Role of the ESCRT-III complex in controlling integrity of the Salmonella-containing vacuole

Vera Göser1 | Alexander Kehl1,2,3 | Jennifer Röder1 | Michael Hensel1,3

1Abt. Mikrobiologie, Fachbereich Biologie/Chemie, Universität Osnabrück, Osnabrück, Germany
2Institut für Hygiene, Universität Münster, Münster, Germany
3CellNanOs, Center for Cellular Nanoanalytics, Fachbereich Biologie/Chemie, Universität Osnabrück, Osnabrück, Germany

Abstract
Intracellular pathogens need to establish specialised niches for survival and proliferation in host cells. The enteropathogen Salmonella enterica accomplishes this by extensive reorganisation of the host endosomal system deploying the SPI2-encoded type III secretion system (SPI2-T3SS). Fusion events of endosomal compartments with the Salmonella-containing vacuole (SCV) form elaborate membrane networks within host cells enabling intracellular nutrition. However, which host compartments exactly are involved in this process and how the integrity of Salmonella-modified membranes is accomplished are not fully resolved. An RNA interference knockdown screen of host factors involved in cellular logistics identified the ESCRT (endosomal sorting complex required for transport) system as important for proper formation and integrity of the SCV in infected epithelial cells. We demonstrate that subunits of the ESCRT-III complex are specifically recruited to the SCV and membrane network. To investigate the role of ESCRT-III for the intracellular lifestyle of Salmonella, a CHMP3 knockout cell line was generated. Infected CHMP3 knockout cells formed amorphous, bulky SCV. Salmonella within these amorphous SCV were in contact with host cell cytosol, and the attenuation of an SPI2-T3SS-deficient mutant strain was partially abrogated. ESCRT-dependent endolysosomal repair mechanisms have recently been described for other intracellular pathogens, and we hypothesise that minor damages of the SCV during bacterial proliferation are repaired by the action of ESCRT-III recruitment in Salmonella-infected host cells.

KEYWORDS
intracellular bacteria, membrane repair, multivesicular body, pathogen-containing compartment

1 INTRODUCTION
Intracellular pathogens have evolved highly specialised mechanisms to survive and proliferate within their eukaryotic host. One strategy represents the inhibition of maturation of the phagosome to create intracellular niches favouring bacterial replication. This can be accomplished by modifying the identities of the phagosome in order to exploit host cell trafficking pathways (Brumell & Scidmore, 2007). The enteropathogen Salmonella enterica resides in a specialised membrane-bound compartment termed Salmonella-containing vacuole.
(SCV), which possesses late endosomal characteristics and allows bacterial survival and proliferation (Haraga, Ohlson, & Miller, 2008). What is crucial for intracellular survival of *Salmonella* is the function of the type III secretion system (T3SS) encoded on *Salmonella* pathogenicity island II (SPI2) (Hensel et al., 1998). The SPI2-T3SS translocates a set of effector proteins across the SCV membrane into the host cytosol in order to manipulate host cell functions (Figueira & Holden, 2012).

A specific characteristic of host manipulation by *Salmonella* is the extensive reorganisation of the endosomal system and the formation of endosome-derived tubular structures extending from the SCV (Liss & Hensel, 2015). These *Salmonella*-induced tubules comprise various structures whereas the best-studied tubules are termed *Salmonella*-induced filaments (SIF) which are characterised by the presence of lysosome-associated membrane glycoprotein 1 (LAMP1) (Garcia-del Portillo, Zwick, Leung, & Finlay, 1993; Schroeder, Mota, & Meresse, 2011). The induced formation of an extensive SIF network by *Salmonella* provides bacterial nutrition enabling proliferation in cell-based models (Liss et al., 2017). The process of SIF formation is dependent on specific SPI2-T3SS effector proteins and their fine-tuned interaction with host cell proteins. The precise involvement of *Salmonella* effector proteins and host interaction partners is still not fully elucidated, but undoubtedly the best understood T3SS effector is SifA (Stein, Leung, Zwick, Garcia-del Portillo, & Finlay, 1996). SifA interacts with various host proteins, among them SKIP (SifA and kinesin-interacting protein) (Boucrot, Henry, Borg, Gorvel, & Meresse, 2005; Dumont et al., 2010), also referred to as PLEKHM2 (pleckstrin homology domain-containing protein family member). SIFA together with SKIP and kinesin-1 forms a complex that mediates elongation of SIF along microtubules. The lack of either SifA or SKIP leads to a deficiency in SIF formation, affecting SCV integrity and resulting in bacterial release into the host cell cytosol. This leads to cytosolic hyper-replication in epithelial cells and reduced intracellular proliferation in macrophages (Beuzon et al., 2000a; Boucrot et al., 2005; Zhao et al., 2015). The reorganisation of the host endocytic pathway leads to a recruitment of endosomal compartments to the SCV. Therefore, various host endosomal marker proteins, among these predominantly Rab GTPases, were found decorating the SCV and resulting in bacterial release into the host cell cytosol. The ESCRT system undoubtedly the best understood T3SS effector is SifA (Stein, Leung, Zwick, Garcia-del Portillo, & Finlay, 1996). SifA interacts with various host proteins and interacts with CHMP2 (Whitley et al., 2003). SifA together with SKIP and kinesin-1 forms a complex that mediates elongation of SIF along microtubules. The lack of either SifA or SKIP leads to a deficiency in SIF formation, affecting SCV integrity and resulting in bacterial release into the host cell cytosol. This leads to cytosolic hyper-replication in epithelial cells and reduced intracellular proliferation in macrophages (Beuzon et al., 2000a; Boucrot et al., 2005; Zhao et al., 2015). The reorganisation of the host endocytic pathway leads to a recruitment of endosomal compartments to the SCV. Therefore, various host endosomal marker proteins, among these predominantly Rab GTPases, were found decorating the SCV and SIF network (Brumell & Scidmore, 2007).

To identify further host cell proteins that are required for SCV and SIF biogenesis, we previously performed an RNA interference (RNAi) knockdown (k/d) screen targeting ca. 500 host cell proteins involved in cellular logistics using small interfering RNAs (siRNA) (Kehl et al., 2019). The screen deployed live cell imaging (LCI) of *Salmonella*-infected host cells and evaluation of the siRNA-induced effects on SCV biogenesis and SIF formation. Among the hits of this screen were subunits of the ESCRT-III (endosomal sorting complexes required for transport) complex, which was investigated in detail in this study.

ESCR comprise five distinct complexes (ESCRT-O, -I, -II, -III, and the Vps4 complex) which assemble into a multisubunit machinery that performs unique membrane bending and scission reactions away from the cytosol. The ESCRT complexes are known to act in concert to facilitate the multivesicular body (MVB) pathway, cytokinesis, and virus budding (McCullough, Colf, & Sundquist, 2013), but recently other functions were identified, such as nuclear envelope (NE) resealing (Hurley, 2015). Moreover, it was demonstrated that the ESCRT system is also involved in repair of endomembranes, in particular of endolysosomal damage, and therefore holds a protective function for the cell (Radulovic et al., 2018; Skowrya, Schlesinger, Naismith, & Hanson, 2018). The MVB pathway represents a process where proteins destined for degradation are incorporated into intraluminal vesicles that bud from the membrane of late endosomes creating a MVB. By fusion of MVB with lysosomes, the intraluminal vesicles are delivered to the degradative pathway (Piper & Katzmann, 2007).

The ESCRT-III complex represents the central membrane scission machinery at the heart of the ESCRT system (Wollert, Wunder, Lippincott-Schwartz, & Hurley, 2009). The subunits of the ESCRT-III complex are soluble monomers that assemble into tightly bound filaments on endomembranes (Ghazi-Tabatabai et al., 2008). The complex consists of four core subunits CHMP2 (Vps2), CHMP3 (Vps24), CHMP6 (Vps20), and CHMP4 (Snf7/Vps32) with CHMP2 and CHMP4 having two and three isoforms in humans, respectively, and the accessory proteins CHMP1 (with two isoforms), CHMP5, CHMP7, and IST1/CHMP8 (McCullough et al., 2013). The exact sequence of recruitment remains unclear, but the main molecular interactions between CHMP subunits have been resolved. CHMP6 is recruited by ESCRT-II and interacts with CHMP4, which then binds to CHMP3 and interacts with CHMP2 (Whitley et al., 2003).

Here, we show the impact of the ESCRT-III complex on *Salmonella* intracellular lifestyle by use of host cells devoid of functional ESCRT-III. For that purpose, we created, using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system, a HeLa knockout (k/o) cell line lacking CHMP3, a core protein of the ESCRT-III complex. We demonstrate that a functional ESCRT system is necessary for SCV biogenesis, since mutant host cells showed a high prevalence of amorphous SCV with defects in separation of intracellular *Salmonella* from the host cell cytosol.

