The chlamydial deubiquitinase Cdu1 supports recruitment of Golgi vesicles to the inclusion

Daniela Auer1 | Sophie D. Hügelschäffer1 | Annette B. Fischer1 | Thomas Rudel1,2

1Department of Microbiology, University of Wuerzburg Biocenter, Wuerzburg, Germany
2Helmholtz Institute for RNA-based Infection Research (HIRI), Würzburg, Germany

Abstract

Chlamydia trachomatis is the main cause of sexually transmitted diseases worldwide. As obligate intracellular bacteria Chlamydia replicate in a membrane bound vacuole called inclusion and acquire nutrients for growth and replication from their host cells. However, like all intracellular bacteria, Chlamydia have to prevent eradication by the host’s cell autonomous system. The chlamydial deubiquitinase Cdu1 is secreted into the inclusion membrane, facing the host cell cytosol where it deubiquitinates cellular proteins. Here we show that inactivation of Cdu1 causes a growth defect of C. trachomatis in primary cells. Moreover, ubiquitin and several autophagy receptors are recruited to the inclusion membrane of Cdu1-deficient Chlamydia. Interestingly, the growth defect of cdu1 mutants is not rescued when autophagy is prevented. We find reduced recruitment of Golgi vesicles to the inclusion of Cdu1 mutants indicating that vesicular trafficking is altered in bacteria without active deubiquitinase (DUB). Our work elucidates an important role of Cdu1 in the functional preservation of the chlamydial inclusion surface.

KEYWORDS

autophagy, Cdu1, ChlaDUB1, Chlamydia trachomatis, DUB, Golgi, xenophagy

1 INTRODUCTION

Chlamydia trachomatis is an obligate intracellular human pathogen and the most common transmitted sexually disease worldwide (Newman et al., 2015). Infection with the pathogen can manifest in a spectrum of diseases like ectopic pregnancy, infertility but also in the blinding eye disease trachoma (Bartlett, Levison, & Munday, 2013; Bébéar & de Barbeyrac, 2009; Wright, Turner, & Taylor, 2008). Chlamydia replicate in a biphasic developmental cycle. Chlamydial elementary bodies (EBs) infect epithelial cells via the endocytic pathway and transform into replicative reticulate bodies (RBs) within a vacuole, the chlamydial inclusion (Engel, 2004). After several rounds of replication inside the inclusion, RBs complete their life cycle and transform back into infectious EBs which are released from the host cell via extrusion or cell lysis (Hybiske & Stephens, 2007). Chlamydia secrete several effectors into the host cell by the type III secretion system (Subtil et al., 2005; Valdivia, 2008) to orchestrate their developmental cycle, receive host cell metabolites and prevent damage or premature death of their host cell. The natural ribbon-like Golgi architecture is destroyed in cells infected with C. trachomatis and vesicular mini stacks originating from fragmented Golgi closely associate with the chlamydial inclusion (Heuer et al., 2009). Host cell sphingolipids are required for the intracellular growth of C. trachomatis in epithelial cells, with sphingomyelin attained via the intersection of the chlamydial inclusion with these Golgi-derived vesicles (Hackstadt, Rockey, Heinzen, & Scidmore, 1996).

As all pathogenic bacteria, Chlamydia has to subvert the host’s immune system to establish a successful infection and both, the adaptive and the innate immune response are effective to control chlamydial infections (Fischer & Rudel, 2016). The cell autonomous immune defence present in all human cells is particularly relevant for an obligate
intracellular pathogen like Chlamydia (Fischer & Rudel, 2016). A very efficient arm of cell autonomous defence is the removal of pathogens via autophagy. Foreign particles like intracellular bacteria in the cytosol or in vacuoles are specifically marked by ubiquitination. In eukaryotic cells poly-ubiquitination of proteins via lysine 63 (K63) is mainly involved in autophagic events like mitophagy or the removal of Salmonella from the cytoplasmia via xenophagy, a form of autophagy specifically targeting bacteria (Geisler et al., 2010; Van Wijk et al., 2012).

K48 poly-ubiquitination of proteins often serves as a signal for proteasomal degradation (Hershko & Ciechanover, 1998; Vucic, Dixit, & Wertz, 2011). Ubiquitination of any kind happens via a cascade of several proteins (Kleiger & Mayor, 2014). This posttranslational modification is tightly regulated but also reversible by removal of the ubiquitin by deubiquitinating enzymes (DUBs).

