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How to do business with lysosomes: *Salmonella* leads the way

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Pathogens have devised various strategies to alter the host endomembrane system towards building their replicative niche. This is aptly illustrated by *Salmonella* Typhimurium, whereby it remodels the host endolysosomal system to form a unique niche, also known as *Salmonella*-containing vacuole (SCV). Decades of research using *in vitro* cell-based infection studies have revealed intricate details of how *Salmonella* effectors target endocytic trafficking machinery of the host cell to acquire membrane and nutrients for bacterial replication. Unexpectedly, *Salmonella* requires host factors involved in endosome-lysosome fusion for its intravacuolar replication. Understanding how *Salmonella* obtains selective content from lysosomes, that is nutrients, but not active hydrolases, needs further exploration. Recent studies have described heterogeneity in the composition and pH of lysosomes, which will be highly relevant to explore, not only in the context of *Salmonella* infection, but also for other intracellular pathogens that interact with the endolysosomal pathway.

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Introduction

Salmonella enterica serovar Typhimurium (*S.* Tm) is a facultative intracellular bacterial pathogen that has been studied for decades as a model organism for host-pathogen interaction studies. Part of its allure from a cell biology perspective lies in its ability to induce a network of remarkable membrane tubules in infected host cells, known as *Salmonella*-induced filaments (SIFs). Notwithstanding the interest in its intracellular lifestyle, *S.* Tm is also the leading cause of foodborne illness and diarrheal disease worldwide [1]. *S.* Tm generally causes localized self-limiting gastroenteritis in the human host, but could also lead to systemic infection in immune-compromised patients [2]. To establish infection in the gastrointestinal tract, the pathogen actively invades the intestinal epithelial cells and induces inflammation, which helps in breaching the epithelial cell barrier. The inflammatory conditions, while impeding pathogen survival, also facilitate recruitment and subsequent infection of phagocytes including macrophages [3]. The ability to survive and replicate within the host phagocytes largely determines whether the pathogen disseminates from the intestinal site to establish a systemic infection [4].

Decades of research on *S.* Tm pathogenesis have allowed us to uncover the intricate mechanisms employed by this pathogen to build a replicative niche inside the host cells. *S.* Tm resides and replicates intracellularly in a unique membrane-bound compartment termed the *Salmonella*-containing vacuole (SCV), although a subpopulation of bacteria can escape the nutrient-limited environment of vacuole and hyper-replicate in the cytosol of epithelial cells [5,6]. SCV biogenesis and maturation require pathogen-encoded type III secretion systems (T3SS), T3SS-1 and -2, which act as molecular syringes to inject more than 40 effector proteins into the host cell cytosol [7]. The T3SS-1 is encoded by the *Salmonella* pathogenicity island (SPI)-1 genomic locus and injects effectors across the host cell plasma membrane to enable bacterial invasion and SCV biogenesis. The T3SS-2, encoded by the SPI-2 locus, injects effectors across the vacuolar membranes, which in turn facilitate SCV maturation, SIF formation and intravacuolar *Salmonella* replication. We refer the reader to excellent reviews for extensive information on the identity and known functions of *S.* Tm effectors, although the host target for several effectors remains to be characterized [7–9].

Living inside a vacuole has its pros and cons, as it protects the pathogen from the cytosolic bactericidal stress response pathways, especially in macrophages, but also limits access to the nutrients from the cytosolic pool. To build a replicative niche, *S.* Tm requires a continuous supply of membrane and nutrients to its vacuole, which as discussed here, is primarily derived from the endolysosomal pathway of the host cells. This short review aims to provide an overview of strategies used by *S.* Tm to modulate the host endolysosomal pathway with a focus on its unusual interaction with late endosomes and lysosomes.

