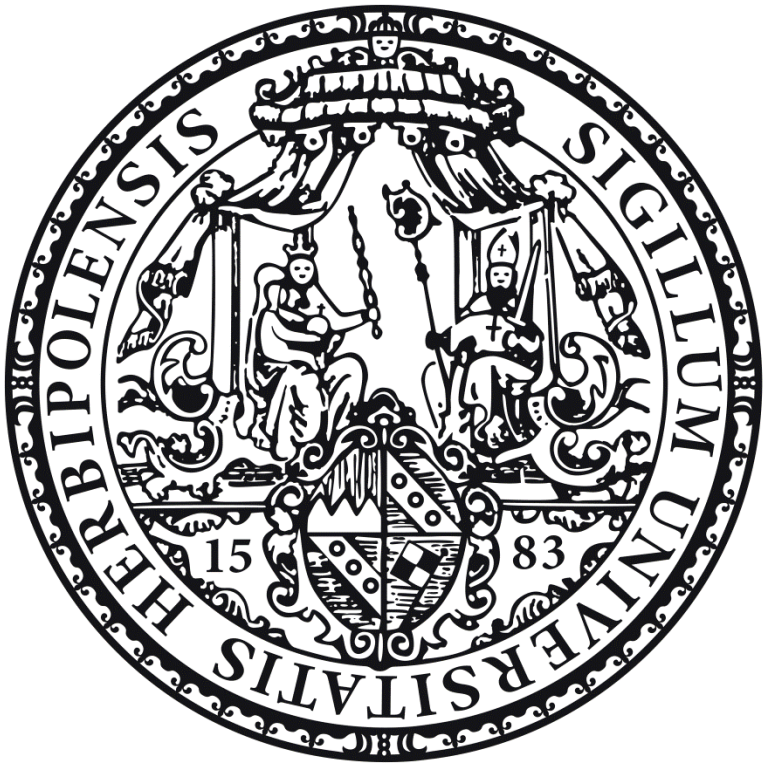
Impact of the chlamydial deubiquitinase *Chla*DUB1 on host cell defense



Dissertation zur Erlangung des

naturwissenschaftlichen Doktorgrades

der Julius-Maximilians-Universität Würzburg

Eingereicht an der Fakultät für Biologie

an der Julius-Maximilians-Universität Würzburg

Eingereicht von Daniela Auer
geboren in Bad Reichenhall

Würzburg, Januar 2019

Eingereicht am:

Mitglieder der Promotionskommission

Vorsitzender:

Erstgutachter: Prof. Dr. Thomas Rudel

Zweitgutachter: Prof. Dr. Alexander Buchberger

Tag des Promotionskolloquiums:

Doktorurkunde ausgehändigt am:

Content

Abstract

The human pathogen *Chlamydia trachomatis* is the main cause of sexually transmitted infections worldwide. The obligate intracellular bacteria are the causative agent of several diseases that reach from conjunctivitis causing trachoma and blindness as well as salpingitis and urethritis which can lead to infertility if left untreated.

In order to gain genetically engineered *Chlamydia* that inducible knock down specific gene expression, the CRISPRi system was established in *C. trachomatis*. In a proof of principle experiment it was shown that *C. trachomatis* pCRISPRi:gCdu1III target *Chla*DUB1 expression and reduce the protein amount up to 50 %. Knock-down of the DUB did not influence protein levels of anti-apoptotic Mcl-1 and did not make cells susceptible for apoptosis. However, reduced dCas9 protein size, bacterial growth impairment and off target effects interfering with the GFP signal, form obstacles in CRISPRi system in *Chlamydia*. For routinely use of the CRISPRi method in *C. trachomatis* further investigation is needed.

Since the bacterial life cycle includes two morphological and functional distinct forms, it is essential for chlamydial spread to complete the development cycle and form infectious progeny. Therefore, *Chlamydia* has evolved strategies to evade the host immune system in order to stay undetected throughout the developmental cycle. The bacteria prevent host cell apoptosis via stabilization of anti-apoptotic proteins like Mcl-1, Survivin and HIF-1α and activate pro-survival pathways, inhibiting invasion of immune cells to the site of infection. The host cell itself can destroy intruders via cell specific defense systems that involve autophagy and recruitment of professional immune cells. In this thesis the role of the chlamydial deubiuqitinase *Chla*DUB1 upon immune evasion was elucidated. With the mutant strain *Ctr* Tn-*cdu1* that encodes for a truncated DUB due to transposon insertion, it was possible to identify *Chla*DUB1 as a potent opponent of the autophagic system. Mutant inclusions were targeted by K48 and K63 chain ubiquitination. Subsequently the inclusion was recognized by autophagic receptors like p62, NBR1 and NDP52 that was reversed again by complementation with the active DUB. Xenophagy was promoted so far as LC3 positive phagosomes formed around the inclusion of *Ctr* Tn-*cdu1*, which did not fuse with the lysosome. The detected growth defect in human primary cells of *Chlamydia* missing the active DUB was not traced back to autophagy, but was due to impaired development and replication. It was possible to identify Ankib1, the E3 ligase, that ubiquitinates the chlamydial inclusion in a siRNA based screen. The activating enzyme Ube1 and the conjugating enzyme Ube2L3 are also essential in this process. *Chlamydia* have a reduced genome and depend on lipids and nutrients that are translocated from the host cell to the inclusion to proliferate. Recruitment of fragmented Golgi stacks to the inclusion surface was prevented when *Chla*DUB1 was inactive, probably causing diminished bacterial growth. Additionally, the modification of the inclusion by Ankib1 and subsequent decoration by autophagic markers was not only present in human but also murine cells. Comparison of other *Chlamydia* strains and species revealed Ankib1 to be located at the proximity of the inclusion in *C. trachomatis* strains only but not in *C. muridarum* or *C. pneumoniae*, indicating that Ankib1 is specifically the E3 ligase of *C. trachomatis*. Moreover, the role of *Chla*DUB1 in infected tissue was of interest, since *Chla*DUB1 protein was also found in early EB stage and so might get in contact with invading immune cells after cell lysis. While bacteria spread and infect new host cells, *Chlamydia* can also infect immune cells. Infection of human neutrophils with *Ctr* Tn-*cdu1* shows less bacterial survival and affirms the importance of the DUB for bacterial fitness in these cells.

Zusammenfassung

Chlamydia trachomatis ist weltweit der häufigste Auslöser von sexuell übertragenen Krankheiten. Das obligat intrazelluläre Bakterium manifestiert sich in diversen Krankheitsbildern, darunter Konjunktivitis, die zu einem Trachom oder sogar Erblindung führen kann und Salpingitis oder Urethritis, die unbehandelt unfruchtbar macht.

Das CRISPRi System wurde in *C. trachomatis* etabliert, um genetisch veränderte Bakterien zu bekommen, in denen induzierbar die spezifische Genexpression herunter gefahren werden kann. Es wurde gezeigt, dass in *C. trachomatis* pCRISPRi:gCdu1III, einem Stamm, der mit der Genexpression von *Chla*DUB1 interferiert, die Menge an *Chla*DUB1 um bis zu 50 % reduziert ist. Die Sensitivität für Apoptose durch sinkende Mcl-1 Proteinmengen wurde dadurch jedoch nicht wieder hergestellt. Das verkürzte dCas9 Protein, vermindertes bakterielles Wachstum, sowie Effekte auf andere Genexpressionen, wie z.B. das GFP Signal zeigen die Problematik des CRISPRi Systems in *C. trachomatis*. Um CRISPRi als Routinemethode für genetische Transformation in Chlamydien zu etablieren, stehen noch weitere Untersuchungen an.

Der Lebenszyklus von Chlamydien zeichnet sich durch zwei morphologisch und funktionell unterschiedliche Stadien aus, weshalb die Vollendung des Lebenszyklus und die Produktion infektiöser Partikel essenziell sind. Daher haben die Pathogene Strategien entwickelt, um dem Immunsystem des Wirts zu entgehen und sich unerkannt in der Zelle zu entwickeln. Die Bakterien verhindern Apoptose infizierter Zellen durch die Stabilisierung von anti-apoptotischen Proteinen wie Mcl-1, Survivin und HIF-1α und aktivieren Überlebens-Signalwege, die die Invasion von Immunzellen in das infizierte Gewebe unterdrücken. Die Wirtszelle selbst ist in der Lage bakterielle Eindringlinge durch die eigenen Abwehrmechanismen wie Autophagie und die Rekrutierung von professionellen Immunzellen zu zerstören. In dieser Arbeit wurde die Rolle der chlamydiellen Deubiquitinase *Chla*DUB1 auf die Vermeidungsstrategien vor dem Immunsystem untersucht. Mit Hilfe der Mutante *Ctr* Tn-*cdu1*, die durch Insertion eines Transposons für eine verkürzte und inaktive Deubiquitinase codiert, konnte gezeigt werden, dass *Chla*DUB1 ein Gegenspieler des Autophagiesystems ist. Die Inklusionen der Mutante wurden mit K48 und K63 Ubiquitinketten modifiziert, was die Rekrutierung von Autophagiemarkern wie p62, NBR1 und NDP52 zur Folge hatte. Die Rekomplementierung mit aktivem *Chla*DUB1 Protein hob die Modifikation der Inklusion wieder auf. Jedoch wurde die Xenophagie so weit vorangetrieben, bis sich LC3 positive Phagosomen um die Inklusionen von *Ctr* Tn-*cdu1* bildeten, die allerdings nicht mit dem Lysosom verschmolzen. Das beobachtete Wachstumsdefizit in Chlamydien, die keine funktionelle Deubiquitinase exprimieren, konnte nicht auf die Autophagie zurückgeführt werden, sondern war voraussichtlich aufgrund verlangsamter Entwicklung und Replikation entstanden.

In einem siRNA basierten Experiment konnte die E3 Ligase Ankib1 für die Ubiquitinierung der *Ctr* Tn-*cdu1* Inklusion identifiziert werden. Des Weiteren sind das Ubiquitin aktivierende Enzym Ube1 und das Ubiquitin konjugierende Enzym Ube2L3 essentiell für die Modifikation der Inklusion. Da Chlamydien ein reduziertes Genom haben und nicht für alle Enzyme selbst kodieren, sind sie auf Lipide und Metabolite der Wirtszelle für ihr Wachstum angewiesen. Die Rekrutierung der fragmentierten Glogi-Membranen zur Inklusionsoberfläche wurde durch inaktives *Chla*DUB1 Protein verhindert, das wahrscheinlich die bakterielle Entwicklung negativ beeinflusst. Des Weiteren ubiquitinierte Ankib1 nicht nur Inklusionen in humanen, sondern auch in murinen Zellen, was auch hier die Bindung von Autophagiemarkern zur Folge

hatte. Der Vergleich unter verschiedenen chlamydiellen Serotypen und Arten zeigte, dass Ankib1 nur an Inklusionen von *C. trachomatis* zu finden war, nicht aber für *C. muridarum* oder *C. pneumoniae*. Des Weitern wurde die Rolle von *Chla*DUB1 in infiziertem Gewebe genauer betrachtet, da die Protease auch während frühen EB Phasen nachgewiesen wurde, in denen sie Kontakt zu immigrierenden Immunzellen haben könnte. Während der Zelllyse werden Bakterien frei gesetzt, die neue Wirtszellen, aber auch Immunzellen, infizieren können. Die Infektion von humanen Neutrophilen mit *Ctr* Tn-*cdu1* zeigte vermindertes bakterielles Wachstum und verdeutlicht die Bedeutung von *Chla*DUB1 für das Überleben in diesen Immunzellen.

1 Introduction

1.1 Chlamydia

1.1.1 Chlamydial Taxonomy

Halberstädter and Prowazek published the first description of *Chlamydia* in 1907. The authors characterized *Chlamydozoa*, which is the Greek word for 'cloak' or 'mantle', as a virus-like particle forming small compartments in the cytosol of epithelial cells as the cause for trachoma (Halberstaedter and Von Prowazek, 1907). Further studies in the following decades revealed the possibility to inoculate the infectious agent in hens eggs (T'ang, et al., 1957). With the cultivation and upcoming molecular techniques it became clear that in fact gram-negative bacteria were the causative agent (Moulder, 1966).

Chlamydia evolved with humankind for many centuries and adapted to the host cells. Therefore, a variety of chlamydial species is known that specialized to vertebrate but also non-vertebrate hosts. To the first identified species *Chlamydia trachomatis* (*C. trachomatis*) and *Chlamydia psittaci* (*C. psittaci*) soon other species like *Chlamydia pneumoniae* (*C. pneumoniae*), the causative agent of pneumonia (Grayston, et al., 1986), and *Chlamydia pecorum* (*C. pecorum*) were added (Fukushi and Hirai, 1992). The newly found organisms *Simkania negevensis* (Kahane, et al., 1995) and *Parachlamydia acanthamoebae* (Amann, et al., 1997) showed similarities to the 16S and 23S ribosomal RNA of *Chlamydia* species and were added to the order of *Chlamydiales* (Figure 1.1) (Everett, et al., 1999).

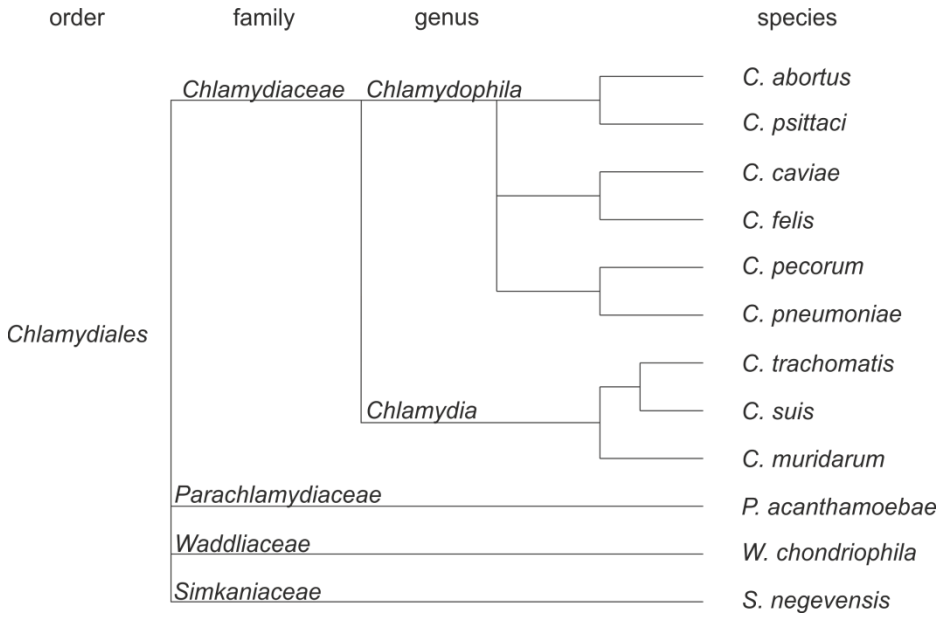


Figure 1.1. Taxonomy of the order of *Chlaymdiales* according to 16S rRNA genes. *Simkaniaceae*, *Waddliaceae* and *Parachlamydiaceae* are families in the order of *Chlamydiales* that harbor only one known species. *Chlamydiaceae* is the largest family with two genera *Chlamydophila* and *Chlamydia* and together nine species. Modified after Everett et al. (Everett, et al., 1999).

1.1.2 Clinical relevance of *C. trachomatis*

Chlamydial species like *C. psittaci* and *C. pecorum* are specialized animal pathogens, nevertheless they can infect humans as zoonosis, which makes them relevant for clinical research (Dean, et al., 2013). *C. pneumoniae* is the causative agent of respiratory diseases like community acquired pneumonia and upper tract respiratory infections that can lead to chronic disease like asthma (Hammerschlag, 2000, Kuo, et al., 1995). However, new infection sites apart from lung tissue for *C. pneumoniae* are discussed that can be a cause for arteriosclerosis (Kuo, et al., 1993), coronary heart disease and multiple sclerosis (Balin, et al., 1998, Maass, et al., 1998).

C. trachomatis, the second human pathogen in the order *Chlamydiales*, counts among the most common sexually transmitted disease worldwide and infects epithelial cells in the eye or urogenital tract. Which cells get infected and consequently which disease can establish depends on the bacterial serovar. Serovar A-C mostly infects cells of the eyelid, leading to conjunctivitis and the establishment of a trachoma. The continuous infection can lead to the scarring of the cornea through roughness of the conjunctival tissue and hence to blindness (Manavi, 2006). Genital tract infection is caused by *C. trachomatis* serovar D-K and leads to urethritis or salpingitis. In men 50 % and in women 70-80 % of genital *C. trachomatis* infection proceeds asymptomatic and therefore gets no treatment (Malhotra, et al., 2013). Untreated upper genital tract infection can lead to pelvic inflammatory disease, which can consequently end in ectopic pregnancy, pelvic pain and infertility. Recently a link between *C. trachomatis* infection and cervical cancer was found but needs further elucidation (Malhotra, et al., 2013). The serovar L1-L3 is the cause of lymphogranuloma venereum (LGV) which leads to inflammation in the lymph nodes. When the lymphatic drainage is blocked due to bacterial inflammation, painful buboes develop in the groin (Manavi, 2006).

Yet there is no vaccination against *C. trachomatis* available, nevertheless research teams all over the world address this issue. Meanwhile *Chlamydia* can be treated with macrolides, tetracycline and penicillin, which are still too expensive for the extensive use in developing countries.

1.1.3 Life cycle

In 1932 Bedson and colleges first described the biphasic developmental cycle of *C. psittaci* that is common for all chlamydial species (Bedson and Bland, 1932). The chlamydial life cycle can be divided into two phases. First the infectious elementary bodies (EBs) bind to the host cell and induce their internalization through endocytosis (Figure 1.2) (Clifton, et al., 2004, Engel, 2004). EBs show reduced metabolic activity, but in contrast to earlier studies are now known to be able to synthesis proteins and metabolite glucose-6-phosphate (Omsland, et al., 2012). After infection of the host cell EBs are engulfed by a membrane and form the so-called chlamydial inclusion in which *Chlamydia* will stay throughout their development cycle (Moulder, 1991). The inclusion shows no markers for the lysosomal pathway and blocks fusion with endosomes and lysosomes from the beginning (Sinai and Joiner, 1997). Approximate 6-8 hpi EBs differentiate into reticulate bodies (RBs) which are non-infectious but strongly replicative (Stephens, 1999). RBs are with 0.8-1 µm bigger than EBs with 0.3 µm diameter (Friis, 1972) and lead to the propagation of the bacteria through binary fission (Stephens, 1999). For bacterial growth and replication, energy and nutrients are required in big quantity. The metabolism of RBs relies on ATP as an energy source that is mainly provided by the host cell (Hatch, et al., 1985, Omsland, et al., 2012). However, it can also be synthesized by the bacteria itself since it encodes for all required enzymes for glycolysis and consequently ATP production (Iliffe-Lee and McClarty, 1999, Stephens, et al., 1998). Heuer

and colleges showed the fragmentation and recruitment of the Golgi apparatus in infected cells, which helps to provide the bacteria with lipids and nutrients (Heuer, et al., 2009). To acquire lipids the chlamydial inclusion recruits Rab6, Rab11 and Rab14 (Damiani, et al., 2014) for lipid acquisition from the Golgi apparatus as well as Rab39 for lipids originating from multivesicular bodies (Beatty, 2006). The fusion with vesicles from the Golgi apparatus requires different SNAREs, syntaxins and VAMPs on the host cell side and chlamydial Inc proteins on the other side, that mimic SNAREs motifs (Kabeiseman, et al., 2013, Lucas, et al., 2015, Pokrovskaya, et al., 2012, Ronzone, 2014). After several replication cycles RBs asynchronously differentiate back into EBs when they reach a threshold size, nevertheless the whole mechanism of transition needs to be elucidated (Lee, et al., 2018). Eventually the host cell will release the infectious particles into the extracellular space through extrusion or cell lysis, from where the next infection cycle starts (Figure 1.2) (Hybiske and Stephens, 2007).

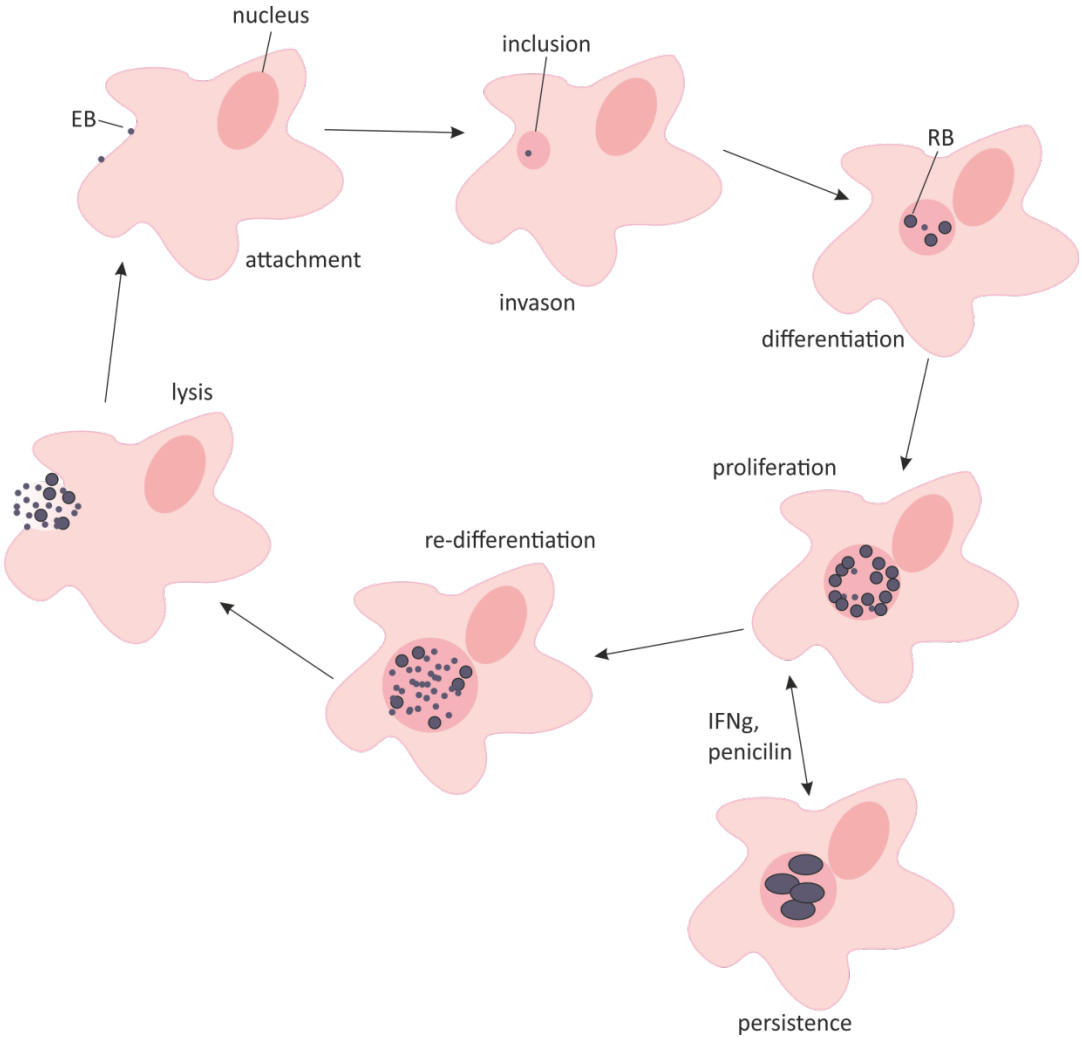


Figure 1.2. Chlamydial developmental cycle. Infectious elementary bodies (EBs) attach to the host cell membrane and enter the cell via endocytosis. The bacterium is engulfed by the so called inclusion and prevents fusion with the lysosomal pathway. In the early phase of infection EBs transform into reticulate bodies (RBs). RBs replicate inside the inclusion before asynchronous re-differentiation into EBs takes place. Bacterial particles are released into the extracellular space via extrusion or cell lysis and a new infection cycle can start. In case of IFNγ presence or treatment with antibiotics like penicillin *Chlamydia* enter a persistent state that is reversed when the inducer for persistency is removed. Modified after Elwell et al. (Elwell, et al., 2016).

Premature cell rupture would be of tremendous costs for the bacteria since the RBs are not capable to infect cells and start a new infection cycle. Therefore, *Chlamydia* have evolved strategies to prevent host cell apoptosis to complete their life cycle (Sharma and Rudel, 2009). The limitation of nutrients but also the presence of host cytokines like IFNɣ or special antibiotics like penicillin triggers persistency in *Chlamydia* (Shemer and Sarov, 1985, Skilton, et al., 2009). During persistence, the bacteria often form enlarged aberrant bodies (ABs) that grow in size but are unable to divide. When the inducer for persistency is removed ABs can transform back into RBs, complete the infection cycle and produce infectious progeny (Byrne and Ojcius, 2004).

1.1.4 Chlamydial genetics

The genome of *C. trachomatis* only comprises 1.0 Mbp and a 7.5 kb plasmid, which is rather small compared to other bacteria (Stephens, et al., 1998). The genome only encodes for 897 coding regions and consequently lacks some essential genes (Stephens, et al., 1998). Nonetheless, *C. trachomatis* evolutionary adapted its genome to its host and reduced all genes that became more and more negligible. Since the bacteria consume many nutrients and lipids from the host, it can lack genes in these synthesis pathways making it more dependent on its host (Stephens, et al., 1998).

