquely either at the 30 min SCV (only 30 min SCV, red bars) or at the 3 h SCV (only 3 h SCV, green bars) or for the proteins enriched at both time-points (both 30 min + 3 h SCV, orange bars). The graphs show the percentage of host proteins enriched at the SCV, relative to the total number of proteins identified in each condition. Statistics were performed by determining the $P$-values (EASE score, as mentioned in the Experimental procedures) and show the robustness of gene-term enrichment for each condition. ND, non-enriched factors in comparison with the control.

C. Selected proteins enriched in the early (30 min) or maturing (3 h) SCV are shown with a '+' symbol.

| Known SCV associated proteins | Early SCV | Maturing SCV |
|------------------------------|----------|--------------|
| Dynactin                     | n.d.     | +            |
| Dynein                       | +        | +            |
| Galectin-3                   | +        | –            |
| Kinesin light chain 1        | n.d.     | +            |
| Kinesin light chain 2        | n.d.     | +            |
| Kinesin-1 heavy chain        | +        | +            |
| Lysosomal acid phosphatase   | +        | –            |
| Rab-4a                       | +        | n.d.         |
| Rab-7a                       | +        | n.d.         |
| VATPase                      | +        | n.d.         |

| ER-derived proteins          | Early SCV | Maturing SCV |
|------------------------------|----------|--------------|
| Calnexin                     | +        | +            |
| Reticulon-4                  | +        | n.d.         |
| VAP-A                        | +        | –            |
| VAP-B                        | +        | –            |

| COPII-complex proteins       | Early SCV | Maturing SCV |
|------------------------------|----------|--------------|
| Sec13                        | +        | n.d.         |
| Sec23                        | +        | +            |
| Sec24                        | +        | n.d.         |
| Sec31                        | +        | n.d.         |

| Lysosome-derived proteins    | Early SCV | Maturing SCV |
|------------------------------|----------|--------------|
| Cathepsin B                  | +        | –            |
| Cathepsin Z                  | +        | –            |
| Cl Man-6-P receptor          | +        | n.d.         |
| Dipeptidyl peptidase 2       | +        | –            |

| Proteins known to be involved in Salmonella infection | Early SCV | Maturing SCV |
|------------------------------------------------------|----------|--------------|
| Arp2/3 complex                                       | +        | n.d.         |
| Actin                                                | +        | n.d.         |
| Annexin A2                                           | +        | n.d.         |
| Filamin                                              | +        | +            |
| Tropomyosin-4                                        | +        | n.d.         |
| Tubulin                                              | n.d.     | +            |

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The precise nature of the SCV–ER interactions could not be determined by light microscopy due to its resolution limit. Therefore, we applied an emerging technique, termed corelative-focused ion beam/scanning electron tomography (C-FIB/SEM). Fluorescence microscopy and large-volume ultrastructural tomography are combined in a single three-dimensional (3D) data set, allowing for precise identification of molecules of interest within the ultrastructural volume. This technique was previously used in our laboratory to characterize the host cell environment around the Shigella-containing vacuole (Melloul et al., 2014). In short, cells were infected with Salmonella for 30 min and fixed. The ER was labelled with a reticulon-4 antibody followed by indirect immunofluorescence; DNA (bacteria and host cell nuclei) was stained and cells were imaged by confocal microscopy followed by FIB/SEM tomography at the exact same location (Fig. 3E, see Experimental procedures for details). The two data sets were then correlated and combined into a single three-dimensional (3D) data set presented here. DAPI and reticulon-4 fluorescent signals were segmented by thresholding (left middle and lower panels) and the corresponding FIB/SEM data (upper middle and right panels) show the segmentation of bacteria (blue) and of the SCV lumen (yellow). After superimposing the ER fluorescent signal with the FIB/SEM data, we could segment the ER ultrastructure (red). Strikingly, in all data sets \((n = 4)\) the SCVs were surrounded by ER (Fig. 3E, lower right panel). Moreover, in all data sets we could observe membrane interactions between the ER and SCVs (Fig. 3F and Fig. S4B show different data sets; in Video Clip S1, ER contacts with multiple SCVs are also observed) that resembled membrane contact sites (MCS) (Orci et al., 2009; Eden et al., 2010; Stefan et al., 2013). Therefore, we conclude that there is membrane contact between the early SCV and the ER.

Interestingly, we found that the early SCV proteome was enriched in all constituents of the COPII complex, namely Sec13, Sec23, Sec24 and Sec31 [proteins form a complex leading to vesicle budding from the ER membrane and the transport of cargo to the Golgi or the cell surface (Haucke, 2003; Sato, 2004; Lord et al., 2013)]. Using immunofluorescence microscopy, we observed the accumulation of COPII complex around a small percentage of intracellular Salmonella (8–15%), as seen by Sec13-positive bacteria (Fig. 4B and C and Fig. S4C). This confirmed our proteomic data and also suggested a potential link between the ER and some of the early SCVs, possibly via COPII-coated vesicles.

To examine if COPII affects the intracellular Salmonella lifestyle, we measured bacterial growth within epithelial cells using gentamicin assays (Elsinghorst, 1994), after inhibiting COPII function via Sec13 siRNA treatment. Salmonella entry into HeLa cells, measured 1 h p.i., was not affected after Sec13 knockdown (data not shown). Strikingly, Sec13-depletion impaired intracellular bacterial growth from 3 h p.i. onwards, as compared with the control (Fig. 4D). These data show that the COPII complex is crucial for bacterial growth within the host cell. Then, we tested if there was a functional link between the
COPII complex and bacterial hyper-replication within the cytosol, which requires SCV rupture (Knodler et al., 2010; 2014; Malik-Kale et al., 2012). By 6 and 9 h p.i., we found COPII localizing to bacterial-shaped structures in 100% of the cells with hyper-replicating Salmonella (Fig. S4D and E), although only surrounding some of the bacteria. These structures were specific to bacterial hyper-replication, as they were absent in infected cells not containing...
hyper-replicating bacteria. This suggested that the COPII complex is directly implicated in promoting *Salmonella* hyper-replication. To test this, cells were transfected with *Sec13* siRNA and the percentage of infected cells containing hyper-replicative bacteria was determined 6 h p.i. by fluorescence microscopy. *Sec13* depletion significantly decreased the number of cells with hyper-replicating *Salmonella* (Fig. 4E) as compared with the control. To assess if COPII vesicles were specifically associated with SCVs that undergo rupture, we used fluorescent gaelectin-3 as a marker for vacuolar lysis (Paz et al., 2010; Ehsani et al., 2012). SCV rupture was observed as soon as 30 min p.i., occurring in SCVs that were also positive for the COPII complex (Fig. 4B). Moreover, we could not detect any gaelectin-3 positive bacteria by 10 min p.i., suggesting that vacuolar damage happens after the initial accumulation of COPII vesicles at the early SCV.