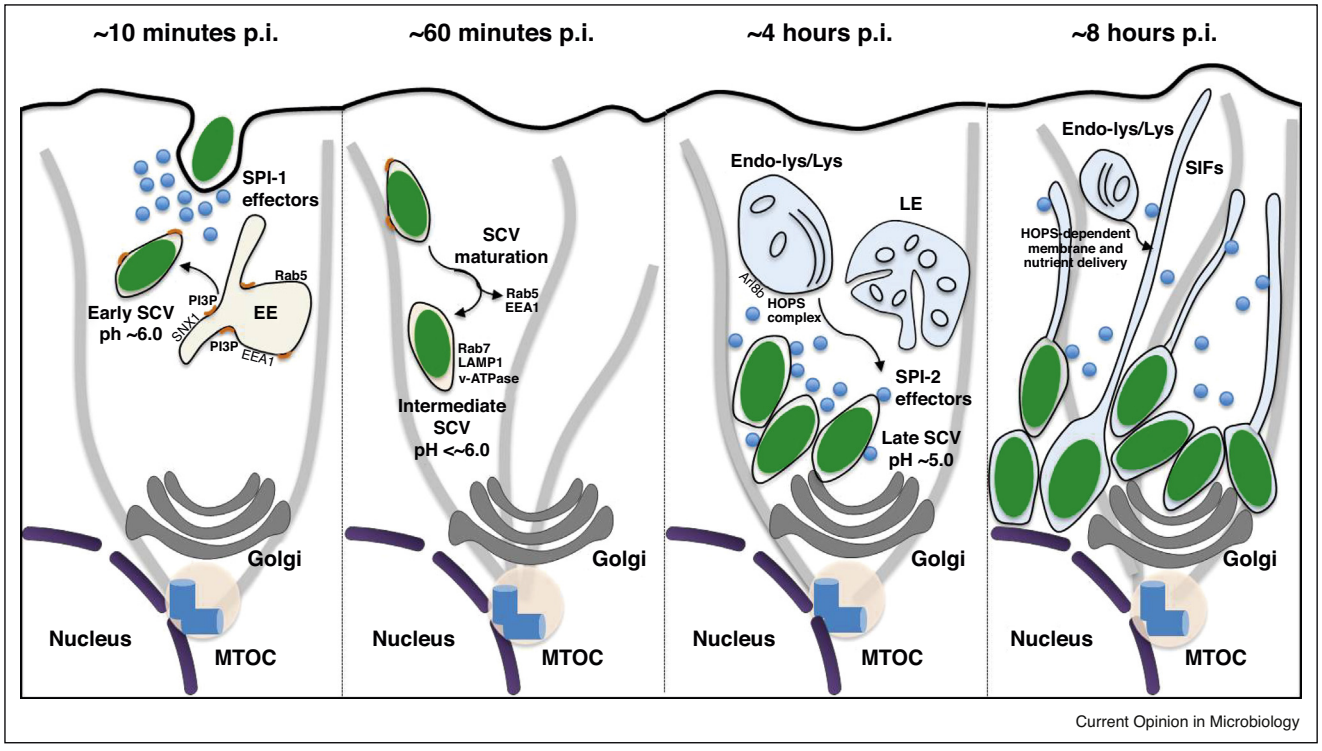
SCV maturation: the usual suspects

The maturation program of the SCV in mammalian cells follows the general scheme of the early to late endosome maturation pathway. Within few minutes of internalization, the SPI-1-T3SS effector SopB promotes Rab5 and Vps34 (a subunit of PI-3-kinase) recruitment to the SCV, leading to transient phosphatidylinositide-3 phosphate (PtdIns(3)P) accumulation on the early SCV membranes [10••,11•]. This, in turn, recruits Rab5 effector and PtdIns(3)P-binding protein EEA1 onto SCV membranes [12], which likely promotes SCV fusion with early endosomes (Figure 1) [13]. Early SCVs also accumulate regulators of the fast and slow recycling pathway, including Rab4, Syntaxin 13 and Rab11 that mediate recycling of proteins from the vacuolar membranes [14]. SCV maturation, which happens over the next 30–60 minutes, is akin to endosome maturation and is marked by loss of Rab5 and its associated effectors and recruitment of Rab7, lysosomal glycoproteins (LAMP1/LAMP2) and v-ATPases to the SCV membranes (Figure 1) [15,16•].

Notably, the role of Rab5 effector Mon1-CCZ1 complex that acts as Rab7 guanine exchange factor (GEF) and promotes endosome maturation remains unexplored in the context of SCV maturation. Rab7 localization is crucial for LAMP1 recruitment to the SCV and SCV positioning to the perinuclear region [16•]. The Rab7 effector RILP that binds and recruits the minus-end-directed microtubule motor protein dynein-dynactin complex regulates this distinct positioning phenotype [17]. While SCVs mature into this late endosomal-like compartment, proteomic studies have shown that the SCV at this stage remains devoid of lysosomal hydrolases and the cation-independent mannose-6-phosphate receptor (CI-M6PR) [18]. At least one factor for this selective removal of CI-M6PR is the presence of sorting nexin 1 (SNX1) on the early and intermediate SCV that promotes retrieval of CI-M6PR from the endosomes-to-trans-Golgi network [19].