Ubiquitination of the bacterial membrane or the surrounding vacuole membrane then recruits autophagy adapter proteins to the ubiquitin molecules (Kirkin, Lamark, Johansen, & Dikic, 2009; Svenning & Johansen, 2013). As a consequence, ubiquitin bound adapters recruit autophagy-related gene 8 (ATG8) family proteins like the light chain 3 (LC3) protein which mediate the formation of the autophagosome around the intruder. Finally, autophagosomes fuse with lysosomes resulting in the destruction of the engulfed bacteria in the formed autolysosomes (Dikic, 2017).

However, bacteria have evolved strategies to interfere with the autophagy system in order to stay alive and proliferate. In Salmonella infection one third of all bacteria are targeted for lysosomal degradation through xenophagy (Birmingham, Smith, Bakowski, Yoshimori, & Brumell, 2006). This affects free Salmonella in the host cytosol as well as bacteria engulfed in the Salmonella containing vacuole (SCV). The SCV membrane often gets punctured which leads to the recruitment of the autophagy system (Thurston, Wandel, von Muhlinen, Foeglein, & Raniewicz, 2011). In order to prevent clearance Salmonella has evolved mechanisms to repair the damaged SCV (Kreibich et al., 2015). Moreover, Salmonella secretes SopA into the cytosol, a HECT E3 ligase with yet unknown targets (Zhang, Higashide, McCormick, Chen, & Zhou, 2006). Also Legionella, E. coli and Shigella are known to secrete effector proteins into the host cell that interfere with the ubiquitination system, often by mimicking E3 ligases (Huibregtse & Rohde, 2014). Chlamydia has also established mechanisms to evade autophagosomal degradation. Early studies revealed that C. trachomatis inclusion formation at the chlamydial inclusion. Despite phagophore formation at the cdu1 mutant inclusion, autophagy was not effective in interfering with chlamydial development. The lack of active Cdu1 prevents Golgi vesicle recruitment and slows the transition of RB to EB resulting in decreasing progeny.

2 | RESULTS

2.1 | Chlamydia require Cdu1 for growth in primary cells

We previously demonstrated that a transposon insertion mutant of cdu1 (Ctr Tn-cdu1) expressing truncated Cdu1 protein is attenuated in a mouse infection model and in cell lines challenged with interferon-γ (IFN-γ) (Fischer et al., 2017). In the absence of IFN-γ Ctr Tn-cdu1 grew without significant difference in several tumour cell lines (Fischer et al., 2017). Tumour cell lines are known to have altered cell autonomous defence including autophagy (Kimmelman, 2011). We therefore asked whether primary cells permit the development of Ctr Tn-cdu1 like previously observed for tumour cells. Primary human fallopian tube cells (Fimb) and human umbilical vein endothelial cells (HUVEC) were infected with wild-type and mutant bacteria and the relative infectivity was determined by transferring bacteria after 48 hr from these primary infections to fresh cells (here referred to as infectivity assay). Fimb and HUVEC infected with Ctr Tn-cdu1 produced less infectious chlamydial particles compared to wild-type infected cells (Figure 1a). In contrast, no deficiency in growth was detected in tumour cell lines like HeLa under these conditions. A detailed analysis of later infection time points revealed a delayed development of Ctr Tn-cdu1 compared to wild-type bacteria (Figure 1b). Moreover, electron microscopy (EM) pictures revealed that Ctr Tn-cdu1 inclusions in Fimb cells harbour less chlamydial particles 30 hr post infection than wild-type Chlamydia (Figure 1c, d). The ratio of EBs in Ctr Tn-cdu1 infected cell was reduced compared to wild-type Chlamydia (Figure 1d). Therefore, the transition of RB to EB seems to be stalled in the mutant infection. The differentiation of RB to EB is not completely understood, nevertheless, the size of RBs is discussed to be important in this process (Lee et al., 2018). We measured the average size of RBs in chlamydial inclusions after 30 hr in Fimb cells and found that RBs of wild-type Chlamydia were significantly smaller
than RBs of the bacteria lacking an active Cdu1 deubiquitinase (0.8236 μm ± 0.065) (Figure 1e). Thus, *Chlamydia* lacking active Cdu1 are developing slower, resulting in less infectious EBs compared to the wild-type bacteria.