The chlamydial plasmid is present in seven chlamydial species (e.g. *C. trachomatis, C. pneumoniae, C. muridarum*) in 4-10 copies per bacteria (Pickett, et al., 2005, Rockey, 2011). It encodes for 8 open reading frames (ORF) (Pickett, et al., 2005, Rockey, 2011) and is essential for the glycogen accumulation of *Chlamydia* (Wang, et al., 2011). The role of some ORFs on the plasmid was elucidated by now. ORF5 or *pgp3* for example is a secreted virulence factor that plays a role in apoptosis prevention (Lei, et al., 2017). ORF1 (*pgp7*) and ORF2 (*pgp8*) are homologs of integrase and recombinase and together with ORF3 (*pgp1*), a helicase, are important for plasmid replication (Comanducci, et al., 1994, Li, et al., 2008). The accumulation of glycogen goes back to Pgp4 protein, encoded by ORF6, that functions as a transcriptional regulator for genome and plasmid encoded genes (Song, et al., 2013, Song, et al., 2014). However, clinical strains lacking the plasmid, were isolated meaning that the genes encoded by the plasmid are not essential for infection establishment (Farencena, et al., 1997, Ripa and Nilsson, 2006, Russell, et al., 2011) but increase infectivity and virulence (Ramsey, et al., 2014).

Due to the obligate intracellular life style of *Chlamydia* genetic transformation and manipulation proved to be difficult. The first successful transformation of *C. trachomatis* used the chlamydial plasmid as a backbone as well as chloramphenicol as a selection marker (Tam, et al., 1994). Transformants can be gained through electroporation, however, the plasmid was not stable and transformants lost resistance after some passages due to mutation in *pgp1* (Tam, et al., 1994), which is important for plasmid maintenance (Gong, et al., 2013). It took some time before the next transformation protocol was published in 2011. The idea of a shuttle vector with the chlamydial plasmid backbone was borrowed from Tam et al. nevertheless a new transformation method using $CaCl_2$ was presented that was taken over by subsequent publications. Moreover, penicillin was introduced as a new selection marker and it was shown that the artificial plasmid could cure the bacteria of the native cryptic plasmid (Wang, et al., 2011). Until now a variety of selection markers like Blasticidin (Ding, et al., 2013) and chloramphenicol (Xu, et al., 2013) and fluorescent marker proteins (e.g. GFP, mCherry, CFP) (Agaisse and Derré, 2013, Wang, et al., 2011) were established in chlamydial genetics as well as promotors that are Tet inducible

(Wickstrum, et al., 2013) or originate from *Chlamydia* itself (Agaisse and Derré, 2013). Though the $CaCl_2$ method turned out to be the most successful used transformation method a dendrimer based approach was published that directly delivered DNA to the inclusion but could not establish in the community (Gérard, et al., 2013, Kannan, et al., 2013). With chlamydial strains carrying different antibiotic resistances DeMars and colleges proofed that *Chlamydia* are capable of horizontal gene transfer and homologous recombination (DeMars, et al., 2007) not only between strains but also species (Suchland, et al., 2009). A completely new genetic approach was the reverse genetics by using chemical mutagenesis. The chemical mutagen EMS lead to mutations in the chlamydial genome. Subsequent sequencing established a library of different mutants and phenotypes (Kokes, et al., 2015). However, this method is intensive in labor since a single mutation is rather rare and the validation of mutants requires re-complementation. Recently a small library of mutants was developed by transposon insertion by the lab of Scot Hefty. The random insertion of a Stop codon in the genome of *C. trachomatis* lead to shortened gene products and more often to dis-functional proteins whereas chemical mutagenesis with single nucleotide exchanges often lead to less severe amino acid exchange. The only specific gene mutation system on the chlamydial genome currently known is the TageTron (Sigma Aldrich) system that utilizes a mobile group II intron (Johnson and Fisher, 2013, Zhong, et al., 2003).
Recently a publication by Ouellette showed the possibility to inducible knock-down the chlamydial *incA* gene using CRISPR interference together with an inactive dCas9 protein form *Staphylococcus aureus* (Ouellette, 2018).

1.2 CRISPR/Cas9 system

1.2.1 The bacterial defense system

Other than the eukaryotic cells, prokaryotes do not possess an innate immune system to deal with intruders. However, both types have evolved strategies to cope with viral DNA. In humans B- and T-cells are capable to recognize proteins from pathogens and destroy them. In archaea and bacteria other mechanisms act against viruses like bacteriophages by blocking uptake or prevention and degradation of injected DNA. One system that deals with invading DNA or RNA involves the genomic region called clustered regularly interspaced short palindromic repeats (CRISPR) and the neighboring CRISPR-associated (*cas*) genes (Figure 1.3) (Barrangou, et al., 2007). The cas genes often encode for helicases, nucleases and polymerases (Horvath 2010). Some bacteria and archaea can store parts of viral DNA as spacer in the CRISPR region separated by repeat sequences. In order to do so, viral DNA is recognized when it enters the cell and a suitable protospacer is integrated into the genome (Figure 1.3). If the cell was already in contact with the virus the primed spacer acquisition involving Cas3 starts. If the virus is new to the cell a naïve mechanism takes place that involves a protein complex consisting of Cas1 and Cas2 proteins, the complete system nevertheless is still unknown (Rath, et al., 2015). In the next step Cas proteins and the pre-CRISPR-RNA (crRNA), including the spacer sequence are expressed. Mature crRNA emerges through processing by Cas proteins and targets specifically the viral DNA in the bacterial cells.

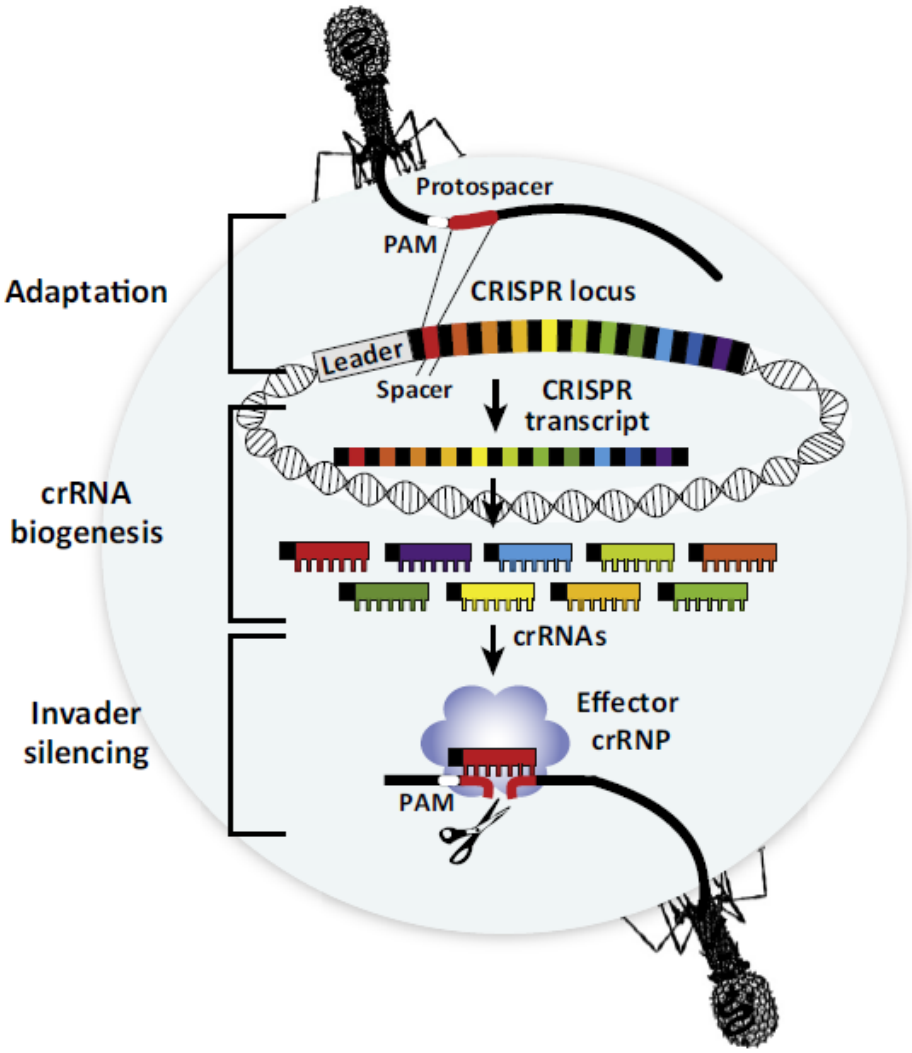


Figure 1.3. Scheme of bacterial CRISPR locus and silencing of viral invaders. After invasion of viral DNA a specific sequence adjacent to a PAM motif can be inserted into the bacterial genome as a so called protospacer. The cluster of different protospacers and bacterial spacers is referred to as the CRISPR locus. Protospacers get transcribed, processed and bind as crRNA specifically to viral DNA invading the bacterial cell. In combination with bacterial nucleases the crRNA cleaves intruder DNA, preventing the second viral attack. Modified after Terns and Terns (Terns and Terns, 2014).

The binding of the crRNA and Cas activity leads to the destruction of the viral DNA (Figure 1.3) (Rath, et al., 2015). Here three types can be distinguished. Type I needs the helicase and nuclease activity of Cas3 together with the Cascade protein complex and a corresponding protospacer adjacent motif (PAM) sequence to degrade the DNA (Rath, et al., 2015). The PAM sequence is a short conserved DNA sequence that is adjacent to the protospacer on the viral DNA and serves to distinguish self and non-self DNA. The type II also uses a PAM sequence to distinguish between the own CRISPR locus and viral DNA and cleaves DNA double strands via the two nuclease domains HNH and RuvC of Cas9. In order to bind the crRNA, Cas9 uses a trans-activating crRNA (tracrRNA) that is partially complementary to the crRNA and bridges between the Cas9 protein and the crRNA (Deltcheva, et al., 2011). The only type that does not utilize a PAM sequence is type III in which a multi-protein complex of six to seven proteins degrades DNA or RNA in a yet unknown mechanism (Rath, et al., 2015, Terns and Terns, 2014).

About 84 % of archaea and 45 % of bacteria have a CRISPR locus either on the chromosome or on a

plasmid (Rath, et al., 2015). The length of the spacer as well as the length of the repeat sequence differs from species to species. Important for the functionality of the system is the palindromic sequence of the repeat region which forms double stranded regions and hairpins and recruits Cas1 and Cas2 (Kunin, et al., 2007, Nuñez, et al., 2015). The insertion of new spacers into the genome is inherited by the offspring, protecting them against bacteriophages that got in contact with the ancestors (Rath, et al., 2015). Apart from the benefits of viral protection the system brings costs for its host. The maintenance and production of spacer costs energy and can cover as much as 1 % of the genome as for *S. tokodaii* (Lillestøl, et al., 2006, Rath, et al., 2015). Furthermore, the rapid development of viruses leads to spacer elements with obsolete target sequences, making them redundant or in the worst case spacers can target the bacterial DNA adverse to the cells fitness.

1.2.2 Application area of the Cas9 system

Since the CRISPR-Cas system is capable of specifically cleaving DNA and RNA it was a matter of time until it was used as a genetic tool for genome editing. Since the crRNA and tracrRNA can be combined to one guide RNA (gRNA) (Jinek, et al., 2012) the type II system is the most commonly used type. The Cas9 protein is a nuclease that cleaves both DNA strands creating blunt ends. The cell repairs these ends again by either non homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ often leads to frameshift by depletion of some nucleotides, creating truncated or incorrect proteins whereas HDR can be used to insert a sequence of interest that is flanked by homologous sequence of the double strand break (Rath, et al., 2015). Meanwhile many Cas9 proteins from different organisms are used for gene editing in eukaryotic and prokaryotic cells and tissue, despite this the first known Cas9 of *S. pyogenes* is still widely used and studied.

The Cas9 system can be used to knock-out and knock-in genes and multiple gRNAs can be used simultaneously. In cases where a gene knock-out would be lethal the knock-down of gene expression using CRISPR interference (CRISPRi) can be used. The mutation of the two active sites of Cas9 HNH and RuvC leads to a loss of nuclease activity but does not affect the specific location of the protein on the gRNA-complement DNA sequence. The attachment of the catalytic inactive or so called dead Cas9 (dCas9) to the promoter region of a gene of interest blocks it for accessibility for the transcription machinery resulting in less mRNA level of the gene. When the gRNA is directed against a sequence in the coding region of a gene the dCas9 protein inhibits the elongation of mRNA synthesis leading to a premature end of the mRNA.

The fusion of transcriptional activator or repressor domains to Cas9 directs the activator or repressor specific to the gene of interest and interferes with the transcription, regulating the frequency of gene expression (Bikard, et al., 2013, Cheng, et al., 2013).

The protospacer and PAM motif are responsible for the specificity of the CRISPR system, nevertheless, off target effects where untargeted DNA gets cleaved happen. In most cases the targeted sequence varies in some nucleotides to the gRNA so that even some mutations lead to a successful knock-out (Terns and Terns, 2014). An advantage of the CRISPR system is that also more than one site can be changed by using more gRNAs e.g. five sites in parallel were shown to work in mouse (Wang, et al., 2013).

The genome of *Protochlamydia* also encodes for the CRISPR/Cas system, nontheless, this is the only known representative in the order of *Chlamydiales* naturally harboring the bacterial defense system (Bertelli, et al., 2016). The CRISPR/Cas system has reached standard technique status for eukaryotic and

prokaryotic cells. Recently Ouellette showed as a proof of principle that genetic editing using CRISPRi also is possible for *C. trachomatis*. He proofed that the chlamydial inclusion membrane protein IncA can be knocked-down by a specific gRNA in combination with the dCas9 from *S. aureus* (Ouellette, 2018).

1.3 Host ubiquitin system

1.3.1 Ubiquitination cascade

Ubiquitin is a 76 amino acid big, highly conserved protein present in all eukaryotic species and tissues (Schlesinger, et al., 1975). It is conjugated to proteins as a posttranslational modification, which influences the protein fate. In 1978 one function of ubiquitin was elucidated, then it was linked to the ATP dependent proteolytic depletion of proteins (Ciehanover, et al., 1978, Hershko, et al., 1980). Apart from protein stability ubiquitin is a signal for a variety of processes in the cell e.g. endocytic trafficking, inflammatory response and DNA repair (Hofmann and Pickart, 1999, Pickart and Fushman, 2004, Staub and Rotin, 2006). The mechanism of ubiquitination is a multi-step process that includes three enzymes. In human cells the two E1 ubiquitin activating enzymes UBE1 and UBE1L2 (in yeast Uba1 and Uba6) are known to initialize the major ubiquitination cascade (Groettrup, et al., 2008). Under ATP hydrolysis the C-terminal carboxyl group of ubiquitin gets adenylated and the E1 active cysteine is linked to ubiquitin via a thioester bond (Figure 1.4). In the next step the E1 enzyme transfers ubiquitin to one of the 40 human E2 ubiquitin conjugating enzymes (Tokgöz, et al., 2012). The small ubiquitin molecule is bound to the active cysteine via a non-covalent thioester bond. The E2 enzyme together with the E3 ubiquitin ligase is determining the ubiquitin linkage type. In the last step the E3 ligase binds the specific substrate and transfers the ubiquitin to the lysine residue forming an isopeptide bond (Figure 1.4) (Pickart, 2001).
The fate of the protein conjugated with ubiquitin depends on mono-, or polyubiquitination as well as the chain linkage type (Komander and Rape, 2012). Monoubiquitination always describes the linkage of one ubiquitin molecule to a lysine of a substrate, which can happen at several lysines simultaneously. Polyubiquitination is more diverse and can vary in its length and linkage type. Ubiquitin encodes for seven lysines (K) and one start-methionine that are capable of binding the C-terminus of a second ubiquitin molecule. Due to that a polyubiquitin chain can be linked via K6, K11, K27, K29, K33, K48, K63 and linear via methionine (M1) (Figure 1.4). Moreover, it is also possible that a polyubiquitinated chain contains several different linkage types, is branched or modified by phosphorylation or acetylation itself (Swatek and Komander, 2016). K48 ubiquitin chains were identified to be involved in proteasomal degradation (Chau, et al., 1989), as well as K11 chains formed by the Anaphase-Promoting Complex (APC/C) (Jin, et al., 2008). The linkage via K63 is often a signal for DNA repair (Hofmann and Pickart, 1999, Zhou, et al., 2009) or signaling (Tokunaga, et al., 2009) as well as clearance of misfolded proteins through autophagy (Olzmann and Chin, 2008).

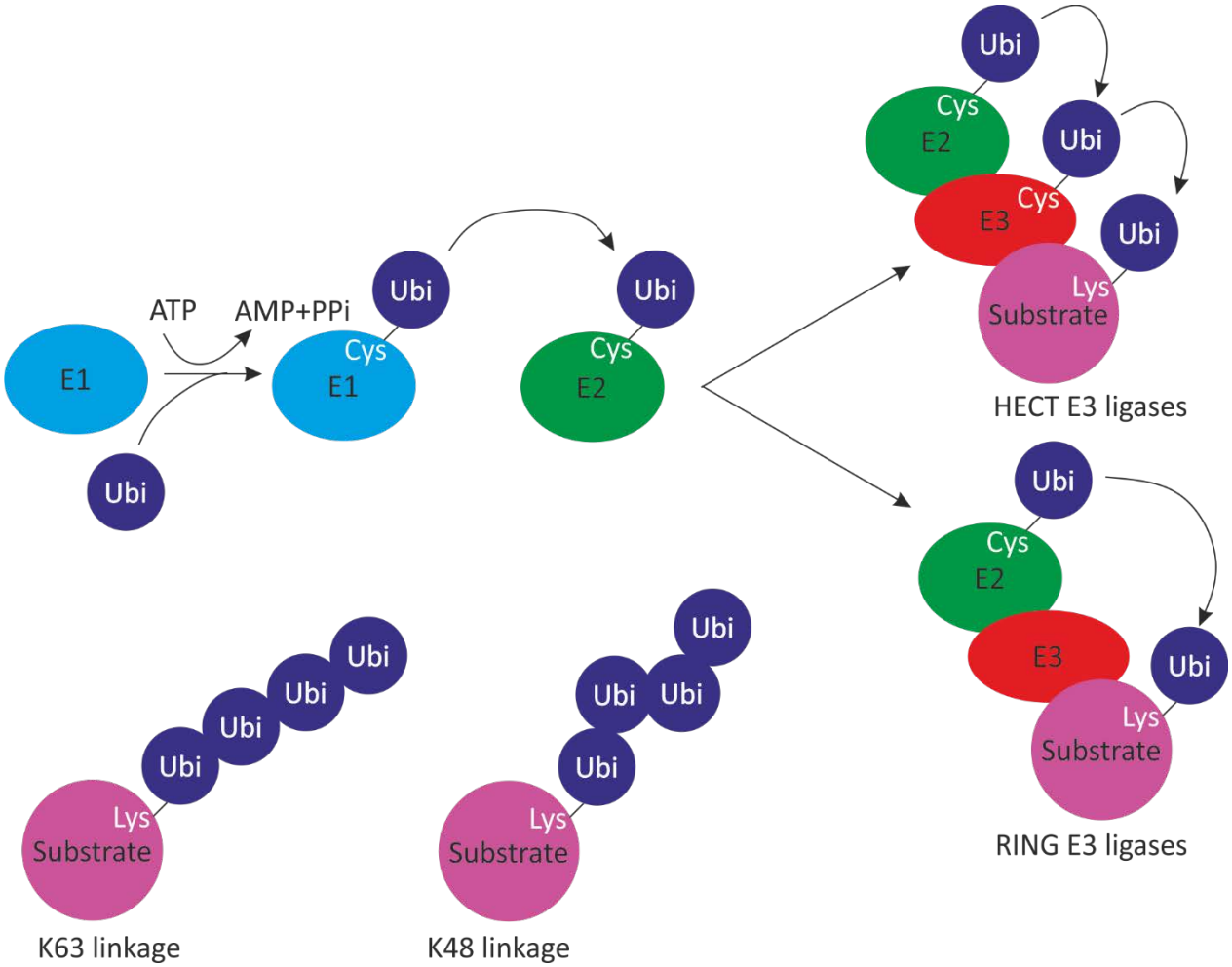


Figure 1.4. Transfer of ubiquitin on substrates by the cascade of enzymes. The E1 activating enzyme binds ubiquitin on a cysteine under ATP consumption. Ubiquitin then gets transferred to the cysteine of an E2 conjugating enzyme before it is bound to the lysine of the substrate by the E3 ligase. The mechanism differs between the families of E3 ligases. While HECT E3 ligases intermediate bind the ubiquitin molecule, RING E3 ligases skip this step and transfer ubiquitin directly to the lysine of the substrate. Polyubiquitination occurs through multiple cycles of the ubiquitination cascade, thereby ubiquitin chains can be conjugated via different lysines, e.g. K48, K63, etc. creating a different fate for the substrate. Modified after Lippai and Löw (Lippai and Lőw, 2014).

1.3.2 E3 ubiquitin ligases

For the E3 ligase the classes of homologous to the E6AP carboxyl terminus (HECT), really interesting new gene (RING), U-box and PHD-finger E3 ligase can be distinguished. The first HECT type E3 ligase described was the E6-AP ligase that targets damaged proteins for proteasomal degradation (Huang, et al., 1999). HECT type E3 ligases are single proteins that bind ubiquitin in an intermediate state via the active cysteine before it is transferred to the substrate (Figure 1.4) (Huibregtse, et al., 1995). The preferred ubiquitin chain linkage type of each HECT E3 ligase is determined by the last 60 amino acids of the C-lobe (Kim and Huibregtse, 2009). Phosphorylation of HECT E3 ligases can either regulate the activity of the HECT E3 ligases like for Itch (Gallagher, et al., 2006) or the substrate availability and binding like for Nedd4 (Debonneville, et al., 2001).

The vast majority of E3 ligases belong to the type of RING E3 ligases. These ligases have a RING domain in common with two bound zinc ions and they can function as a monomeric or dimeric ligase or as a multi-subunit complex (Metzger, et al., 2012). Ubiquitin is not directly bound to the E3 enzyme but with the

simultaneous binding of E2 and substrate the E3 ligase complex facilitates the transfer of ubiquitin to the substrate (Figure 1.4) (Metzger, et al., 2012).
The multi-subunit RING ligase family contains among others the cullin RING ligases (CRL) that comprises the SCF (Skp1, cullin, F-box protein) ligases. The regulation of CRL ligases happens via the neddylation state of the cullin. Neddylated cullins change conformation so that the ubiquitin-E2 enzyme and the substrate are in spatial proximity and ubiquitin gets transferred (Duda, et al., 2008).
U-box E3 ligases are quite similar to RING ligases and share a common U-box domain that is crucial for the binding and transfer of ubiquitin to a substrate (Hatakeyama, et al., 2001). At least one U-box protein, Ufd2, functions as a polyubiquitin ligase which is currently the only member of E4 enzymes. Together with an E1, E2 enzyme and a HECT E3 ligase Ufd2 transfers polyubiquitin chains to substrates (Koegl, et al., 1999).

Recently a new group of ring between ring (RBR) ligases was elucidated. The well-studied E3 ligases Parkin and HOIP/HOIL-IL (subunits of E3 ligase LUBAC) but also rather neglected enzymes like Ankyrin repeat and IBR-domain containing protein1 (Ankib1) belong to this subgroup. RBR ligases act in a two-step mechanism like RING and HECT ligases through transfer of the ubiquitin from the E2 enzyme to a RING domain and consequently transfer the ubiquitin to the substrate (Uchida and Kitagawa, 2016). LUBAC is the currently only known E3 ligase that links ubiquitin via the first methionine, producing a linear ubiquitin chain (Kirisako, et al., 2006). The E3 ligase Parkin is not only involved in mitophagy (Narendra, et al., 2008) but also in xenophagy of *Mycobacterium tuberculosis* in infected cells (Manzanillo, et al., 2013). The RBR type E3 ligase Ankib1 resembles Parkin in structure and domains (Miller, et al., 2004). Ankib1 among others was found to be partially deleted in patients suffering from cerebral cavernous malformation (CCM) (Muscarella, et al., 2010). In chicken infected with *Salmonella enterica* the promotor of Ankib1 is less methylated and therefore more active (Wang, et al., 2017). However, the naïve function of Ankib1 is still not known and needs further investigation.
The human genome encodes for 40 E2 ubiquitin conjugating enzymes. For a smooth procedure one E2 enzyme has to serve multiple E3 ligases. UBE2L3 for example or the yeast homologue UbcH7 is known to interact with several ligases like the HECT type E3 ligase E6-AP (Kumar, et al., 1997) or the RBR type E3 ligase Parkin (Shimura, et al., 2000).