## RESULTS

### 2.1 Defects in ESCRT-III affect SCV formation

We previously performed an LCI-based RNAi k/d screen for host cell functions contributing to intracellular proliferation of *Salmonella enterica* serovar Typhimurium (STM), and formation of SCV and SIF in STM-infected HeLa cells (Kehl et al., 2019). Transfection with control siAllStars had no effect on SCV formation (see Figure 1(a) and Movie 1). The k/d of CHMP7 had moderate effects on SIF formation and intracellular proliferation of *Salmonella* in HeLa cells; however, an aberrant SCV morphology was observed. Whereas the SCV membrane is usually tightly enclosing *Salmonella* (Eswarappa, Negi, Chakraborty, Chandrasekhar Sagar, & Chakravortty, 2010), the CHMP7 k/d caused the SCV to expand during the course of infection, culminating in noticeably spacious SCV (see Figure 1(b) and Movie 2). Interestingly, a similar phenotype was recently observed upon depletion of PLEKHM1, also resulting in reduced intracellular replication of STM (McEwan, Richter, et al., 2015).
Mammalian CHMP7 is a poorly characterised accessory protein of ESCRT-III (Horii et al., 2006). Though recently, a nonendosomal role specifically for CHMP7 was discussed (Bauer, Brune, Preiss, & Kölling, 2015), the numerous endosomal interactions of *Salmonella* (Knuff & Finlay, 2017; Liss & Hensel, 2015; Tuli & Sharma, 2019) hint at a possible role of the ESCRT pathway in SCV and/or SIF biogenesis in this context. In line with this, both isoforms of the AAA ATPase VPS4, responsible for disassembly of ESCRT-III, were high-ranking hits in the

**FIGURE 1** Legend on next page.

Mammalian CHMP7 is a poorly characterised accessory protein of ESCRT-III (Horii et al., 2006). Though recently, a nonendosomal role specifically for CHMP7 was discussed (Bauer, Brune, Preiss, & Kölling, 2015), the numerous endosomal interactions of *Salmonella* (Knuff & Finlay, 2017; Liss & Hensel, 2015; Tuli & Sharma, 2019) hint at a possible role of the ESCRT pathway in SCV and/or SIF biogenesis in this context. In line with this, both isoforms of the AAA ATPase VPS4, responsible for disassembly of ESCRT-III, were high-ranking hits in the

**FIGURE 1** Legend on next page.
RNAi screen, and ESCRT-0 component HGS/HRS was the highest-ranking hit (Kehl et al., 2019). However, so far, the only involvement of ESCRTs in Salmonella pathogenesis shown was the SopB-dependent impairment of proper trafficking of the epidermal growth factor receptor (EGFR) to the lysosome, a process dependent on the ESCRT pathway (Dukes et al., 2006).

Thus, we tested whether the k/d of another accessory ESCRT-III component or a core component would lead to similar phenotypes. The efficient k/d of all analysed CHMP components was verified by RT-PCR (see Figure 1f). In fact, we observed that k/d of CHMP1B (see Figure 1(c) and Movie 3) or CHMP4B (Figure 1(d) and Movie 4) both resulted in SCV phenotypes comparable to k/d of CHMP7. These observations strengthen the involvement of the complete ESCRT-III in SCV biogenesis.

2.2 | ESCRT-III subunits are present at membranes of SCV and SIF

To probe the potential interaction of ESCRT-III with Salmonella-modified membranes, we investigated the localisation of various components of ESCRT-III in Salmonella-infected host cells. HeLa cells expressing both fluorescent protein-tagged LAMP1 and subunits of the ESCRT-III complex were infected with Salmonella, and LCI was performed (Figure 2(a)). For CHMP3, we did not succeed to detect fusion proteins in transfected cells. We found that core subunits CHMP6 and CHMP4A both decorated SIF and an almost complete colocalization with LAMP1 was observed. Association with membranes of SIF and SCV was also observed for accessory component CHMP5 but not for CHMP1B and CHMP7.

In conclusion, we observed that core components of the ESCRT-III system responsible for membrane fission in the MVB pathway are recruited to SIF and SCV in Salmonella-infected cells.

2.3 | Generation and validation of monoclonal knockout HeLa cell lines

To further analyse the role of ESCRT-III in the intracellular lifestyle of Salmonella and Salmonella-induced endosomal remodelling, we generated a k/o HeLa cell line devoid of ESCRT-III. To achieve complete loss of ESCRT-III function, ablation of a core protein was required. CHMP3 represents a suitable target, as the protein had no isoforms in humans and is localised at the core of the filamentous protein chain representing the ESCRT-III complex (Figure 2(b)). We decided to also generate a k/o cell line of a known Salmonella effector interaction partner, the host protein SKIP. Monoclonal CHMP3 or SKIP k/o HeLa cell lines were created using the clustered regularly interspaced short palindromic repeats (CRISPR) system. We first created dedicated vectors expressing GFP, Cas9, and an single-stranded guide RNA (sgRNA) with specific target sequences against either the human CHMP3 or SKIP gene (see Figure S1). After transfection of HeLa cells, the cell population was subjected to flow cytometry, and after expansion of sorted single cell HeLa populations, monoclonal cell lines were validated (Figure 3). Cell lysates of six individual CRISPR/Cas9-treated HeLa cell lines were analysed and synthesis of CHMP3 or SKIP, respectively, was detected by Western blot.

The efficient k/o of all analysed CHMP components was verified by RT-PCR (see Figure 1f). In fact, we observed that k/d of CHMP1B (see Figure 1(c) and Movie 3) or CHMP4B (Figure 1(d) and Movie 4) both resulted in SCV phenotypes comparable to k/d of CHMP7. These observations strengthen the involvement of the complete ESCRT-III in SCV biogenesis.

**FIGURE 1** Knockdown of CHMP subunits causes formation of amorphous SCV. LAMP1-GFP-expressing HeLa cells were reverse transfected with siRNA for negative control (AllStars, a), and for knockdown of CHMP7 (b), CHMP1B (c), or CHMP4B (d) and incubated for 72 hr. Subsequently, cells were infected with Salmonella WT constitutively expressing mCherry with an MOI of 15. Live cell imaging was performed from 1 to 7 hr p.i. on an SDCM. Grey arrowheads indicate normal SCV, and white and yellow arrowheads indicate development of representative spacious SCV. The SCV indicated by yellow arrowheads are shown in magnified details. Scale bar, 10 μm. Time lapse series are shown in Movie 1, Movie 2, Movie 3, and Movie 4. (e) Quantification of SCV morphologies in siRNA-transfected cells after infection with Salmonella WT. SCV containing single bacteria with LAMP1-positive membranes tightly enclosing single bacteria were scored as “normal SCV.” SCV containing multiple bacteria, and/or loose association with bacteria, and/or increased luminal space were scored as “amorphous SCV.” For each condition, at least 100 SCV were scored, combining images from two independent siRNA k/o screens. (f) Validation of CHMP knockdown. HeLa LAMP1-GFP cells were reverse transfected with AllStars or the indicated siRNA, and incubated for 72 hr. Total RNA was extracted and mRNA reverse transcribed. The generated cDNA was used in RT-PCR targeting the corresponding host factor with GAPDH serving as reference gene. Depicted is the mean with standard deviation of three biological replicates (n = 3) each performed in triplicates. Statistical analysis was performed with Student’s t test and indicated as **p < .001**
using the carboxyfluorescein succinimidyl ester (CFSE) assay. CFSE (ex. 492/em. 517) stably labels nuclear DNA, and each cell division results in a sequential twofold dilution of fluorescence intensities (Lyons & Parish, 1994). Cells were treated with CFSE, and fluorescence was monitored by flow cytometry over the course of 3 days. As a control for nonproliferating cells, cell lines were additionally treated with nocodazole to arrest cell proliferation. When comparing the maximum fluorescence intensity of cell populations at different time points, a reduction of fluorescence over time is detectable (Figure 3(b)). This reduction ceased after day 1 when cells were treated with nocodazole. When quantifying the factor of daily fluorescence decrease, similar values were obtained for either HeLa, HeLa SKIP k/o, or HeLa CHMP3 k/o cells (Figure 3(c)) indicating a normal proliferation of CRISPR/Cas9-treated cells.

To further validate the HeLa CHMP3 k/o line, an EGFR internalisation assay was performed. Upon binding its ligand EGF, EGFR undergoes internalisation and endocytic trafficking. Some receptors recycle back to the plasma membrane, whereas others are degraded via the endocytic pathway cycling from late endosomes to lysosomes (Sorkin & Goh, 2008). Depletion of CHMP3 leads to impaired ligand-induced degradation of EGFR, resulting in an arrested shuttling of EGFR from early to late endosomes/lysosomes (Bache et al., 2006). HeLa and HeLa CHMP3 k/o cells were stimulated with EGF, and after a 6-hr chase, cells were fixed and immunostained against EGFR, early

**FIGURE 2**  Colocalization of ESCRT-III subunits with SCV and SIF. (a) HeLa cells either stably transfected with LAMP1-GFP (green) or transiently transfected with LAMP1-mCherry (red) were cotransfected with various plasmids for the synthesis of fusion proteins of CHMP1B, CHMP4A, CHMP5, CHMP6, and CHMP7 with GFP (green) or mRuby (red). Cells were infected with STM WT expressing mCherry (red) and living cells were imaged by CLSM 6–9 hr p.i. Scale bars, 10 μm. (b) Schematic depiction of the multivesicular body pathway and the involvement of the ESCRT system. The subunits of ESCRT-III complex are shown in detail and levels of localisation with SIF are indicated by label colours: green, full colocalization; yellow, partial colocalization; red, no colocalization; black, not tested.
endosome antigen 1 (EEA1) and LAMP1 (Figure S2). Confocal laser scanning microscopy (CLSM) revealed lack of EGFR-positive vesicles in HeLa cells. In contrast, colocalization of EGFR-positive vesicles with the early endosomal marker EEA1 but not with the late endosomal/lysosomal marker LAMP1 was found in HeLa CHMP3 k/o cells (Figure S2). These results indicate that the loss of subunit CHMP3 is sufficient to inhibit ESCRT-III-dependent endocytic protein degradation. We did not observe gross alterations in the LAMP1-positive endosomal compartment of CHMP3 k/o cells.

Taken together, we created CRISPR/Cas9-mediated monoclonal HeLa k/o cell lines devoid of functional expression of either SKIP or CHMP3. The similar proliferation of HeLa and k/o cell lines indicates that the mutations did not result in gross growth defects and results obtained with these cells lines should be specific to the respective k/o.

2.4 Attenuation of SPI2-T3SS mutant strains is reduced in CHMP3 k/o cells

We next investigated the role of CHMP3 for intracellular proliferation of Salmonella. HeLa or HeLa CHMP3 k/o cells were infected with
STM WT, ssaV, and sifA mutant strains. Intracellular CFU were determined at 1 and 16 hr p.i., and the x-fold intracellular replication was determined (Figure 4(a)). The sifA strain lacks an effector protein required for maintaining SCV integrity and remodelling of the host cell endosomal system (Beuzon et al., 2000a). SsaV is an integral subunit of the SPI2-T3SS and ssaV-deficient strains are impaired in T3SS assembly, cannot translocate effector proteins, and showed reduced intracellular replication in cell-based models (Hansen-Wester, Stecher, & Hensel, 2002; Hensel et al., 1998; Klein & Jones, 2001; Nikolaus et al., 2001). We observed a moderate reduction of replication of STM WT in HeLa CHMP3 k/o cells compared with HeLa cells. In contrast, intracellular replication of the STM ΔssaV and ΔsifA strains was increased in HeLa CHMP3 k/o cells, and the proliferation of the ΔsifA strain exceeded that of STM WT in HeLa cells.