We then asked if COPII function was involved in SCV rupture. By 1 h p.i., *Sec13* knockdown significantly reduced the percentage of infected cells with ruptured SCVs (Fig. 4F), which were identified by the presence of gaelectin-3-positive bacteria. These results suggest that COPII complex assembly is important for SCV rupture and *Salmonella* hyper-replication. To confirm this, we overexpressed two different *Sec13* mutants, *Sec13*T39N or *Sec13*H79G, which are, respectively, constitutively inactive or active (Ward et al., 2001; Schindler and Schekman, 2009). In this way, it is possible to perturb either the first step of COPII complex assembly or the final step of COPII vesicle release. Interestingly, inhibition of either of these steps resulted in impairment of *Salmonella* hyper-replication (Fig. 4E), and the upstream event of SCV rupture (Fig. 4F), in a similar manner as *Sec13* knockdown.

Altogether, these data indicate that COPII vesicles accumulate at the early SCV and the latter physically interacts with the ER. Moreover, COPII assembly promotes SCV rupture followed by bacterial hyper-replication in the host cytosol. Salmonella growth within the late SCV is regulated by interactions with lysosome-like vesicles

Our quantitative proteomics data confirmed that the SCV also interacts with lysosomes (Fig. 2B and C) (Oh et al., 1996; Drecktrah et al., 2007). Associated proteins included cathepsins, the cation-independent mannose-6-phosphate receptor (CI-M6PR), dipeptidyl peptidase 2, palmitoyl protein thioesterase 1 (PPT-1) and Rab-7a. Especially, they were enriched in the early SCV and then depleted in the maturing SCV (Figs 2C and 5A). We also observed the same trend for VAMP7 (see also Fig. 4A), a lysosomal v-SNARE protein essential for heterotypic, late endosome-lysosome fusion (Luzio et al., 2007). This protein was interesting to us as it has been associated with intracellular growth of other bacteria, such as *Chlamydia trachomatis* and *Coxiella burnetii* in epithelial cells (Delevoye et al., 2008; Campoy et al., 2013). Moreover, in the early SCV, we observed the enrichment of syntaxin-4, a t-SNARE that binds VAMP7 leading to lysosomal fusion with the PM (Luzio et al., 2010). By using fluorescently labelled VAMP7, we found that VAMP7-positive vesicles were recruited to the early SCV, as most of the bacteria were positive by 30 min p.i. (Fig. 5B, arrows and 5C). Additionally, the number of VAMP7-positive bacteria significantly decreased during SCV maturation, corroborating our quantitative proteomics data. Co-immunostaining for the lysosomal marker Lamp-1 showed colocalization with VAMP7 on cytosolic vesicles and around bacteria (Fig. 5B and D). By time-lapse microscopy, we observed that VAMP7-positive vesicles accumulating around the early SCV are acidic, as they were positive for LysoTracker (Fig. 5S; Video Clips S2–S4). Interestingly, as the SCV matures, we observed that VAMP7 either transiently dispersed from the SCV (Fig. S5A and B; Video Clips S2 and S3) or not (Fig. S5C; Video Clip S4). However, in all cases we observed a diminishing of LysoTracker signal around the SCV (Fig. S5; Video Clips S2–S4). Together with the proteomics data, this suggests that vesicles with...
Fig. 5. The early SCV is enriched in lysosomal proteins.
A. The graph shows the log, fold-change in lysosomal protein abundance in the SCV-enriched fraction compared with the non-infected control from five independent experiments. Relative protein abundances were considered different when the log, fold-change > 0.387 (red line) or < -0.387 (green line) with a P-value < 0.05. Statistics are relative to the non-infected control for each protein.
B–D. Cells were transfected with VAMP7-RFP, infected with \textit{Salmonella} and fixed at different time-points. In (B), infected cells were immunostained for Lamp-1 and bacteria were labelled with DAPI. Arrows and arrowheads point to VAMP7-positive or -negative \textit{Salmonella} respectively. Representative confocal microscopy images are shown. The graph in (C) shows the percentage of VAMP7-positive \textit{Salmonella} by counting at least 100 intracellular bacteria from three independent experiments performed in triplicate. Line-scan analysis (D) of VAMP7-RFP and Lamp-1-positive SCVs at 30 min p.i. The graph shows the mean ± SEM relative fluorescence intensity from 25 SCVs. P-values were determined using the two-way analysis of variance test for multiple comparisons from three independent experiments performed in triplicate. Scale bars represent 10 µm.
lysosomal characteristics directly interact with the SCV at early stages of its maturation, followed by depletion of lysosomal content. Lysosome-like vesicle fusion with the SCV were directly visualized by C-FIB/SEM. Cells were transfected with VAMP7-RFP, infected with Salmonella for 30 min and fixed. Figure 6A (Video Clip S5) shows that VAMP7-and Lamp-1-positive structures in the bacterial vicinity (upper panels) correlate to vesicles that surround and interact with the SCVs, as seen by overlaying the fluorescence signals with the ultrastructural data (middle and lower panels). Importantly, detailed analysis of different areas within the C-FIB/SEM data (Fig. 6B) depicts an SCV fused with a VAMP7/Lamp-1-positive vesicle (left panels, yellow arrows). Moreover, we also observed membrane invagination of a VAMP7/Lamp-1-positive vesicle into a SCV (right panels, red arrows), most likely representing the early steps of vesicle fusion. Thus, C-FIB/SEM data clearly show that the early SCV interacts and fuses with lysosome-like vesicles in its vicinity.