Between 1–3 hours post infection (p.i.), SCVs are rapidly acidified by the v-ATPase present on the vacuolar membrane. The acidic conditions promote association of T3SS-2 with the vacuolar membrane, and consequently secretion of SPI-2 effectors into the host cytosol (Figure 1) [20,21]. Based on the prior findings that RILP regulates

Figure 1



Schematic representation of SCV interaction with the endocytic pathway. Within 5-10 minutes p.i., *S*. Tm vacuole is marked by the presence of early endosomal proteins, Rab5 and EEA1. SPI-1 effectors, especially SopB, plays a crucial role in the recruitment of Rab5 and PI-3-kinase that mediate PtdIns(3)P production on the SCV membranes. Exchange of Rab5 to Rab7 facilitates SCV maturation that happens at ~1 hour p.i. and SCVs at this stage are marked by late endosomal proteins including LAMP1 and v-ATPase. Rab7 recruits its effector RILP to position SCV in a dynein-dependent manner near the perinuclear region, apposed to the Golgi and MTOC. SCVs rapidly acidify in the next 1-2 hours that promotes secretion of SPI-2 effectors. SPI-2 effector SifA mediates SCV interaction with late endosomes and lysosomes by recruiting multisubunit tethering factor HOPS complex to SCV membranes. This interaction with the late endocytic compartments is quintessential for SIF formation, which in turn enable nutrient access to the vacuolar bacteria, promoting *S*. Tm replication.

v-ATPase recruitment to late endosomes [22], it is plausible that the Rab7-RILP complex is required for v-ATPase localization on SCV membranes. As described in the next sections, SPI-2 effectors play crucial roles in SCV interaction with the host endocytic and exocytic vesicles, providing access to membranes and nutrients for the intravacuolar replication of *Salmonella*.

Let the replication begin: onset of SIF formation

With the onset of SPI-2 effector translocation into the host cytosol, what started out as a "typical" endosome maturation process now takes a surprising turn, with the appearance of highly dynamic tubules emanating from the perinuclear-positioned SCVs. These characteristic tubules, most commonly visualized by staining for the late endosomal/lysosomal protein-LAMP1, are known as *Salmonella*-induced filaments or SIFs [23]. Besides LAMP1, late endosomal/lysosomal proteins, including LAMP2, Rab7, Arl8b, and v-ATPase localize to SIFs, hinting at a late endosomal/lysosomal origin of these tubules (Figure 1) [24,25,26•]. Intriguingly, SIFs are devoid of lysosomal hydrolases and CI-M6PR [15,16•]. These observations indicate that *S.* Tm modulates cargo trafficking to deliver particular content uniquely for SIF formation. SIFs are not the only tubules formed upon SCV maturation; studies have reported LAMP1-negative tubules (LNT) [27] and *Salmonella*-induced SCAMP3 tubules (SIST) [28] form at later time points of infection. Nevertheless, SIFs remain by far the best characterized regarding their structure, composition and factors (both *Salmonella* effectors and host proteins) required for their biogenesis. In HeLa cells, SIF formation begins at ~5 hours p.i. and by 8–10 hours p.i. they appear as an extensive interconnected network of highly stable tubules with little to no LAMP1 now present on vesicular membranes [29•,30•]. Recent advances in fluorescence and electron microscopy and EM tomography have shed light on SIF ultrastructure [31•]. Nascent SIFs were reported to be highly dynamic single membrane tubules of ~120 nm diameter that by 8–10 hours p.i. forms a double membrane network of stable tubules of ~220 nm diameter. Interestingly, only the outer lumen of the double membrane SIF is accessible to the endocytic probes and is a continuum with the luminal space of the SCV, while the inner lumen contains portions of the host cytosol. This structural arrangement is likely to prevent antimicrobial factors of the host cytosol to access the vacuolar space of the pathogen [31•].