1.3.3 Deubiquitinating enzymes

1.3.3.1 Human deubiquitinating enzymes

Ubiquitination is a reversible posttranslational modification. Approximately 100 human genes encode for deubiquitinases (DUBs), but not all of those are specific for ubiquitin or functional (Nijman, et al., 2005). DUBs can either work as exo- or endo-DUBs according to their structure. Exo-DUBs cleave ubiquitin from the distal end of an ubiquitin chain generating monoubiquitin whereas endo-DUBs can hydrolyze the bond between ubiquitin molecules internal of a chain, releasing a longer ubiquitin chain and ensuring a faster editing (Komander, et al., 2009). All DUBs have at least one ubiquitin binding domain (UBD) in common. Until now five classes of DUBs can be distinguished that belong to the family of cysteine proteases with a catalytic triade (Cys, His, Asp) in the active center or the metalloproteases that need zinc ions for activity. The biggest family, the cysteine proteases, covers ubiquitin specific proteases (USP), ubiquitin C-terminal hydrolases (UCH), ovarian tumor proteases (OUT), Machado-Joseph disease

proteases (MJD) (Nijman, et al., 2005) and the newly described MIU-containing novel DUB family (MINDY) (Rehman, et al., 2016). The only member of the metalloprotease family is the JAB1/MPN/Mov34 (JAMM) domain protease (Nijman, et al., 2005).

The task of deubiquitination is almost as diverse as of ubiquitination. As antagonists of E3 ligases DUBs are essential to regulate various cellular processes in cells. Some DUBs are in complex with the 26S proteasome and recycle ubiquitin from proteins that are degraded, filling up the free ubiquitin pool in the cell (Borodovsky, et al., 2001). Proteins can also be rescued from degradation by removal of the K48 ubiquitin chain, which is faster than synthesis of new proteins. Deubiquitination brings substrates back into the initial state and serves as a tool for regulation of cellular processes. Thereby deubiquitination interferes with signaling processes by editing or removing the ubiquitin code. Other DUBs are needed to cleave single ubiquitin molecules from newly synthesized precursor ubiquitin chains or free chains that were cleaved *en bloc* from the substrate by another DUB (Komander, et al., 2009). Some DUBs are associated with ubiquitin E3 ligases and reverse the tendency to auto-ubiquitinate themselves, like USP8 deubiquitinates the E3 ligase Nrdp1 (Wu, et al., 2004).

DUBs can be specific for one K-linkage, however, some hydrolases have a wider spectrum of linkage types or other ubiquitin like molecules (Gong, et al., 2000, McGouran, et al., 2013). This specificity towards K- linkage types is due to the different three-dimensional structure of the linked ubiquitin chains. There are around 20.000 possible ubiquitination sites in a cell antagonizing roughly 100 DUBs (Udeshi, et al., 2013). Therefore, some DUBs that cleave monoubiquitin from substrates are capable to interact with several different target proteins.

The regulation of DUB activity is also divers. Some DUBs can be regulated on transcriptional levels, interaction with scaffold proteins or through ubiquitination. The modification with ubiquitin can either activate or inhibit DUB activity (Todi, et al., 2009). Interestingly some DUBs can counteract the modification by auto-deubiquitination (Leznicki and Kulathu, 2017, Zhang, et al., 2012).

1.3.3.2 Chlamydial deubiquitinating enzymes

The ubiquitin system is limited to eukaryotic cells only. However, some viruses and prokaryotes have developed strategies to interfere with the ubiquitin system in order to increase their fitness. In the genome of *C. trachomatis* two genes encoding for deubiquitinases were identified and called *Chla*DUB1 (Cdu1) and *Chla*DUB2 (Cdu2). Analysis of the transcription levels of *Chla*DUB1 revealed that expression starts in mid phase around 16 hpi and increases until late phases (Belland, et al., 2003). Since *Chla*DUB1 and *Chla*DUB2 show similarity to the active center of cysteine deubiquitinases the group of Misaghi showed that *in vitro* the two chlamydial DUBs could cleave an ubiquitin probe and another ubiquitin like modifier, Nedd8 (Misaghi, et al., 2006). Although *Chla*DUB1 can cleave pro-Nedd8 it cannot cleave Nedd8 conjugated to cullin substrate (Pruneda, et al., 2016). However, the deneddylating activity of *Chla*DUB1 was not verified during infection yet. Furthermore *Chla*DUB1 indeed stabilizes IκBα by removal of conjugated ubiquitin and prevention of its degradation by the proteasome. Since IκBα forms a complex in which NFκB is inactive, the stabilization of IκBα prevents NFκB activation and consequently inflammatory response of the cell (Le Negrate, et al., 2008). As a chlamydial protein the DUB needs to get in close contact with its host cell substrates. Despite contradictory publications *Chla*DUB1 is secreted into the inclusion membrane, anchoring with the transmembrane domain and facing the host cell cytosol with the active site (Fischer, et al., 2017, Wang, et al., 2018). With this orientation the DUB interferes with host cell proteins like Mcl-1. By deubiquitinating Mcl-1, the anti-apoptotic protein gets stabilized

and supports apoptosis inhibition and consequently chlamydial survival (Fischer, et al., 2017). The second chlamydial deubiquitinase *Chla*DUB2 on the other side was found to be located in the host cell plasma membrane (Wang, et al., 2018). The structure of *Chla*DUB1 was solved and reveals that it belongs to the cysteine proteases with Cys, Asp and His in the catalytic center and a specificity for K48 and K63 ubiquitin chains and to a lesser extent for K11 (Fischer, et al., 2017, Pruneda, et al., 2016). *Chla*DUB1 is present in several chlamydial strains like *C. trachomatis*, *C. muridarum*, *C. suis* but not in *C. pneumoniae*. However, another deubiquitinase called *Chla*OUT was identified in the genome of *C. pneumoniae*, *C. caviae*, *C. abortus*, *C. felis*, *C. pecorum* and *C. psittaci* but not in *C. trachomatis*. *Chla*OUT is secreted into the host cell cytoplasm and cleaves K48 and K63 polyubiquitin chains at the chlamydial entry site (Furtado, et al., 2013). However, the substrates of *Chla*OUT are still unknown.

1.4 Cell autonomous defense

1.4.1 Mechanisms of cell autonomous defense

Chlamydia have evolved for thousands of years which lead to perfect adaption of the pathogen to its host cell. Bacteria have strategies to infect cells and feast on their nutrients. On the other side cells have established mechanisms to detect and destroy unwanted intruders, the cell autonomous defense. In the first step the cell has to recognize the bacteria's presence. This happens by pattern-recognition receptors (PRR). Different PRRs can bind different bacterial molecules the so called pathogen-associated molecular patterns (PAMP) like lipopolysaccharides (LPS) or damage associated molecular pattern (DAMP). The PRRs can be on the plasma membrane or endosome membrane and are for instance toll like receptors (TLR), mainly binding to extracellular or invading bacteria or in the cytosol like nucleotide binding oligomerization domain (NOD) receptors. The activation of NOD receptors can lead to pro-inflammatory cytokine release via NFκB or inflammasome activation (Akira, et al., 2006). The multi protein complex of the inflammasome includes caspase-1 and caspase-5 that lead to IL-1β and IL-18 release (Martinon, et al., 2002). The binding to TLR in addition leads to the activation of the antigen-specific acquired immunity and T-helper cell recruitment or to cytokine secretion and type I IFN secretion release (Akira, et al., 2006). Type I IFN and more important IFNɣ that is secreted during pathogen infection, lead to the onset of expression of several genes in the cell, among them the group of guanylate-binding proteins (GBPs). GBPs are GTPases that can hydrolyze GTP, bind other proteins and target membrane structures. These features are important for their function during infection. In human cells seven GBPs are known whereas mouse cells have 13 GBPs in total (Man, et al., 2017). Apart from facilitating the rupture of pathogen membranes GBPs also activate the inflammasome (Finethy and Coers, 2016). Another group of IFNɣ inducible proteins are the immunity related GTPases (IRG) that can be divided into two subgroups. The GKS are not present in human cells, but in mouse cells and specifically bind to pathogen membranes, reducing the membrane integrity. The second group, IRGM proteins, were also found in human cells. IRGM proteins only target host membranes and protect them from destabilization through GKS proteins (Haldar, et al., 2013). The lack of IRGM protein on membranes is recognized as a 'missing-self' signal that leads to GKS annealing, consequent ubiquitination and rupture of the membrane (Haldar, et al., 2015). How this mechanism works in detail needs further elucidation. Eukaryotic cells can produce cytotoxic molecules that are called reactive oxygen species (ROS) or reactive nitrogen species (RNS) during infection. These molecules target and damage for example bacterial DNA and lipids that leads to destruction of the intruder (MacMicking, 2012).

Another mechanism that helps the cell to survive is autophagy. On the one hand unselected autophagy ensures sufficient supply of nutrients for the cell in times of starvation or limitation. On the other hand selective autophagy can remove and recycle damaged or unwanted organelles or bacteria from the cytosol (Figure 1.5) (Weidberg, et al., 2011). Depending on the structure that is attacked by the autophagic system one discriminates between mitophagy for removal of damaged mitochondria, xenophagy for destruction of intracellular bacteria, peroxyphagy for targeting the peroxysome and so on (Gomes and Dikic, 2014). During xenophagy the bacterial target membrane is ubiquitinated with K48 or K63 chains by E3 ligases. Subsequent autophagy marker like sequestosome1 (p62), Neighbor of BRCA1 gene 1 (NBR1), calcium-binding and coiled-coil domain-containing protein 2 (NDP52) and optineurin can bind to the modification (Kirkin, et al., 2009, Svenning and Johansen, 2013). A double membrane, the so called phagophore engulfs the complex and closes, forming the so called autophagosome (Figure 1.5). The double membrane consists of ATG8 homologues (LC3A, LC3B, LC3C, GABARAP, GABARAPL1 or GABARAPL2) that are lipidated with phosphatidylethanolamine (PE) via an enzyme cascade and integrated into the membrane (Mizushima, et al., 2011). The autophagosome will eventually fuse with the lysosome forming the autolysosome in which the bacteria and proteins are degraded by proteases (Dikic, 2017). Many bacteria are entering the cell via endocytosis and are engulfed by a membrane derived from the host cell membrane. Bacteria can either escape from the membrane into the cytosol of the cell or proliferate inside this vacuole. In case of LC3-assisted phagocytosis (LAP) this membrane gets decorated with LC3 molecules as soon as TLR have detected the intruder (Randow and Youle, 2014). The closed LC3 autophagosome is degraded by fusion with endosomes and lysosomes. Rupture of the pathogen containing vesicle gives access of cytosolic galectins to the glycan in the inside of the vesicle membrane. The binding of galectin 8 to glycan triggers the recruitment of NDP52 and induces autophagy (Thurston, et al., 2012). Another receptor that recognizes infection in the cytosol is stimulator of interferon genes (STING). It binds the bacterial second messenger molecules cyclic dinucleotides like di-GMP and di-AMP and induces autophagy formation (Watson, et al., 2012).

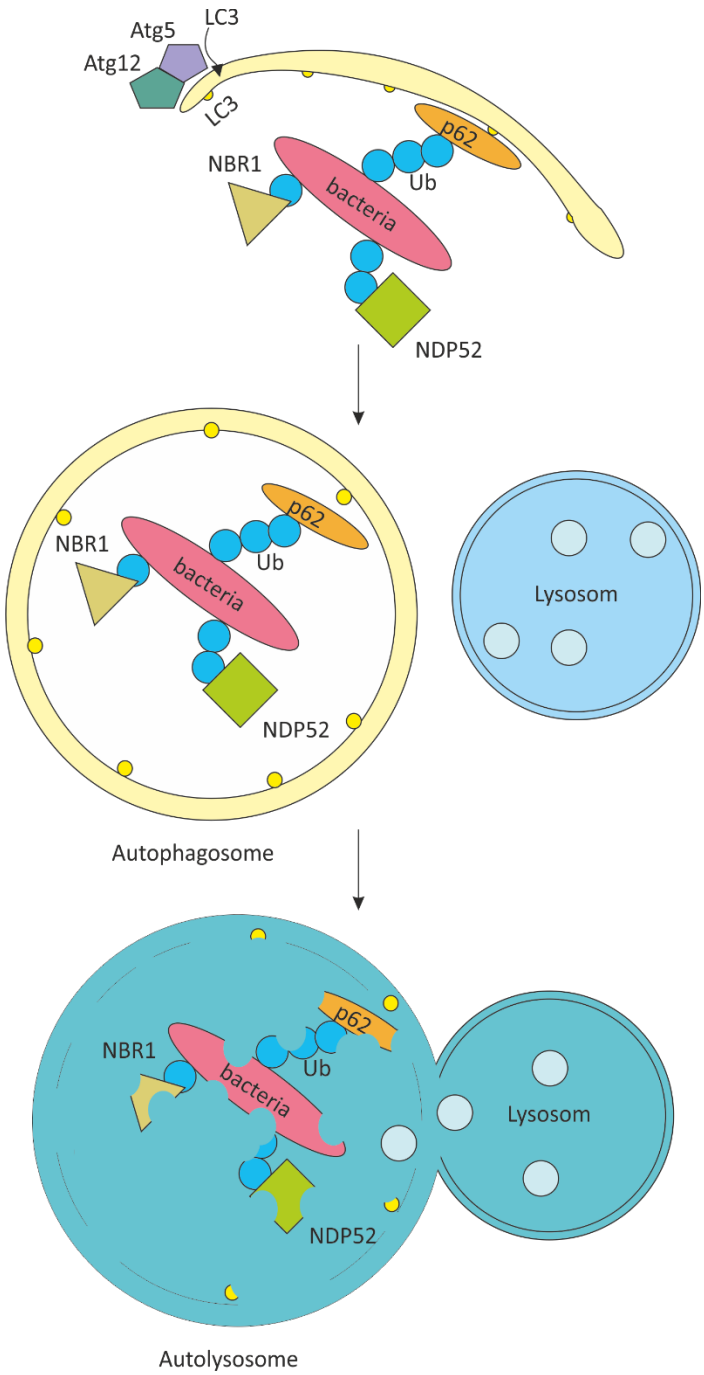


Figure 1.5. The process of autophagy. The damaged organelle, invading bacteria or protein aggregate is recognized by E3 ligases and gets ubiquitinated. Autophagy marker like p62, NBR1 and NDP52 bind to the ubiquitin and trigger the formation of the LC3 containing phagophore that forms around the complex. The closed autophagosome eventually fuses with the lysosome, which leads to degradation of the content in the autolysosome. Modified after Lippai and Löw (Lippai and Lőw, 2014).

1.4.2 Cell autonomous defense in *Chlamydia* infection

Cell autonomous immunity acts against bacterial infection. However, *Chlamydia* have adapted counter strategies to block host cell defense and to survive and proliferate. In early infection *C. trachomatis* prevents the fusion of the inclusion with the lysosome and therefore degradation of the bacteria

(Eissenberg and Wyrick, 1981). Moreover, *C. trachomatis* blocks host apoptosis, since premature lysis leads to the total loss of infectious progeny. This is achieved on the one hand through stabilization of the anti-apoptotic Mcl-1 protein via *Chla*DUB1 (Fischer, et al., 2017) and on the other hand by inhibition of Cytochrome c release and mitochondria permeability (Sharma and Rudel, 2009) as well as activation of survival pathways like PI3K/AKT (Su, et al., 2004).

Furthermore, IFNɣ that is secreted upon infection also affects the amino acid synthesis of the cell. Upon IFNɣ presence indole-2,3-dioxygenase (IDO) depletes tryptophan in human cells making it no more accessible for *C. trachomatis*. Since the bacteria are auxotroph for this amino acid the depletion leads to limitation of their growth (Finethy and Coers, 2016). However, genital tract strains of *C. trachomatis* are capable to reverse this growth deficiency by synthesis of tryptophan using indole as a substrate (Caldwell, et al., 2003). Murine cells have other defense mechanisms than human cells. This starts with the fact, that mouse cells do not express IDO. Furthermore the reaction upon IFNɣ is different. When *C. trachomatis* infects murine cells GBP1 and GBP2 (Haldar, et al., 2015) bind to the inclusion as well as the GSK proteins Irga6 (Al-Zeer, et al., 2009) and Irgb10 (Coers, et al., 2008) in the presence of IFNɣ. This recruits the E3 ligases TRAF6 and Trim21 which leads to ubiquitination of the chlamydial inclusion (Haldar, et al., 2015). Consequently the inclusion suffers from integrity loss and the bacteria are cleared from the cell through the autophagosomal pathway. Despite some similar mechanisms in human cells *C. trachomatis* is protected from ubiquitination and subsequent clearance (Haldar, et al., 2016). TLR2, TLR4 and NOD1 recognize *C. trachomatis* infection in the human cell leading to chemokine secretion and recruitment of immune cells like macrophages (Buchholz and Stephens, 2008, Darville and Hiltke, 2010). Therefore, the interleukin secretion leads to bacterial clearance via inflammation but also to tissue damage and scarring. Infection of epithelial cells triggers inflammasome activation and Caspase-1 processing which is not leading to interleukin secretion but is required from the bacteria for lipid acquisition (Abdul-Sater, et al., 2009). On the contrary in immune cells the Caspase-1 activation triggers secretion of IL-1β and IL-18 promoting the immune response (Abdul-Sater, et al., 2010). For both cell types ROS is needed to activate the inflammasome. It is reported that low levels of ROS is required for successful establishment of *C. trachomatis* infection, but also damages the host cell DNA (Chumduri, et al., 2013). Moreover, the presence of *C. trachomatis* DNA in the cell is detected by cGAS which produces cyclic dinucleotides that activate STING and boosts the type I IFN secretion (Zhang, et al., 2014).

Chlamydial proteins interact with the host cell and prevent anti-bacterial properties. For example some chlamydial Inc proteins that are located in the chlamydial inclusion are essential for the stability of the inclusion (Weber, et al., 2017). Furthermore loss of this Inc proteins leads to clearance of the bacteria through autophagosomes and STING activation (Weber, et al., 2017). When it comes to autophagy it was shown that *C. trachomatis* infected cells have increased level of lipidated LC3 in mid phase but not in early time point of infection (Pachikara, et al., 2009). However, the autophagosomes are not forming around the chlamydial vacuole but are present in the cytosol, which is probably a reaction to the nutrient starvation (Pachikara, et al., 2009). Another publication by Al-Younes describes LC3 in cooperation with MAP1 around the chlamydial inclusion. In this case LC3 accumulation is not linked with autophagy but rather with the stability of the microtubule, which happens to be in close proximity to the inclusion (Al-Younes, et al., 2011).

1.5 Golgi rearrangement during infection

The Golgi apparatus is a membranous structure in the cytoplasm of the cell forming stacked cisternae network that are functionally distinct due to the enzymatic composition of glycosylation enzymes (Dunphy and Rothman, 1985). Proteins and lipids get processed, sorted into vesicles and transported to their operational area while traversing the Golgi network (Dunphy and Rothman, 1985). Thereby the *cis*-Golgi is close to the ER, the major place for protein synthesis, whereas the *trans*-Golgi membranes releases the packed vesicles to their destination (Shorter and Warren, 2002). In order to maintain the structure of the Golgi, specific proteins like golgins and GTPases are essential (Short, et al., 2005). The membrane network, however, is flexibel and capable of rearrangements during mitosis (Sütterlin, et al., 2002) and cell death like apoptosis (Nozawa, et al., 2002).

Also during *Chlamydia* infection the Golgi plays an important role and undergoes structural changes. Heuer et al. proofed Golgi fragmentation and assembly of Golgi derived ministaks around the chlamydial inclusion to be essential for chlamydial growth (Heuer, et al., 2009). Moreover, the cleavage of host golgin-84 was identified to be essential for the Golgi rearrangement during infection (Heuer, et al., 2009). By manipulation of the Golgi network *C. trachomatis* address lipid filled vesicles to the inclusion, where they are consumed by the bacteria (Heuer, et al., 2009, Lipinski, et al., 2009). Not only vesicles directly from the Golgi but also recycling endosomes are reported to fuse with the chlamydial inclusion, as Transferrin and the Transferrin receptor were found at the inclusion (Al-Younes, et al., 2011, Rzomp, et al., 2003). The GTPases Rab6 and Rab11 were identified as regulators of *Chlamydia* induced Golgi fragmentation, since the knock-down of either one decreases the development and growth of *Chlamydia* via inhibition of Glogi fragmentation (Lipinski, et al., 2009). Since Rab6 and Rab11 are involved in the acquisition of lipids for the chlamydial inclusion, they are also found to localize around it (Damiani, et al., 2014, Lipinski, et al., 2009).

1.6 Aim of the work

The aim of this work was to establish the first CRISPRi knock-down system for *C. trachomatis* with a genetic approach. Genetic manipulation of *Chlamydia* was published in many papers and becomes more and more routine work (Gérard, et al., 2013, Kannan, et al., 2013, Wang, et al., 2011, Wickstrum, et al., 2013). However, an inducible knock-down system suitable for *C. trachomatis* was still missing at the beginning of this work. The CRISPRi system established in this thesis proofs functional, nevertheless can only be the start.

The second part of this thesis deals with the mechanisms that *C. trachomatis* uses to intervene with pathways of the host cell defense and characterizes the chlamydial mutant *C. trachomatis* Tn-*cdu1*. We showed with the inactive *Chla*DUB1 mutant *Ctr* Tn-*cdu1* that the protease is also involved in prevention of detection by the host defense pathway. The goal here was to further investigate *Chla*DUB1 during infection regarding its role in prevention of detection by the host defense system and to identify proteins and pathways that interfere with *Chla*DUB1. Since *Chla*DUB1 is present in other *Chlamydia* species, the DUBs were hold in comparison. Furthermore the task of *Chla*DUB1 in *C. trachomatis* infection in immune cells like neutrophils was elucidated.

2 Material and Methods

2.1 Material

2.1.1 Bacterial strains

Table 1 *E.coli* strains

Strains	Properties
E.coli DH5α	Cloning
E. coli XL1blue	Cloning
E. coli JM110	Dam and Dcm methylases deficient
E.coli pCRISPRi:gCdu1III DH5α	Transformed with the pCRISPRi:gCdu1III plasmid
E.coli pCRISPRi:gGFP DH5α	Transformed with the pCRISPRi:gGFP plasmid

Table 2 *C. trachomatis* strains

Strain	Serovar	Properties	Origin
C. trachomatis	LGV L2/Bu434		ATCC® VR-902B™
C. trachomatis Tn-*cdu1*	LGV L2/Bu434	*C. trachomatis* with inserted transposon in the *Chla*DUB1 gene, leading to an inactive truncated *Chla*DUB1 protein	(Fischer, et al., 2017)
C. trachomatis pCRISPRi:gCdu1III	LGV L2/Bu434	*C. trachomatis* transfected with pCRISPRi: gCdu1III	Dr. A. Fischer
C. trachomatis pCRISPRi:gCdu1III A8 pool	LGV L2/Bu434	*C. trachomatis* transfected with pCRISPRi: gCdu1III, single clone	This work
C. trachomatis pCRISPRi:gCdu1III A8.2	LGV L2/Bu434	*C. trachomatis* transfected with pCRISPRi: gCdu1III, single clone	This work
C. trachomatis pCRISPRi:gCdu1III A8.3	LGV L2/Bu434	*C. trachomatis* transfected with pCRISPRi: gCdu1III, single clone	This work
C. trachomatis pTet:GFP	LGV L2/Bu434	*C. trachomatis* transfected with pTet:GFP	(Auer, 2014)
C trachomatis A	A		ATCC® VR-571B™
C. trachomatis D	D		ATCC® VR-885™
C. pneumoniae	CWL-029		ATCC® VR-1310™
C. muridarum	Nigg II		ATCC® VR-123™

2.1.2 Cell lines

Table 3 Cell lines

Cell line	Properties	Medium	Source
HeLa 229	Human cervix adenocarcinoma cells	RPMI 1640, 10 % FCS	ATCC® CCL-2.1™
Fimb	Human fimbriae	RPMI 1640, 20 %FCS	biopsies
HUVEC	Human umbilical vein cells	Medium 200, LSGS supplement	ATCC® CRL-1730™
HeLa 229 Flp-In™ T-Rex™	HeLa229 cell containing the empty pcDNA5/FRT/TO plasmid	RPMI 1640, 10 % FCS, 250 µg/ml Hygromycin	Thermo Fischer scientific
HeLa 229 Flp-In™ T-Rex™ Cdu1-Flag	HeLa229 cell with integration of Cdu1-Flag gene in the genome due to pcDNA5/FRT/Cdu1-Flag plasmid transfection	RPMI 1640, 10 % FCS, 250 µg/ml Hygromycin, 4.5 µg/ml Blasticidin	made by Dr. Annette Fischer
HeLa 229 Flp-In™ T-Rex™ Cdu1Mut-Flag	HeLa229 cell with integration of Cdu1Mut-Flag gene in the genome due to pcDNA5/FRT/Cdu1Mut-Flag plasmid transfection	RPMI 1640, 10% FCS, 250 µg/ml Hygromycin, 4.5 µg/ml Blasticidin	made by Dr. Annette Fischer
HeLa Parental	Knock-out in *parkin*	RPMI 1640, 10 % FCS	(Lazarou, et al., 2015)
HeLa PentaKO	Knock-out in *parkin*, *ndp52*, *optineurin*, *nbr1*, *tax1bp1*, *p62*	RPMI 1640, 10 % FCS	(Lazarou, et al., 2015)
McCoy	Murine fibroblast	RPMI 1640, 10% FCS	ATCC® CRL-1696™
Mef	Murine fibroblast	DMEM, 10 % FCS	ATCC® SCRC-1040™

FCS was heat inactivated before addition to medium.