The competitive index (CI) assay is a method that allows to measure more subtle difference in virulence. Host cells were coinfected with STM WT and a mutant strain, and the intracellular replication after 16 hr p.i. was determined, with the ratio of WT to mutant strain representing the CI. As both strains are exposed to the same conditions, mixed infection allows the direct measurement of intracellular replication of a mutant with a decrease of variability encountered between independent trials. In cells coinfected with STM WT and ΔssaV, the CI index is significantly reduced in either HeLa k/o cell line in comparison to nontreated HeLa cells (Figure 4(b)). The CI index decreased from 14 in infected HeLa cells to 6 in infected HeLa SKIP k/o cells, and to 5 in infected HeLa CHMP3 k/o cells. In the case of CHMP3 k/o, the reduced CI is a product of a significantly increased replication of the ssaV mutant strain, whereas the replication of WT was not changed in HeLa CHMP3 k/o cells (Figure S3). When comparing the CI index of STM WT and ΔsifA in HeLa and HeLa CHMP3 k/o or SKIP k/o cells, respectively, the CI index showed no significant alteration (Figure 4(b)).

In summary, the data suggest that in a mixed infection of HeLa CHMP3 k/o cells with STM WT and ΔssaV, the mutant strain gained advantage in intracellular replication, manifesting in a decreased CI index in comparison to parental HeLa cells.

### 2.5 CHMP3 k/o HeLa cells form amorphous SCV after *Salmonella* infection

To monitor phenotypes induced by STM infection of HeLa and HeLa CHMP3 k/o cells, LCI was performed. As a marker for SCV morphology and *Salmonella*-induced remodelling of the host cell endosomal system, cells were transfected for expression of LAMP1-GFP. In line with previous observation, STM WT in HeLa cells is mostly found in SCV that contain single bacteria and was characterised by minimal distance between the bacteria and the SCV membrane (Figure 5(a), HeLa). In STM WT-infected HeLa CHMP3 k/o cells, such normal SCV morphologies were also observed, as well as the appearance of amorphous SCV (Figure 5(a), HeLa CHMP3 k/o). These SCV resembled the amorphous SCV phenotypes observed after siRNA k/d of CHMP1B, CHMP4B, or CHMP7 (Figure 1). Amorphous SCV were heterogeneous in appearance and characterised by a bulky morphology, often harbouring multiple *Salmonella*, and an increased distance between the SCV membrane and *Salmonella*. We quantified the distance between SCV membranes flanking *Salmonella* as indicated in Figure 5(b) and compared SCV diameters in Figure 5(c). The SCV formed in HeLa cells showed a rather homogeneous diameter of 0.72 ± 0.08 μm (mean ± standard deviation). SCV with normal morphology in HeLa

---

**FIGURE 4** Intracellular proliferation of *Salmonella* in HeLa CHMP3 k/o and SKIP k/o cell lines. (a) HeLa or CHMP3 k/o cell lines were infected with *Salmonella* WT, or strains deficient in ssaV or sifA as indicated at an MOI of 1. The x-fold replication was determined as the ratio of CFU 16 to 1 hr p.i. obtained by plating lysates onto agar plates for CFU determination. (b) Competitive index (CI) assays of HeLa and k/o cell lines. Cells were coinfected with WT in combination with either the ssaV or sifA mutant strain at an overall MOI of 1. The replication at 16 hr p.i. was determined as the ratio of CFU 16 to 1 hr p.i. and CI factor of replication of WT to mutant strain was calculated. Shown are means and standard deviations of three biological replicates done in triplicates. Statistical analyses were performed by Student’s t test and significances are indicated as follows: n.s. not significant; *p < .05; **p < .01
CHMP3 k/o cells had similar diameters (0.71 ± 0.07 μm), while amor-
phous SCV had increased diameters within a large range (1.56 ± 0.62 μm).

To follow the SCV development, time-lapse LCI was performed
with HeLa cells and HeLa k/o cells transiently transfected with
LAMP1-mCherry to visualise SCV and SIF network and infected with
STM WT expressing eGFP (Figure S4). LCI was performed starting
6 hr p.i. by spinning disc confocal microscopy (SDCM) over the course
of 10 hr, and representative time points of infection are shown. In
HeLa cells, infection with STM WT resulted in the formation of SCV
and SIF network clearly visible 10 hr p.i. (Movie 5). Infection of HeLa
SKIP k/o cells did not lead to SIF formation, and hyper-replicating bac-
teria were detected in the cytosol 16 hr p.i. (Movie 6). Bacteria
infecting HeLa CHMP3 k/o cells were still able to induce SIF at 6 to
16 hr p.i. Starting 10 hr p.i., the SCV enlarged to bulky SCV (Movie 7),
thus recapitulating the phenotype observed for cells targeting other
CHMP subunits by siRNA (Figure 1).

The effect of CHMP3 k/o on development of SCV containing WT
or SPI2-T3SS-deficient STM is shown in Figure 6. Both STM WT and
ΔssaV lead to the development of two distinct infection phenotypes
in HeLa and HeLa CHMP3 k/o cells. In WT-infected HeLa CHMP3
k/o cells, bacteria were either enclosed by individual tightly fitting
SCV membranes, or amorphous bulky SCV were encountered. Here,
the SCV membrane was loosely surrounding individual bacteria, or a
cluster of Salmonella cells completely enclosed in one large compart-
ment containing multiple bacteria (Figure 6(a)). The same was
observed for cells infected with the ΔssaV mutant (Figure 6(b)). A sig-
nificant increase of infected cells with bulky SCV was observed for
CHMP3 k/o cells after infection by either WT or ΔssaV strains. When
infected with STM WT, the occurrence of bulky SCV increased from
around 5% in HeLa to around 15% in CHMP3 k/o cells (Figure 6(c)).
An increased frequency of amorphous SCV was also observed in HeLa
CHMP3 k/o cells infected with the ΔssaV strain.

Collectively, our observation revealed an increase of amorphous
SCV formation in the absence of CHMP3 in infected HeLa cells.

2.6 Presence of SCV-bound population

Normal SCV are tight-fitting vacuolar compartments that govern com-
plete separation of Salmonella from the cytosol of host cells. To test if
bulky SCV also provide segregation from the cytosol, we deployed
STM strains harbouring a reporter indicating exposure to host cell
cytosol. The reporter is based on the promoter PuhpT fused to sfGFP
with the promoter being activated by exogenous glucose-6-phosphate
(G6P) which is exclusively found in the host cell cytosol. In order to
detect all intracellular Salmonella, the reporter also expresses a consti-
tutive red fluorophore, i.e. DsRed (Röder & Hensel, 2019).

HeLa and HeLa k/o cells were infected with STM strains
expressing the cytosolic reporter and pulse-chased with Alexa Fluor

![Figure 5](image-url)
FIGURE 6 Legend on next page.
positive for induction of the cytosolic reporter, we found a
Comparing the amount of HeLa and HeLa SKIP k/o cells infected with
proliferation, ciprofloxacin was added at various time points after
degree of SCV damage, we infected HeLa and HeLa CHMP3 k/o cells
(Figure 7
sfGFP-positive bacteria did not increase over time of infection
higher percentage compared with HeLa cells. The proportion of
positive bacteria was also significantly increased, with an app. twofold
increase in the latter. The presence of cytosol-exposed bacteria
found in a time-dependent manner (Figure 8(c)). The increased frequency of cytosol-exposed Salmonella in HeLa CHMP
k/o cells was increased at various time points with or without addition of ciprofloxacin.

Taken together, these results show that in HeLa CHMP3 k/o
cells, Salmonella is in contact with cytosolic G6P while still being con-
fining by a SCV membrane. This indicates a membrane disintegration of the SCV without resulting in the outbreak and hyper-replication of Salmonella in the cytosol.

3 | DISCUSSION

Here, we describe the impact of the ESCRT-III complex in general, and
of the core component CHMP3 in particular, on Salmonella intracellu-
lar lifestyle. An siRNA k/d screen revealed the involvement of ESCRT-
III core proteins in proper SCV biogenesis. Such core proteins were
recruited to SCV and SIF continuum during intracellular proliferation. Further, the frequency of host cells with
cytosol-exposed Salmonella was determined. As shown in Figure 8, the number of infected host cells with cytosol-exposed Salmonella
was about twofold higher in HeLa CHMP k/o cells compared with
HeLa cells. Ciprofloxacin inhibited intracellular proliferation (Figure 8 (b)) and led to decreased frequency of host cells harbouring cytosol-
exposed Salmonella in a time-dependent manner (Figure 8(c)).

To further correlate the intracellular proliferation of STM with the
degree of SCV damage, we infected HeLa and HeLa CHMP3 k/o cells
with STM WT harbouring the cytosolic reporter. To stop bacterial proliferation, ciprofloxacin was added at various time points after
infection. For infected cells fixed at 8 hr p.i., flow cytometry was used
to determine the levels of constitutively expressed DsRed as proxy
for Salmonella proliferation. Further, the frequency of host cells with
cytoplasm-exposed Salmonella was determined. As shown in Figure 8, the number of infected host cells with cytosol-exposed Salmonella
was about twofold higher in HeLa CHMP k/o cells compared with
HeLa cells. Ciprofloxacin inhibited intracellular proliferation (Figure 8 (b)) and led to decreased frequency of host cells harbouring cytosol-
exposed Salmonella in a time-dependent manner (Figure 8(c)). The increased frequency of cytosol-exposed Salmonella in HeLa CHMP
k/o cells was increased at various time points with or without addition of ciprofloxacin.