We then investigated the involvement of VAMP7 on Salmonella growth during longer time-courses of epithelial cell infection. Depletion of this protein did not affect bacterial entry into host cells (data not shown) and did not alter intracellular Salmonella growth until 6 h p.i. (Fig. 7A). In contrast, VAMP7 knockdown stalled bacterial growth at later time-points (> 6 h p.i.). This effect was not due to impaired bacterial escape from the SCV and replication in the cytosol, as VAMP7 knockdown did not affect Salmonella hyper-replication (Fig. S6A). As described earlier (Figs 2B and 5A), we obtained decreased lysosomal protein content in the maturing SCV (around 3 h). Interestingly, time-lapse microscopy indicated a resurging, massive, VAMP7 accumulation around the late SCV (> 5 h p.i.), often forming tubular structures resembling SIFs (Fig. S5B and C; Video Clips S3 and S4), but these did not label with LysoTracker. We evaluated and confirmed this hypothesis, as VAMP7 colocalizes with Lamp-1 in the SIFs (Fig. 7B, time-lapse microscopy is shown in Fig. S6B and Video Clip S6). Moreover, VAMP7 is important for SIF formation, as we observed a significant decrease in the number of infected cells with SIFs after VAMP7 depletion (Fig. 7C).

Together, we show that the early SCV fuses with VAMP7-positive lysosome-like vesicles, but these do not seem to be important for the initial steps of intracellular Salmonella growth. Moreover, the lysosomal content is quickly depleted from the SCV during its maturation process. At later time-points, VAMP7-positive vesicles with reduced acidic activity are recruited to the late SCV, where they contribute to SIF formation and establishment/maintenance of the replicative bacterial niche.

**Discussion**

In this work, a quantitative proteomics approach was used to identify novel host proteins associated with the SCV. We show how the dynamic interaction between this unique organelle and different host cell compartments regulates bacterial growth during SCV maturation in different ways (Fig. 7D). Within the first hours of infection, the COPII complex regulates SCV integrity, inducing Salmonella access to the host cell cytosol and hyper-replication. At later stages, interactions between the SCV and lysosome-like vesicles promote SIF formation and intravacuolar bacterial growth.

We used fractionation methodologies based on density gradients to successfully isolate early (modified mostly by the bacterial T3SS1) or maturing SCVs (modified by the bacterial T3SS1 and T3SS2). Vacuoles in late stages of maturation were not isolated, as bacterial replication and the complex structure of membrane tubules formed by the SCV would interfere with the biochemical purification. The isolated SCVs were highly enriched and largely free from other organelles, most importantly from mitochondria-derived contaminants. We assessed SCV integrity through a non-biased ELISA assay, rather than the electron microscopy (EM) used in other studies (Gagnon et al., 2002; Shevchuk et al., 2009), due to the challenges of handling, preparation and image interpretation of isolated cellular fractions for EM. To circumvent the limitations of biochemical SCV isolation and incomplete organelle separation (Rogers and Foster, 2007; Walther and Mann, 2010), we performed a label-free quantitative subtractive proteomics analysis from five independent infection experiments. The inventory of the target SCV fraction was compared with a related non-infected control fraction. The remaining proteins enriched in the SCV fraction can be considered specific SCV components. Our stringent quantification has some limitations, potentially missing some true hits if they are also present to some extent as contaminants in the control fraction. A likely example is Lamp-1, which is known to localize to maturing and late SCVs (Steele-Mortimer, 2008), but was excluded by our quantification procedure. Still we identified about 400 host proteins enriched in the SCV fraction at different stages of maturation, including many factors not previously known to associate with this compartment. Interestingly, we found that the SCV proteome is enriched in factors derived from the host ER, COPII vesicles and lysosomes. Therefore, we investigated how the interactions between these subcellular compartments and the SCV affect intracellular bacterial growth.

Although it remains debated, several proteomics studies provided evidence that the ER associates with the phagosome and is an important source of membrane for phagocytosis (Gagnon et al., 2002; Guermonprez et al.,

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Fig. 6. The early SCVs fuse with lysosome-like vesicles in their vicinity. Fluorescence confocal microscopy was followed by large-volume FIB/SEM of the same Salmonella-infected cell. A. Confocal image of an infected cell is shown in the upper left panel, together with the region that was imaged by FIB/SEM (white box). Corresponding zoomed images for DAPI and VAMP7 or DAPI and Lamp-1 are shown in the upper middle and right panels respectively. A xz-view FIB/SEM section is shown in the middle left panel, together with the overlay with VAMP7 or Lamp-1 fluorescent signals (middle and right panels respectively). B. Zoomed images of different FIB/SEM sections are shown, together with the respective segmentations, both in the xz and xy views. In the left panels, yellow arrows point to the site of SCV fusion with a lysosome (bacteria were segmented in blue; SCV and fused lysosome in yellow). In the right panels, red arrows point to the site of lysosomal membrane invagination into the SCV (bacteria were segmented in blue; SCV in yellow and lysosome in red). Amira software was used to present the data. The figure shows one representative C-FIB/SEM data set (n = 3).