**2.2 Inclusions of Ctr Tn-cdu1 are ubiquitinated**

Recently we showed that the deubiquitinase Cdu1 is secreted into the inclusion membrane with the active site located on the host cell.
CTr Tn-cdu1 inclusion is ubiquitinated. HeLa (a) or Fimb cells (b) were infected with CTr WT or CTr Tn-cdu1 for 24 hr at a MOI of 1 or 0.3 respectively. Cells were fixed with 4% paraformaldehyde (PFA)/sucrose followed by indirect immunofluorescence. Chlamydial HSP60 (pink) as well as K48-linked ubiquitin (green, left panel) or K63-linked ubiquitin (green, right panel) was detected. Scale bar, 10 μm. (c) HeLa Cdu1-Flag cells were infected with CTr WT or CTr Tn-cdu1 at a MOI of 1 for 24 hr with/without addition of AHT (50 ng/ml) for 4 hr. Cells were fixed with 4% PFA/sucrose and indirect immunofluorescence was performed with ubiquitin (green) and Cdu1 (pink). The asterisk shows the chlamydial inclusion. Scale bar, 10 μm. (d) Quantification of (c) showing ubiquitination of CTr WT or CTr Tn-cdu1 inclusions in HeLa Cdu1-Flag and HeLa Cdu1(C345A)-Flag cells upon AHT treatment. Pictures shown in (c) from three independent experiments were used to count 100 inclusions per replicate with and without ubiquitin signal around the chlamydial inclusion. ND, not detected. (e) HeLa cells were infected with CTr WT and CTr Tn-cdu1 at a MOI of 1 for 24 hr before cells were fixed using 4% PFA/sucrose. Indirect immunofluorescence was performed against Cdu1 (green), ubiquitin (pink) and 4’,6-Diamidino-2-phenylindol (DAPI) for DNA (blue). Cell contours are marked in white. Scale bar, 10 μm.
FIGURE 3  Ubiquitinated inclusions of Ctr Tn-cdu1 are recognised by the host cell autophagy system. (a) HeLa cells were infected with Ctr WT or Ctr Tn-cdu1 at a MOI of 1 for 24 hr. Cells were fixed with 4% PFA/sucrose and indirect immunofluorescence microscopy was performed with K63 ubiquitin (green), p62/SQSTM1 (red) and DAPI for DNA. Scale bar, 10 μm. (b) HeLa cells were infected with the chlamydial strains Ctr WT and Ctr Tn-cdu1 at a MOI of 1 for 24 hr followed by fixation with 4% PFA/sucrose. For indirect immunofluorescence microscopy cells were stained for cHSP60 (green) and light chain 3 (LC3) (pink). Scale bar, 10 μm. (c) Immunofluorescence images of Ctr WT and Ctr Tn-cdu1 infected HeLa cells were analysed by counting inclusions positive for p62 or LC3 recruitment. 34 pictures of three independent experiments were counted. Significance was calculated by Student’s t test. * p < .05, ** p < .001. (d) HeLa cells were infected with Ctr WT or Ctr Tn-cdu1 at a MOI of 1 for the indicated time points. Cells were then lysed in SDS-sample buffer and expression of p62, LC3, chHSP60 and beta-Actin was detected by immunoblot. (e) The levels of the indicated proteins from the immunoblots shown in panel (d) were quantified for the 24 hpi time point. Shown are the mean from three independent experiments ±SD. Significance was tested using the Student’s t test. * p ≤ .05, ** p ≤ .01. (f) Fimb cells infected with Ctr WT or Ctr Tn-cdu1 at a MOI of 0.3 for 30 hr were fixed for EM preparation. Representative images are shown. The white arrow appoints to a double membrane forming around the chlamydial inclusion. Scale bar, 1 μm
FIGURE 4  Growth and replication defect of Ctr Tn-cdu1 in primary cells is independent of autophagy. (a) HeLa cells were infected with Ctr WT or Ctr Tn-cdu1 at a MOI of 1 for 24 hr before cells were fixed with 4% PFA/sucrose. Cells were stained for Lamp1 (green) and cHSP60 (pink). Scale bar, 10 μm. (b) Primary Fimb cells were infected with Ctr WT or Ctr Tn-cdu1 at a MOI of 0.3 for 24 hr and treated with or without 10 nM Bafilomycin A1 for 7 hr. Cells were lysed in SDS-sample buffer and immunoblot was performed. (c) Relative growth of Ctr WT and Ctr Tn-cdu1 after 24 hr in Fimb cells treated with or without Bafilomycin A1 for 7 hr. Bacterial load was determined by quantifying chlamydial OmpA levels and normalisation to GAPDH (b). Significance was calculated using the Student’s t test. * p < .05. (d) Fimb cells were treated with a control siRNA (siControl) or sip62 for 24 hr before infection with Ctr WT or Ctr Tn-cdu1 at a MOI of 0.3. Lysates were taken 24 hpi and analysed by immunoblotting for p62, OmpA and actin. (e) Infectivity assay in PentaKO and Parental cells was performed. Cells were infected with Ctr WT and Ctr Tn-cdu1 with a MOI of 1 and lysates from these primary infections were taken 24 hpi (Primary). For progeny infection cells were lysed 48 hpi and fresh HeLa cells were infected with the infections supernatant. Lysates for progeny infection were taken 24 hpi. Both, primary and progeny infection were analysed for chlamydial OmpA and cHSP60. (f) Quantification of immunoblots showing relative bacterial growth in PentaKO and Parental cells. Bacterial load was determined by quantifying chlamydial HSP60 (cHSP60) levels and normalisation to actin (e). Growth of wild-type Chlamydia was set to 100%. Results of three independent experiments are shown. Significance was calculated using the Student’s t test. * p < .05, ** p < .01.
proximity to the protease (Figure S3a). However, at sites of contact a deubiquitinate other inclusions in the same cell that are not in direct contact revealed that active Cdu1 in the inclusion membrane cannot remove ubiquitin from chlamydial inclusions. We then asked if the Cdu1 deubiquitinase activity is required for the removal of ubiquitin from the cytoplasm and the removal of ubiquitin from the inclusion surface (Figure S3c) demonstrating that wild-type Chlamydia can complement the deubiquitination defect of Ctr Tn-cdu1 from inside the inclusion. We conclude from these results that the chlamydial inclusion is constantly ubiquitinated in the absence of Cdu1 and the high deubiquitinating activity of Cdu1 removes ubiquitin from the surface of the inclusion.