Figure 6: SCV phenotypes in HeLa CHMP3 k/o cells after infection by WT or ΔssaV strains. HeLa and HeLa CHMP3 k/o cells were infected
with Salmonella WT (a) or ΔssaV strains (b). At 16 hr p.i., cells were then fixed with 3% PFA and immuno-stained against LPS (red) and LAMP1
(green) and imaged by CLSM. (c) Quantification of SCV phenotypes in HeLa and HeLa CHMP3 k/o cells. Per condition, at least 100 infected HeLa
cells were identified and scored for normal or amorphous, bulky SCV phenotypes. Depicted are the means and standard deviation of three
biological replicates done in triplicates. Statistical analyses were performed by Student’s t test and significances are indicated as follows: **p < .01;
***p < .001. Scale bars, 1 and 5 μm in overview and details, respectively.
FIGURE 7  Analysis of the presence of cytosol-exposed *Salmonella* in HeLa and HeLa k/o cells. (a) HeLa, CHMP3, and SKIP k/o cells were pulse-chased with dextran-Alexa647 (blue) and infected with *Salmonella* expressing a cytosolic reporter for 8 hr at an MOI of 50. Shown are representative SDCM images. Cytosol-exposed *Salmonella* (green) and total *Salmonella* (red) were quantified by flow cytometry. (b) Hela, CHMP3 k/o, and SKIP k/o cells were infected with *Salmonella* strains expressing a cytosolic reporter with MOI 5 and fixed 4, 8, and 16 hr p.i. with subsequent flow cytometry measurements of infected cells with sfGFP-positive (cytosol-exposed) STM. Shown are the means and standard deviation of three biological replicates done in triplicates. Statistical analyses were performed by Student's t test and significances are indicated as follows: *p* < .05; **p** < .01; ***p** < .001. Scale bar, 10 μm.
enlarged SCV, very similar to the aberrant SCV described in this study, has been observed before. In host cells depleted of pleckstrin homology domain-containing protein family member 1 (PLEKHM1), a profound defect in SCV morphology with multiple bacteria accumulating in enlarged amorphous SCV was determined (McEwan, Richter, et al., 2015). PLEKHM1, an interaction partner of SifA, can directly bind the small GTPase Rab7 and components of the homotypic fusion and protein sorting (HOPS) complex (McEwan, Popovic, et al., 2015). Depletion of Rab7, HOPS, or PLEKHM1 led to aberrant SCV morphology. The authors suggested that hijacking of endogenous membranes via the PLEKHM1/HOPS/Rab7/SifA complex ensures SCV integrity, as membranes are delivered for growth of the SCV (McEwan, Richter, et al., 2015).

Given the fact that Salmonella accumulates factors of the ESCRT system on SCV and SIF membranes, we surmised that these proteins can be specifically recruited to the SCV-SIF continuum. By disruption of the ESCRT-III complex, also a source for SCV extension by endomembranes, in particular MVBs, would be depleted. In this case, the amorphous SCV phenotype would represent a general consequence of shortage of endomembrane supply to SCV. Nevertheless, some recent studies suggest alternative explanations involving membrane repair mechanisms at various locations in the cell. On the one
hand, the general repair of plasma membrane wounds (Jimenez et al., 2014), as well as specifically membrane pores caused by pyroptosis was accredited to the ESCRT-III complex (Ruhl et al., 2018). On the other hand, nuclear envelope (NE) resealing is dependent on ESCRT-III specifically recruited by CHMP7 (Vietri et al., 2015) via the inner nuclear membrane protein LEMD2/LEM2 (Gu et al., 2017).

Additionally, membrane instability due to ESCRT defects was attributed to endomembrane repair mechanisms by this system (Skowyra et al., 2018). In this recent study, repair of minor endolysosomal damage by the ESCRT system, in particular the ESCRT-III complex, was reported. This raises the question if pathogens also use the ESCRT system to repair damage on phagosomal membranes. In HeLa cells expressing CHMP4B-eGFP and infected with the obligate intracellular pathogen Coxiella burnetii, pathogen-containing vacuoles became positive for CHMP4B before later being negative for the marker (Radulovic et al., 2018). These phenomena repeated several times and vacuole expansion, a sign for an intact replicative niche, occurred after CHMP4B recruitment. Accordingly, the authors hypothesised that membranes of the Coxii containing vacuole are prone to sporadic ruptures, which are repeatedly repaired by the ESCRT machinery. Various ESCRT-III proteins are also recruited to phagosomes harboring Mycobacterium tuberculosis (Mittal et al., 2018). Here, the authors speculate that the ESCRT-III system is responding to phagosomal membrane damage inflicted by the ESX-1 secretion system (T7SS) to maintain membrane integrity and allow phagosome expansion. Work by Lopez-Jimenez et al. (2018) revealed the recruitment of ESCRT-I subunit Tsg101, Chmp4, and Vps4 to damaged M. marinum-containing vacuoles in Dictyostelium discoideum. Moreover, RNAi screens and follow-up studies demonstrated that ESCRT is important in mycobacterial growth restriction (Mehra et al., 2013; Philips, Porto, Wang, Rubin, & Perrimon, 2008; Philips, Rubin, & Perrimon, 2005). This could be shown for fast-growing Mycobacteria M. fortuitum and M. smegmatis, as well for slow-growing M. bovis BCG and M. tuberculosis. Even though M. fortuitum, M. smegmatis, and M. bovis do not possess dedicated systems to breach phagosomal membranes, still minor perturbations are caused.

In light of these findings, we propose that Salmonella exploits the ESCRT system in a similar way. Lesions of the SCV in infected cells could be mended by the specific recruitment and subsequent action of the ESCRT system (Figure 9d)). This recruitment most likely does not only encompass the core ESCRT-III, as indicated by phenotypes of CHMP3 k/o cells, but also the accessory proteins CHMP7 and CHMP1B as indicated by the phenotypes caused by their k/d, even though both proteins did not show strong localisation with SCV or SIF. This discrepancy might be explained by only transient interactions of these proteins with Salmonella-modified membranes. CHMP7 might thus play a similar role in recruitment of ESCRT-III as in NE resealing, in which possibly a Salmonella effector might substitute the host adapter LEMD2. Interestingly, whereas CHMP7 interacts with IST1 to recruit the microtubule (MT)-severing AAA ATPase spastin in NE sealing (Vietri et al., 2015), specifically CHMP1B recruits spastin in cytokinesis (Reid et al., 2005; Yang et al., 2008).

Hence, this additionally implies that MT participate in regulating SCV integrity as already demonstrated by several interactions of Salmonella effectors with the MT network (Leone & Meresse, 2011; Rajashekar & Hensel, 2011). Consequently, in HeLa CHMP k/d or k/o cells, membrane damages of the SCV cannot be repaired due to a dysfunctional ESCRT-III complex and SCV lesions remain, allowing the influx of cytosol (Figure 9b)). We reason that STM inflicts minor SCV membrane damage, either by the T3SS or as a side effect of bacterial proliferation and resulting mechanical tension on the SCV. However, proliferation of Salmonella WT in CHMP3 k/o cells was not impaired.

Using CI assays, we examined intracellular replication of mutant strains. Independent of the host cell genotype, the CI for mixed infections with WT and sifA mutant strain was app. one, showing a balanced replication. This phenotype is based on the capability of Salmonella sifA mutant strains to escape instable SCV and proliferate in the cytosol of epithelial cells (Beuzon, Salcedo, & Holden, 2002; Brumell, Tang, Zaharia, & Finlay, 2002). The knockout of either CHMP3 or SKIP in HeLa cells did not change CI indices, indicating

**FIGURE 9** Model of the impact of ESCRT-III complex on Salmonella intracellular lifestyle. (a, b) Infected cells devoid of functional ESCRT-III complex induce the formation of membrane-damaged amorphous SCV allowing the influx of cytosol. (a) The ssaV mutant strain does not form SIF and gains an advantage because leaky SCV mutant membranes ensure the access of nutrients from the cytosol. Harmful antimicrobial molecules do not accumulate in the SCV. (b, d) The WT strain induces formation of SIF that provide nutritional supply and reduction of antimicrobial factors. The additional access to the cytosol in CHMP3 k/o host cells does not enhance the effect of SIF formation. (c, d) Infected host cells induce the formation of functional, tight-fitting SCV without contact to the host cytosol. (c) Lack of SIF formation of the ΔssaV mutant strain is disadvantageous, as nutrients cannot be accessed and stress factors accumulate within the SCV. EE, early endosome; MVB, multivesicular body; Ly, lysosome.
that these host cell functions did not augment the mutant strain. Interestingly, we observed altered CI indices of HeLa k/o cells when coinfectected with *Salmonella* WT and ΔssaV strains. Mutant strains lacking a functional SPI2-T3SS fail to induce SIF formation, resulting in nutrient starvation as the formation of SIF acts as means of nutrient supply for *Salmonella* (Liss et al., 2017). Because the ssAV mutant strain proliferates better in CHMP3-deficient than in CHMP3-proficient host cells, we speculate that a partially instable SCV membrane supports nutrient access to the ssAV mutant strain. Additionally, proteomic analyses in macrophages revealed that ΔssaV mutant strain showed signatures of increased exposure to stress by host defence mechanisms, in particular reactive oxygen species (Noster et al., 2019). Therefore, the beneficial effect of a leaky SCV membrane might be a result of access to nutrients in the host cytosol, as well as reduced exposure to damaging factors in the SCV (Figure 9). This hypothesis refutes that minor SCV lesions are caused by the SPI2-T3SS, as the secretion system is absent in the ΔssaV strain. Thus, we favour the model that minor damages of SCV membranes result from mechanical forces due to bacterial proliferation during later stages of infection. In contrast, SCV damage at early stages of *Salmonella* infection induced by SPI1-T3SS activity is not repaired by the ESCRT system but by autophagy-dependent membrane repair mechanisms (Kreibich et al., 2015; Owen & Casanova, 2015). In general, this indicates that SCV damage caused by the SPI1-T3SS is more severe than by bacterial proliferation. Endogenous endosomal membrane wounds recruit the autophagy or the ESCRT system, respectively, depending on whether the wound is large or small (Skowyra et al., 2018). Although defective endogenous membrane is degraded by autophagy, the SCV is repaired in the late stages of intracellular lifestyle.