2003; Houde et al., 2003). Our data support this hypothesis, as the early SCV was enriched in ER proteins. Moreover, we observed the recruitment of ER protein calnexin to the bacterial entry site, which could suggest that the ER provides membrane for PM ruffles (Gagnon et al., 2002) and Salmonella uptake. We additionally speculate that calnexin accumulation at the entry site could be important for Salmonella uptake, as this protein was previously shown to control phagocytosis of particles (Müller-Taubenberger et al., 2001) and L. pneumophila (Fajardo et al., 2004), possibly by modulating actin. Other quantitative proteomics studies support the hypothesis of ER-phagosome association by showing that the ER accounts for approximately 20% of the early phagosome proteome (Campbell-Valois et al., 2012), in agreement with our analysis in which the same percentage of ER protein enrichment was found at the early SCV. Finally, our C-FIB/SEM approach resolves the controversy about ER interactions with the SCV as we could visualize them directly. We hypothesize that the ER and the SCV are connected via MCS, which might regulate several functions at the interface, such as lipid transfer or modification, Ca²⁺ signalling, phosphatidylinositol metabolism (English and Voeltz, 2013; Stefan et al., 2013) or membrane fission (Rowland et al., 2014). Moreover, ER-SCV MCS could be promoted through interactions between Rab7 and the ER protein VAP-A. We found these two proteins enriched in our early SCV proteome and they were previously described to act as a scaffold for the formation of ER-late endosome MCS (Rocha et al., 2009). Even though we did not observe fusion between the SCV and the ER, it cannot be excluded and should be further investigated. We also found similarities between the proteomes of the SCV and the LCV (Hoffmann et al., 2014). ER-derived proteins such as the inositol 1,4,5-trisphosphate, atlastin-3, Rab-2a, Sec20, Sec22b, reticulon-4, VAP-A and VAP-B are enriched in the vacuoles containing Salmonella or Legionella, pointing to possible similar mechanisms of interaction with the ER.

A striking piece of evidence pointing to the existence of SCV-ER communication is that all the components of the COPII complex were enriched in the early SCV proteome. Importantly, COPII assembly regulates Salmonella replication within epithelial cells via Sar1 GTPase activity. We propose that COPII accumulation on the SCV destabilizes vacuolar integrity, through a mechanism that needs to be determined, promoting Salmonella release into the cytosol and hyper-replication. Intraacellular L. pneumophila growth is also promoted by Sar1-mediated COPII activity. However, in this case, COPII activity enables exiting vesicles from the ER to fuse with the LCV to promote the formation of a replicative organelle (Kagan and Roy, 2002; Robinson and Roy, 2006). Thus, it seems that both Salmonella and L. pneumophila co-opt COPII vesicle activity for different vacuolar maturation outcomes (rupture vs. creation of a replicative organelle) that result in a similar advantage for the bacterium: intracellular growth. It has been recently shown for different bacterial pathogens that host proteins are involved in vacuolar rupture (Akimana et al., 2010; Davis et al., 2012; Mellouk et al., 2014). In the case of Shigella flexneri, which enters host cells similarly to Salmonella, vacuolar lysis and bacterial escape into the cytosol is mediated by several host GTPases, such as Cdc42, Rab-3b, Rab-4a, Rab-5a and Rab-11a (Mellouk et al., 2014). However, the COPII component Sec13 does not seem to be involved in the rupture of the Shigella vacuole. It would be interesting to address the common host proteins targeted by Shigella and Salmonella for destabilization of vacuolar membrane integrity. It is thought that ER tubules could be preferred sites for the formation of COPII vesicles at the ER exit sites (ERES) (Friedman and Voeltz, 2011). Moreover, markers for ERES are often found in close juxtaposition with other organelles (Hughes and Stephens, 2008; Kurokawa et al., 2014), which might allow fast, efficient and targeted routes of secretory transport (Budnik and Stephens, 2009). Taken together with our FIB/SEM data, we propose that the close proximity between the ER and the SCV facilitates COPII vesicle activity between ERES and the SCV. Recruitment of ER-derived vesicles to the SCV could be facilitated by Sec22b and syntaxin-4, which were enriched on the early SCV proteome. These proteins were previously described to act together, mediating the recruitment of ER components to phagosomes in dendritic cells (Cebrian et al., 2011).
Lysosomal interaction with the SCV has remained a matter of debate. Initial studies pointed to the SCV avoiding fusion with late endosomes and lysosomes, as hydrolases and the M6PR were reported to be absent from the late SCV (Buchmeier and Heffron, 1991; Garcia-del Portillo and Finlay, 1995; Rathman et al., 1997; Hashim et al., 2000). This is, however, controversial (Oh et al., 1996) and has been recently questioned, as extensive dynamic interactions between the lysosomal system and the SCV were observed during vacuole maturation (Drecktrah et al., 2007). Intriguingly, our proteomics data show that the early SCV is enriched in lysosomal proteins, pointing to a model where the early SCV remains accessible to incoming lysosomal content that does not take place at later time-points of vacuole maturation. Such direct interactions were visualized by C-FIB/SEM, in which we observed fusion events of VAMP7/Lamp-1-positive vesicles with the early SCV. Thus, fusion between
lysosome-like vesicles and the early SCV could be part of a normal mechanism of vacuole maturation. When the vacuole reaches the stage of the maturing SCV, it contains fewer lysosomal proteins, such as VAMP7. One possible explanation is that Salmonella could control recycling pathways in the host cell to remove unwanted proteins from the SCV in order to avoid degradation, as previously suggested (Bujny et al., 2008). Moreover, interaction with the ER might modulate SCV maturation, as the acquisition of ER-derived membranes to phagosomes was shown to alter normal phagosomal maturation in dendritic cells (Cebran et al., 2011). Interestingly, we show here that interactions between SCVs and VAMP7-positive vesicles do not play a role in the establishment of the early and maturing SCV.

Surprisingly, at later time-points (> 5 h p.i.), we verified that VAMP7-positive but LysoTracker-negative vesicles are recruited to the late SCV and also to SIFs. Our data also show that VAMP7 is important for SIF formation and Salmonella replication exclusively within the late SCV. Due to the role of VAMP7 on vesicle fusion, we hypothesize that this cellular mechanism could provide membrane for SIF elongation and bacterial replication. Despite the late SCV being again enriched in lysosomal proteins such as VAMP7 and Lamp-1, it is LysoTracker negative. This confirms the hypothesis that the early SCV interacts with lysosomes, and that Salmonella actively reduces the lysosomal content/activity of its replicative niche during vacuole maturation (McGourty et al., 2012). Moreover, as SIFs display reduced acidity, they might be involved in diluting the lysosomal proteins that are delivered to the SCV (Schroeder et al., 2011). In the future, it will be interesting to identify bacterial effectors that control the successive cycles of interactions between the SCV and the lysosomal system. It could also be informative to compare our data with SCV remodelling in macrophages, as SCV-lysosome fusions have been reported in those cells (Oh et al., 1996), or with intestinal epithelia, where the mechanisms of SCV maturation are largely unknown.