2.1.3 Plasmids and constructs

Table 4 Plasmids and constructs

Name	Properties	Source
pcDNA5/FRT/TO	Expression vector of inducible proteins in mammalian cells	Invitrogen
pOG44	Plasmid harboring the Flp recombinase	Invitrogen
pcDNA5/FRT/Cdu1-Flag	Expression of a Flag-tagged *Chla*DUB1 protein	Dr. A. Fischer
pcDNA5/FRT/Cdu1Mut-Flag	Expression of a Flag-tagged inactive *Chla*DUB1 protein	Dr. A. Fischer
pCRISPRi:gCdu1III	Expression of dCas9 and gRNA targeting *Chla*DUB1 in *C. trachomatis*	Dr. A. Fischer
pTet:GFP	Expression of inducible GFP in *C. trachomatis*	(Auer, 2014)

2.1.4 Oligonucleotides

Table 5 Oligonucleotides for sequencing

Name	Sequence 5'-> 3'	Features
Seq dCas9 rev 1	ctggaagttgttgtcgaacta	pCRISPRi
Seq dCas9 frw1	ggctcccctatcagcttc	pCRISPRi
Seq dCas9 rev 2	caaactatcatcatggatcagc	pCRISPRi
Seq dCas9 frw 2	gtattagggataagcaatctg	pCRISPRi
Seq dCas9 rev 3	cttgctcagacttagcaatc	pCRISPRi
Seq dCas9 frw 3	gcgtatctaaatgccgtcg	pCRISPRi
Seq pgRNA frw	cttggagctcccgctgct	pCRISPRi
Seq pgRNA rev	cagtgagcgaggaagcgg	pCRISPRi
Seq pCRISPRi::SW2	ggagctgcatgtgtcagag	pCRISPRi
T7 frw	taatacgactcactatagg	pcDNA3
SP6 rev	cattagtgtcacctaaat	pcDNA3
BGH rev	tagaaggcacagtcgagg	pCDNA4TO
CMV frw	cgcaaatgggcggtaggcgtg	pCDNA4TO

Table 6 Oligonucleotides for RT-PCR

Name	Sequence 5'-> 3'
qRT RNF214 frw	ctggatagactcaagaatcag
qRT RNF214 rev	ctctttttctgcttcttctag
qRT BRPF3 frw	ggcgactcccctaga
qRT BRPF3 rev	ttgagggagccactca
qRT Ankib1 frw	atatgctgcattagacaaacg
qRT Ankib1 rev	agctcgaagtaaagcttcag
qRT UBE1 frw	agctgcgagtgggtg
qRT UBE1 rev	agtggctgctcccc
qRT UBEK2 frw	taccagaaacatacccatttaa
qRT UBEK2 rev	ctgcccattgatctttca
qRT UBE2L3 frw	tgatgaaggagcttgaagaa
qRT UBE2L3 rev	gattctgaaggctcccttat
qRT GAPDH frw	gagccccagccttctccatg
qRT GAPDH rev	gaaatcccatcaccatcttccag
qRT GFP frw	gacacaacattgaagatggaa
qRT GFP rev	gaaagggcagattgtgtg
qRT gRNA frw	gtctttgaagttgagttggtg
qRT gRNA rev	taacggactagccttattttaact
qRT dCas9 frw	ggaagcgactcgtctcaaac
qRT dCas9 rev	atgcgctaaggccaaataga
qRT Cdu1 frw	gcctatcaaaggccct
qRT Cdu1 rev	agctagctgtttcatagtctgc
qRT Cdu2 frw	tcatcatagcgctattcctc
qRT Cdu2 rev	agacgtcatctagttgttgtaaaa
qRT Ctr frw	atgggaaggtttcggc
qRT Ctr rev	cacccatttggaattcttta

Table 7 siRNA

siRNA	Ordering number	Company
HIP2/UBE2K	SI03105165, SI04949203	Qiagen
UBE2L3	SI03188562, SI04131575	Qiagen
UBE1	SI00754306, SI03239096	Qiagen
AKIB1	SI02777880, SI00295813	Qiagen
BRPF3	SI00313565, SI00313579	Qiagen
RNF214	SI04214679, SI04325573	Qiagen
P62	GS8878 pool of 4	Qiagen
ATG5	GS9474 pool of 4	Qiagen
Control	D-001810-10-05	Dharmacon
siRNA pool E3 ligase (589 different targets)	Pool of 4 siRNAs per gene	Dharmacon

2.1.5 Antibodies

Table 8 Primary antibodies

Antibody	Origin	Dilution	Company and order number
Actin	Mouse monoclonal	1:5000 WB	Sigma Aldrich #A5441
Ankib1	Rabbit monoclonal	1:200 IF 1:1000 WB	Thermo Fischer #PA5-54506
ATG12	Rabbit monoclonal	1:100 IF	Cell Signaling #2010
Cdu1	Rabbit polyclonal	1:1000 WB 1: IF	Self made (Huber, 2014)
cHSP60	Rabbit polyclonal	1:800 IF	Self made
cHSP60	Mouse polyclonal	1:1000 WB 1:300 IF	Santa Cruz #sc-57840
dCas9	Rabbit polyclonal	1:1000 WB	Clontech #632607
Flag	Mouse monoclonal	1:500 IF	Sigma Aldrich #F1805
GAPDH	Rabbit polyclonal	1:1000 WB	Santa Cruz #sc-25778
GBP1	Rat monoclonal	1:200 IF	Santa Cruz #sc-53857
Lamp1	Mouse monoclonal	1:250 IF	Santa Cruz #sc-18821
LC3	Rabbit polyclonal	1:1000 WB	Sigma Aldrich #I7543
LC3B	Rabbit polyclonal	1:50 IF	Cell signaling #2775
MxA	Mouse monoclonal	1:1000 WB	Dr. Schneider-Schaulies (Schneider-Schaulies, et al., 1994)
NBR1	Mouse monoclonal	1:500 IF	Santa Cruz #sc-130380
NDP52	Mouse monoclonal	1:500 IF	Santa Cruz #sc-376540
OmpA	Rabbit polyclonal	1:1000 WB	Self made
P62	Mouse monoclonal	1.1000 WB 1:1000 IF	Santa Cruz # sc-28359
UBE1	Rabbit polyclonal	1:1000 WB 1:100 IF	Abcam #ab34711
UBE2L3	Rabbit polyclonal	1:1000 WB 1:100 IF	Thermo Fischer #PA5-21598

Ubi FK2	Mouse monoclonal	1:200 IF	Enzo # BML-PW8810-0500
Ubi K48	Rabbit monoclonal	1:500 IF	Millipore #05-1307
Ubi K63	Rabbit monoclonal	1:100 IF	Millipore #05-1308

Table 9 Secondary antibodies and dyes

Antibody	Origin	Dilution	Company and order number
ECL anti-mouse IgG HRP linked	Goat	1:3000	Santa cruz #sc -516102
ECL anti-rabbit IgG HRP linked	Goat	1:3000	Dianova #111-035-144
Anti-rabbit Cy5 linked	Goat	1:300	Dianova #111-175-144
Anti-mouse Cy3 linked	Goat	1:300	Dianova #115-165-146
Anti-mouse Cy5 linked	Goat	1:300	Dianova #115-175-146
Anti-rabbit Cy3 linked	Goat	1:300	Dianova #111-165-144
Anti-rat Cy2 linked	Goat	1:300	Dianova #112-225-143

2.1.6 Enzymes, Markers and Kits

Table 10 Enzymes

Enzyme name	Supplier
Taq DNA Polymerase	Genaxxon
Reprofast DNA Polymerase	Genaxxon
Phusion High-Fidelity DNA Polymerase	Thermo Fischer
T4 DNA Ligase	Thermo Fischer
DNase I	Thermo Fischer
Restriction enzymes	Thermo Fischer

Table 11 Marker and Ladders

Name	Supplier
GeneRuler™ 1 kb DNA ladder	Thermo Fischer
PageRuler™ Prestained Protein Marker	Thermo Fischer

Table 12 Kits

Name	Supplier
GeneJET™ Gel Extraction Kit	Thermo Scientific
NucleoBond®PC100	Machery Nagel
NucleoSpin®Plasmid	Machery Nagel
miRNeasy Mini Kit	Qiagen
miScript II RT Kit	Qiagen
RevertAid First Strand cDNA Synthesis Kit	Thermo Fischer
RNase-free DNase Set	Qiagen

2.1.7 Antibiotics

Table 13 Antibiotics

Name	Dilution	Comment
Ampicillin (Amp)	100 µg/ml	*E.coli* selection
Anhydrotetracyclin (AHT)	1 µg/ml eukaryotic cells 50 ng/ml prokaryotic cells	Induction of protein expression
Blasticidin	4.5 µg/ml	Selection of transfected cells
Chloramphenicol (Cam)	20 µg/ml	*E.coli* selection
Cycloheximide (CHX)	1 µg/ml	Selection of transformed *C. trachomatis*
Hygromycin	250 µg/ml	Selection of transfected cells
Penicillin G (PenG)	1 U/ml	Selection of transformed *C. trachomatis*
Zeocin	350 µg/ml	Selection of transfected cells

All antibiotics were purchased from Sigma Aldrich or Roth.

2.1.8 Buffers and Medium

Table 14 Bacterial culture medium and buffers

Buffer/medium	Ingredients
LB medium	10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl
LB agar plates	10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 15 g/l agar
TfBI	30 mM $C_2H_3KO_2$, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$, 15 % (v/v) glycerin, pH 5.8
TfBII	10 mM MOPS, 75 mM $CaCl_2$, 10 mM KCl, 15 % (v/v) glycerin, pH 7.0
SOC medium	2 % (w/v) bacto-tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose
SPG buffer	75 g/l sucrose, 0.52 g/l KH_2PO_4, 1.22 g/l Na_2HPO_4, 0.72 g/l L-glutamic acid, pH 7.4

Table 15 Buffer for biochemical methods

Buffer	Ingredients
SDS upper buffer	500 mM Tris HCl pH 6.8, 0.04 % (w/v) SDS
SDS lower buffer	1.5 M Tris HCl pH 8.8, 0.04 % (w/v) SDS
10 % SDS lower gel solution	for 10 ml: 2.5 ml SDS lower buffer, 3.4 ml 30 % acrylamide, 4.1 ml H2O, 75 µl 10 % APS, 7.5 µl TEMED
13 % SDS lower gel solution	for 10 ml: 2.5 ml SDS lower buffer, 4.3 ml 30 % acrylamide, 3.2 ml H2O, 75 µl 10 % APS, 7.5 µl TEMED
upper gel solution	for 10 ml: 2.5 ml SDS upper buffer, 1.25 ml 30 % acrylamide, 6.25 ml H2O, 100 µl 10 % APS, 20 µl TEMED
SDS sample buffer (2X)	100 mM Tris HCl pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol, 1.5 % (v/v) β-mercaptoethanol, bromophenol blue
1x SDS-PAGE Running buffer	3.03 g/l Tris, 14.41 g/l glycine, 1.0 g/l SDS
1x Semi Dry Transfer buffer	192 mM glycine, 0.1 % (w/v) SDS, 25 mM Tris, 20 % (v/v) methanol
1x TBS-T	6.05 g/l Tris, 8.75 g/l NaCl, 0.5 ml/l Tween20, adjust to pH 7.5 with HCl
IB blocking solution	5 % (w/v) dry milk powder or BSA in 1x TBS-T
ECL solution 1	100 mM Tris HCl pH 8.6, 2.5 mM Luminol, 0.4 mM p-coumaric acid

ECL solution2	100 mM Tris HCL pH 8.6, 0.02 % H_2O_2
50x TAE	242 g/l Tris, 57.1 ml/l acetic acid, 37.2 g/l EDTA

Table 16 Buffer for immunofluorescence and TEM

Buffer	Ingredients
4 % PFA/sucrose	4 % (w/v) PFA, 4 % (w/v) sucrose in 1x PBS, adjust to pH 7.4
Permeabilization solution	0.02 % (v/v) Triton X-100 in PBS
Blocking solution	2 % (v/v) FCS in PBS
Mowiol	2.4 g Mowiol 4-88, 6 g glycerol, 6 ml H2O, 12 ml 0.2 M Tris HCl pH 8.5
TEM fixation solution	2.5 % glutaraldehyde, 50 mM KCl, 50 mM cacodylate, pH 7.2

Table 17 Medium and supplement for cell culture

Name	Supplier/ingredients
2x HBS	50 mM Hepes pH 7.05, 140 mM NaCl, 1.5 mM Na2HPO4
$CaCl_2$	Sigma Aldrich
Chloroquine	25 mM in PBS, Sigma Aldrich
Collagen	2 mg/ml in 0.25 % acetic acid, Thermo Fischer
DMEM	Sigma Aldrich
DPBS	GIBCO
FCS	PAA
Hanks' Balanced Salt Solution (HBSS)	Gibco
Low serum growth supplement	Thermo Fischer
Medium 200	Thermo Fischer
OptiMEM® reduced serum medium	GIBCO
RPMI 1640	GIBCO
Stocking medium	FCS +10 % (v/v) DMSO
TrypLE™ Express	GBICO

2.1.9 Chemicals

Table 18 Chemicals

Name	Supplier
Acrylamid Rotiphorese Gel 30 (37.5:1)	Roth
Albumin Fraktion V (BSA)	Roth
Alexa Fluor 555 Phalloidin	Thermo Fischer
Ammonium persulfate (APS)	Merck
Bafilomycin A1	Thermo Fischer
Chloroform	Roth
cOmplete EDTA free protease inhibitor cocktail	Roche
Coumaric acid	Sigma Aldrich
DAPI	Sigma Aldrich
Dimethyl sulfoxide (DMSO)	Roth
Dry milk powder	Oliver Öhrlein
Ficoll® Paque Plus	Sigma Aldrich
FITC Dextran 10 kDa	Sigma Aldrich

IFNα	Sigma Aldrich
IFNγ	Sigma Aldrich
Intas HD Green	Intas
Isopropyl-β-D-thiogalactopyranosid (IPTG)	Roth
Lipofectamine RNAiMAX	Thermo Fischer
Loading dye 6 x	Thermo Fischer
Luminol	Roth
Polyethylenimine (PEI)	Polyscience
QIAzol Lysis Reagent	Qiagen
RestoreTM Plus Western Blot Stripping buffer	Thermo Fischer
SYBR Green	VWR
Tetramethylethylenediamine (TEMED)	Fluka Analytica
TNFα	Cell Signaling
Transferrin-CF448	Hölzel Diagnostika
Triton X-100	Roth
Tryptophan	Sigma Aldrich
Tween 20	Roth

2.1.10 Technical equipment

Table 19 Technical equipment

Equipment	Supplier
2720 Thermal Cycler	Applied Biosciences
Autoclave	WEBECO, Sytec VX 150
AvantiTM J-25T centrifuge	Beckham Coultier
Chemiluminescence camera system	Intas
Cold centrifuge 5417R	Eppendorf
DMIL light microscope	Leica
Electric balance ABS-80-4	Kern
FACSaria III	BD
Gene Pulser™ electroporator	Biorad
Hera Cell 240i incubator	Thermo Fischer
ImageScanner III Labscan™ 6.0	GE Healthcare
Megafuge 1.0R	Heraeus
NanoDrop 1000 spectrophotometer	Peqlab Biotechnology
Operetta LCSTM	Perkin Elmer
PerfectBlue Dual Gel Twin PAGE chambers™	Peqlab Biotechnology
PerfectBlue™ Semi-Dry Elektroblotter	Peqlab Biotechnology
pH electrode SenTix	WTW series inolab
Plate reader infinite 200	Tecan
Shaker TR125	Infors HT
Sonifier	Branson VWR
Spectrometer Ultrospec 3100 pro	Amersham Bioscience
Step One Plus real-time PCR system	Applied Biosystems
TCS SPE confocal microscope	Leica
Thermal cycler 2720	Applied Biosystems
Thermo mixer comfort	Eppendorf
Transmissionselectronmicroscope (EM900)	Carl Zeiss

2.1.11 Software

Windows 7, Office 2010, EndNote X7, Corel Draw X5, LAS AF confocal microscopy software, ImageJ, LabImage Chemostar (Intas), Codon Code Aligner 3.7.1, Serial Cloner 2.5, Harmony® High Content Imaging and analysis Software (Perkin Elmer), Step One Software v2.3, Seaview 3.2.

2.2 Methods

2.2.1 DNA Cloning methods

Polymerase Chain Reaction (PCR)

To obtain DNA fragments for cloning PCR was performed.

5 µl	Buffer (10x)
1 µl	dNTPs (10 mM)
1µl	Primer frw (10 mM)
1µl	Primer rev (10 mM)
0.25 µl or	Reprofast DNA Polymerase or
0.5 µl	Phusion High-Fidelity DNA Polymerase
50 ng	Template DNA
Fill up to 50µl	H_2O

The PCR program started with an initial separation step at 94°C for 5 min. The following cycle was repeated 35 times. First DNA strands were separated at 94°C for 30 sec, before primer could anneal at 58°C for 30 sec. The elongation of the PCR fragments was performed at 72°C and took 1 min/kb for Reprofast or 30 sec/kb for Phusion High-Fidelity DNA polymerase. Finally the PCR tubes were incubated at 72°C for 5 min to fill up all PCR products before the tubes were cooled down to 4°C. The PCR amplicon was loaded onto an agarose gel to check for DNA bands.

Colony PCR

In order to identify transformed bacteria from untransformed Colony PCR was performed. Single colonies were picked, diluted in 20 µl H_2O and heated up to 94°C for 10 min to release DNA into the supernatant. Cells were centrifuged at 13 000 g for 1 min to separate the cell debris from the DNA. 2.5 µl of supernatant was mixed with following ingredients.

2.5 µl	Buffer (10x)
0.5 µl	dNTP (10 mM)
0.5 µl	Primer frw (10 mM)
0.5 µl	Primer rev (10 mM)
0.25 µl	Taq DNA Polymerase
18.25 µl	dH_2O

Double strands were initially separated by 94°C for 5 min followed by a 25 times repeated cycle of 94°C for 30 sec, 58°C for 30 sec to anneal the primer to the template and 72°C accordingly to the length of the PCR product (1 min/kb for Taq DNA Polymerase). The PCR program was completed with a last elongation step of 5 min at 94°C before cooling down to 4°C. The PCR product was checked for bands in gel electrophoresis.

Gel electrophoresis and DNA purification

In order to obtain a 1 % agarose gel 1 g agarose was heated up in 100 ml 1x TAE buffer. After cooling down to approximately 40°C 5 µl HD Green was added to the gel and purred into an appropriate gadget. PCR products were mixed with 6x loading dye and loaded onto the agarose gel. The bands were separated by applying 120 V and visualized under UV light. Bands were cut out under UV light and DNA

was purified using the GeneJET™ Gel Extraction Kit as described by the manufacturer. DNA amount was measured using the NanoDrop 1000 spectrometer.

Plasmid isolation

Plasmids were isolated from bacterial culture using the NucleoSpin®Plasmid kit. Therefore, 5 ml overnight bacterial culture was centrifuged at 13 000 g for 1 min and the pellet was used as instructed by the manufacturer. To obtain larger amounts of plasmids the NucleoBond®PC100 kit was used. According to the manufacturer 50 ml of bacterial overnight culture was centrifuged at 4000 g for 10 min and continued as described in the manual. DNA was used for cloning or transfection of cells and bacteria.

Restriction of plasmids and PCR amplicon

In order to obtain single strand overhangs for cloning, plasmids and PCR products had to be digested with restriction enzymes. Usually 1 µg plasmid or the whole PCR amplicon was incubated with 1 U of the restriction enzyme and the corresponding buffer at 37 °C for 1 h. The digestion solution was purified using the GeneJET™ Gel Extraction Kit following the manufacturer's instructions.

2.2.2 Cell culture methods

Cell cultivation

HeLa 229, McCoy and PentaKO cells as well as the parental strain were grown in RPMI-1640 supplemented with 10 % FCS. Murine Mef cells proliferate in 10 % FCS DMEM and primary Fimb cells were cultivated in collagen coated flasks with 20 % FCS RPMI 1640. Endothelial HUVEC cells were grown in Medium 200 supplemented with low serum growth supplement. All cells were cultivated at 37°C and 5 % CO_2 and were splitted every 2-3 days into T75 flasks or microplates as needed. Therefore, cells were washed with PBS once and incubated with 1 ml trypsin until cells detached. Fresh medium containing FCS was used to stop the trypsin and cells were distributed on new flasks or microplates.

Stock preparation of cells

80 % confluent cells in T75 flasks were detached from the flask as described above. Cells were taken up in medium and pelleted for 10 min at 1000 g. The pellet was re-suspended in 10 % DMSO in FCS and gradually frozen at -80°C before transfer into the liquid nitrogen tank for long term storage.

Transfection of plasmid DNA

Cells were seeded in 12-well microplates. The next day 2 µl transfection reagent Pei and 1 µg plasmid DNA were diluted in 50 µl OptiMEM respectively. After 5 min incubation at room temperature the two solutions were combined and incubated at room temperature for 20 min. The medium of the cells was reduced to 400 µl 5 % FCS RPMI 1640 and the transfection mix was trickled on the cells. After 5 h the medium was again exchanged for 10 % FCS RPMI 1640. In case of inducible plasmids the gene expression was triggered by addition of 1 µg/ml Dox for uninfected and 1 µg/ml AHT for *Chlamydia* infected cells.

Single cell selection

HeLa 229 cells were infected with a multiplicity of infection (MOI) of 0.1 with *C. trachomatis* pCRISPRi:gCdu1 III and cultivated for 24 h. Cells were trypsinized, pelleted and re-suspended in PBS. Single infected cells were identified with the FACSaria III and sorted in a 96-well plate. The infected single

cell was then combined with HeLa 229 cells with the result that infectious EBs could infect new cells and proliferate. For further selection the cultivation was performed in the presence of PenG. Procedure was repeated for *C. trachomatis* pCRISPRi:gCdu1III A8 pool and resulting single clones positive for dCas9 expression were called *C. trachomatis* pCRISPRi:gCdu1III A8.2 and A8.3 respectively.

siRNA knock-down and screen

For the siRNA knock-down screen HeLa 229 cells were seeded with a density of 1,8 *10^3 cells per well in 96-well microplates and incubated over night at 37 °C and 5 % CO_2. For transfection siRNA was diluted in 10 µl OptiMEM to a concentration of 300 nM and incubated for 5 min. From the transfection reagent Lipofectamine RNAiMAX 0.2 µl per well was mixed with 10 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 80 µl 5 % FCS RPMI 1640 medium. The siRNA Lipofectamine mixture was added to the cells in triplicates to a final concentration of siRNA of 30 nM. After 7 h the medium was exchanged with fresh 5 % FCS RPMI 1640 medium. Two days later the transfected cells were infected with *Ctr* Tn-*cdu1* or *Ctr* WT with an MOI of 0.8 for 3 h before medium was exchanged to 5 % FCS RPMI 1640. For immunofluorescence staining preparation cells were washed with DMSO and fixed with 4 % PFA 24 hpi for 20 min. After staining of the cells, plates were analyzed by the automatic Operetta microscope (Perkin Elmer). For each well 20 pictures were taken automatically and analyzed. Number of cells, inclusions and p62 dots at the inclusion were counted and number of p62 dots per inclusion were calculated for each well.

Apoptosis assay

Chlamydia are known to inhibit apoptosis in order to complete their live cycle in the host cell. Active *Chla*DUB1 was found to have an important function in the prevention of apoptosis (Fischer et al, 2017). In order to check the ability to inhibit apoptosis in cells lacking active *Chla*DUB1 HeLa 229 cells were seeded in 12-well multiplates. When cells reached a confluency of 60 % cells were infected with *C. trachomatis* pTet:GFP, *C. trachomatis* pCRISPRi:gCdu1III A8.2 or *Ctr* Tn-*cdu1*, *Ctr* WT at an MOI of 1 or left uninfected for 16 h. For apoptosis induction medium was then supplemented with 5 µg/ml CHX and 5 ng/ml TNFα or CHX alone as a negative control. After 8 h of induction supernatant was collected, cells were washed with PBS, trypsinized and combined with the supernatant. Cells were pelleted by centrifugation at 600 g for 5 min at 4°C and re-suspended in SDS buffer. Apoptosis resistance was investigated by the cleavage of PARP in immunoblot.

Neutrophil isolation

Human neutrophils from peripheral blood were isolated by Franziska Solger. First 10 ml blood was taken from a donor and diluted 1:4 with PBS. In a 50 ml falcon 10 ml Ficoll® was poured on top of 10 ml PBS before the blood was carefully layered on top of the Ficoll®. The falcon was centrifuged at 400 g at 22°C for 30 min without brakes so that a gradient could form. The upper yellow plasma section as well as the cloudy phase and the Ficoll® were aspirated hence leaving a colorless phase containing granulocytes and a red section with erythrocytes in the falcon. Next 30 ml of PVA was added and carefully pipetted to get a homogenous mixture which was incubated at room temperature for 45 min. Most erythrocytes sank to the bottom leaving the neutrophils in the supernatant. Carefully the supernatant was harvested and neutrophils were pelleted at 1000 g for 5 min with brakes. The supernatant was discarded and 16 ml of

sterile water was mixed with the neutrophils for 30 sec in order to lyse remaining red blood cells. Lysis was stopped by addition of 4 ml 5x PBS and neutrophils were again pelleted at 100 g for 5 min before the supernatant was discarded and purified neutrophils were re-suspended in 1x HBSS. Cells were directly used for infection.