2.3 The autophagy machinery is recruited to the ubiquitinated inclusion

Ubiquitination via lysine 48 is often a signal for protein degradation by the proteasome, whereas a linkage via K63 preferably induces autophagy (Hershko & Ciechanover, 1998; Olzmann & Chin, 2008; Vucic et al., 2011). Autophagy adapters like sequestosome1 (hereafter referred to as p62) bind to ubiquitin K63 and subsequently recruit autophagy initiation proteins like LC3. Compared to inclusions of wild-type bacteria, inclusions of Ctr Tn-cdu1 contained high amounts of p62 visible as surface-associated ring structures on these inclusions (Figure 3a, c). Furthermore, p62 protein levels were upregulated in cells infected with Ctr Tn-cdu1 starting around mid-phase of the developmental cycle (Figure 3d, e) and accumulation of all LC3 variants (LC3A, LC3I and lipidated and active LC3II increased during the mid-stage of the cycle (Figure 3d, e) and accumulation of all LC3 variants (LC3A,
LC3B, LC3C) (Figure S4 i–k) as well as the ATG8 family members GABARAP, GABARAPL1 and GABARAPL2 (Figure S4 f–h) were detected around the inclusion (Figure 3c, Figure S4 f–k) which indicates the formation of an autophagophore that was observed for the mutant but not the wild-type Chlamydia (Figure 3b, f). In addition the lipidation of LC3I to form lipidated LC3II initiating autophagophore formation depends on a multi-protein complex which also includes ATG12. ATG12 was also identified at the inclusion of Ctr Tn-cdu1 (Figure S4e). These experiments confirmed the recruitment of autophagy receptors to and the formation of a LC3 positive double membrane around the inclusion of Ctr Tn-cdu1 during infection.