Although SIFs have been studied for a long time; their function in *S.* Tm replication was not understood until recently. The appearance of SIFs correlates with the onset of *Salmonella* intracellular replication, indicating a role for these tubular structures in establishing infection [23,32]. Indeed, *S.* Tm strains defective in SIF formation are highly attenuated in their ability to replicate in cultured cell lines and in establishing systemic infection in a mouse model [24,33]. Previous studies have shown that fluid-phase cargo, such as labeled probes that are pinocytosed in endosomes and finally traffic to lysosomes, reaches the lumen of these tubules [29•,30•,31•]. These findings suggest that material exchange occurs between the endolysosomal system and the SIF lumen. More recently, studies by Hensel and colleagues have demonstrated that SCV and SIFs are in a continuum, that is the SIF lumen is accessible to the pathogen present in the vacuole [31•]. Accordingly, auxotrophic *S.* Tm strains defective in synthesis of particular amino acids were able to access nutrients from the host cells only if they were capable of SIF formation [34••,35•]. In line with this, bacteria connected to the SIF network are significantly more metabolically active than those in the SIF-defective strains [34••]. Taken together, these studies suggest an essential role of SIFs in providing nutrient access to the generally nutrient-deficient vacuolar niche of the pathogen.

This begs the question of how are these remarkable tubular structures formed? What is the role of *S.* Tm effectors and host cell factors in SIF formation? Mounting experimental evidence (as described below) indicates that membranes for SIF biogenesis are primarily derived from SCV fusion with late endosomes and lysosomes. Expectedly, in this scenario, vacuolar *S.* Tm has to devise strategies to ensure selective delivery of nutrients, but not the active hydrolases present in late endosomes/lysosomes. We discuss this exciting aspect of *S.* Tm intracellular life in the subsequent sections.

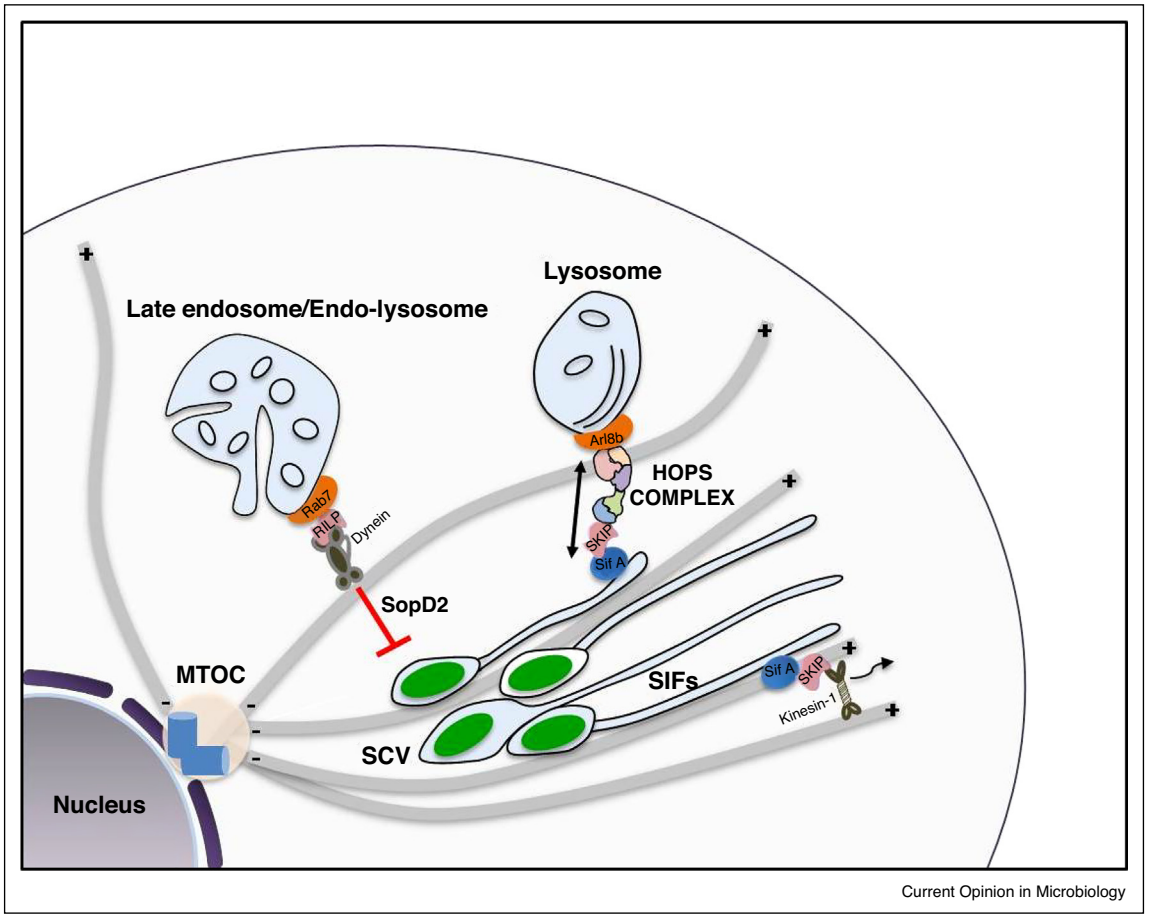
Molecular players involved in SIF formation and *Salmonella* replication