In summary, quantitative proteomic analysis of the SCV combined with cell biology techniques revealed that this unique and specialized organelle interacts with several host cell compartments. We demonstrate that Salmonella growth within epithelial cells is regulated by the interactions between the SCV and either the ER or the lysosomal system, in distinct ways. ER-derived COPII-vesicle activity promotes SCV rupture and Salmonella hyper-replication within the cytosol, while successive interactions between the SCV and VAMP7-positive vesicles regulate bacterial growth within the SCV, by promoting formation of SIFs.

Experimental procedures

Bacterial strains

The following Salmonella strains were used: SL1344 (wild type), SL1344 pM965 (Salmonella-GFP) and SL1344 expressing dsRed (Salmonella-dsRed). Bacteria were grown in lysogeny broth (LB) medium supplemented with 0.3 M NaCl at 37°C in an orbital shaker. LB was supplemented with streptomycin (50 μg ml⁻¹) and, when appropriate, with ampicillin (50 μg ml⁻¹).

Plasmids, siRNAs and cell transfection

HeLa cells were plated either on 12-well plated containing glass coverslips (1 x 10⁵ cells per well) or into 96-well glass bottom plates (Greiner) (7 x 10³ cells per well) 24 h before plasmid transfection. Cells were then transfected with one or two expression plasmids using the X-tremeGENE 9 DNA Transfection Reagent (Roche) for 24 or 48 h, according to the manufacturer’s instructions. The pRFP-VAMP7 plasmid was kindly provided by Thierry Galli (Institut Jacques Monod, Université Paris 7). The plasmids encoding the CFP-tagged Sar1, Sar1[T39N] and Sar1[H79G] were a kind gift from Franck Perez (Institut Curie). All siRNAs SMARTpool were obtained from Dharmacon: VAMP7 (6845), Sec13 (6396), non-specific non-targeting pool. Cells were reversed transfected for 72 h with Lipofectamine RNAiMAX (Life Technologies) reagent according to the manufacturer’s instructions. Protein knockdown efficiency was assessed by Western blot, by lysing cells with RIPA buffer at 4°C. Equal protein
amounts were separated in a SDS-PAGE gel, transferred to a nitrocellulose membrane and immunoblotted with Sec13 or VAMP7 specific antibodies. An actin antibody was used as loading control.

Cell culture and infection assays

All cell culture reagents were purchased from Invitrogen unless otherwise stated. Human epithelial HeLa cells (clone CCL-2 from the American Type Culture Collection (ATCC)) were cultured in Dulbecco’s modified eagle’s medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C, 5% CO₂. All live-cell fluorescence microscopy and infection assays were performed in EM buffer (120 mM NaCl, 7 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 5 mM glucose, 25 mM HEPES, pH 7.3). For invasion experiments, overnight bacterial cultures were subcultured 1/20 and grown until late exponential/early stationary phase. Before infection, bacteria were gently washed with PBS and resuspended in EM buffer. Except for live-cell microscopy, bacteria were added to the cells at a multiplicity of infection (MOI) of 100, incubated for 5 min at room temperature and then at 37°C for 10 min, so that a synchronized infection could be followed. Non-internalized bacteria were washed three times with warm EM buffer and incubated up to 30 min at 37°C. Extracellular bacteria were killed by adding EM containing 50 μg ml⁻¹ gentamicin for 1 h. The concentration of gentamicin was then decreased to 10 μg ml⁻¹ for the remainder of the experiment and 10% FBS was added to the medium. At the desired time-points, the cells were either processed for fractionation, enumeration of intracellular bacteria or fixed for immunofluorescence analysis. To count the number of intracellular bacteria, infected cells were gently washed with PBS and lysed with ice-cold distilled water containing 0.2% Triton X-100 for 5 min. Bacteria were then serially diluted and plated onto LB agar.

Cell fractionation and isolation of the SCV

For the isolation of the SCV, approximately 6 × 10⁷ HeLa cells were used in T225 flasks. Cells were infected as described before and, at the selected time-points (30 min or 3 h), they were extensively washed with ice-cold homogenization buffer (HB: 250 mM sucrose, 0.5 mM ethylene glycol tetracetic acid (EGTA), 20 mM HEPES-KOH pH 7.4, supplemented with complete protease inhibitors and 5 μg ml⁻¹ cytochalasin-D), detached and then homogenized in HB with a Dounce homogenizer. All steps were performed at 4°C. Between 30 and 40 strokes were performed, until more than 80% free nuclei were visible. Nuclei and intact cells were removed by performing three sequential centrifugations, at 100 g (1000 rpm) for 5 min each, in order to obtain the PNS. In parallel, a non-infected control was prepared. In order to separate all the subcellular organelles, the PNS was loaded on top of a 10–25% (1.06–1.15 g cm⁻³) linear OptiPrep (Sigma) gradient with a 50% (1.22 g cm⁻³) cushion, in a 14 × 89 mm ultracentrifuge tube (Beckman), and then centrifuged at 210000 g (35000 rpm) for 4 h at 4°C, in a SW-41 swinging bucket rotor, with low acceleration and slow brake. Approximately 5 × 10⁹ bacteria were added to the non-infected control PNS. After ultracentrifugation, 1.0 ml fractions were collected from the top to the bottom of the gradient and each fraction was analysed by measuring its refractive index, the number of bacteria by CFUs and the organelle separation efficiency by Western blot. Equal amount of proteins in each fraction were analysed by Western blot. The following primary antibodies were used: mouse anti-EEA-1 (1:2500, BD Biosciences), mouse anti-Rab5 (1:2000, BD Biosciences), rabbit anti-Lamp-1 (1:2000, Abcam), mouse anti-GM130 (1:1000, BD Biosciences), rabbit anti-Catalase (1:2500, Abbcam), mouse anti-Calreticulin (1:2000, Abcam), mouse anti-TOM22 (1:2000, Sigma), rabbit anti-VAMP7 (1:2000, Pierce), rabbit anti-Salmonella Lipopolysaccharide (LPS) (1:20000, Abbcam) and mouse anti-reticulon-4 (1:2000, Thermo-Scientific). The secondary antibodies were diluted 1:10000 (anti-mouse-HRP and anti-rabbit-HRP, Amersham).