2.4 The growth defect of Ctr Tn-cdu1 in primary cells is independent of autophagy

Phagophore formation and recruitment of the autophagy machinery to bacteria containing vacuoles are clear indications of active xenophagy. We therefore tested whether autophagosomes forming around the Ctr Tn-cdu1 inclusion fuse with lysosomes. However, co-localization with the lysosomal marker protein Lamp1 could not be observed (Figure 4a). As the autophagy system is recruited to the inclusion we tested whether autophagy actively degrades the bacteria which would explain the observed growth defect of the mutant in primary cells. The lysosomal inhibitor Bafilomycin A1 interferes with the degradation of bacteria by inhibition of the H+-ATPase and prevention of the acidification of the lysosomal lumen. To determine the bacterial load upon inhibitor treatment, levels of bacterial OmpA or HSP60 protein were detected by immunoblotting. These assays revealed that Bafilomycin A1 treatment could not rescue the growth defect in Ctr Tn-cdu1 (Figure 4b, c). To further corroborate that reduced infectivity of the mutant depends on autophagy we also targeted the early stages of autophagy. Knock down of the key autophagy marker p62 in primary Fimb cells did not rescue the growth defect of Ctr Tn-cdu1 (Figure 4d). Several different autophagy receptors in addition to p62 were identified at the inclusion of Ctr Tn-cdu1 that could compensate
for reduced p62 levels. Therefore, a PentaKO cell line with deletions in the autophagy markers p62, NBR1, NDP52, Optineurin and TAX1BP1 was used to test the involvement of any known autophagy marker (Lazarou et al., 2015). The PentaKO cell line as well as the parental strain are also negative for Parkin E3 ligase. Knock out of all these autophagy genes did not rescue the growth of Ctr Tn-cdu1 in primary or progeny infection (Figure 4e, f). In line with previous results we therefore claim that the growth defect of Ctr Tn-cdu1 is not due to the autophagy and degradation by the lysosome but rather a slowed replication and transition of chlamydial RBs to EBs.

2.5 | Altered Golgi fragmentation of Ctr Tn-cdu1 can be rescued

Reasons for impaired development in Chlamydia can be various. As obligate intracellular bacteria Chlamydia are known to consume metabolites and nutrients from the host cell. In wild-type Chlamydia infected cells the Golgi is fragmented and recruited to the inclusion surface for enhanced lipid acquisition by the bacteria (Heuer et al., 2009). In Ctr Tn-cdu1 infected HeLa cells we find the Golgi also fragmented, however, recruitment to the chlamydial inclusion is impaired and the fragmented Golgi vesicle appeared dispersed around the inclusion (Figure 5a, b). To demonstrate that this reduced Golgi vesicle recruitment is due to a defect in Cdu1 activity in cells infected with mutant Chlamydia we expressed active Cdu1 in the cytoplasm of the cell. Cells complemented with Cdu1 Golgi stacks were recruited to the Ctr Tn-cdu1 inclusion as in wild-type infected cells (Figure 6a, b). Golgi fragmentation was hardly visible in infected Fimb cells (Figure 5S) suggesting that defects in Golgi fragmentation do not account for the growth difference of wild-type and mutant Chlamydia in these cells. Furthermore, acquisition of ceramide appeared neither affected in HeLa nor in Fimb cells (Figure 6S), ruling out a defect in ceramide uptake as a reason for the developmental defect of Ctr Tn-cdu1. All in all our data suggest that Cdu1 is required for the efficient recruitment of Golgi vesicles to the inclusion surface of Chlamydia, an effect that appears to be host cell dependent and independent of ceramide uptake into the inclusion. Although Chlamydia does not reside in the cytosol of host cells for extended times, a leaky or perforated inclusion membrane during late stage infections or as a consequence of cell autonomous immune attack may offer targets for activating autophagy (Fischer & Rudel, 2018; Weber et al., 2017a). In Ctr Tn-cdu1, but not in wild-type infected cells we find the inclusion decorated with ubiquitin and recruitment of autophagy proteins already during the replicative phase of the bacteria (24 hpi) when the inclusion is still intact (data not shown). We therefore postulate that the chlamydial inclusion is constantly targeted by the autophagy machinery but the chlamydial deubiquitinase Cdu1 efficiently interferes with this process at the initial steps by removing ubiquitin from the inclusion surface. This role of Cdu1 is supported by the observation that substitution of active Cdu1 in the cell cytoplasm removes the ubiquitin moieties from the inclusion membrane, emphasising the importance of the active DUB for keeping the inclusion free of ubiquitin (Figure 2c, d). In infected cells, this process is restricted to the inclusion surface where the deubiquitinase is anchored, since wild-type Cdu1 is not able to deubiquitinate neighbouring mutant inclusions; the involvement of soluble cytosolic factors can be excluded.