2.2.3 RNA methods

RNA isolation and cDNA synthesis

RNA was isolated from infected 6-well microplates using the miRNeasy Mini Kit. 700 µl QIAzol Lysis Reagent was added to the cells and incubated at room temperature for 5 min. 140 µl chloroform was added, mixed and incubated or another 2 min. After 15 min of centrifugation (4°C, 12.000 g) the aqueous phase was mixed with 100 % ethanol and transferred to the binding column. Bound DNA was digested with the RNase-free DNase Set. 10 µl DNase I stock solution in 70 µl RDD buffer was incubated directly on the column for 30 min at room temperature. The column was washed with RWT buffer and RPE buffer before RNA was eluted in RNase-free water. The purity was verified with the NanoDrop 1000 spectrometer. RNA was transcribed into DNA using the miScript II RT Kit and the HiFlex buffer for bacterial RNA or RevertAid First Strand cDNA Synthesis Kit for eukaryotic RNA.

qRT-PCR

Quantitative RT-PCR was performed with primer pairs listed above. 10 µl SYBR Green was added with 1.8 µl of each Primer (10 µM) and 1.4 µl H_2O. The amount of cDNA used was tested empirically. In the Step One Plus RT PCR system technical triplicates were analyzed and interpreted with the Step One Software v2.3.

2.2.4 Bacterial culture methods

Stock preparation of *E. coli*

In order to gain electrocompetent bacteria liquid overnight culture of *E. coli* was prepared. Therefore, some *E. coli* material was inoculated with 20 ml of LB media and the appropriate antibiotics. The next day the bacteria were grown in 500 ml salt free LB medium to an OD_{600} of 0.8. The bacteria were harvested by centrifugation at 4000 g and 4°C for 10 min. The pellet was carefully washed in ice cold H_2O and centrifuged as before. After disposal of the supernatant the bacteria were re-suspended in 2.5 ml 10 % glycerol, aliquoted in 200 µl aliquots and sored at -80 °C until transformation.

Stocks of transformed bacteria were obtained by mixture of 850 µl bacterial overnight culture with 150 µl 100 % glycerol and immediate freezing at -80°C.

Transformation of *E. coli* using electroporation

Electrocompetent *E.coli* DH5α was chosen for cloning and expression of recombinant plasmids. *E. coli* JM110 lacking Dcm and Dam methylase was taken for plasmid production used for *C. trachomatis* transformation. Therefore, a 200 µl *E. coli* aliquot was thawed on ice and 5 µl ligation solution or 0.5 µl purified plasmid was added. After 10 min incubation time on ice the bacteria was transferred to an electroporation cuvette and transformed applying 2.4 kV, 25 µF and 200 Ω. Bacteria were immediately

mixed with 800 µl pre-warmed SOC medium and incubated at 37 °C and 800 rpm for 1 h. The appropriate antibiotics containing LB-agarplate was coated with the bacteria and incubated over night at 37°C. The next day colony PCR was performed to identify positive clones and bacteria were prepared for storage.

AHT induction of *E. coli*

E. coli transformed with pCRISPRi:gCdu1III were induced with AHT in order to check for successful protein expression. Therefore, a liquid *E. coli* culture supplemented with ampicillin was grown to an OD_{600} of 0.5 before 100 ng/ml AHT was added for 4 h. 500 µl of sample was harvested by centrifugation at full speed for 2 min and re-suspended in SDS sample buffer. In immunoblot protein expression of dCas9 and GFP was analyzed.

Stock preparation of *Chlamydia trachomatis*

For proliferation of *C. trachomatis* a T75 flask of HeLa 229 cells was infected with an MOI of 1 and incubated at 35°C and 5 % CO_2 for 48 h. Infected cells were scraped form the flask and transferred into a falcon containing sterile glass beads. The falcon was vortexed to disrupt the cell wall and to set free the chlamydial particles into the medium. From this crude lysate 1 ml was used to infect a 150 cm^2 dish seeded with HeLa 229 cells (70-80 % confluency). Cells were again incubated as described before for 48 h. In order to get the infectious particles, cells were again scraped form the dish and vortexed together with glass beads. The suspension was centrifuged at 1000 g for 10 min and 4°C until the cell debris was pelleted at the bottom. The infectious supernatant was centrifuged at 30 000 g and 4°C for 30 min. After washing of the pelleted bacteria with ice cold 1x SPG buffer, the suspension was again centrifuged as described before. The harvested pellet was re-suspended in 1 ml 1x SPG buffer per 150 cm^2 dish and pressed through hollow 18 G and 20 G needles to separate bacteria. The infectious liquid was aliquoted and stored at -80°C until further need. To determine the MOI of the new stock a 12-well microplate of HeLa 229 cells was seeded and infected with different amount of bacteria. After the number of inclusions and cells was counted with the help of a bright field microscope 24 hpi the concentration of infectious particles was calculated.

Infection with *Chlamydia strains*

Human and murine cells were seeded as needed and incubated overnight. When cell density reached 70-80 % medium was changed for 5 % FCS medium mixed with the appropriate amount of chlamydial EBs to reach the desired MOI. To facilitate the infection with *Ctr* serovar A, *Ctr* serovar D, *C. muridarum* and *C. pneumoniae* cells containing the infectious medium were centrifuged at 910 g for 1 h at 35°C. For investigation of early infection time points a MOI of 20 was used for HeLa cell infection. The cells were centrifuged at 910 g for 1 h at 4°C in order to synchronize the infection. Cells were then incubated at 35°C and 5 % CO_2 until further investigation.

Chlamydial infection of human neutrophils

Immune cells were isolated as described above and 5*10^5 cells were seeded in a 12-well microplate in triplicates. Cells were centrifuged for 10 min at 1000 g at room temperature to settle down. Subsequent cells were infected with *Ctr* Tn-*cdu1* or *Ctr* WT with an MOI of 10 for 4 h at 35°C. After incubation the cells were centrifuged as before and washed twice with sterile PBS. 500 µl of 10 % FCS RPMI 1640 was added together with sterile glass beads to each well and the plate was carefully shaken until all cells

were lysed. The *Chlamydia* containing supernatant was then used to re-infect a fresh 12-well microplate of HeLa 229 cells. Cells were incubated at 35°C until lysates were taken 24 hpi and chlamydial growth was visualized by immunoblot.

Generation of transformed *Chlamydia trachomatis*

Chlamydial EBs were transformed with pCRISPRi gCdu1III according to the method published by the group of Ian Clarke in 2011 with some changes. 15 µg plasmid was incubated with 1.6 *10^7 IFU EBs in 200 µl $CaCl_2$ buffer for 30 min at room temperature. A T75 flask of McCoy or HeLa 229 cells was washed with PBS trypsinized and washed twice with $CaCl_2$ buffer. The cells were given to the *Chlamydia* and incubated at room temperature for another 30 min with occasional mixing, before distributing them in a T75 flask. 1 U/ml PenG and 1 µg/ml CHX was provided 16 h post transformation. The cells were lysed every 48hpi with sterile glass beads, centrifuged and the supernatant was given to fresh cells with 1 U/ml PenG and 1 µg/ml CHX for propagation. The procedure was repeated until persistent inclusions vanished and active populations grew.

Treatment with inhibitors and chemicals

Primary Fimb or HeLa 229 cells were infected with *C. trachomatis* at an MOI of 0.3 or 1 respectively and incubated for 7-17 h as described. Then 2 µM MLN4924, 10 nM Bafilomycin A1 or the same volume of DMSO as a control was added directly to the cells. For treatment with IFNs HUVEC, Fimb and HeLa 229 cells were also infected as described and incubated with 100 U/ml IFNα, 5 ng/ml IFNγ or 40 µg/ml tryptophan after 6 h of infection. After 24 hpi lysates for immunoblot analysis were taken or cells were fixed with 4 % PFA for immunofluorescence staining.

Chlamydial infectivity assay

Cells were seeded in two 12-well multiplates and grown to a confluency of 70 %. Cells were infected with appropriate *C. trachomatis* strains with a MOI of 1. The first plate was incubated for 24 hpi until lysates for primary infection were taken. For each well of the second plate a lysate was prepared 48 hpi with sterile glass beads, which was used to re-infect another 12-well multiplate seeded with fresh cells. Lysates for immunoblots were taken 24 hpi from the secondary infection and growth was analyzed in immunoblots.

Chlamydial growth behavior

HeLa 229 cells were seeded in 6-well multiplates and infected with *C. trachomatis* pCRISPRi gCdu1 III A8.2 or *C. trachomatis* pTet:GFP. At defined time points 50 ng/ml AHT was added to the cells. After 2 h of incubation lysates were taken as described. In immunoblots the chlamydial HSP60 level was analyzed. For Operetta experiments HeLa cells were seeded in 96-well microplates and infected with *Ctr* WT, *Ctr* pCRISPRi:gCdu1 III A8.2 or A8.3 in triplicates. After 8 hpi cells were treated with 5 µM tetracycline or 50 ng/ml AHT for 20 h. Cells were washed with PBS and fixed with 4 % PFA/sucrose for immunofluorescence staining afterwards. Chlamydial particles were stained with HSP60 antibody (1:1000) and cellular cytoskeleton with Phalloidin (1:300) and analyzed by the Operetta microscope. Average area of cells and inclusions was measured from 6 individual pictures per well.

Inclusion integrity test

The inclusion harbors the chlamydial particles and is the platform for exchange between host cell and

bacteria. In order to test the integrity of the inclusion HeLa 229 and Fimb cells were seeded in 12-well multiplates and infected with *Ctr* Tn-cdu1 or WT with an MOI of 1 for HeLa 229 or 0.3 for Fimb cells respectively. FITC-dextran was diluted 1:200 in 10 % FCS RPMI 1640 and given to the cells 9 hpi for 1 h when medium was exchanged again to 10 % FCS RPMI 1640. The distribution of FITC-dextran was observed from 24 hpi onward using the fluorescent microscope.

2.2.5 Biochemical methods

GFP fluorescence analysis using FACS

In order to check for the presence of fluorescent GFP in *C. trachomatis* pCRISPRi:gCdu1 III A8 and *C. trachomatis* pTet:GFP upon AHT induction, HeLa 229 cells were infected with chlamydial strains at an MOI of 0.5. After 22 hpi cells were treated with 50 ng/ml AHT for 2 h before cells were washed with PBS once and trypsinized. Infected cells were re-suspended in PBS and analyzed for GFP fluorescence using the FACSaria III.

Antibody purification using cyanogen bromide activated sepharose beads

In order to purify self-made antibodies from serum cyanogen bromide activated sepharose beads (CnBr) were used. Therefore, 0.14 g CnBr beads were soaked in 1 mM HCl for 30 min, centrifuged at 600 g for 2 min at room temperature and washed with 0.2 M carbonate buffer. For binding to the beads 100 µg antigen in 0.2 M carbonate buffer was incubated together with the beads at 4°C on a rotary shaker overnight. On the next day the antigen coupled beads were washed with carbonate buffer three times and transferred into 500 mM NaCl in PBS. The antibody containing serum was diluted 1:1 with 7 ml PBS and incubated together with the beads at 4°C at the rotary shaker for another night. The following morning the antigen antibody coupled beads were separated from the serum by loading on a column. The serum was stored at -20 °C for further purification since unbound antibody was still present. The column was washed twice with 500 ml NaCl in PBS. For elution of the antibody 10 ml of 0.1 M glycine with a pH of 2.5 was given to the beads in 500 µl aliquots. Each aliquot was immediately neutralized by addition of 100 µl 1 M Tris HCl pH 7.5. The aliquots were pooled, concentrated and diluted in PBS using the Vivaspin 10 kDa cut off centrifugation tubes. The purified antibody was diluted 1:1 in glycerol, stored at -20°C and tested in immunofluorescence and immunoblotting for working concentrations.

Preparation of lysates for SDS-PAGE

Cells seeded in 12-well multiplates and infected with *C. trachomatis* were prepared for lysates. The supernatant was removed and cells were immediately put on ice to inhibit protease activity and protein degradation. 100 µl of 2x SDS sample buffer was given to the cells, scraped from the bottom and transferred to a fresh tube. The lysates were heated up to 94°C for 10 min and used for SDS-PAGE.

SDS-PAGE and immunoblotting

Proteins were separated with a 10 % or 13 % SDS PAGE and transferred to a PVDF membrane. Membranes were blocked with 5 % dry milk powder in TBS-T and incubated with the primary antibodies anti-Cas9 1:1000, anti-*Chla*DUB1 1:1000, anti-cHSP60 1:1000, anti-beta-Actin 1:5000, anti-GFP 1:1000 , Anti-LC3 1:1000 overnight at 4°C. The membrane was washed with TBS-T for 30 min and incubated with the HRP-coupled secondary antibody 1:3000 in 5 % milk in TBS-T for 1 h. After washing the membrane

was developed with an ECL solution at the Intas Chem HR 16-3200 reader. Quantification was done by ImageJ software.

Indirect immunofluorescence

HeLa 229 or primary Fimb cells were seeded on glass cover slips and infected with *C. trachomatis* as described for the desired time. For fixation cells were washed with PBS and fixed with 4 % PFA/sucrose for 30 min at room temperature or with ice cold methanol for 20 min at -20°C. The cells were permeabilised with 0.2 % Triton X 100 in PBS for another 30 min for HeLa 229 and 15 min for Fimb cells and blocked with 2 % FCS in PBS for 1 h. Primary antibodies were diluted in 2 % FCS/PBS and cells were stained for 1 h in a humid chamber to avoid draining of the cells. After washing of the cells with PBS the secondary antibody Cy2/3/5-ms/rb/rat (Dianova) was added in a 1:300 ratio in 2 % FCS/PBS. Coverslips were washed again with PBS and sticked to a glass slide with Mowiol.

Transferrin-448 staining

HeLa 229 cells were infected with *Ctr* WT and *Ctr* Tn-*cdu1* as indicated. After 24 hpi medium was exchanged for 5 % FCS/RPMI medium for 30 min. Cells were washed with cold PBS once and stained with 50 µM Transferrin-CF448 (Hölzel Diagnostika) in 0.1 % BSA in PBS for 90 min at 4°C in the dark. In order to remove the dye, cells were washed twice with cold PBS and incubated for 0, 15, 30 or 60 min at 35°C before fixation with 4 % PFA/sucrose and staining with cHSP60 as described above.

Transmission electron microscopy

Primary Fimb cells were grown on glass slides and infected with *Ctr* Tn-*cdu1* or *Ctr* WT with an MOI of 0.3. After 24 hpi cells were fixed with TEM fixation solution (2.5 % glutaraldehyde, 50 mM KCl, 50 mM cacodylate) for 1 h at room temperature before washing in 50 mM cacodylate buffer. The glass slide was transferred into 2 % OsO4/cacodylate buffer for 1 h, washed with H_2O and incubated in 0.5 % uranyl acetate overnight. Cells were dehydrated by washing with increasing ethanol concentration from 50 - 100 % and embedded in Epon 812. Samples were cut into sections and analyzed by transmission electron microscopy TEM900 (Zeiss).

3 Results

3.1 CRIPSRi system in *C. trachomatis*

3.1.1 Cloning of pCRISPRi:gCdu1III

The CRISPR system is applicable in various eukaryotic and prokaryotic species promoting the rapid change in gene expression. At the beginning of this thesis there was no reliable knock-out or knock-down system for *C. trachomatis* available. The aim was to establish a CRISPRi-based method for inducible knock-down of chlamydial genes during infection. The plasmid pCRISPRi:gCdu1III expresses the dCas9 protein derived from *S. pyogenes* and the guide RNA (gRNA) which is targeting *Chla*DUB1 in *C. trachomatis* (Figure 3.1 A). The gRNA is constantly expressed and was designed to bind to the *Chla*DUB1 gene shortly after the start codon, adjacent to a PAM motif (Figure 3.1 B). The expression of the dCas9 protein is inducible and under control of a tetracycline inducible promoter. For selection in bacterial cells a beta-lactamase resistance cassette was integrated into the backbone as well as a GFP-CAT cassette for better identification of bacterial transformants (Figure 3.1 A). The plasmid can be replicated in *E. coli*, for replication in *C. trachomatis* the plasmid was fused with the chlamydial SW2 plasmid. Design and cloning of this plasmid was performed by Dr. Annette Fischer.

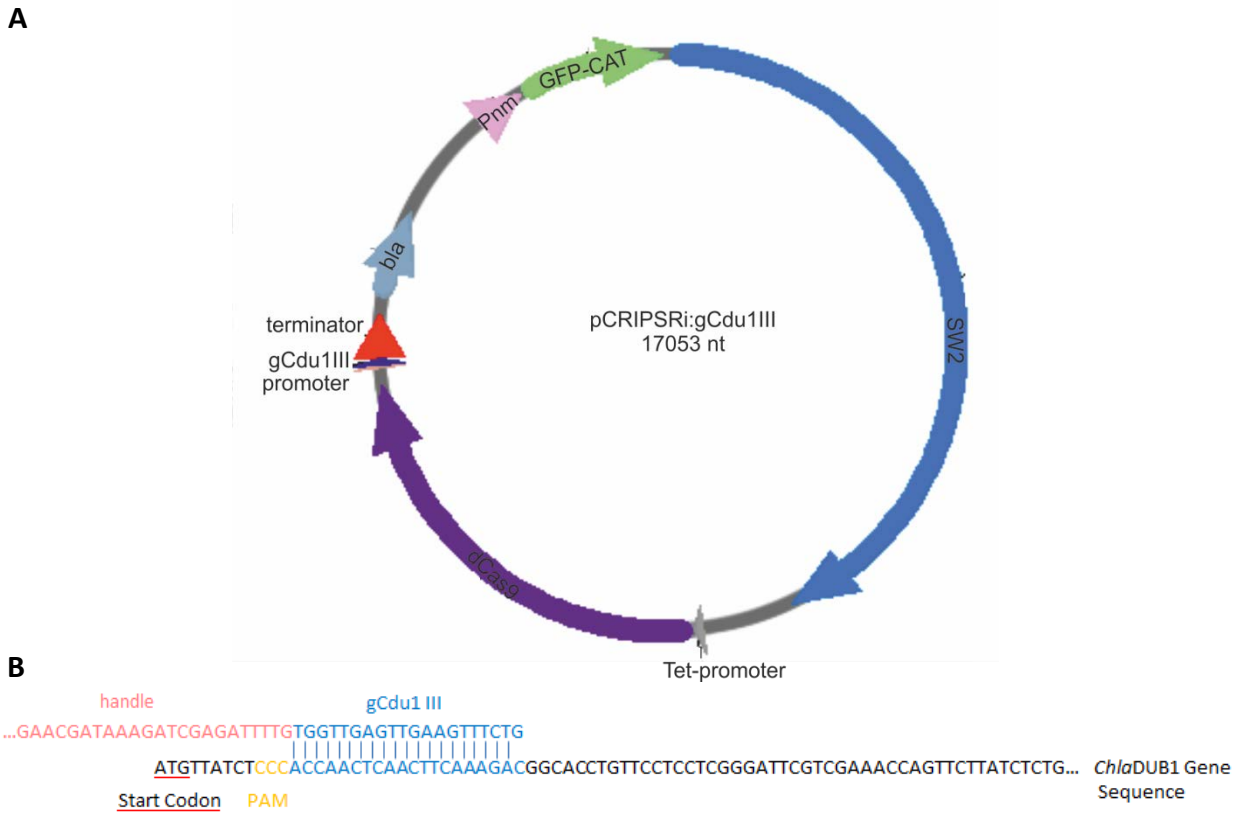


Figure 3.1. A. Graphic map of pCRISPRi:Cdu1III and binding of the gRNA to the *Chla*DUB1 gene. A. The plasmid encodes for a beta-lactamase for selection with ampicillin and penicillin. The GFP-CAT gene is under control of a constantly active promoter

and facilitates selection. The dCas9 gene expression is controlled by a tetracycline inducible promoter. The gRNA gCdu1III targets 20 nt of the *Chla*DUB1 gene and is constantly expressed. For successful transformation into *C. trachomatis* the plasmid was fused with the chlamydial SW2 plasmid, which also harbors the origin of replication. B. Scheme of the binding of the gRNA on the *Chla*DUB1 gene. The gRNA gCdu1III targets 20 nt of the *Chla*DUB1 gene (blue) directly after a PAM sequence (yellow). The gCdu1III sequence is followed by a handle sequence (pink) and subsequent terminator (not shown). The binding of the gRNA takes place in the beginning of the coding region leading to an inhibition of transcription.

3.1.2 The CRIPSRi system in *E. coli* DH5α

In order to test the successful expression of the dCas9 protein under AHT induction pCRISPRi:gCdu1III and pCRISPRi:gGFP plasmids were transformed into *E. coli* DH5α. The plasmid pCRISPRi:gGFP is constructed as show above with a gRNA targeting GFP. Positive *E. coli* transformants were selected using ampicillin and sequence identity of the plasmids was verified by sequencing. *E. coli* liquid cultures were treated with 100 ng/ml AHT for 4 h before lysates were taken and analyzed for dCas9 and GFP expression. The immunoblot reveals the successful expression of GFP and dCas9 protein upon AHT induction in *E. coli* pCRISPRi:gCdu1III (Figure 3.2). The GFP signal in *E.coli* pCRISPRi:gGFP decreases as the gRNA targets the GFP sequence encoded on the plasmid backbone (Figure 3.2).

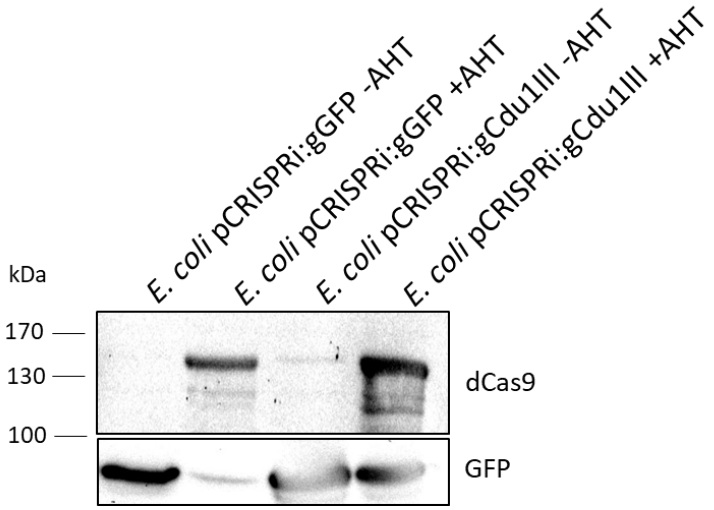


Figure 3.2. Immunoblot analysis of *E. coli* transformed with pCRISPRi:gCdu1III or pCRISPRi:gGFP. Liquid *E. coli* cultures were grown to an OD_{600} of 0.5 and induced with 100 ng/ml AHT for 4 h. Lysates were taken and dCas9 expression and GFP amount was investigated in immunoblot.

3.1.3 Transformation of pCRISPRi:gCdu1III in *C. trachomatis*

Since the pCRISPRi:gCdu1III plasmid successfully expresses dCas9 in *E.coli* the plasmid was transformed into *C. trachomatis* by Dr. Annette Fischer. After several rounds of selection a pool of transformed and untransformed *C. trachomatis* was obtained. To get a pure culture single *C. trachomatis* clones were FACS sorted, grown and again sorted for single clones. Single clones were tested for dCas9 expression by induction with 50 ng/ml AHT 20 hpi for 4 h and analyzed by immunoblot. The clones positive for dCas9 expression were called pCRISPRi:gCdu1III A8 pool for the first sort and pCRISPRi:gCdu1III A8.2 or A8.3 for the second single cell sort. The immunoblot reveals that the initial pool of transformed *C. trachomatis*

expressed full length dCas9 protein as well as a smaller approximately 130 kDa dCas9 protein whereas the double single sorted *C. trachomatis* clones express the truncated dCas9 band and only clone A8 pool expresses traces of the full length protein too (Figure 3.3 A). Repeated induction of *C. trachomatis* pCRISPRi:gCdu1III A8 pool did not show full length dCas9 protein anymore. Renewed screening for single clones expressing full length dCas9 protein was not successful. Sequencing of the pCRISPRi:gCdu1III plasmid out of the A8.3 clone affirms the correct full length DNA sequence for dCas9. As shown in the immunoblot only induction with AHT leads to dCas9 protein expression, proving a rather tight regulation of the promoter (Figure 3.3 B).