ELISA for quantification of intact SCVs

The bottom of the wells of an ELISA plate (Nunc) was coated with a polyclonal rabbit anti-Salmonella antibody (Abcam) in PBS and incubated overnight at 4°C. Blocking was carried out by incubating the wells with a 2% BSA solution at room temperature for 90 min. Samples from the PNS or from F6 to F8 were added to the wells and incubated for 1 h at room temperature. As a control, an aliquot from the same sample was subjected to osmotic shock by incubation with distilled water. Standard curves were generated by adding known amounts of bacteria in 1:2 dilution series. Bacteria were then detected by incubation with the same antibody, biotinylated in 2% BSA. Signal was quantified at 450 nm after sequential incubation with streptavidin peroxidase (Sigma) and o-Phenylenediamine dihydrochloride (Sigma).

Sample preparation for MS, data processing and analysis

Each experiment was carried out in five biological replicates. After density centrifugation, 150 μl of fraction F6, which was highly enriched in intact SCVs, was subjected to methanol/chloroform protein precipitation (Wessel and Flügge, 1984). Proteins were then separated in one-dimensional SDS-PAGE in order to eliminate OptiPrep contaminations, each lane was cut into 10 slices and in-gel tryptic digestion was performed as described previously (Wilm et al., 1996). Peptides were finally extracted in 50 mM NH₄HCO₃/acetonitrile/formic acid (42.5/42.5/5), dried down and reconstituted in H₂O/acetonitrile/formic acid (98/2/0.1) before LC-MS/MS analysis. Tryptic digests were analysed by nanoLC-MS/MS using an Ultimate 3000 RSLC system (Dionex, Thermo-Scientific, Waltham, MA, USA) coupled to the nanoelectrospray ion source of a Q-Exactive mass spectrometer (Thermo-Scientific, Bremen, Germany). One microgram of each digest was loaded on a C-18 µ-precolumn (C-18 PepMap100, 5 μm, 100 Å, Dionex, Thermo-Scientific, Waltham, MA, USA) at a flow rate of 30 μl min⁻¹ of solvent A and the separation was performed using an in-house packed 15 cm nano-HPLC column (75 μm inner diameter) with C-18 resins (3 μm particles, 100 Å pore size, Reprosil C-18, Dr. Maisch GmbH). Peptides were separated at a flow rate of 300 nI min⁻¹ using a gradient of 2% to 55% solvent B for 30 min, followed by a 10 min washing step at 100% solvent B and a reconditioning step at 2% B for 20 min. Solvent A was H₂O/acetonitrile/formic acid (98/2/0.1) and solvent B was H₂O/acetonitrile/formic acid (20/80/0.08). NanoLC-MS/MS experiments were conducted in data-dependent acquisition mode. A resolution of 70 000 (m/z 400) was used for MS scans. The 10 most intense ions were selected for HCD frag-
mentation and fragments were analysed in the orbitrap. A dynamic exclusion window of 30 s was used. Raw files were processed with MaxQuant software (version 1.3.0.5) (Cox and Mann, 2008; Luber et al., 2010). Protein identification was carried out using Andromeda (Cox et al., 2011) against a concatenated database including Salmonella strain SL1344 proteins (tax 216597 – 4657 proteins) and human proteins (tax 9606 – 20233 proteins). Trypsin was chosen as specific enzyme with a maximum number of two miscleavages. Possible modifications included carbamidomethylation (Cys, fixed), oxidation (Met, variable) and N-terminal acetylation (variable). Mass tolerance for MS was set to 20 ppm for the first search then 6 ppm for the main search and 10 ppm was used for MS/MS. The ‘match between run’ option was selected with a maximal retention time window of 2 min. Five amino acids were required as minimum peptide length. A false discovery rate of 1% was used for the identification.

Salmonella, reverse and contaminant proteins were excluded and only proteins identified with a minimum of two peptides were considered. Statistical relative protein quantification was performed with the peptide intensities extracted from the ‘peptides.txt’ MaxQuant output file using MSstats R package (Choi et al., 2014). MSstats enables protein significance analysis between different conditions (here control, 30 min and 3 h p.i.) and statistical protein quantification from label-free LC-MS experiments. The gene ontology analysis was performed with DAVID/EASE tools (Huang et al., 2009a,b) (http://david.abcc.ncifcrf.gov/).

Microscopy and image analysis

Time-lapse microscopy of living cells was performed at 37°C in a PerkinElmer UltraView spinning disk confocal microscope with a 40×/1.3 NA oil objective. Every 10, 20 or 30 min, a stack of 15 z-planes (500 nm step size) was acquired sequentially. In 4% paraformaldehyde (PFA)-fixed samples, the following antibodies were used after cell permeabilization with 0.5% saponin for 10 min: mouse anti-Sec13 (1:100, Abnova), mouse anti-reticulin-4 (1:300, Thermo Scientific), rabbit anti-calnexin (1:100, Stressgen), rabbit anti-Lamp-1 (1:500, Abcam), Alexa Fluor 488-conjugated anti-mouse or anti-rabbit (1:200, Life Technologies) and Cy5-conjugated anti-rabbit (1:200, Life Technologies). Z-stacks of 300 nm step size were acquired with a 60×/1.2 NA water objective. The following excitation lasers were used: 405, 488, 561 or 640 nm. Fluorescence emission was detected with 445 (W60), 525 (W50), 615 (W70) or 705 (W90) nm filters respectively. All images were further analysed with ImageJ and FIJI softwares. All confocal microscopy derived images shown correspond to maximum 3D projections.