In contrast to our observations, LC3 has been shown to be recruited to C. trachomatis inclusions in other studies (Al-Younes et al., 2004; Al-Younes et al., 2011). These authors used a polyclonal goat antiserum (which is no longer available) which was directed against the unmodified LC3 form. It is therefore possible that the modified active LC3 preferably detected by the antibody used in this study is recruited only to the ubiquitinated inclusion. Despite the strong ubiquitination and the recruitment of autophagy receptors and effectors for phagophore formation to the inclusion in mutant infected cells, autophagy appears not to be a major mechanism by which host cells restrict chlamydial infection. Interestingly, p62 and LC3 are increased during the mid-phase of infection, when Cdu1 protein levels are also upregulated (Fischer et al., 2017). Since we neither find ubiquitination nor the recruitment of autophagy marker during the early phase of infection before Cdu1 is expressed, the inclusion may either not be detected or detection by the autophagic system may be actively prevented independent of Cdu1. Moreover, inhibition of autophagy neither in early phases by knock down of autophagy receptors (Figure 4d, e, f) nor in late phases by Bafilomycin A1 inhibitor treatment (Figure 4b, c) could rescue the growth defect of Ctr Tn-cdu1. Also inclusions of mutant Chlamydia in HeLa cells that do not restrict growth and progeny infection are also decorated with autophagy markers (Figures 2a and 3a, Figure S4a, b). Finally, no fusion with lysosomes was detected indicating that Ctr Tn-cdu1 is not degraded in autolysosomes (Figure 4a). These findings therefore strongly suggest that the growth defect of Ctr Tn-cdu1 is not due to clearance of infection by autophagy.

Our electron microscopic analyses revealed a reduced number of EBs in Ctr Tn-cdu1 compared to wild-type bacteria (Figure 1c, d) in line with impaired progeny infection of the mutant. We therefore postulate that redifferentiation of RBs to EBs is affected in Ctr Tn-cdu1. Recently redifferentiation has been correlated to the RB size which is reduced in every replication cycle until a certain threshold is reached and EBs are formed (Lee et al., 2018). Analysis of bacterial RB size
demonstrated that mutant bacteria are larger (Figure 1e) consistent with a disturbed differentiation to EBs. Although the complete mechanism of RB to EB transition is still unknown, the observed phenotype could be explained by slowed growth and replication of Ctr Tn-cdu1. Since bacteria depend on host metabolites for growth that they also take from organelles like the Golgi apparatus, the structure of the Golgi was further analysed. Indeed, Golgi membranes were less recruited to the Ctr Tn-cdu1 than the wild-type inclusions (Figure 5a, b). Reduced availability of Golgi membranes at the chlamydial inclusion is known to limit chlamydial growth (Heuer et al., 2009; Lipinski et al., 2009). However, we could not observe a major difference in the uptake of ceramide into their inclusions. Recently, Ctr Tn-cdu1 was suggested to avoid Golgi fragmentation (Pruneda et al., 2018). In contrast to their data we did see Golgi fragmentation in the cytoplasm of Ctr Tn-cdu1 infected cells but reduced association of Golgi vesicles with the inclusion. Recruitment of Golgi vesicles to the inclusion could be rescued by expression of active Cdu1 (Figure 6a, b) consistent with a central role of Cdu1 in this process. Although our data argue against a central role of Golgi fragmentation or Golgi vesicle recruitment to the inclusion as major reason for the growth defect of the Cdu1 mutant, Cdu1 appears to be a key factor required to keep the inclusion surface susceptible for interaction with host vesicular trafficking and possibly nutrient acquisition.

4 | EXPERIMENTAL PROCEDURES

4.1 | Cell lines and cultivation

HeLa 229 cells (ATCC Cat# CCL-2.1; RRID:CVCL_1276; tested negative for mycoplasma contamination by polymerase chain reaction (PCR)), primary Fimb cells (primary cells of human fimbriae obtained from biopsies of patients undergoing medically indicated surgery. According to the hospitalisation contract patients agreed to scientific use of biopsies after anonymization of samples; tested negative for mycoplasma contamination by PCR), U2OS cells expressing GFP (green fluorescent protein)-LC3 or GFP-GABARAP (kind gift of Alexandra Stolz) and PentaKO or Parental cells (kind gift of Michael Lazarou) were cultured in RPMI (Roswell Park Memorial Institute)-1640 medium (Gibco, Waltham, MA) supplemented with 10% fetal calf serum (FCS) (Paa, Germany). HUVEC (ATCC Cat# CRL-1730; tested negative for mycoplasma contamination by PCR) were grown in medium 200 supplemented with low serum growth supplement (Thermo Fischer, Waltham, MA). Cells were cultured by 37°C and 5% CO2. HeLa cells were seeded in 12-well plates and incubated over night at 37°C and 5% CO2. For transfection siRNA was diluted in 50 μl OptiMEM to a concentration of 300 nM and incubated for 5 min. From the transfection reagent Lipofectamine RNAiMAX 0.8 μl per well was mixed with 50 μl OptiMEM and incubated at room temperature for 5 min. The siRNA mixture and the transfection reagent was mixed and incubated at room temperature for another 20 min. Supernatant was removed from the cells and overlayed with 5% FCS RPMI 1640 medium. The siRNA Lipofectamine mixture was added to the cells to a final concentration of siRNA of 30 nM. After 7 hr the medium was exchanged with fresh 20% FCS RPMI 1640 medium. Two days later the transfected cells were infected with Ctr Tn-cdu1 or Ctr WT with an MOI of 0.3.