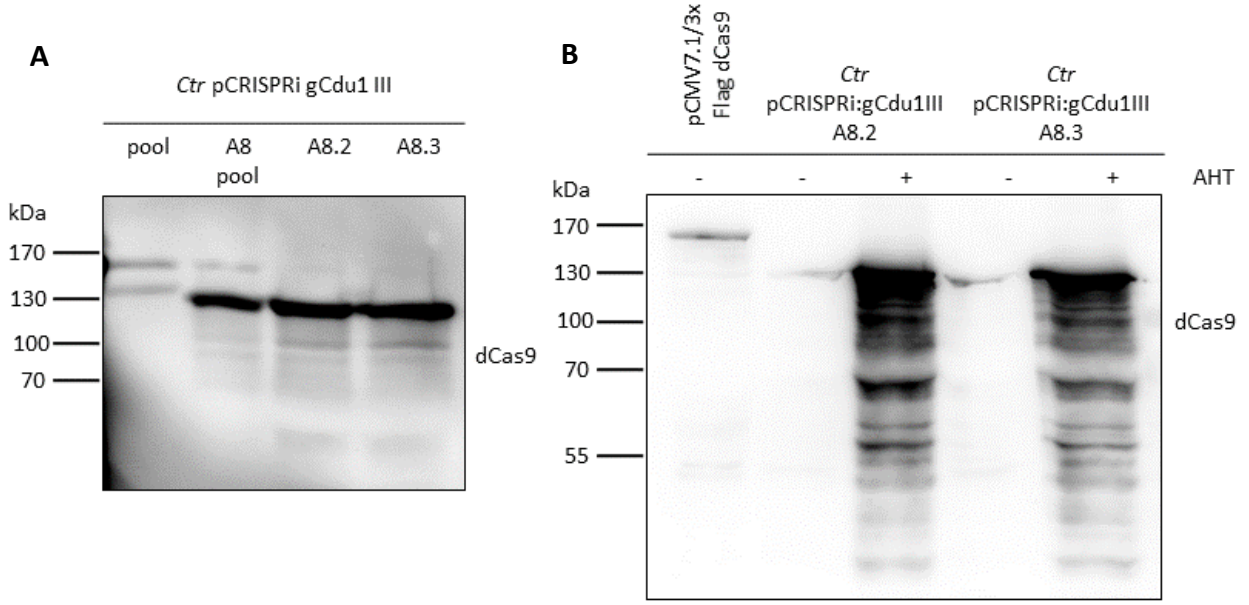


Figure 3.3. DCas9 expression in *Ctr* pCRISPRi:gCdu1III infected HeLa 229 cells. A. HeLa 229 cells were infected with *Ctr* pCRISPRi:gCdu1III pool and the single clones A8 pool as well as A8.2 and A8.3. After 22 h of infection dCas9 expression was induced by addition of 50 ng/ml AHT for 2 h. Lysates were taken and analyzed in immunoblot for dCas9 expression. B. HeLa 229 cells were infected with an MOI of 1 with *Ctr* pCRIPRi:gCdu1III single clones A8.2 or A8.3. After 22 h dCas9 expression was induced by treatment with 50 ng/ml AHT for 2 h. Transfection of Hela cells with pCMV7.1/3xFlag dCas9 served as a positive control for dCas9 size. Lysates were used for immunoblot to investigate dCas9 expression upon induction.

For better selection of transformed bacteria a GFP cassette was integrated into the pCRISPRi:gCdu1III plasmid. In flow cytometry the GFP signal was investigated in uninduced and induced *C. trachomatis* A8 pool. GFP is under control of a constantly active promoter with the result that uninduced bacteria show GFP fluorescence in FACS analysis (Figure 3.4 A left panel, 13.9% GFP positive). The induction with AHT, however, diminishes the GFP fluorescence radically (Figure 3.4 A middle panel, 0.1% GFP positive), which can also be seen in immunoblot (Figure 3.4 B). The reduction in GFP fluorescence is not due to quenching by the addition of AHT since induction of *C. trachomatis* pTet:GFP leads to strong GFP signal (Figure 3.4 A right panel).

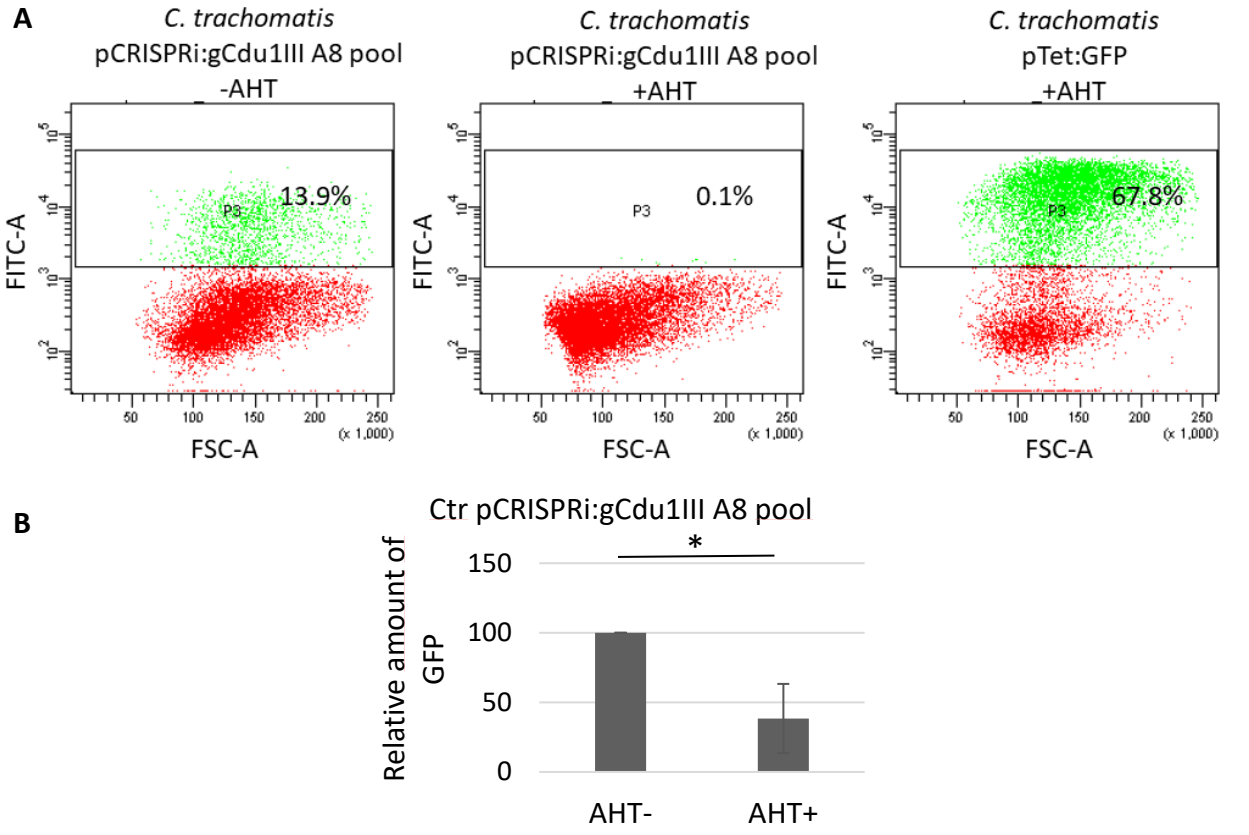


Figure 3.4. Analysis of GFP signal in *C. trachomatis* pCRISPRi:gCdu1III upon AHT induction. A. Flow cytometry analysis of GFP signal in *C. trachomatis* pCRISPRi:gCdu1III A8 pool. HeLa 229 cells were infected with *Ctr* pCRISPRi:gCdu1III A8 pool or *Ctr* pTet:GFP and treated with 50 ng/ml AHT at 20 hpi. Cells were detached and analyzed for GFP fluorescence by flow cytometry 24 hpi. B. Quantification of immunoblots of HeLa 229 cells infected with *C. trachomatis* pCRISPRi:gCdu1III A8 pool and treated with 50 ng/ml AHT for 2 h. Lysates were taken 24 hpi. GFP signal of samples without AHT treatment were set 100 %. Mean values of five independent experiments are shown. * p<0.05

3.1.4 Growth deficiency of *Ctr* pCRISPRi:gCdu1III

Expression and moreover massive overexpression of non-chlamydial proteins in *C. trachomatis* can negatively influence the bacterial growth or development. In order to rule out that the expression of dCas9 protein and the transcription of the gRNA have a negative influence on the replication of *C. trachomatis* a growth assay was performed. Therefore, inclusion size of infected cells treated with or without AHT was analyzed by the automatic Operetta microscope. Treatment with AHT alone does not influence the inclusion size in wildtype *Chlamydia*, whereas the addition of tetracycline, an antibiotic that works as a protein translation inhibitor, restrains chlamydial growth and consequently diminishes the inclusion size (Figure 3.5). In *Ctr* pCRISPRi:gCdu1III single clones A8.2 and A8.3 the induction with AHT impairs the inclusion size significantly (Figure 3.5). *Ctr* pCRISPRi:gCdu1III shows a growth deficiency in the presence of AHT during infection.

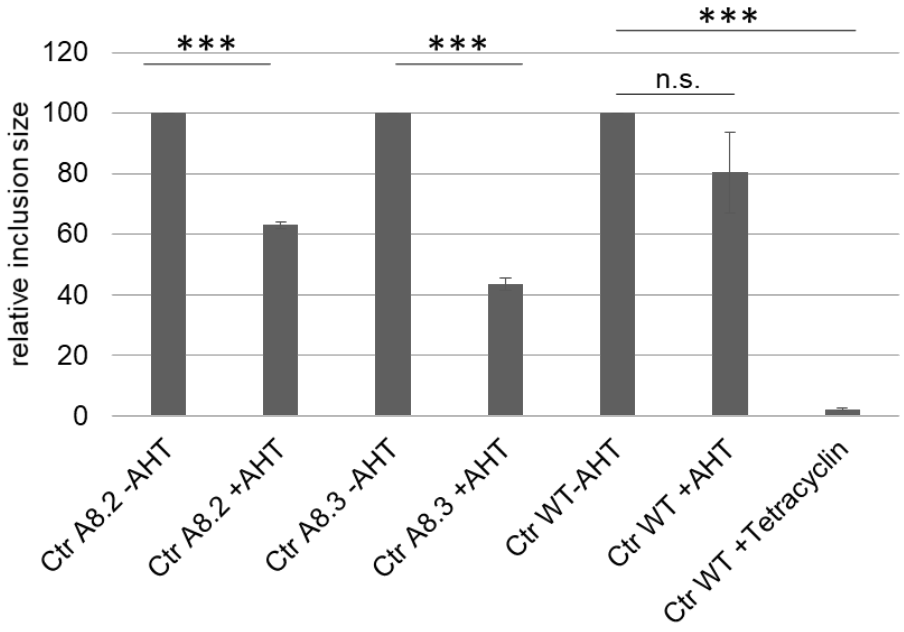


Figure 3.5. Inclusion size of *Ctr* pCRISPRi:gCdu1III decreases in infected cells upon AHT treatment. HeLa 229 cells were infected with *Ctr* pCRISPRi:gCdu1III single clone A8.2 or A8.3 or *Ctr* WT for 8 h before treatment with 50 ng/ml AHT for 20 h. The negative control was treated with 5µM tetracycline 8 hpi. After 28 hpi cells were fixed with 4 % PFA/sucrose and stained for chlamydial HSP60 and Phalloidin. Inclusion size was automatically measured using the Operetta microscope. Inclusion size of chlamydial strains without AHT treatment was set 100 %. Mean values of three independent experiments are shown. n.s. not significant, *** p<0.001

3.1.5 Knock-down of *Chla*DUB1 by CRISPRi

The reason for the expression of the truncated dCas9 protein is not clear, nevertheless, the influence of the CRISPRi system on the *Chla*DUB1 protein level was investigated. Since *Chla*DUB1 is expressed in mid to late phase of chlamydial infection a time course experiment was performed with dCas9 induction using AHT for 2 h at different time points of infection. The chlamydial strain *Ctr* pTet:GFP was used as a control since the expression of GFP is also under control of the Tet-inducible promoter. A truncated dCas9 protein with approximately 130 kDa that lacks around 30 kDa for full length protein is expressed upon induction (Figure 3.6 A). Moreover, the protein level of *Chla*DUB1 decreases significantly when dCas9 is present whereas AHT induction in *Ctr* pTet:GFP infected cells does not influence the protein level of *Chla*DUB1 (Figure 3.6 B). Analysis of immunoblots affirms the significant knock-down of *Chla*DUB1 in single clones A8.2 and A8.3 to 69 % and 49 %, respectively (Figure 3.6 B).

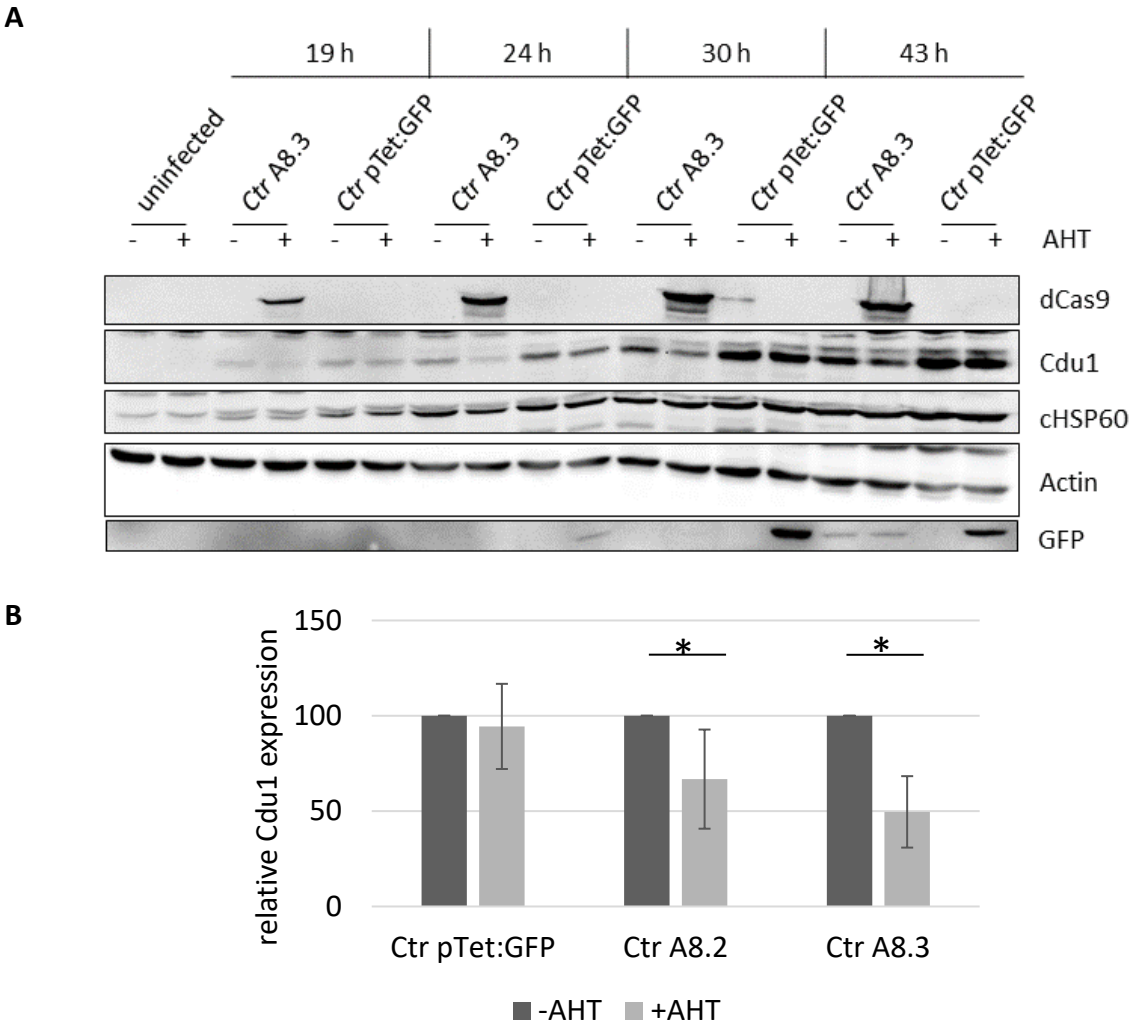


Figure 3.6. Knock-down of *Chla*DUB1 in *Ctr* pCRISPRi:gCdu1III infected cells. A HeLa 229 cells were infected with *Ctr* pCRISPRi:gCdu1III A8.3 or *Ctr* pTet:GFP respectively. 20 hpi cells were treated with 50 ng/ml AHT for 4 h until lysates were taken. Protein abundance was investigated on immunoblots. B. Immunoblots of infected *Ctr* pCRISPRi:gCdu1III A8.2 (Ctr A8.2) and A8.3 (Ctr A8.3) at time point 24 hpi were analyzed according to the *Chla*DUB1 protein amount upon knock-down. Cells infected with *Ctr* pTet:GFP serve as a negative control and were set 100 % for the sample without AHT treatment. Mean values of four independent experiments are shown. * p<0.05.

To assure that the knock-down of *Chla*DUB1 is due to CRISPRi the expression of the gRNA targeting *Chla*DUB1 was investigated. Therefore, the RNA levels of the gRNA, *Chla*DUB1, dCas9 and a chlamydial internal control (OmpA) were analyzed by RT-PCR using the primer pairs qRT gRNA frw, qRT gRNA rev, qRT Cdu1 frw, qRT Cdu1 rev, qRT dCas9 frw, qRT dCas9 rev, qRT Ctr frw and qRT Ctr rev, respectively. Expression levels were normalized to the chlamydial control. The results not only confirm the expression of dCas9 in *Ctr* pCRISPRi:gCdu1III A8 pool and single A8.3 clone but also the expression of the gRNA (Figure 3.7). Corresponding with the previous results the induction of *Ctr* pCRISPRi:gCdu1III with AHT leads to the expression of both the gRNA targeting *Chla*DUB1 and the dCas9 protein leading to the knock-down of *Chla*DUB1 protein level.

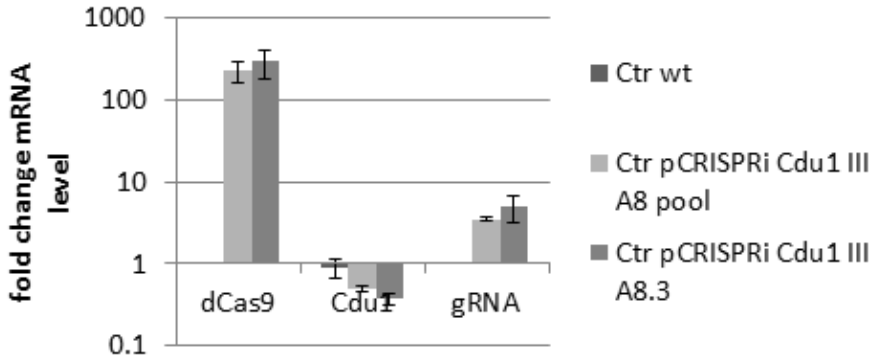


Figure 3.7. Levels of mRNA of dCas9, Cdu1 and the gRNA in *Ctr* WT, *Ctr* pCRISPRi:Cdu1III A8 pool and A8.3 upon AHT treatment. HeLa cells were infected with *Ctr* WT or *Ctr* pCRISPRi:Cdu1III A8 pool or single clone A8.3 for 22 h before addition of 50 ng/ml AHT. 24 hpi RNA was harvested, cDNA transcribed and qRT-PCR was performed. The fold change of the dCas9, Cdu1 and gRNA expression is shown above.

3.1.6 Influence of *Chla*DUB1 knock-down on apoptosis resistance

Knock-down of *Chla*DUB1 was shown to work upon AHT induction in *Ctr* pCRISPRi:gCdu1III even though up to 69 % of *Chla*DUB1 protein is still present in *Chlamydia* (Figure 3.6 B). Some targets of *Chla*DUB1 were recently published. Apart from the inhibitory subunit of NFκB, IκBα (Le Negrate, et al., 2008), also the anti-apoptotic protein Mcl-1 was shown to be stabilized by deubiquitination through *Chla*DUB1 (Fischer, et al., 2017). Immunoblot of Mcl-1 after knock-down of *Chla*DUB1 in infected cells did not show a reduction in abundance of the anti-apoptotic protein (Figure 3.8 A). In order to check whether the knock-down of *Chla*DUB1 still leads to an increase in apoptosis an apoptosis assay was performed. Uninfected cells quickly undergo apoptosis upon treatment with TNFα and CHX, regardless of AHT presence (Figure 3.8 B+C). In contrast cells are protected against apoptosis by infection with *Ctr* pTet:GFP and *Ctr* pCISPRi:gCdu1III A8.2 even when treated with AHT (Figure 3.8 B+C). The slight knock-down of *Chla*DUB1 in *Ctr* pCISPRi:gCdu1III A8.2 infected cells does not lead to Mcl-1 degradation and apoptosis sensitivity.

The establishment of the *C. trachomatis* pCRISPRi:gCdu1III was performed to gain bacteria that lack active *Chla*DUB1 protease. Experiments show the expression of a truncated dCas9 protein upon induction and reduction of *Chla*DUB1 protein. However, in the meantime a transposon mutant called *Ctr* Tn-*cdu1* obtained by Prof. Dr. Scott Heftys group was available that completely lacks active *Chla*DUB1. Therefore, the subsequent experiments were performed with this mutant.

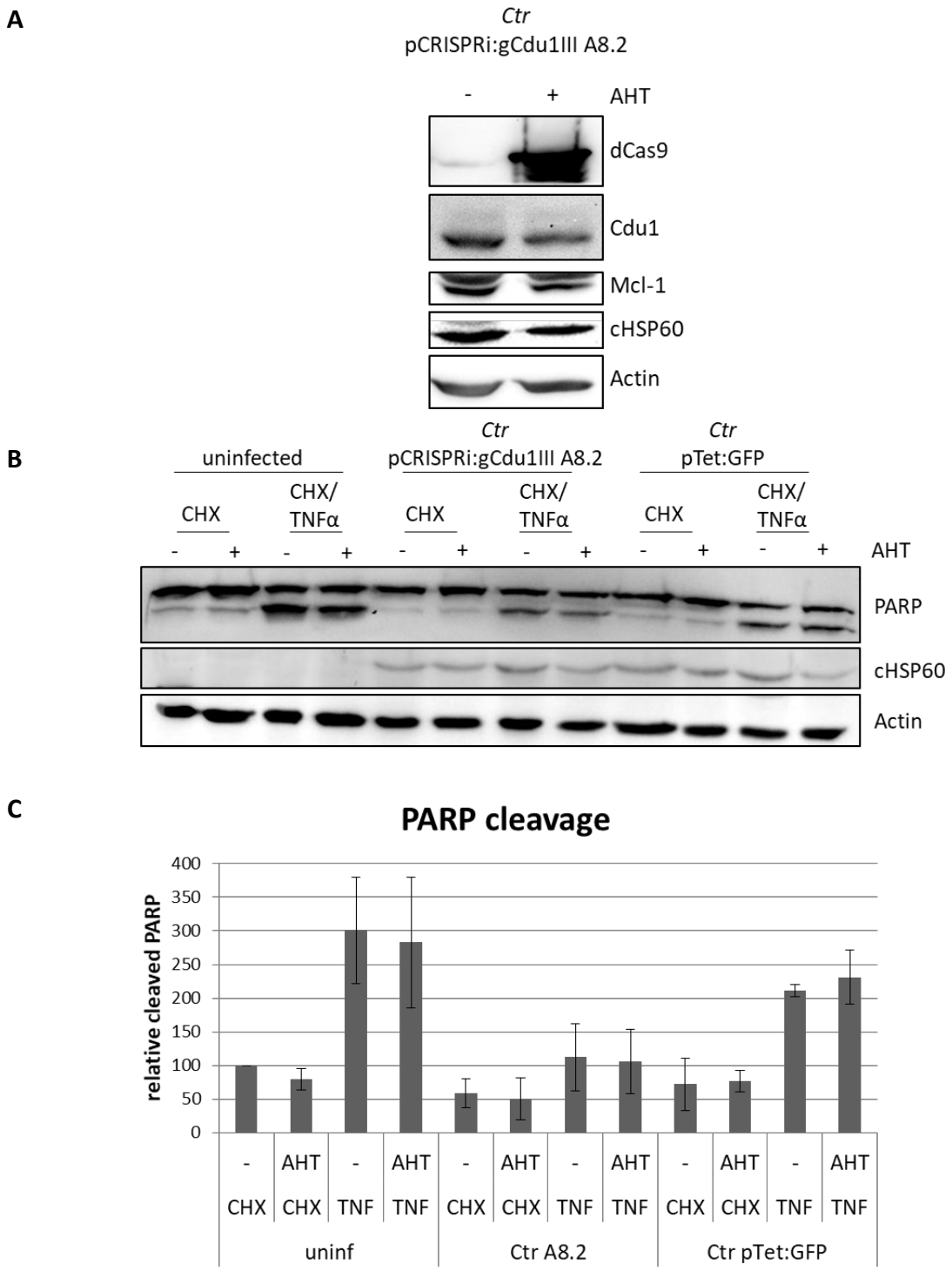


Figure 3.8 Apoptosis assay in *Ctr* pCRISPRi:gCdu1III infected HeLa 229 cells. A. HeLa 229 cells were infected with *Ctr* pCRISPRi:gCdu1III A8.2 for 24 h and protein induction was started with AHT for 4 h. Protein level of dCas9, Cdu1, Mcl-1, chlamydial HSP60 and Actin was checked with and without AHT treatment in immunoblot. B. HeLa 229 cells were infected with *Ctr* pCRISPRi:gCdu1III A8.2 or *Ctr* pTet:GFP for 16 h before apoptosis was induced by addition of 5 ng/ml TNFα and 5 µg/ml CHX or CHX alone. After 8 h of induction lysates were taken and analyzed by immunoblot for cleaved PARP. C. Quantification of cleaved PARP in apoptosis assays in HeLa cells infected with *Ctr* pCRISPRi:gCdu1III A8.2 or *Ctr* pTet:GFP. Uninfected cells treated without AHT but with CHX were set 100 %. Mean values of three independent experiments are shown.