Overall, we present evidence that the ESCRT system, in particular ESCRT-III, is involved in the maintenance of SCV membrane integrity at later stages of the infection, implicating a complex exploitation of host membrane repair mechanisms for the intracellular lifestyle of *Salmonella*. These observations give rise to new questions. Are components of the ESCRT-III complex also repeatedly recruited to the SCV membrane during the infection representing multiple repair steps as damage to the SCV membrane occurs over time? Are other host membrane repair mechanisms exploited to further stabilise SCV membrane in the advanced infection? Further analyses of host-pathogen interactions will likely contribute to answer these questions.

## 4 EXPERIMENTAL PROCEDURES

### 4.1 Bacterial strains and growth conditions

*Salmonella enterica* serovar Typhimurium (STM) was used as wild-type (WT) strain and mutant strains were isogenic to WT (Table 1). STM strains were routinely grown on LB agar or in LB broth containing 50 μg mL⁻¹ carbenicillin for maintenance of plasmids at 37°C using a roller drum at 60 rpm for aeration.

### 4.2 Cell lines and cultivation

Experiments were performed using a HeLa cell line (ATCC No. CCL-2), or a lentivirus-transfected HeLa line for stable expression of LAMP1-GFP (Krieger et al., 2014). Cells were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g L⁻¹ glucose, 4 mM stable glutamine, and sodium pyruvate (Biochrom, Berlin, Germany) supplemented with 10% inactivated fetal calf serum (I LCS; Gibco, Darmstadt, Germany) in an atmosphere of 5% CO₂ and 90% humidity at 37°C.

### 4.3 Reverse transfection with siRNA

For RNAi, siRNA were spotted at final concentrations of 5.2 nM onto 96 well clear bottom black cell culture microplates (Corning, Corning, NY, USA). For mRNA extraction siRNA were spotted to achieve final concentration of 5 nM on standard cell culture 6-well plates (TPP, Trasadingen, Switzerland). The siRNA used in this study are listed in Table S1. Next, a mixture of the transfection reagent HiPerFect (Qiagen, Hilden, Germany) and serum-free cell culture medium was applied, and this was incubated for 5–10 min at room temperature (RT). Then, 5,000 or 125,000 cells per well for 96-well plates and 6-well plates, respectively, in serum-containing medium were added and incubated for 72 hr at 37°C in a humidified atmosphere containing 5% CO₂.

### 4.4 Gene expression quantification

After reverse transfection with different siRNAs, total RNA of cells was extracted using the RNeasy Mini Kit following the manufacturer’s instructions (Qiagen, Hilden, Germany). Homogenisation during extraction was performed using Qiagen QIAshredder columns. Then, 1 μg of RNA digested with DNasel (NEB, Frankfurt a. M., Germany) was used for reverse transcription of mRNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Dreieich, Germany) following the manufacturer’s instructions using the Oligo(dT)18 primer. For RT–PCR 1 μl of cDNA was used with the Thermo Scientific Maxima SYBR Green/Fluorescein qPCR Master Mix (2×). As reference gene, the housekeeping gene GAPDH was selected (Vreeburg, Bastiaan-Net, & Mes, 2011). For control of individual host factors, knockdown primers were used employing the PrimerBank database (Wang & Seed, 2003; Wang, Spandidos, Wang, & Seed, 2012). Primers

---

**Table 1.** *Salmonella enterica* serovar Typhimurium strains used in this study

| Designation   | Relevant characteristics | Source/reference   |
|---------------|--------------------------|--------------------|
| NCTC12023    | Wild type                | Lab stock          |
| MvP1890      | ΔssaV::FRT               | (Noster et al., 2019) |
| MvP503       | ΔsifA::FRT               | (Popp et al., 2015) |
for those as well as GAPDH are listed in Table S2. Primer concentration was 150 nM each and for each primer pair primer efficiency was determined. RT-PCR was performed in an iCycler (Bio-Rad, Munich, Germany) in triplicates in 96-well plates. Relative expression was determined using the $2^{-\Delta\Delta Ct}$ method (Kerr et al., 2010; Town et al., 2009) with GAPDH expression set as 100%. Results were graphed using SigmaPlot 13 (Systat Software, Erkrath, Germany).

### 4.5 Construction of plasmids

Plasmids used in this study were either obtained from Addgene, kind gifts from various laboratories, or cloned by Gibson Assembly or restriction enzyme digests and are listed in Table 2. For the construction of plasmids encoding for host proteins fused to mRuby2 or eGFP, Gibson Assembly was performed. Oligonucleotides for cloning are listed in Table S2. First, N- or C-terminal mRuby2 vectors were cloned. The vectors pEGFP-C1 and pEGFP-N1 were amplified, and mRuby2 was exchanged by eGFP. Genes encoding for host proteins were amplified from vectors obtained from DNASU (Table 2) and then inserted into mRuby2 vectors by Gibson Assembly. Plasmids encoding for host proteins fused to EGFP were constructed using restriction enzyme digest. The vector pEGFP-C3 was digested with KpnI and XbaI or KpnI and BamHI and the larger fragment was recovered. The inserts were treated the same way and fragments were ligated.

#### TABLE 2  Plasmids used in this study

| Designation          | Relevant characteristics | Source/reference                                      |
|----------------------|--------------------------|-------------------------------------------------------|
| pEGFP-C1             | $P_{CMV}$::EGFP-C1       | Clontech                                              |
| pEGFP-C3             | $P_{CMV}$::EGFP-C3       | Clontech                                              |
| pEGFP-N1             | $P_{CMV}$::EGFP-N1       | Clontech                                              |
| pENTR223_CHMP1B      | CHMP1B                   | DNASU 16967 (HsCD00506851)                            |
| pENTR223_CHMP4A      | CHMP4A                   | DNASU 16967 (HsCD00508510)                            |
| pENTR223_CHMP5       | CHMP5                    | DNASU 16967 (HsCD00507015)                            |
| pENTR223_CHMP7       | CHMP7                    | DNASU 16967 (HsCD00512112)                            |
| pCHMP6-GFP           | CHMP6::mEGFP             | Addgene #31806 (Guizetti et al., 2011)                |
| p3432                | $P_{CMV}$::hLAMP1::eGFP  | (Rajashekar et al., 2008)                             |
| p4056                | pFPV mNeptune 2.5        | This study                                            |
| p3451                | $P_{CMV}$::hLAMP1::mCherry| (Vorwerk, Krieger, Deiwick, Hensel, & Hansmeier, 2015) |
| pFPV25.1             | $P_{pam}$::eGFPmut3      | (Valdivia & Falkow, 1996)                             |
| pX458                | $P_{U6}$::sgRNA scaffold | Addgene #4813B                                        |
| p3463                | $P_{U6}$::sgCHMP3        | This study                                            |
| p3467                | $P_{U6}$::sgSKIP $P_{SV40}$::Cas9 $P_{T2A}$::EGFP | This study |
| p4889                | $P_{CMV}$::DsRed $P_{pyr}$::cGFP | (Roder & Hensel, 2019)                              |
| p4080                | $P_{CMV}$::mRuby2-C1     | This study                                            |
| p4081                | $P_{CMV}$::mRuby2-N1     | This study                                            |
| p4090                | $P_{CMV}$::CHMP1B::mRuby2| This study                                            |
| p4093                | $P_{CMV}$::mRuby2::CHMP4A| This study                                            |
| p4100                | $P_{CMV}$::CHMP5::mRuby2 | This study                                            |
| p4133                | $P_{CMV}$::mRuby2::CHMP7 | This study                                            |
| pcDNA3-mRuby2        | $P_{CMV}$::mRuby2        | Addgene #40260 (Lam et al., 2012)                     |
| p5401                | mCherry::LC3B in pLX304  | This study                                            |

### 4.6 Generation of CRISPR/Cas9 k/o cell lines

Generation of k/o cell lines was performed as previously described (Ran et al., 2013). Briefly, HeLa cells were seeded in surface-treated 6-well plates (TPP). In the following day, cells were transfected with 1:2 FuGENE®HD transfection reagent and 2 µg plasmid DNA (Table 2) expressing a single-stranded guide RNA against CHMP3 or SKIP, respectively, the Cas9 nuclease and EGFP. Guide RNAs have been designed using the CRISPR Design tool provided by the Zhang lab (Cong et al., 2013; Hsu et al., 2013). A 20 bp guide sequence was chosen either targeting human CHMP3 or SKIP preceding a protospacer adjacent motif (PAM; Figure S1). Cloning was done using the vector pX458 (Addgene) and two complementary primers.
containing guide RNA sequence and BbsI ligation adapters synthesized by IDT (Table S2). Plasmids were created by a single-step digestion and ligation reaction where digestion with BbsI is combined with ligation by T7 DNA ligase in one step. After letting the cells expand for 5–7 days, flow cytometry sorting was performed for each polyclonal cell line and cells were sorted in surface-treated 96-well plates (TPP). After incubation with DMEM media containing 20% FCS and 30% conditioned media (spent media harvested from cultured HeLa cells), cells were expanded for 2–3 weeks.

4.7 | On- and off-target screen

Genomic DNA from individual monoclonal CRISPR/Cas-treated, as well as nontreated control HeLa cell lines was isolated using the DNeasy Blood & Tissue Kit (Qiagen). The on-target regions were amplified using specific oligonucleotides (Table S2) and sequenced. As heterozygous mutations lead to the amplification of different PCR products, the results cannot be analysed using alignment to the HeLa nontreated control. For this purpose the TIDE (Tracking of Indels by DEcomposition) Web-Tool was used (Brinkman, Chen, Amendola, & van Steensel, 2014). To analyse off-target Cas9 effects on the monoclonal k/o HeLa cell lines, the online tool Cas-OFFinder (Bae et al., 2014) was used and a mismatch number of three nucleotides was allowed. For the resulting off-targets (Table 3) oligonucleotides (-Table S2) were designed and gene sections amplified, sequenced and checked for indel incorporation.