Correlative-focused ion beam/scanning electron large-volume tomography

HeLa cells grown on MatTek dishes with a finder grid were fixed with 0.1% glutaraldehyde (GA) and 4% PFA for 15 min. After confocal microscopy imaging using a 60×/1.3 NA water objective, positions of interest were marked at 10× magnifications. Then cells were fixed overnight with 2.5% GA in 0.1 M HEPES pH 7.2 and post fixed in 1% osmium/1.5% potassium ferrocyanide in 0.1M HEPES for 1 h. Samples were treated for 30 min with 1% tannic acid and 1 h with 1% osmium tetroxide, rinsed in water and dehydrated in an ethanol series of 25%, 50%, 75%, 90% and 100% (5 min each). Cells were embedded in hard Epon resin. After resin polymerization, Epon blocks were mounted on a SEM stub and coated with a 25 nm layer of gold/palladium. Samples were placed in an Auriga FIB/SEM system (Zeiss) and the site of interest previously visualized by light microscopy was relocated using the imprint of the gridded dish in the Epon. Site was prepared and data acquired using ATLAS 3D software (Zeiss) using the backscatter detector at 2 kV with pixel sizes of 5 nm (Fig. 2E and F) or 10 nm (Fig. 5 and Fig. S4B). Slice thickness was 10 nm in all acquisitions. Stack alignment was performed with ImageJ and 3D visualization, data correlation, manual segmentation and video clips with Amira (FEI).

Quantification of cells containing hyper-replicating bacteria

Identification of cells containing hyper-replicating bacteria was performed using fluorescence microscopy. HeLa cells were infected with dsRed-expressing Salmonella and fixed at 6 h p.i., stained with DRAQ5 to enable computer segmentation and imaged on a Perkin Elmer, Opera spinning disk microscope. Images were analysed using Columbus software (Perkin Elmer) to quantify the red intensity (representing bacteria) per cell and defining intensity thresholds for uninfected, infected and hyper-replicating cells. The percentage of hyper-replication was defined as the number of cells containing hyper-replicating bacteria divided by the total number of infected cells.

Statistical analysis

Statistical analyses were performed in GraphPad Prism software v6, or MSstats R package for MS data. Significance was referred as *, **, *** and **** for P-values < 0.05, < 0.01, < 0.001 and < 0.0001 respectively.

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Author contributions

JCS, JC-R and JE designed the study. JCS performed all experiments with the help of JF and AW, except mass...
spectrometry performed by MD. AM and CS performed electron microscopy sample preparation and data acquisition. Data were analysed by JCS, MD, JF, AW, MM and VH. JCS and JE wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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Relative protein abundances at the 30 min SCV compared with the control fraction and the 30 min SCV fraction (B) or the 3 h SCV fraction (A), as a function of statistical significance. The SCV contacts with the Salmonella distribution was assessed with a PMol Microbiol 248–259.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. (Related to Fig. 1). Validation of the ELISA assay and density gradients quality.

A–B. The bottom of the wells of an ELISA plate was coated with an anti-Salmonella antibody and known amounts of bacteria were added in 1:2 dilution series. Salmonella were then detected by incubation with a biotinylated anti-Salmonella antibody and streptavidin peroxidase. Signal was quantified at 450 nm, correlated to number of bacteria per ml and plotted as linear-linear (A) or log-log (B).

C. After ultracentrifugation of the different PNSs, 12 fractions were collected (F1–F12) from the top to the bottom of the gradients. The refractive index was measured in each fraction, using a refractometer, and then the density (in g cm−3) was calculated. The graph shows the mean ± SEM from 15 different gradients from five independent experiments.

Fig. S2. (Related to Fig. 1). Subcellular organelle distribution in the 3 h infection fractionation.

All fractions F1 to F12 and the PNS were tested using markers for the following compartments: early endosomes (EEA-1 and Rab5), late endosomes and lysosomes (Lamp-1), Golgi (GM130), peroxisomes (catalase), mitochondria (TM22) and ER (calreticulin). Salmonella distribution was assessed with a specific anti-Salmonella LPS antibody.

Fig. S3. (Related to Fig. 2). Relative protein quantitative differences between the different protein subsets and gene ontology analysis.

A–B. Volcano plots of relative protein abundance differences between the control fraction and the 30 min SCV fraction (A) or the 3 h SCV fraction (B), as a function of statistical significance. Relative protein abundances were considered different when the log2 fold-change >0.387 or <−0.387, with a P-value <0.05. Proteins with no statistically significant abundance difference between conditions are represented in gray. Proteins enriched at the 30 min SCV are depicted in red (A), whereas proteins enriched at the 3 h SCV are shown in green (B). In both graphs, factors enriched in the control non-infected fraction are shown in blue. Values correspond to the average fold-changes of five independent experiments.

C. The host proteins enriched in the SCV were subjected to gene ontology analysis and grouped according to their biological process. For each term, the analysis was performed for the proteins enriched uniquely either at the 30 min SCV (only 30 min SCV, red bars) or at the 3 h SCV (only 3 h SCV, green bars) or for the proteins enriched at both time-points (both 30 min + 3 h SCV, orange bars). The graphs show the percentage of host proteins enriched at the SCV, relative to the total number of proteins identified in each condition. Statistics were determined by determining the P-values (EASE score) and show the robustness of gene-term enrichment for each condition. ND, non-enriched factors in comparison with the control.

Fig. S4. (Related to Figs 3 and 4). The SCV contacts with the host cell ER and the COP II complex accumulates around intracellular Salmonella.

A. Equal amounts of protein from subcellular fractions F3 to F7 were immunoblotted, using markers for reticulon-4 or VAMP7.