3.2 Autophagy is induced in *C. trachomatis* Tn-cdu1

3.2.1 Recruitment of ubiquitin and ubiquitin-binding proteins in *C. trachomatis* Tn-*cdu1* infection

CRISPR experiments to knock-down *Chla*DUB1 were stopped when the chlamydial mutant *Ctr* Tn-*cdu1* became available that completely lacks active protease. The chlamydial mutant *Ctr* Tn-*cdu1* is lacking an active *Chla*DUB1 enzyme and only expresses a truncated *Chla*DUB1 protein due to transposon insertion in the sequence of the active site during infection of HeLa cells (Fischer, et al., 2017). The expression of truncated *Chla*DUB1 can also be visualized by immunofluorescence (IF) and was tested in infection of primary Fimb cells. Only in *Ctr* WT the *Chla*DUB1 protein can be clearly detected around the chlamydial inclusion, for *Ctr* Tn-*cdu1* a very faint signal co-localizing with the chlamydial HSP60 signal is detected (Figure 3.9).

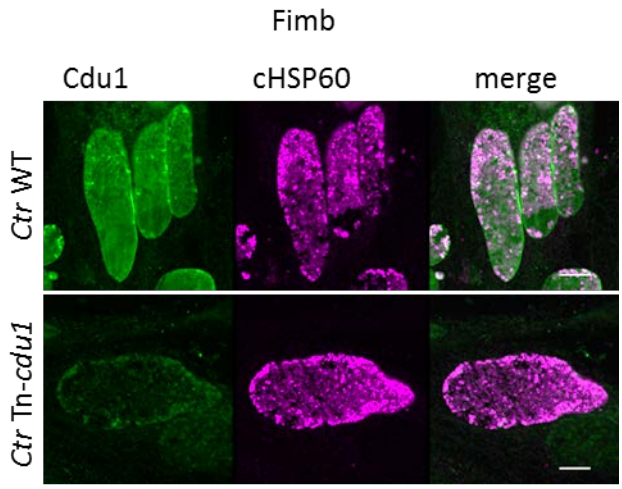


Figure 3.9. *Chla*DUB1 of *Ctr* Tn-*cdu1* does not localize with the inclusion. IF of *Chla*DUB1 distribution in primary Fimb cells infected with *Ctr* WT or *Ctr* Tn-*cdu1*. Fimb cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* with a MOI 0.3 for 24 h. Cells were fixed with 4 % PFA/sucrose and stained for *Chla*DUB1 (green) and chlamydial HSP60 (magenta). Scale bar, 10 µm.

As a deubiquitinase (DUB) *Chla*DUB1 removes the posttranslational modification, ubiquitin, form substrates. If the DUB is inactive a change in ubiquitination pattern will be the consequence. Hence we showed that infection with *Ctr* Tn-*cdu1* leads to accumulation of K48 ubiquitination around the inclusion in HeLa (Figure 3.10 A) (Fischer, et al., 2017) but also in primary Fimb cells (Figure 3.10 C). Further investigation also revealed K63 ubiquitin linkage around the *Ctr* Tn-*cdu1* inclusion in both HeLa and primary Fimb cells (Figure 3.10 B+D).

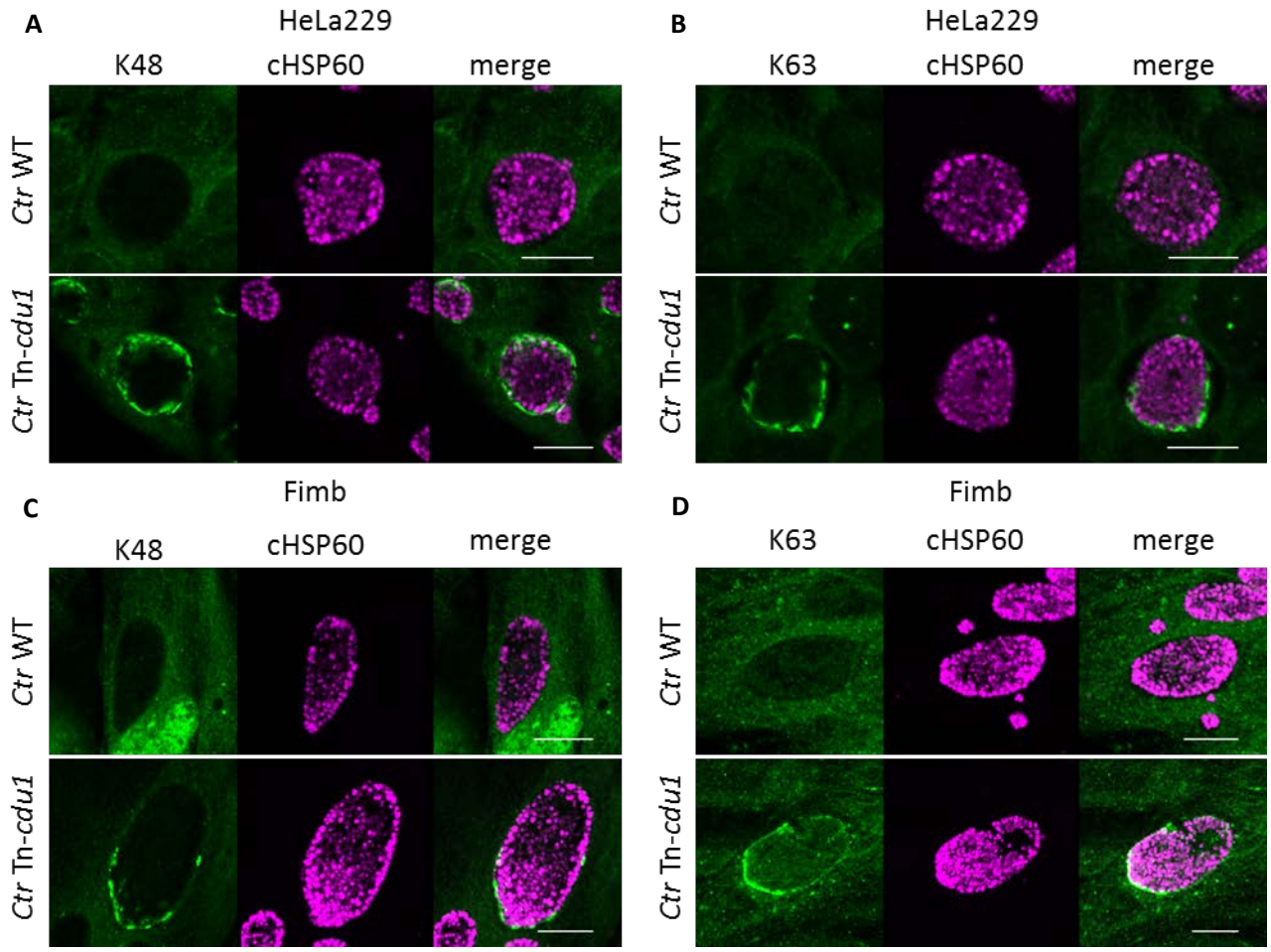


Figure 3.10. Ubiquitin chains K48 and K63 are at the inclusion of *Ctr* Tn-*cdu1*. HeLa (A+B) and Fimb (C+D) cells were infected with *Ctr* WT and *Ctr* Tn-*cdu1* with an MOI of 1 for HeLa and MOI of 0.3 for Fimb cells. After 24 h of infection cells were fixed with ice cold MetOH for 20 min. Cells were stained for ubiquitin chains K48 (A+C) or K63 (B+D) (green) and chlamydial HSP60 (magenta). Scale bar, 10 µm.

The different linkage types of ubiquitination serves as an information tag, determining the fate of the modified protein. Conjugation of ubiquitin via K48 is often a signal for proteasomal degradation (Chau, et al., 1989), whereas a K63 linkage is involved in different signaling pathways and in autophagy (Olzmann and Chin, 2008). In autophagy, the damaged organelle is usually ubiquitinated before autophagy markers get recruited and a phagophore separates the target from the cytosol. The destruction of the phagophore cargo happens through fusion with endosomes and lysosomes (Dikic, 2017). Hence, the presence of autophagy receptors on the inclusion of *Ctr* Tn-*cdu1* were investigated. IF of sequestosome 1, hereafter referred to as p62, revealed that p62 is accumulated around the inclusion of *Ctr* Tn-*cdu1*, but not in wild type *Chlamydia* in HeLa and in Fimb cells (Figure 3.11 A). Analysis of IF pictures indicates that 75 % of all *Ctr* Tn-*cdu1* inclusions in HeLa cells are decorated with p62 at 24 hpi (Figure 3.11 B). Since *Chla*DUB1 expression starts in mid phase of infection and lasts until late phase a time course experiment was performed. P62 protein levels support the previous findings that p62 is accumulated in *Ctr* Tn-*cdu1* infected cells and increases with time (Figure 3.11 C).

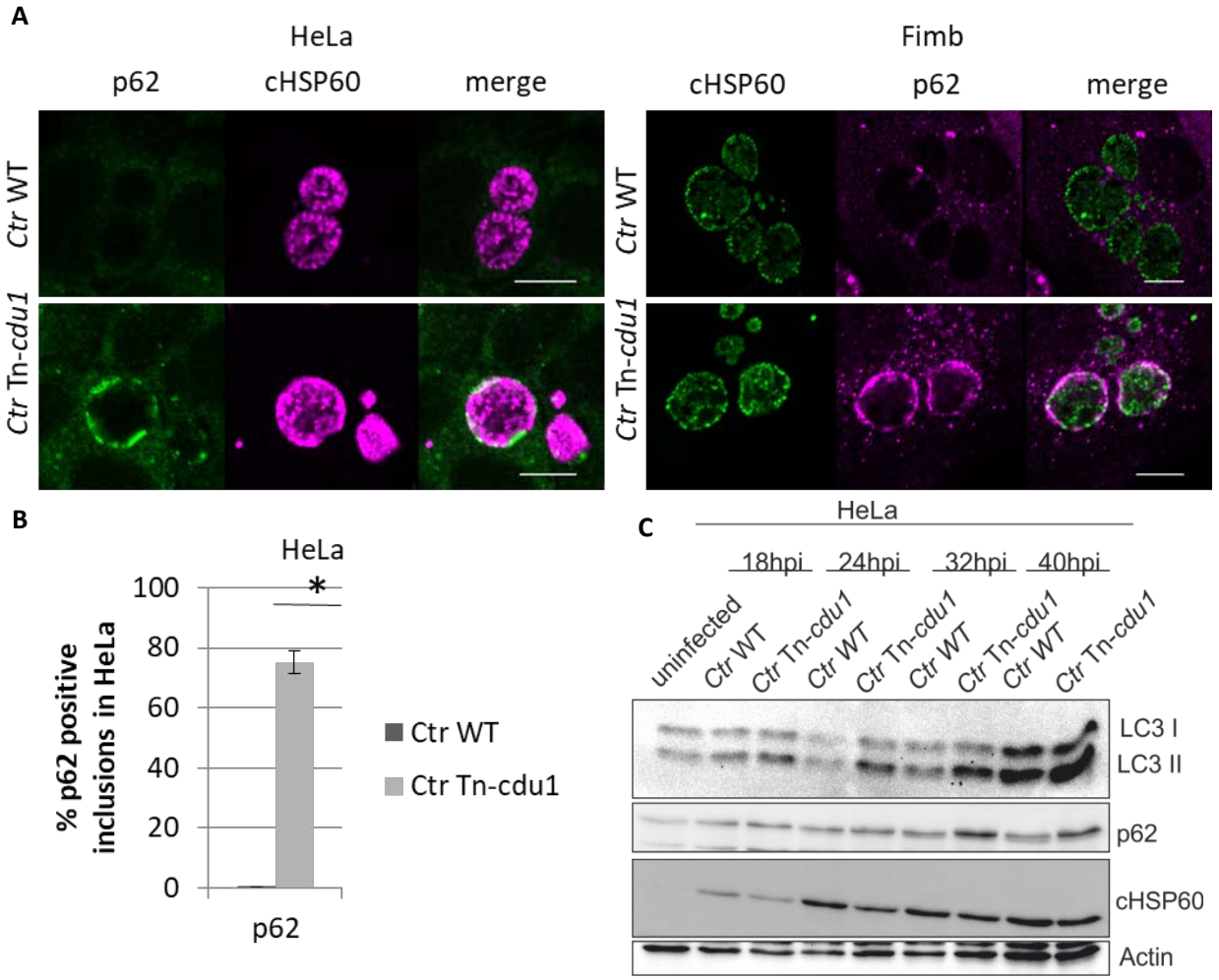
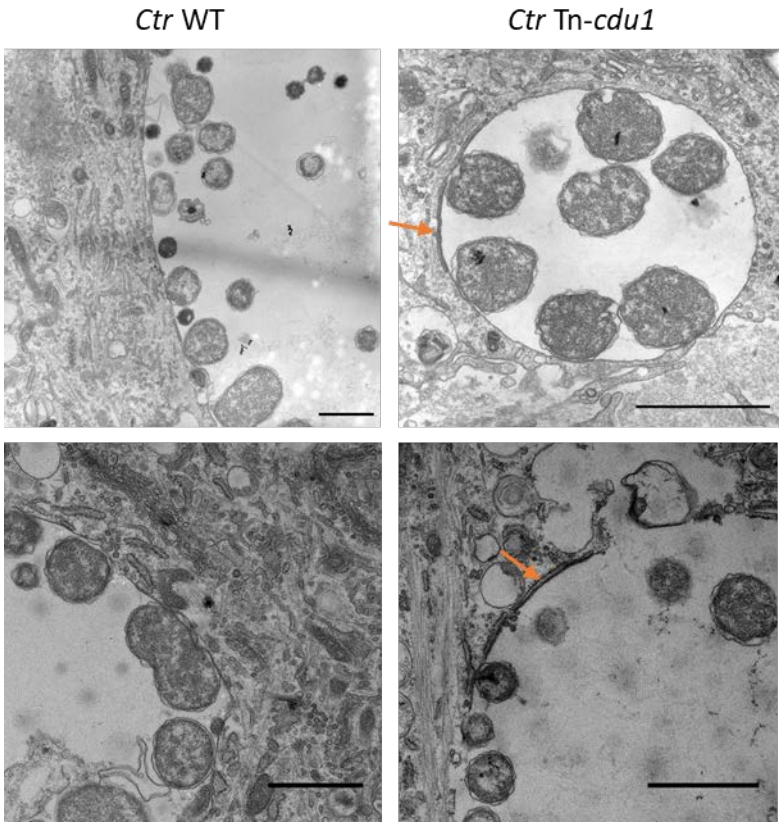


Figure 3.11. Localization and quantification of p62 in HeLa and Fimb cells. A. HeLa or Fimb cells were infected with *Ctr* Tn-*cdu1* or wildtype *Chlamydia* at an MOI of 1 for HeLa and 0.3 for Fimb respectively. After 24 hpi cells were fixed with 4 % PFA/sucrose and stained for chlamydial HSP60 and p62. Scale bar, 10 µm. B. Quantification of the IF pictures regarding the p62 signal at the inclusion of *Ctr* WT and *Ctr* Tn-*cdu1*. Mean values of three independent experiments are shown. * p< 0.05. C. HeLa cells were infected with *Ctr* WT and *Ctr* Tn-*cdu1* MOI 1 for indicated time. Lysates were taken and immunoblot was performed for LC3, p62, cHSP60 and beta-actin.

During autophagy ubiquitinated proteins get recognized by adaptor proteins like p62, which initiate the LC3 membrane formation. In order to see whether the recruitment of autophagy markers leads to phagophore formation in *Ctr* Tn-*cdu1* infection, LC3B accumulation around inclusions were investigated in HeLa and in primary Fimb cells. Pictures taken by transmission electron microscopy confirm the formation of a double membrane around *Ctr* Tn-*cdu1* inclusions in primary cells (Figure 3.12 A). Moreover, in both cell types membranes positive for LC3B are visible around *Ctr* Tn-*cdu1* inclusions (Figure 3.12 B). The quantification of IF pictures in HeLa cells shows that approximately 45 % of all *Ctr* Tn-*cdu1* inclusions are positive for LC3B (Figure 3.12 C).

A



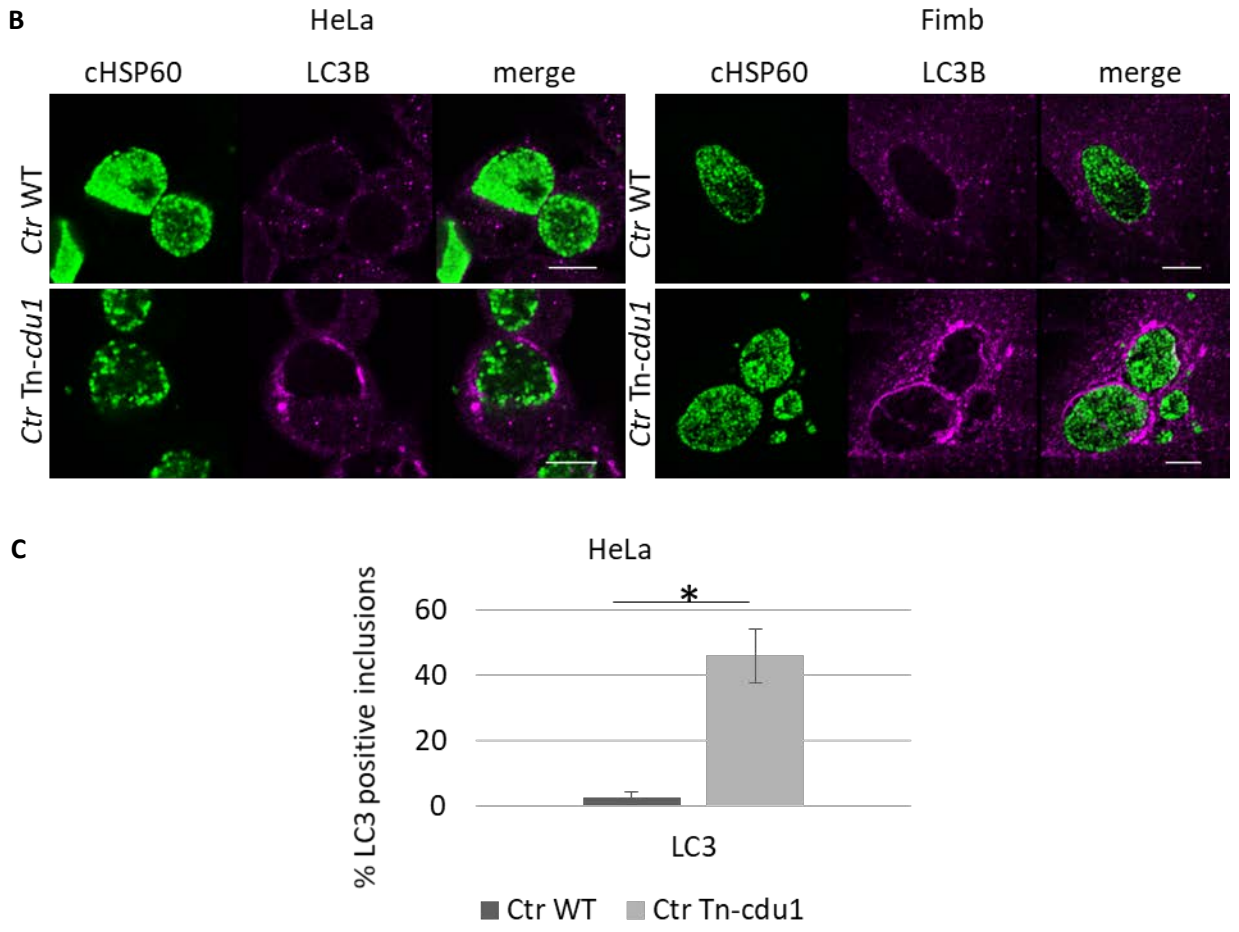


Figure 3.12. Formation of LC3 positive membrane around *Ctr* Tn-*cdu1* inclusion. A. Fimb cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* at an MOI of 0.3. After 24 hpi cells were fixed and prepared for electron microscopy. Orange arrows indicate the double membrane. Scale bar, 1 µm. B. IF pictures of LC3 membrane formation around *Ctr* Tn-*cdu1* inclusion. HeLa and Fimb cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* at an MOI of 1 or 0.3, respectively. After 24 hpi cells were fixed with ice cold MetOH for 20 min. Cells were stained for chlamydial HSP60 (green) and LC3B (magenta). Scale bar, 10µm. C. Quantification of LC3 positive inclusions according to IF pictures. Mean values of four independent experiments are shown. * p<0.05.

Apart from p62 various adaptor proteins are known to be recruited to ubiquitin. In xenophagy the autophagy receptors NBR1, NDP52 and optineurin are often associated with bacterial intruders and promote their degradation (Kirkin, et al., 2009, Svenning and Johansen, 2013). Therefore, the presence of these autophagy receptors was checked in *Ctr* Tn-*cdu1* infected cells. IF pictures clearly show the accumulation of NBR1 and NDP52 on *Ctr* Tn-*cdu1* inclusions whereas in *Ctr* WT infected cells the inclusion stays clear of autophagy markers (Figure 3.13 A+B). This was also observed in primary Fimb cells (Figure 3.13 C+D). Furthermore the recruitment of the LC3 membrane depends on the lipidation of LC3I to LC3II with phosphatidylethanolamine (PE) through an enzyme cascade. In the last step of this lipidation a complex of ATG16L1, ATG5 and ATG12 is formed that facilitates the transfer of PE to LC3I. IF analysis reveals that ATG12 is present on the inclusion of *Ctr* Tn-*cdu1* infected cells (Figure 3.13 E, F), which is in line with the finding of LC3 membrane forming around the mutant inclusion (Figure 3.12).

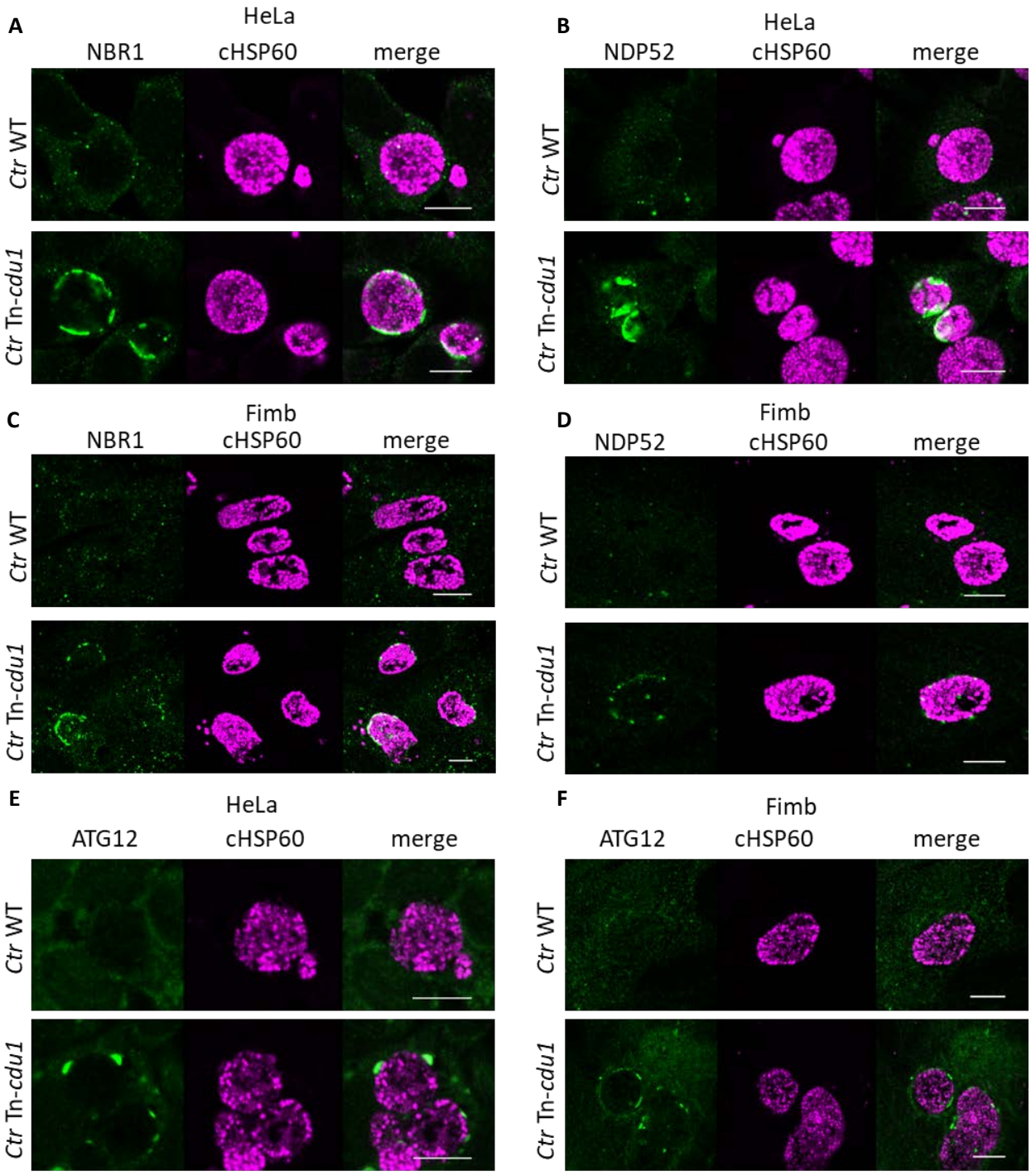


Figure 3.13. Accumulation of autophagy marker around *Ctr* Tn-*cdu1* in infected cells. HeLa cells (A,B,E) were infected with *Ctr* WT and *Ctr* Tn-*cdu1* at MOI 1 and Fimb cells with an MOI of 0.3 (C,D,F) for 24 h. After fixation with 4 % PFA/sucrose cells were stained against chlamydial HSP60 (magenta) and NBR1 (A,C), NDP52 (B,D) or ATG12 (E,F) (green). Example of majority is shown for each sample. Scale bar, 10 µm.