4.8 | Western blot analyses

Whole cell lysates were prepared using a lysis buffer (1% Triton X-100, 5% glycerol in phosphate-buffered saline (PBS)) from CRISPR/Cas-treated monoclonal and nontreated HeLa control cell lines. After centrifugation, the protein concentration in the supernatant was determined by the BCA Protein Assay (ThermoFisher Scientific) and acetone precipitation for 1.5 hr at −20°C was performed. Protein pellets were dissolved in SDS-PAGE loading buffer containing 0.1% glycine/HCl, pH 2.2 and boiled for 5 min. Circa 15 μg of precipitated whole cell protein was loaded on a 15% SDS-PAGE and after electrophoresis, samples were blotted onto a 0.22 μm nitrocellulose membrane using a semi-dry electrophoretic transfer unit (BioRad). Blots were incubated with a primary antibody directed against CHMP3 (1:2,000) or SKIP (1:100), respectively, and γ-Tubulin (1:1,000; Table S3) as loading control. Secondary antibodies coupled to horse-radish peroxidase were chosen according to donor species of primary antibodies and diluted 1:10,000. Detection was achieved by an ECL detection kit (ThermoFisher Scientific), and blots were visualised with a ChemiDoc imaging system (BioRad).

4.9 | EGFR internalisation assay

HeLa cells were seeded on coverslips (VWR) in a surface-treated 24-well plate (TPP) and treated with DMEM media containing 10 μg x ml⁻¹ cycloheximide for 1 hr at 37°C. Cells were then nontreated, or the media was replaced with media containing 10 μg x ml⁻¹ cycloheximide and 100 ng x ml⁻¹ recombinant murine epidermal growth factor (EGF, PeproTech) for 30 min. After washing the cells twice with PBS, the cells were further incubated with medium containing cycloheximide for 6 hr. After fixation with 3% paraformaldehyde (PFA), immunostaining was performed as described above using the primary antibodies anti-human EEA1 (1:250), anti-human LAMP1 (1:500), and anti-human EGFR (1:100). Secondary antibodies were selected accordingly (Table S3).

4.10 | CFSE proliferation assay

CFSE was used to monitor the proliferation of CRISPR/Cas-treated and nontreated HeLa cell lines. Cells were seeded in surface-treated 6-well plates (TPP) and stained with 5 μM CFSE in PBS for 20 min at 37°C and washed twice with PBS. As a control for blocked proliferation, cells were treated with 100 nM nocodazole for the ongoing

| Designation | Locus | Mismatches | Sequence |
|-------------|-------|------------|----------|
| On-target   |       |            |          |
| sgSKIP      | Chr1  | 0          | GACCTGTGATGGATCCGGATGG |
| sgCHMP3     | Chr2  | 0          | GAACATGCAGATCCTTCTCGG |
| Off-target  |       |            |          |
| sgSKIP off target 1 | Chr16 | 3 | GACCTTGTGATGGcGGAAGGG |
| sgSKIP off target 2 | Chr19 | 3 | GACCACTGATGATCGCAGAGGG |
| sgSKIP off target 3 | Chr19 | 3 | aACCTTGATGTCAGACAGG |
| sgCHMP3 off target 1 | Chr3  | 3 | GcACTgGAGACATCTCCTTCTG |
| sgCHMP3 off target 2 | Chr4  | 3 | cCAACTACGAGACATTCCTG |
| sgCHMP3 off target 3 | Chr2  | 3 | GAAACTGACTCAGTCTT |
| sgCHMP3 off target 4 | Chr10 | 3 | GcAAGACGACTCCTCTCAG |
| sgCHMP3 off target 5 | Chr9  | 3 | GAACATGCAGATCCTTCTAGG |
experiments. Cells were detached and fixed with 3% PFA days 0–3 after CFSE staining and flow cytometry was performed using the Attune NxT flow system (ThermoFischer Scientific). Cell proliferation correlates with decrease of fluorescence intensity over the generations of cells.

4.11 | Cytosolic reporter

HeLa cells either CRISPR/Cas9-treated or nontreated were seeded in surface-treated 12-well plates (TPP). Cells were infected with Salmonella WT strain harbouring the cytosolic reporter plasmid p4889 (Table 2, Röder & Hensel, 2019) as described above at MOI of 5 for 25 min after centrifugation for 5 min and 500 × g. Cells were detached and fixed with 3% paraformaldehyde (PFA) after 4, 8, and 16 hr. Using the Attune NxT flow cytometry (ThermoFischer Scientific), infected cells were gated by virtue of constitutive red fluorescence of STM, and the intensity of green fluorescence was determined as proxy of exposure to cytosolic G6P.

4.12 | Quantitation by flow cytometry analyses

HeLa cells were infected at a MOI of 5 for 25 min; 1, 2, 4, and 6 hr p.i. 100 ng x ml⁻¹ ciprofloxacin were added to stop bacterial replication. At 8 hr p.i., cells were detached with Biotase and fixed with 3% PFA for subsequent flow cytometry analyses using the Attune NxT flow cytometer (ThermoFischer Scientific). Experiments were performed in triplicates at least three times. Data were analysed with Attune NxT 2.5.

4.13 | Epi-fluorescence microscopy

For confocal laser-scanning microscopy (CLSM) of fixed cells, untreated HeLa or HeLa k/o cells were seeded on coverslips (VWR) in surface-treated 24-well plates (TPP). Salmonella WT andssaV mutant strains (Table 1) were grown overnight in LB broth at 37°C in a roller drum and subcultured 1:31 in fresh LB media for 3.5 hr. Cells were infected at MOI 50 for 25 min at 37°C. After washing thrice with PBS, cells were incubated with media containing 100 μg x ml⁻¹ gentamicin for 1 hr to kill noninvading bacteria and then replaced with media containing 10 μg x ml⁻¹ gentamicin for the rest of the experiment. Sixteen hours p.i. cells were fixed with 3% PFA and immunostained as described (Müller, Chikkaballi, & Hensel, 2012). In short, permeabilization was done using a blocking solution (2% goat serum, 2% BSA, 0.1% saponin) for 30 min at RT. Incubations with primary antibodies against Salmonella LPS O-antigen (1:500), anti-human LAMP1 (1:500), and secondary antibodies (- Table S3) were done for 1 hr at RT.

Epi-fluorescence imaging was performed on a Leica SP5, objective HCX PL APO CS x 100 (NA 0.7–1.4), polychroic mirror TD 488/543/633 (Leica). Furthermore, a Zeiss Cell Observer spinning disc confocal microscope (SDCM) was used for epi-fluorescence imaging. The microscope was equipped with a Yokogawa Spinning Disc Unit CSU-X1a 5000, Evolve EMCCD camera (Photronics, Tucson), objective Alpha Plan-Apochromat x 63 (NA 1.46) and the following filter combinations: GFP with BP 525/50, Cy3 with BP 605/70 and Cy5 with BP 690/50 (Zeiss).

4.14 | Transfection

HeLa cells were cultured in surface-treated 8-well chamber slides (Ibidi, Gräfelfing, Germany) for 1 day. A total of 0.5 μg of plasmid DNA (p5401, p3432) was solved in 25 μl DMEM without iFCS and mixed with 2 μl FUGENE reagent (ratio of 1:2 for DNA to FUGENE). After incubation for 10 min at RT, the transfection mix was added to the cells in DMEM with 10% iFCS for at least 18 hr. Before infection, the cells were provided with fresh medium without transfection mix.

4.15 | Live cell imaging

For SDCM of living cells, HeLa and HeLa k/o cells were seeded in surface-treated 8-well chamber slides (Ibidi). Imaging medium consisting of Minimal Essential Medium (MEM) with Earle's salts, without NaHCO₃, without L-glutamine, without phenol red (Biochrom) and supplemented with 30 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Sigma-Aldrich) pH 7.4 was used (Rajashekar, Liebl, Seitz, & Hensel, 2008). Cells were either transfected with a plasmid encoding LAMP1-mCherry using the FuGENE®HD transfection reagent (Table 2), or pulse-chased with dextran-Alexa647 (10,000 Da molecular weight, Invitrogen). For the latter, cells were pulsed with 200 μg x ml⁻¹ dextran-Alexa647 1 hr p.i. for 4 hr and after washing twice with PBS, cells were chased for 1 hr directly before LCI. Infection was performed as described above with Salmonella WT either harbouring a plasmid encoding GFP or a cytosolic reporter (Table 2). The medium contained 10 μg x ml⁻¹ gentamicin to kill extracellular Salmonella.

The SDCM as described above was used with an automated PZ-2000 stage (Applied Scientific Instrumentation, Eugene, OR, USA), and infrared-based focus system Definite Focus, operated by Zeiss ZEN 2012 software (blue edition). The microscope was equipped with live cell periphery consisting of a custom-made incubation chamber surrounding the microscope body and connected with “The Cube” heating unit (Life Imaging Services, Basel, Switzerland) maintaining 37°C and Incubation System S for CO₂ and humidity supply (PeCon, Erbach, Germany). Images were acquired using the Zeiss LD Plan-Neofluar 40x/0.6 Corr air objective (with bottom thickness correction ring). For acquisition of GFP and mCherry BP 525/50 (Zeiss) and LP 580 (Olympus, Hamburg, Germany) filters, respectively, were applied. All images obtained were processed by ZEN software.