From the pathogen side, the process of SIF formation requires translocation of SPI-2 effectors into the host cytosol. The identity and functional significance of SPI-2 effectors involved in SIF formation have been described in detail in recent reviews [7,36]. The dominant player in SIF biogenesis is the SPI-2 effector SifA, identified as the gene mutation in *S.* Tm strain that failed to induce SIFs in host cells [37]. SifA is thought to play a direct role in the SCV interaction with LAMP1-positive late endosomes and lysosomes, as its plasmid-borne expression induces vacuolation of the host late endocytic compartments [38]. Further, co-expression of SifA and SPI-2 effector-SSeJ, which has lipid modification activity, is sufficient to induce endosomal tubulation [39]. A breakthrough in understanding the mechanism of SifA action was the discovery of its host target, PLEKHM2, also known as SifA and kinesin-interaction protein (SKIP) [40••]. As the name suggests, SKIP also interacts with the plus-end-directed microtubule motor protein, Kinesin-1 [40••]. Thus SifA-SKIP-Kinesin-1 tripartite interaction immediately provided a model for SIF elongation, whereby SifA anchored to the SCV membranes via its

C-terminal isoprenylation anchor recruits the SKIP-Kinesin-1 complex that would generate the pulling forces to mediate elongation of SIFs towards the cell periphery [41]. More recently, SseJ has been shown to co-localize with kinesin, presumably via an interaction with SifA and SKIP [42]. Intriguingly, excessive accumulation of kinesin and SseJ around the SCV has been observed in cells infected with *sifA*-lacking *S.* Tm strain, which hampers membrane integrity of the vacuole likely through the lipid modifying activity of SseJ [40••,42]. This observation also suggest that kinesin recruitment to the SCVs is independent of SifA, and was later attributed to SPI-2 effector PipB2 that directly binds to kinesin light chain and was required for Kinesin recruitment to SCV membranes [40••,43].

Although these studies shed light on mechanisms regulating SIF elongation, how membranes are delivered for SIF formation remained an open question. Our recent study has identified that SifA-SKIP complex interacts with and recruits lysosomal tethering factor HOPS (HOmotypic fusion and Protein Sorting) complex to promote SIF formation and *S.* Tm replication (Figure 2) [26•]. HOPS is a hexameric protein complex conserved from yeast to humans that promotes tethering and homotypic fusion of vacuoles in yeast and heterotypic fusion of late endosomes and lysosomes in mammalian cells [44,45]. The lysosomal association of the HOPS complex is mediated by the small GTPase Arl8b in human cells and by Rab7 in yeast [44–47]. The involvement of Arl8b and the HOPS complex in SIF formation, nutrient delivery to the SCVs as well as *S.* Tm replication, clearly indicates that *S.* Tm acquires content from host late endosomes and lysosomes (Figure 2) [26•]. Our findings support previous observations that SIF biogenesis requires players involved in late endosome-lysosome fusion including the small GTPase Rab7, the Rab7- and Arl8b-dual effector PLEKHM1, and the SNARE protein VAMP7 [16•,18,48,49].

Figure 2



Hypothetical model of selective interaction of *S.* Tm vacuole with Arl8b-positive lysosomes. SPI-2 effectors SifA and SopD2 might facilitate interaction of SCVs with Arl8b-positive lysosomes that are less acidic while inhibiting fusion with the more acidic Rab7-positive late endosomes and endo-lysosomes, respectively. Indeed, Arl8b and its effector HOPS complex are significantly-enriched on SCVs and SIFs at later time points of infection that coincide with bacterial replication [26•], while recruitment of Rab7 effectors RILP and FYCO1 is not observed [54•].