B. Cells infected with Salmonella for 30 min were fixed and imaged by fluorescence confocal microscopy followed by FIB/SEM. Several yz-views are shown, corresponding to different acquisition planes, and depict sites of membrane contact between the SCV and the ER (arrowheads). We show here a different data set from the one shown on Fig. 3E–F. (b, bacteria; s, SCV lumen).

C. Cells were infected with Salmonella-dsRed, fixed at different time-points and immunostained for Sec13 (green). DNA was stained with DAPI (cyan). Representative confocal microscopy images show the association of Sec13 around intracellular bacteria. Arrows point to Sec13-positive bacteria.

D. HeLa cells were infected with Salmonella-dsRed, fixed at different time-points and immunostained for Sec13. DNA was stained with DAPI (cyan). Images show representative infected cells with or without hyper-replicating Salmonella after confocal microscopy analysis. Arrows point to Sec13-positive structures resembling bacterial-shaped membrane remnants.

E. The percentage of infected cells containing Sec13-positive bacterial-shaped membrane remnants was determined by counting 50 cells with or without hyper-replicating bacteria, from triplicate wells. Scale bars correspond to 10 μm.

Fig. S5. (Related to Video Clips S2–S4). VAMP7-positive lysosome-like vesicles are recruited to the early SCV, which then gets depleted in acidic content.

HeLa cells were transfected with VAMP7-RFP for 48 h, treated with LysoTracker deep red at 50 nM for 30 min and infected with GFP-expressing Salmonella at a MOI of 30. Infected cells were then imaged by time-lapse confocal microscopy. After 30 min cells were washed and 50 μg ml−1 gentamicin was added for 1 h. The concentration of gentamicin was then decreased to 10 μg ml−1 for the remainder of the experiment and 10% FBS was added to the medium. Arrows point to intracellular bacteria. Scale bar corresponds to 10 μm. Representative data from five independent experiments are shown.

Fig. S6. (Related to Fig. 7 and Video Clip S6). VAMP7 does not affect Salmonella hyper-replication but is recruited to SIFs.

A. HeLa cells were treated with scramble or VAMP7 siRNA for 72 h and infected with dsRed-expressing Salmonella for 6 h. Cells were then fixed, counterstained with DRAQ5 and imaged by fluorescence microscopy. Statistics were determined using the Student’s t-test, ns, not significant. Data from three independent experiments are shown.

B. Cells were transfected with VAMP7-RFP and Lamp-1-GFP and infected with Salmonella. Gentamicin was added to kill extracellular bacteria and cells were then imaged by time-lapse confocal microscopy every 10 min. Arrows indicate SIF tubules. Representative data from three independent experiments are shown.

Table S1. Relative protein abundances at the 30 min SCV compared with the non-infected control for the complete list of host proteins identified by proteomics.

Table S2. Relative protein abundances at the 3 h SCV compared with the non-infected control for the complete list of host proteins identified by proteomics.
Table S3. Complete list of host proteins enriched in the SCV at 30 min, 3 h or at both time-points.

Table S4. Selected host proteins enriched in the SCV identified by proteomics.

Video Clip S1. (Related to Fig. 3E and F – 38 MB; 1 min and 11 s). Ultrastructural characterization of ER interactions with the early SCV. Successive FIB/SEM sections are shown in the xy-view and show sites of membrane contact between SCVs and the ER (arrowheads). The bacteria (blue), the SCV lumen (yellow) and the ER (red) were segmented using Amira software.

Video Clip S2. (Related to Fig. S5A – 771 KB; 20 s). HeLa cells were transfected with VAMP7-RFP (red) and lysosomes were stained with LysoTracker deep red (purple). Cells were then infected with Salmonella-GFP (green) and imaged by confocal microscopy. VAMP7- and LysoTracker-positive vesicles accumulate around the early SCV, in the first 2 h of infection. Then the SCV loses both markers and by 5 h p.i. gets again enriched in VAMP7 but not LysoTracker. Scale bar corresponds to 10 μm.

Video Clip S3. (Related to Fig. S5B – 1.3 MB; 30 s). HeLa cells were transfected with VAMP7-RFP (red) and lysosomes were stained with LysoTracker deep red (purple). Cells were then infected with Salmonella-GFP (green) and imaged by confocal microscopy. VAMP7- and LysoTracker-positive vesicles accumulate around the early SCV, in the first 2 h of infection. Then the SCV loses both markers and by 5 h p.i. gets again enriched in VAMP7 but not LysoTracker. By 6 h p.i., bacterial replication is observed, together with VAMP7-positive tubules emanating from the SCV. Scale bar corresponds to 10 μm.

Video Clip S4. (Related to Fig. S5C – 1.3 MB; 18 s). HeLa cells were transfected with VAMP7-RFP (red) and lysosomes were stained with LysoTracker deep red (purple). Cells were then infected with Salmonella-GFP (green) and imaged by confocal microscopy. VAMP7- and LysoTracker-positive vesicles accumulate around the early SCV, in the first 2.5 h of infection. Then the SCV loses LysoTracker and by 6 h p.i., bacterial replication is observed, together with VAMP7-positive tubules emanating from the SCV. Scale bar corresponds to 10 μm.

Video Clip S5. (Related to Fig. 6 – 49 MB; 1 min and 23 s). Ultrastructural characterization of the interactions between early SCVs and VAMP7/Lamp-1-positive vesicles. Fluorescence confocal microscopy of DAPI (blue), VAMP7 (red) and Lamp-1 (green) was followed by 3D FIB/SEM at the same cell. Segmentation of the bacteria (blue), vesicles and SCVs lumen (yellow) show that some VAMP7/Lamp-1-positive vesicles interact and fuse with the SCVs. Fluorescence staining of the bacteria was used as correlating fiducials.

Video Clip S6. (Related to Fig. S6B – 1.3 MB; 27 s). HeLa cells were transfected with VAMP7-RFP and Lamp-1-GFP and infected with Salmonella. Lamp-1- and VAMP7-positive SIFs emanate from the SCV 5 h p.i. Scale bar corresponds to 10 μm.