4.2 | siRNA knock-down in Fimb cells

For the siRNA knock-down Fimb cells were seeded in 12-well plates and incubated over night at 37°C and 5% CO2. For transfection siRNA was diluted to 50 μl OptiMEM to a concentration of 300 nM and incubated for 5 min. From the transfection reagent Lipofectamine RNAiMAX 0.8 μl per well was mixed with 50 μl OptiMEM and incubated at room temperature for 5 min. The siRNA mixture and the transfection reagent was mixed and incubated at room temperature for another 20 min. Supernatant was removed from the cells and overlayed with 5% FCS RPMI 1640 medium. The siRNA Lipofectamine mixture was added to the cells to a final concentration of siRNA of 30 nM. After 7 hr the medium was exchanged with fresh 20% FCS RPMI 1640 medium. Two days later the transfected cells were infected with Ctr Tn-cdu1 or Ctr WT with an MOI of 0.3.

4.3 | Cultivation of Ctr and inhibitor treatment

For chlamydial infection cells were grown until a confluency of 70% was reached. Medium was exchanged for RPMI-1640 containing 5% FCS and chlamydial particles resembling a MOI of 1. Cells were incubated at 35°C and 5% CO2 until further needed. For inhibitor treatment 10 nM Bafilomycin A1 (Thermo Fischer) or 2 μM MLN4924 was added to the infected cells 17 or 22 hpi respectively. For stock preparation cells were scraped and lysed with glass beads 48 hpi. Chlamydia were separated by centrifugation at 1000g for 10 min and pelleted at 30,000g for 30 min both at 4°C. After washing the bacteria with 1× sucrose-phosphate-glutamic acid (SPG buffer (75 g/L sucrose, 0.52 g/L KH2PO4, 1.22 g/L Na2HPO4, 0.72 g/L L-glutamic acid, pH 7.4)) Chlamydia were re-suspended in 1× SPG, aliquoted and stored at −80°C until needed. Bacteria were tested for mycoplasma contamination by PCR and inclusion forming unit was determined.

4.4 | Infectivity assays

Fimb, HUVEC HeLa and PentaKO cells were seeded in two 12 well microplates and grown to a confluency of 70%. Cells were infected with Ctr WT or Ctr Tn-cdu1 with an MOI of 1 for HeLa and PentaKO cells and an MOI of 0.3 for primary cells as described before. Primary lysates were taken 24 hpi by adding 2× SDS lysis buffer (100 mM Tris/HCl [pH 6.8], 20% glycerin, 4% SDS, 1.5% 2-mercaptoethanol, 0.2% bromphenolblue) to the cells. For progeny infection infected cells were lysed with glass beads 48 hpi and an aliquot of the supernatant was used to infect a fresh 12 well plate seeded with fresh cells. Lysates of progeny infection were taken 24 hpi as described. For analysis in immunoblot lysates were heated at 94°C for 5 min.

GTAACTCGTCTAGCCAGAAGAGCT and genomic DNA of Chlamydia trachomatis L2 strain 434/Bu as template. Gene expression was initiated by addition of 50 ng/ml AHT.
4.5 | **Indirect immunofluorescence**