4.16 | Competitive index assay

Competitive index (CI) assay was performed as previously described (Segura, Casadesus, & Ramos-Morales, 2004). Briefly, CRISPR/Cas-
treated and nontreated HeLa cell lines were seeded in a surface-treated 24-well plate (TPP) and grown to 80% confluency at the day of infection. WT and aph mutant strains were separately grown overnight in LB and LB containing 50 μg x ml⁻¹ kanamycin and subcultured 1:31 in fresh LB media for 3.5 hr in a roller drum at 37°C. Cells were infected with a 1:1 mixture of WT and mutant strains at an overall MOI of 1 for 25 min. Noninvasive bacteria were killed using media containing 100 μg x ml⁻¹ gentamicin for 1 hr and replaced by media containing 10 μg x ml⁻¹ gentamicin for the ongoing experiment. The numbers of viable intracellular bacteria were determined 1 and 16 hr p.i. by plating on LB and LB kanamycin agar. The CI for bacterial survival is defined as the ratio of x-fold replication of WT to mutant strain.

ACKNOWLEDGEMENTS
This work was supported by grants HE 1964/18-2 and SFB 944 project Z of the Deutsche Forschungsgemeinschaft to M. H. and by grant 315834B, P2 of the Bundesministerium für Bildung und Forschung to M. H. We like to thank Hans-Peter Schmitz (Div. Genetics) for support with flow cytometry and members of the Division Microbiology for continuous support and fruitful discussions. The skilled support in generating of the recombinant DNA by Monika Nietschke and Ursula Krehe is gratefully acknowledged. Plasmids were kindly provided by Addgene and DNASU.

CONFLICT OF INTEREST
The authors declare no conflict of interest. The authors declare no conflict of financial interest.

CONTRIBUTIONS
V. G., A. K. and M. H. conceived the study; V. G., A. K., and J. R. performed experimental work; V. G., A. K., J. R., and M. H. analysed the data; V. G., A. K. and M. H. wrote the manuscript.

ORCID
Vera Göser https://orcid.org/0000-0002-4592-064X
Alexander Kehl https://orcid.org/0000-0002-1127-8847
Jennifer Röder https://orcid.org/0000-0002-1682-6873
Michael Hensel https://orcid.org/0000-0001-6604-6253

REFERENCES
Bache, K. G., Stuffers, S., Malerod, L., Slagsvold, T., Raiborg, C., Lecherdeur, D., & Stenmark, H. (2006). The ESCRT-III subunit hVps24 is required for degradation but not silencing of the epidermal growth factor receptor. Molecular Biology of the Cell, 17(6), 2513–2523. https://doi.org/10.1091/mbc.e05-10-0915
Bae, S., Park, J., & Kim, J. S. (2014). Cas-OFFinder: A fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics, 30(10), 1473–1475. https://doi.org/10.1093/bioinformatics/btu048
Bauer, I., Brune, T., Preiss, R., & Kölling, R. (2015). Evidence for a non-endosomal function of the Saccharomyces cerevisiae ESCRT-III-like protein Chm7. Genetics, 201(4), 1439–1452. https://doi.org/10.1534/genetics.115.178939
Beuzon, C. R., Meresse, S., Unsworth, K. E., Ruiz-Albert, J., Garvis, S., Waterman, S. R., & Holden, D. W. (2000). Salmonella maintains the integrity of its intracellular vacuole through the action of SifA. The EMBO Journal, 19(13), 3235–3249. https://doi.org/10.1093/emboj/19.13.3235
Beuzon, C. R., Salcedo, S. P., & Holden, D. W. (2002). Growth and killing of a Salmonella enterica serovar Typhimurium sifA mutant strain in the cytosol of different host cell lines. Microbiology, 148(Pt 9), 2705–2715.
Boucrot, E., Henry, T., Borg, J. P., Gorvel, J. P., & Meresse, S. (2005). The intracellular fate of Salmonella depends on the recruitment of kinesin. Science, 308(5725), 1174–1178.
Brinkman, E. K., Chen, T., Amendola, M., & van Steensel, B. (2014). Easy quantitative assessment of genome editing by sequence trace decomposition. Nucleic Acids Research, 42(22), e168. https://doi.org/10.1093/nar/gku936
Brumell, J. H., & Scidmore, M. A. (2007). Manipulation of rab GTPase function by intracellular bacterial pathogens. Microbiol Mol Biol Rev, 71(4), 636–652. doi:71/4/636[pii]https://doi.org/10.1128/MMBR.00023-07
Brumell, J. H., Tang, P., Zaharik, M. L., & Finlay, B. B. (2002). Disruption of the Salmonella-containing vacuole leads to increased replication of Salmonella enterica serovar Typhimurium in the cytosol of epithelial cells. Infection and Immunity, 70(6), 3264–3270.
Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., & Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science, 339(6121), 819–823. https://doi.org/10.1126/science.1231143
Dukes, J. D., Lee, H., Hagen, R., Reaves, B. J., Layton, A. N., Galyov, E. E., & Whiteley, P. (2006). The secreted Salmonella dublin phosphoinositide phosphotase, SopB, localizes to PtdIns(3)P-containing endosomes and perturbs normal endosome to lysosome trafficking. The Biochemical Journal, 395(2), 239–247. https://doi.org/10.1042/BJ20051451
Dumont, A., Boucrot, E., Drevensek, S., Daire, V., Gorvel, J. P., Paus, C., & Meresse, S. (2010). SKIP, the host target of the Salmonella virulence factor SifA, promotes kinesin-1-dependent vacuolar membrane exchanges. Traffic, 11(7), 899–911. doi:TRA1069 [pii]https://doi.org/10.1111/j.1600-0854.2010.01069.x
Eswarappa, S. M., Negi, V. D., Chakraborty, S., Chandrasekhar Sagar, B. K., & Chakravorty, D. (2010). Division of the Salmonella-containing vacuole and depletion of acidic lysosomes in Salmonella-infected host cells are novel strategies of Salmonella enterica to avoid lysosomes. Infection and Immunity, 78(1), 60–79. https://doi.org/10.1128/IAI.00668-09
Figueira, R., & Holden, D. W. (2012). Functions of the Salmonella pathogenicity island 2 (SPI-2) type III secretion system effectors. Microbiology, 158(Pt 5), 1147–1161. doi:mic.0.058115-0 [pii]https://doi.org/10.1099/mic.0.058115-0
García-del Portillo, F., Zwick, M. B., Leung, K. Y., & Finlay, B. B. (1993). Salmonella induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells. Proceedings of the National Academy of Sciences of the United States of America, 90(22), 10544–10548.
Ghazi-Tabatabai, S., Saksena, S., Short, J. M., Pobbati, A. V., Veprintsev, D. V., Crowther, R. A., & Williams, R. L. (2008). Structure and disassembly of filaments formed by the ESCRT-III subunit Vps24. Structure, 16(9), 1345–1356. https://doi.org/10.1016/j.str.2008.06.010
Gu, M., Lajoie, D., Chen, O. S., von Appen, A., Ladinsky, M. S., Redd, M. J., & Frost, A. (2017). LEM2 recruits CHMP7 for ESCRT-mediated nuclear envelope closure in fission yeast and human cells. Proceedings of the National Academy of Sciences of the United States of America, 114(11), E2166–E2175. https://doi.org/10.1073/pnas.1613916114
Guizetti, J., Schermelleh, L., Mantler, J., Maar, S., Poser, I., Leonhardt, H., ... Gerlich, D. W. (2011). Cortical constriction during abscission involves
helices of ESCRT-III-dependent filaments. Science, 331(6024), 1616–1620. https://doi.org/10.1126/science.1201847

Hansen-Wester, I., Stecher, B., & Hensel, M. (2002). Type III secretion of Salmonella enterica serovar Typhimurium translocated effectors and SseFG. Infection and Immunity, 70(3), 1403–1409.

Haraga, A., Ohlson, M. B., & Miller, S. I. (2008). Salmonella interplay with host cells. Nature Reviews Microbiology, 6(1), 53–66. https://doi.org/10.1038/nrmicro1788

Hensel, M., Shea, J. E., Waterman, S. R., Mundy, R., Nikolaus, T., Banks, G., ... Holden, D. W. (1998). Genes encoding putative effector proteins of the type III secretion system of Salmonella pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Molecular Microbiology, 30(1), 163–174.

Horii, M., Shibata, H., Kobayashi, R., Katoh, K., Yorikawa, C., Yasuda, J., ... Kerr, M. C., Wang, J. T., Castro, N. A., Hamilton, N. A., Town, L., Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Leone, P., & Meresse, S. (2011). Kinesin regulation by kinesin. EMBO Journal, 30(3), 639–647. https://doi.org/10.1038/ncomms12441

Liss, V., Swart, A. L., Kehl, A., Hermanns, N., Zhang, Y., Chikkaballari, D., ... Hensel, M. (2017). Salmonella enterica remodels the host cell endosomal system for efficient intravacuolar nutrition. Cell Host & Microbe, 21(3), 390–402. https://doi.org/10.1016/j.chom.2017.02.005

Lopez-Jimenez, A. T., Cardenal-Munoz, E., Leuba, F., Gerstenmaier, L., Barisch, C., Hagedorn, M., ... Soldati, T. (2018). The ESCRT and autophagy machineries cooperate to repair ESX-1-dependent damage at the Mycobacterium-containing vacuole but have opposite impact on containing the infection. PLoS Pathogens, 14(12), e1007501. https://doi.org/10.1371/journal.ppat.1007501

Lyons, A. B., & Parish, C. R. (1994). Determination of lymphocyte division by flow cytometry. Journal of Immunological Methods, 171(1), 131–137.