While SIF biogenesis primarily requires fusion with late endosomes/lysosomes, proteomic studies have revealed that proteins from a diverse set of compartments including the Golgi, ER, mitochondria and the nucleus are also-enriched in membrane domains associated with *S.* Tm. Several studies indicate that *Salmonella* recruits exocytic transport vesicles to the SCV, allowing bacteria to obtain nutrients from the secretory pathway [50,51]. SPI-2 effectors SSeF and SSeG tether the SCV to the Golgi and are likely candidates that might regulate SCV fusion with exocytic vesicles [52].

SCV interaction with late endosomes/ lysosomes: the role of lysosomal heterogeneity?

SCV interaction with late endosomes and lysosomes is counterintuitive to the general paradigm of 'pathogen evading lysosomes' for survival within host cells. Holden and colleagues uncovered a possible clue to the puzzle of how SCVs and SIFs become devoid of active proteases. Their study showed that the SifA-SKIP complex impairs M6PR retrograde trafficking from endosomes to the Golgi, disrupting the normal trafficking of M6P-tagged inactive hydrolases to late endosomes [53••]. Thus, it was proposed that SCV fusion with these 'detoxified' lysosomes ensures selective delivery of nutrients, but not antimicrobial factors to the SCV. However, subsequent evidence showed that expression of SifA was not sufficient to inhibit lysosomal hydrolase activity, but rather required an additional SPI-2 effector, SopD2 that was expressed in *S.* Tm infected cells at 5 hours p.i. [54•,55]. SopD2 interacts with Rab7 and prevents binding of its downstream effectors by inhibiting Rab7 activation from the GDP bound state to GTP bound state [54•]. Thus, using this two-pronged approach of mis-routing lysosomal hydrolases and blocking cargo trafficking to lysosomes, *S.* Tm ensures its intravacuolar replication.

Recent studies have documented a previously unappreciated heterogeneity of LAMP1-positive membranes that are generally regarded as lysosomes [56]. LAMP1-positive vesicles might represent one of three populations of late endosomes, endolysosomes and lysosomes. The three populations likely differ in their pH and hydrolase activity. Late endosomes and endolysosomes are more acidic than lysosomes, which primarily act as storage organelles for inactive hydrolases [57]. Although the field currently lacks-specific markers to identify these distinct compartments, a recent study has suggested that Rab7-positive vesicles are more acidic as compared to the Arl8b-positive vesicles [58••]. Considering the previous reports that show Arl8b and its effectors are enriched on the SCV and SIFs, we propose a hypothetical model whereby SCVs might preferentially interact with Arl8b-positive vesicles in a SifA- and HOPS-dependent manner, while SopD2 suppresses interaction with Rab7-positive vesicles (Figure 2). This modulation of the late endocytic GTPases function would likely ensure selective delivery of nutrients, but not active hydrolases, to facilitate intravacuolar *S.* Tm replication.

Concluding remarks and open questions

S. Tm has evolved numerous strategies to manipulate the endolysosomal system of the host cell, directing traffic of membranes and nutrients from these compartments towards its vacuolar niche. Identifying the host targets of *S.* Tm effectors has been a critical step in understanding the virulence mechanisms of this highly successful human pathogen. Only a few such effector-host protein pairs have been identified to date, and therefore, continued work on *S.* Tm effectors and their host targets will reveal fascinating aspects of the *S.* Tm intravacuolar lifestyle. One such profound aspect of *S.* Tm infection is the process of SIF formation, which enables the pathogen to acquire nutrients from the host cell. Several fundamental questions about SIFs remain to be answered, for instance, how are the SIF membranes deformed into tubules? Is the process similar to the formation of tubular recycling endosomes and are common players involved? Does this process involve proteins that can sense and induce membrane curvature (such as BAR domain-containing proteins)? Although we know the proteome of these tubules, the lipids that localize to and are required for SIF formation are presently unknown. Intriguingly, SIF formation bears a resemblance to the recently described process of autolysosome reformation that initiates as a result of lysosome depletion. Could SIF formation, therefore, be a response to lysosome depletion induced by *S.* Tm infection, involving the same molecular players as are involved in lysosome reformation? Understanding these fundamental aspects of the *Salmonella* intracellular lifestyle will not only uncover new candidate players that regulate *Salmonella* pathogenesis, but will also provide new insights into organelle biology of the host.

Conflict of interest statement

Nothing declared.

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