Cells were grown on glass cover slides and infected as described above. For inducible cell lines 50 ng/ml AHT was added 4 hr before harvest. Cells were washed with phosphate-buffered saline (PBS) (Gibco) once and fixed using 4% PFA/sucrose. After permeabilisation with 0.2% TritonX 100 in PBS for 30 min, antigens were blocked with 2% FCS in PBS for 1 hr. Primary antibodies were diluted as follows in 2% FCS in PBS: anti-K48 (Merck #05-1,307, 1:500), anti-K63 (Merck #05-1,308, 1:100), anti-Ubi FK2 (Enzo #BML-PW8810-0500, 1:200), anti-chHSP60 (selfmade, 1:800), anti-Cdu1 (selfmade (Fischer et al., 2017), 1:300), anti-p62 (santa cruz #sc-28,359, 1:1000), anti-LC3B (cell signalling #2775, 1:50), anti-NBR1 (santa cruz #sc-130,380, 1:200), anti-NDPS2 (santa cruz #sc-376,540, 1:200), anti-ATG12 (cell signalling #2010, 1:200), anti-Ankib1 (Thermo Fischer #PA5-54506, 1:200), anti-GM130 (BD #610823, 1:400), anti-Lamp1 (santa cruz #sc-18,821, 1:200). After incubation with the primary antibody for 1 hr at room temperature, remaining antibody was washed away and the secondary Cy3 or Cy5 coupled antibody (Dianova) was diluted 1:300 in 2% FCS in PBS and incubated in the dark for 1 hr. Nucleic acid was stained with DAPI for 30 min.

4.6 | **Ceramide uptake assay by live cell imaging**

Cells were seeded in glass bottom dishes (ibidi, Gräfelfing, Germany) and infected with Ctr WT and Ctr Tn-cdu1 for 24 hr. 1.5 μM SIR-DNA dye (Spirochrome, Stein am Rhein, Switzerland) was added and incubated for 1 hr. Cells were washed three times with PBS, before live cell imaging media (RPMI 1640 (Gibco) + 25 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) (Gibco)) was added. Imaging was performed using a Leica TCS SP5 confocal microscope with a 63x oil immersion objective connected with a pre-warmed (37°C) incubation chamber. 0.5 μM nitrobenzoxadiazole (NBD)-C6-ceramide (Invitrogen, Waltham, Massachusetts, USA) was added to the cells and images were acquired every minute using the LAS software. Recorded pictures were processed with ImageJ.

4.7 | **Immunoblotting**

Lysates were loaded on a 10% SDS PAGE and transferred on a PVDF membrane (Roche, Basel, Switzerland) using the semidyblotter (Thermo Fischer). Membranes were blocked in 5% bovine serum albumin/tris-buffered saline (BSA/TBS) containing 0.05% Tween20 (TBS-T) or 5% milk/TBS-T for 1 hr and incubated with the primary antibody diluted in 5% BSA/TBS-T or 5% milk/TBS-T at 4°C overnight. Primary antibodies were diluted as follows: anti-LC3B (cell signalling #2775, 1:500), anti-chHSP60 (santa cruz #sc-57,840, 1:1000), anti-Actin (Sigma Aldrich #A5441, 1:1000), anti-p62 (santa cruz #sc-28,359, 1:1000), anti-OmpA (selfmade, 1:1000), anti-GAPDH (santa cruz, #sc-25,778, 1:1000). After incubation membranes were washed three times with TBS-T and incubated with the secondary antibody in 5% milk/TBS-T for 1 hr at room temperature. The anti-mouse-horseradish peroxidase (HRP) (santa cruz, #sc-516102) and anti-rabbit-HRP (Dianova, #111-035-144) secondary antibodies were diluted 1:3000. Blots were washed as described and analysed using ECL solutions and the Intas Chem HR 16–3200 reader. Blots were quantified using ImageJ software.

4.8 | **Transmission electron microscopy**

Fimb cells were seeded on glass cover slides and infected with Ctr Tn-cdu1 or Ctr WT with an MOI of 1. After 24 or 30 hr of infection slides were washed once with PBS and fixed using 2.5% glutaraldehyde in 0.05 M cacodylate buffer for 30 min at room temperature. Cells were washed in cacodylate buffer, incubated in 2% OsO4/0.05 M cacodylate buffer for 1 hr and in 0.5% uranylacetate overnight. Samples were dehydrated and embedded in EPON and cut. Images were taken with the JEOL JEM-2100 microscope. For analysis of RB diameter pictures were measured in ImageJ with five inclusions per sample.

4.9 | **Statistical analysis**

Statistical analysis was performed using Excel. Statistical significance between groups was determined using two-tailed Student’s t test. Data are shown as mean ± SE.

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**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest with the contents of this article.

**ORCID**

Thomas Rudel https://orcid.org/0000-0003-4740-6991

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