McCullough, J., Colf, L. A., & Sundquist, W. I. (2013). Membrane fission reactions of the mammalian ESCRT pathway. Annual Review of Biochemistry, 82, 663–692. https://doi.org/10.1146/annurev-biochem-072909-101058

McEwan, D. G., Popovic, D., Gubas, A., Terawaki, S., Suzuki, H., Stadel, D., ... Dikic, I. (2015). PLEKHM1 regulates autophagosome-lysosome fusion through HOPS complex and LC3/GABARAP proteins. Molecular Cell, 57(1), 39–54. https://doi.org/10.1016/j.molcel.2014.11.006

McEwan, D. G., Richter, B., Claudi, B., Wigge, C., Wild, P., Farhan, H., ... Dikic, I. (2015). PLEKHM1 regulates Salmonella-containing vacuole biogenesis and infection. Cell Host & Microbe, 17(1), 58–71. https://doi.org/10.1016/j.chom.2014.11.011

Mehra, A., Zahra, A., Thompson, V., Sirsaengtaksin, N., Wells, A., Porto, M., ... Phillips, J. A. (2013). Mycobacterium tuberculosis type VII secreted effector EsxH targets host ESCRT to impair trafficking. PLoS Pathogens, 9(10), e1003734. https://doi.org/10.1371/journal.ppat.1003734

Mittal, E., Skowyra, M. L., Uwase, G., Tinaztepe, E., Mehra, A., Koster, S., ... Phillips, J. A. (2018). Mycobacterium tuberculosis type VII secretion system effectors differentially impact the ESCRT endomembrane damage response. MBio, 9(6). https://doi.org/10.1128/mbio.01765-18

Müller, P., Chikkaballari, D., & Hensel, M. (2012). Functional dissection of SseF, a membrane-integral effector protein of intracellular Salmonella enterica. PLoS One, 7(4), e35004. https://doi.org/10.1371/journal.pone.0035004

Nikolaus, T., Deivick, J., Rapp, C., Freeman, J. A., Schroder, W., Miller, S. L., & Hensel, M. (2001). See BCD proteins are secreted by the type III secretion system of Salmonella pathogenicity island 2 and function as a translocon. Journal of Bacteriology, 183(20), 6036–6045. https://doi.org/10.1128/JB.183.20.6036-6045.2001

Noster, J., Chao, T. C., Sander, N., Schulte, M., Reuter, T., Hansmeier, N., & Hensel, M. (2019). Proteomics of intracellular Salmonella enterica reveals roles of Salmonella pathogenicity island 2 in metabolism and antioxidant defense. PLoS Pathogens, 15(4), e1007741. https://doi.org/10.1371/journal.ppat.1007741

Owen, K. A., & Casanova, J. E. (2015). Salmonella manipulates autophagy to “Serve and Protect.” Cell Host & Microbe, 18(5), 517–519. https://doi.org/10.1016/j.chom.2015.10.015

Phillips, J. A., Porter, M. C., Wang, H., Rubin, E. J., & Perrimon, N. (2008). ESCRT factors restrict mycobacterial growth. Proceedings of the National Academy of Sciences of the United States of America, 105(8), 3070–3075. https://doi.org/10.1073/pnas.0707206105

Phillips, J. A., Rubin, E. J., & Perrimon, N. (2005). Drosophila RNAi screen reveals CD36 family member required for mycobacterial infection. Science, 309(5738), 1251–1253. https://doi.org/10.1126/science.1116006

Piper, R. C., & Katzmann, D. J. (2007). Biogenesis and function of multivesicular bodies. Annual Review of Cell and Developmental Biology, 23, 519–547. https://doi.org/10.1146/annurev.cellbio.23.090506.123319
Popp, J., Noster, J., Busch, K., Kehl, A., Zur Hellen, G., & Hensel, M. (2015). Role of host cell-derived amino acids in nutrition of intracellular Salmonella enterica. *Infection and Immunity*, 83(12), 4466–4475. https://doi.org/10.1128/IAI.00624-15

Radulovic, M., Schink, K. O., Wenzel, E. M., Nahse, V., Bongiovanni, A., Lafont, F., & Stemark, H. (2018). ESCRT-mediated lysosome repair precedes lysophagy and promotes cell survival. The EMBO Journal, 37(21). https://doi.org/10.15252/embj.201899753

Rajashekar, R., & Hensel, M. (2011). Dynamic modification of microtubule-dependent transport by effector proteins of intracellular Salmonella enterica. *European Journal of Cell Biology*, 90(11), 897–902. https://doi.org/10.1016/j.ejcb.2011.03.008

Rajashekar, R., Liebl, D., Seitz, A., & Hensel, M. (2008). Dynamic remodeling of the endosomal system during formation of Salmonella-induced filaments by intracellular Salmonella enterica. *Traffic*, 9(12), 2100–2116. doi:TRAS821 [pii]. https://doi.org/10.1111/j.1600-0854.2008.00821.x

Ran, F., Hsu, P. D., Wright, J., Agarwala, J., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*, 8(11), 2281–2308.

Reid, E., Connell, J., Edwards, T. L., Duley, S., Brown, S. E., & Sanderson, C. M. (2005). The hereditary spastic paraplegia protein spastin interacts with the ESCRT-III complex-associated endosomal protein CHMP1B. *Human Molecular Genetics*, 14(1), 19–38. https://doi.org/10.1093/hmg/ddi003

Röder, J., & Hensel, M. (2019). Presence of SopE and mode of infection result in increased SCV damage and cytosolic release during host cell infection by Salmonella enterica. *Cell Microbiol*, e13155. doi:https://doi.org/10.1111/cmi.13155

Ruhl, S., Shkarina, K., Demarco, B., Heilig, R., Santos, J. C., & Broz, P. (2018). ESCRT-dependent membrane repair negatively regulates pyropoiesis downstream of GSDMD activation. *Science*, 362(6417), 956–960. https://doi.org/10.1126/science.aar7607

Schoeder, N., Mota, L. J., & Meresse, S. (2011). Salmonella-induced tubular networks. *Trends in Microbiology*, 19(6), 268–277. https://doi.org/10.1016/j.tim.2011.01.006

Segura, I., Casadeus, J., & Ramos-Morales, F. (2004). Use of mixed infections to study cell invasion and intracellular proliferation of Salmonella enterica in eukaryotic cell cultures. *Journal of Microbiological Methods*, 56(1), 83–91.

Skowrya, M. L., Schlesinger, P. H., Naismith, T. V., & Hanson, P. I. (2018). Triggered recruitment of ESCRT machinery promotes endolysosomal repair. *Science*, 360(6384), eaar5078. https://doi.org/10.1126/science.aaar5078

Sorkin, A., & Goh, L. K. (2008). Endocytosis and intracellular trafficking of ErbBs. *Experimental Cell Research*, 314(17), 3093–3106. https://doi.org/10.1016/j.yexcr.2008.08.013

Stein, M. A., Leung, K. Y., Zwick, M., Garcia-del Portillo, F., & Finlay, B. B. (1996). Identification of a Salmonella virulence gene required for formation of filamentous structures containing lysosomal membrane glycoproteins within epithelial cells. *Molecular Microbiology*, 20(1), 151–164.

Town, L., McGlinn, E., Fiorenza, S., Metzis, V., Butterfield, N. C., Richman, J. M., & Wicking, C. (2009). The metalloendopeptidase gene Pitm1 is regulated by hedgehog signaling in the developing mouse limb and is expressed in muscle progenitors. *Developmental Dynamics*, 238(12), 3175–3184. https://doi.org/10.1002/dvdy.22126

Tuli, A., & Sharma, M. (2019). How to do business with lysosomes: Salmonella leads the way. *Current Opinion in Microbiology*, 47, 1–7. https://doi.org/10.1016/j.mib.2018.10.003

Valdivia, R. H., & Falkow, S. (1996). Bacterial genetics by flow cytometry: Rapid isolation of Salmonella Typhimurium acid-inducible promoters by differential fluorescence induction. *Molecular Microbiology*, 22(2), 367–378.

Vietri, M., Schink, K. O., Campsteijn, C., Wegner, C. S., Schultz, S. W., Christ, L., & Stemark, H. (2015). Spastin and ESCRT-III coordinate mitotic spindle disassembly and nuclear envelope sealing. *Nature*, 522(7555), 231–235. https://doi.org/10.1038/nature14408

Vorwerk, S., Krieger, V., Deiwick, J., Hensel, M., & Hansmeier, N. (2015). Proteomes of host cell membranes modified by intracellular activities of Salmonella enterica. *Molecular & Cellular Proteomics*, MCP, 14(1), 81–92. https://doi.org/10.1074/mcp.M114.041145

Vreeburg, R. A., Bastiaan-Net, S., & Mes, J. J. (2011). Normalization genes for quantitative RT-PCR in differentiated Caco-2 cells used for food exposure studies. *Food & Function*, 2(2), 124–129. https://doi.org/10.1039/c0fo00068j

Wang, X., & Seed, B. (2003). A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Research*, 31(24), e154.

Wang, X., Spandidos, A., Wang, H., & Seed, B. (2012). PrimerBank: A PCR primer database for quantitative gene expression analysis, 2012 update. *Nucleic Acids Research*, 40(Database issue), D1144–D1149. https://doi.org/10.1093/nar/gkr1013

Whitley, P., Reaves, B. J., Hashimoto, M., Riley, A. M., Potter, B. V., & Holman, G. D. (2003). Identification of mammalian Vps24p as an effector of phosphatidylinositol 3,5-bisphosphate-dependent endosome compartmentalization. *The Journal of Biological Chemistry*, 278(40), 38786–38795. https://doi.org/10.1074/jbc.M300684200

Wollert, T., Wunder, C., Lippincott-Schwartz, J., & Hurley, J. H. (2009). Membrane scission by the ESCRT-III complex. *Nature*, 458(7235), 172–177. https://doi.org/10.1038/nature07836

Yang, D., Rismachi, N., Renvoise, B., Lippincott-Schwartz, J., Blackstone, C., & Hurley, J. H. (2008). Structural basis for midbody targeting of spastin by the ESCRT-III protein CHMP1B. *Nature Structural & Molecular Biology*, 15(12), 1278–1286. https://doi.org/10.1038/nsmb.1512

Zhao, W., Moest, T., Zhao, Y., Guilhon, A. A., Buffat, C., Gorvel, J. P., & Meresse, S. (2015). The Salmonella effector protein SifA plays a dual role in virulence. *Scientific Reports*, 5, 12979. https://doi.org/10.1038/srep12979

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Göser V, Kehl A, Röder J, Hensel M. Role of the ESCRT-III complex in controlling integrity of the Salmonella-containing vacuole. *Cellular Microbiology*. 2020;22: e13176. https://doi.org/10.1111/cmi.13176