The presence of the phagophore in *Ctr* Tn-cdu1 infected cells suggests that the bacteria are not able to escape the cells autonomous defense. For destruction of captured bacteria the phagophore fuses with endosomes and lysosomes. However, neither in *Ctr* WT nor in *Ctr* Tn-*cdu1* infected cells co-localization of

the inclusion with Lamp1, a lysosomal protein, was observed (Figure 3.14). Consequently the fusion with the lysosome does not occur in *C. trachomatis* infected cells, even when *Chla*DUB1 is inactive.

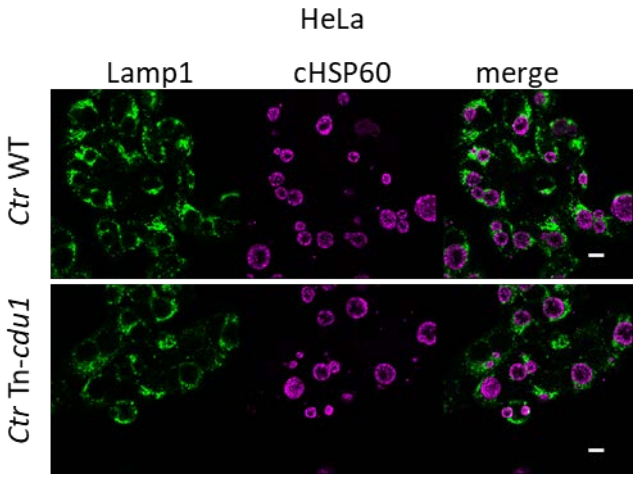


Figure 3.14. Lamp1 positive vesicles do not fuse with chlamydial inclusion during infection. HeLa cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* at an MOI of 1 for 24 h, before cells were fixed with 4 % PFA/sucrose. Using the Lamp1 (green) and cHSP60 (magenta) antibodies the cells were stained and analyzed by microscopy. Scale bar, 10 µm.

All in all inclusions of *Ctr* Tn-*cdu1* are modified with ubiquitin and subsequent autophagy markers like p62, NBR1 and NDP52 that are typical for xenophagy. The mutant inclusion is then engulfed by LC3 positive phagophore, but does not fuse with lysosomes.

3.2.2 Rescue of accumulation of autophagy markers by *Chla*DUB1 expression

In *Ctr* Tn-*cdu1* infected cells ubiquitin, autophagy markers like p62 and LC3 are recruited to the inclusion, whereas the wildtype chlamydial inclusions are lacking those receptors (Figure 3.11 A, 3.12 B). *Ctr* Tn-*cdu1* was sequenced and shows no other mutation except in the *Chla*DUB1 gene in comparison with the wildtype bacteria (Fischer, et al., 2017). To proof that the ubiquitination and subsequent decoration with autophagy receptors is only due to the inactive *Chla*DUB1 protein, active DUB protein was transiently expressed in HeLa 229 Flp-In™ T-Rex™ Cdu1-Flag cells, hereafter referred to as HeLa Cdu1-Flag cells, infected with *Ctr* WT or *Ctr* Tn-*cdu1*, respectively. Therefore, infected HeLa Cdu1-Flag cells were treated with 50 ng/ml AHT for 4 h 20 hpi to induce *Chla*DUB1 expression. IF after 24 h of infection reveals the expression of *Chla*DUB1 in the cell cytoplasm upon AHT treatment (Figure 3.15). Moreover, the ubiquitination of *Ctr* Tn-*cdu1* inclusion vanishes upon active *Chla*DUB1 expression in the cell (Figure 3.15). This finding means that ubiquitination of the *Ctr* Tn-*cdu1* inclusion is reversed by active *Chla*DUB1 protein.

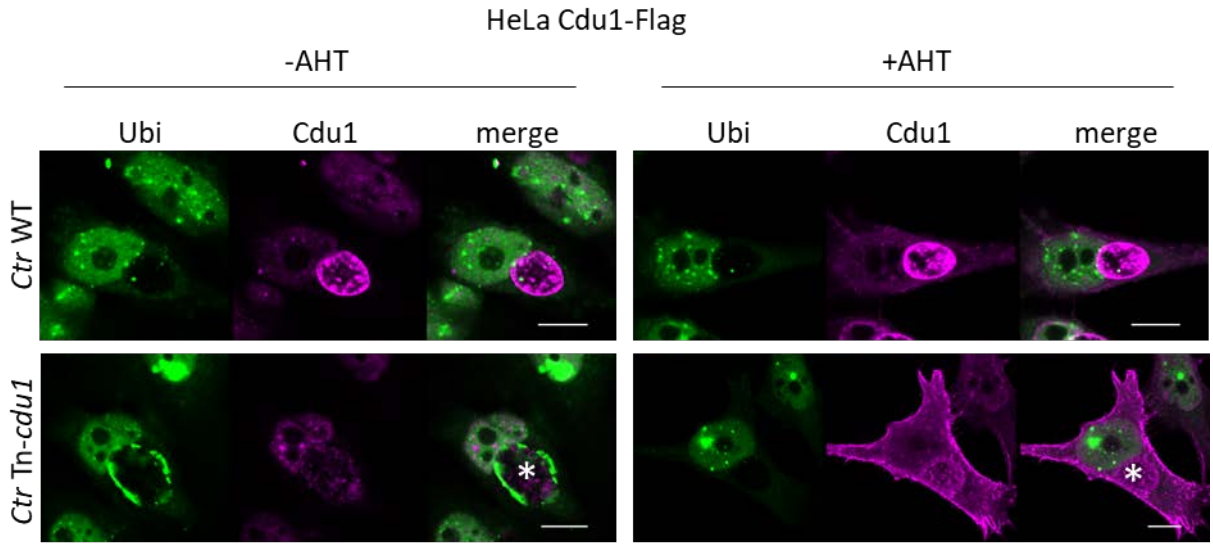


Figure 3.15. Rescue of ubiquitination pattern around *Ctr* Tn-*cdu1* through active *Chla*DUB1 expression. HeLa Cdu1-Flag cells were seeded on glass cover slides and infected with *Ctr* WT or *Ctr* Tn-*cdu1* at MOI 1. After 20 h of infection cells were treated with or without AHT for transient *Chla*DUB1 expression for 4 h, until cells were fixed with 4 % PFA/sucrose. Fixed cells were stained for *Chla*DUB1 expression (magenta) and conjugated ubiquitin (green). The asterisk exposes the inclusion of *Ctr* Tn-*cdu1*. Pictures represent the majority. Scale bar, 10 µm.

3.3 Identification of Ubiquitin E3 ligase

3.3.1 Screening for the E3 ligase

The fact that chlamydial inclusions with inactive *Chla*DUB1 get ubiquitinated raises other questions. One of them is which E3 ligase ubiquitinates the inclusion of *Ctr* Tn-*cdu1*. In order to identify the cascade of E1, E2 and E3 enzymes involved in this process a siRNA-based knock-down screen was performed. All in all 589 different proteins were targeted with four distinct siRNAs, whereby the targets were part of the E1, E2 and E3 enzyme complexes. Cells treated with the siRNAs were infected with *Ctr* Tn-*cdu1* or *Ctr* WT as a control and the inclusion were allowed to grow for 24 h. As the visualization with the ubiquitin antibody (FK2) was not always reliable in previous experiments, p62 antibody was used that binds to the ubiquitinated inclusion. In order to check for successful knock-down of proteins a siRNA targeting p62 was included. The infected cells were fixed and stained for p62 and chlamydial HSP60 and analyzed by the automatic Operetta microscope (Perkin Elmer). For analysis 20 pictures per well were evaluated regarding the p62 dots in the ring shaped structure around the chlamydial inclusion (Figure 3.16 A).

The screen revealed eleven hits to show an at least 30 % reduction in p62 accumulation around the *Ctr* Tn-*cdu1* inclusion. Six out of these eleven hits show a significant reduction in all three replicates (Figure 3.16 B). Among these hits are the E1 enzyme UBE1, the E2 enzymes UBE2L3, HIP2 also known as UBE2K and the E3 enzymes Ankib1, RNF214 and BRPF3 (Figure 3.16 B).

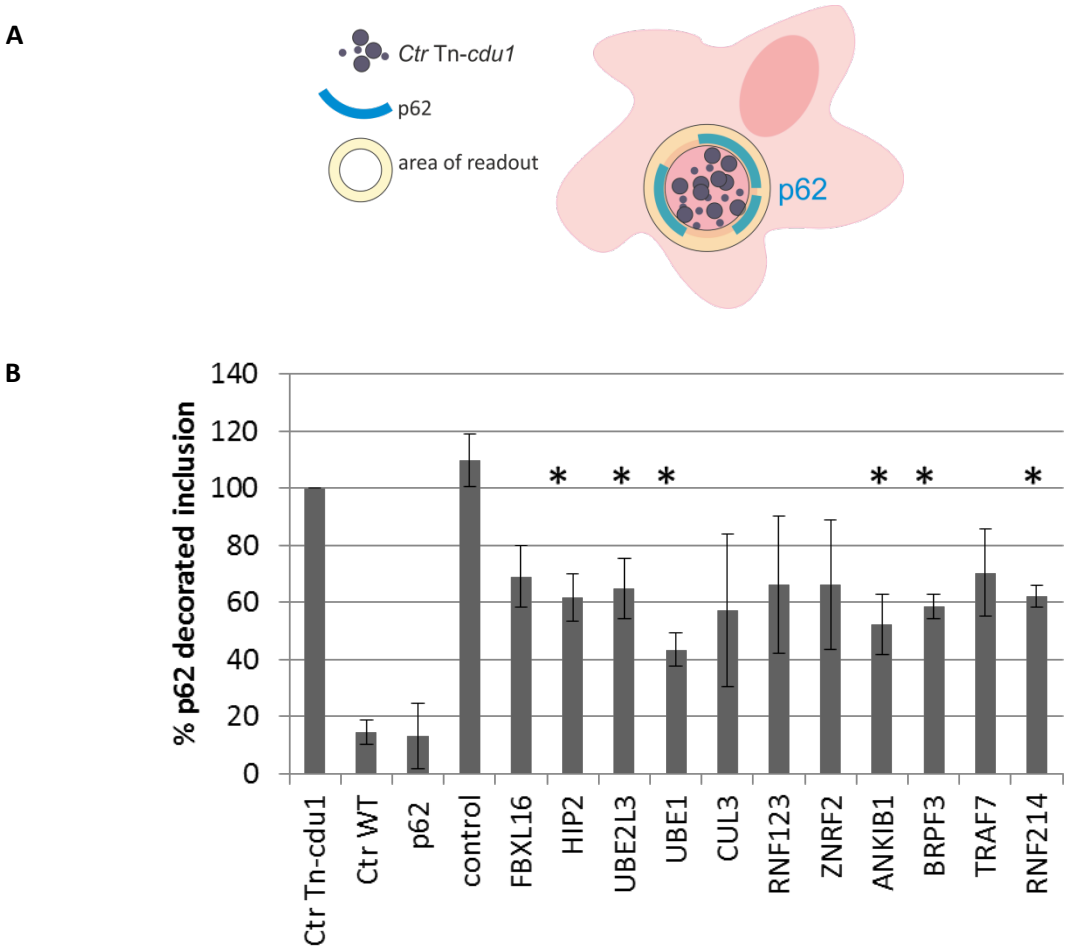


Figure 3.16. Setup and analysis of E3 ligase screen. A. Schematic drawing showing the area of readout of p62 ring around *Ctr* Tn-*cdu1* inclusion of E3 ligase screen. B. A siRNA knock-down E3 ligase screen was performed in HeLa cells using four distinct siRNAs for 24 h. Cells were infected with *Ctr* Tn-*cdu1* or *Ctr* WT as a control. After 24 hpi cells were fixed and stained for p62 and cHSP60 and analyzed by the automatic Operetta microscope. 20 pictures of each well were analyzed according to the p62 accumulation around the inclusion. The figure depicts the list of targets that show at least 30 % reduction in p62 accumulation around *Ctr* Tn-*cdu1* inclusion in siRNA knock-down screen, when ring formation around *Ctr* Tn-*cdu1* was referred to as 100 %. *Ctr* WT infected cells as well as sicontrol treated cells serve as a negative control. *Ctr* Tn-*cdu1* infected cells and cells treated with sip62 serve as a positive control. Data was analyzed using the Harmony® High Content Imaging and analysis software (Perkin Elmer). Mean values of three independent experiments are shown above. * p< 0.05.

Additionally to the siRNA-based screen another experiment was performed to reveal if the family of CRL E3 ligases like cullin 3 that was also identified in the screen, are involved in the ubiquitination of the *Ctr* Tn-*cdu1* inclusion. The activity of the CRLs is regulated via the neddylation status of the central cullin. For activity the cullin needs to get conjugated with Nedd8 via an enzyme cascade similar to the ubiquitination cascade. MLN4924 is a compound that mimics AMP and therefore can bind to NAE, the activating enzyme of the neddylation cascade, which is then no longer accessible for ATP and hence inactive. The inhibition of the neddylation cascade in the first step by MLN4924 inactivates the CRLs and prevents ubiquitination by these E3 ligases. The experiment in which infected cells were treated with MLN4924 helped to limit the number of possible E3 ligases, involved in ubiquitination of *Ctr* Tn-*cdu1* inclusion. Figure 3.17 A shows that treatment with MLN4924 inhibits the neddylation of cullin1 in HeLa cells regardless of the bacterial infection, since the bigger band resembling cullin-Nedd8 is missing in

inhibitor treated samples. In IF of *Ctr* Tn-*cdu1* the activity status of cullins does not influence the ubiquitination of the inclusion (Figure 3.17 B). The treatment with MLN4924 has no effect on *Ctr* WT inclusions (Figure 3.17 B). This finding indicates that CRLs are not involved in the ubiquitination of the *Ctr* Tn-*cdu1* inclusion, but other E3 families have to be considered.

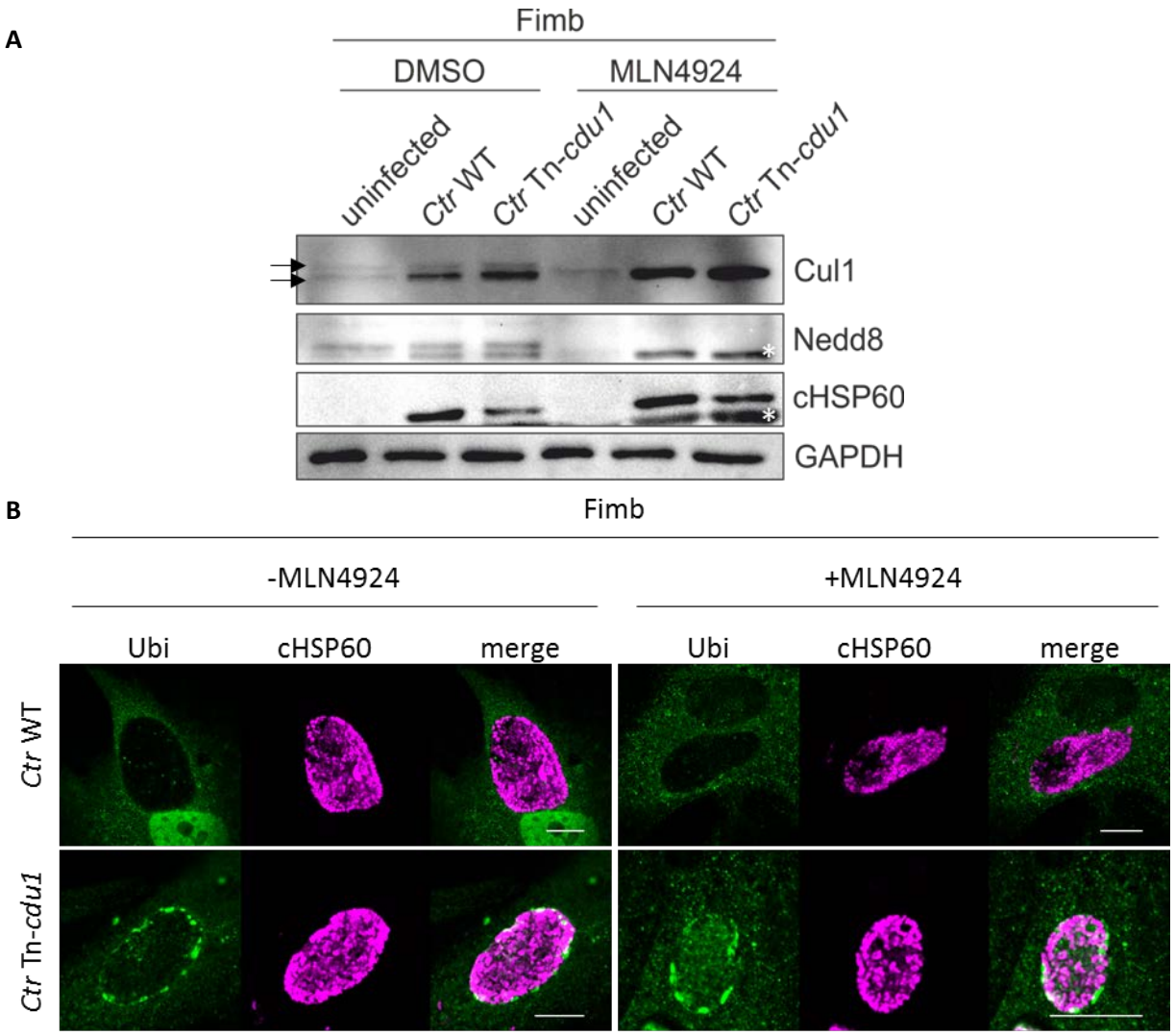


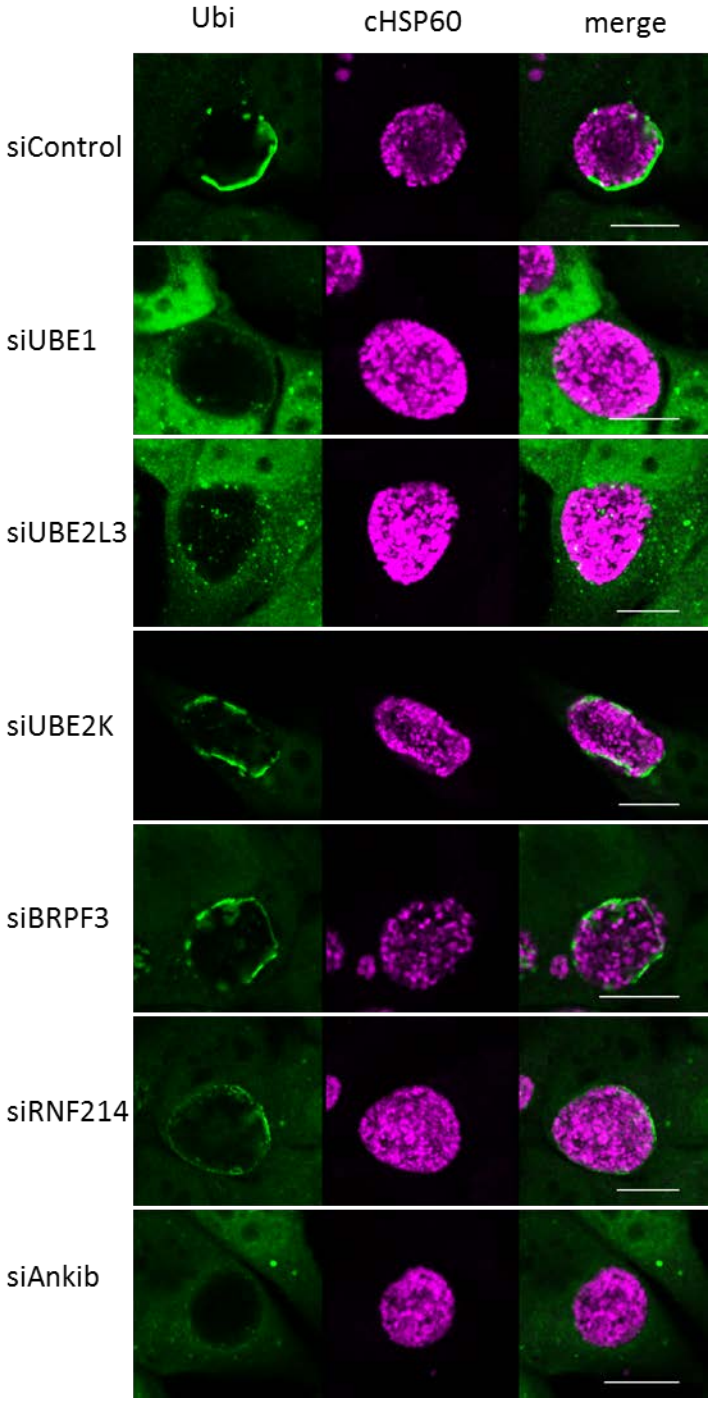
Figure 3.17. Inhibition of CRLs by MLN4924 inhibitor treatment. A. Fimb cells were infected with *Ctr* WT and *Ctr* Tn-*cdu1* with a MOI of 0.3. After 7 hpi cells were treated with 2 µM MLN4924 or the same amount of DMSO as a solvent control. Lysates were taken 24 hpi and immunoblot was performed to investigate the neddylation of cullins. Arrows tag the cullin and neddylated cullin band, asterisks mark unspecific bands. B. Fimb cells infected with *Ctr* WT or *Ctr* Tn-*cdu1* (MOI 0.3) were treated with 2 µM MLN4924 7 hpi. Cells were fixed after 24 hpi with 4 % PFA/sucrose and stained for conjugated ubiquitin with the FK2 antibody (green) and chlamydial HSP60 (magenta). Majority of the pictures is shown here. Scale bar, 10 µm.

3.3.2 Validation of the E3 ligase screen hits

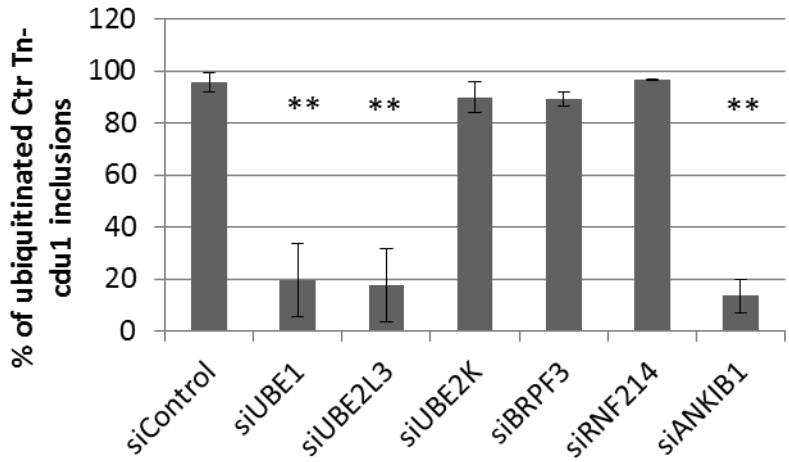
The siRNA-based E3 ligase screen revealed eleven hits that are more than 30 % reduced, among them cullin3 (Figure 3.16 B). Since the results exclude all CRLs and so cullin3 (Figure 3.17 B), only the six significant hits were further investigated. By knock-down of the respective proteins using two different siRNAs, three targets could be validated to be essential for inclusion ubiquitination. The knock-down of the E1 activating enzyme UBE1, E2 conjugating enzyme UBE2L3 as well as E3 ligase Ankib1 results in lack

of conjugated ubiquitin around the inclusion of *Ctr* Tn-*cdu1*-infected cells (Figure 3.18 A+B). The antibody FK2 used in this experiment recognizes conjugated mono- as well as polyubiquitin independent of the linkage type. IF experiments using the linkage specific K48 and K63 antibody revealed that both conjugation types are missing around the inclusion of *Ctr* Tn-*cdu1* when UBE1, UBE2L3 or Ankib1 were knocked down (Figure 3.18 C). Sufficient knock-down for UBE1, UBE2L3 and Ankib1 was validated by simultaneous qRT-PCR analysis (Figure 3.18 D).

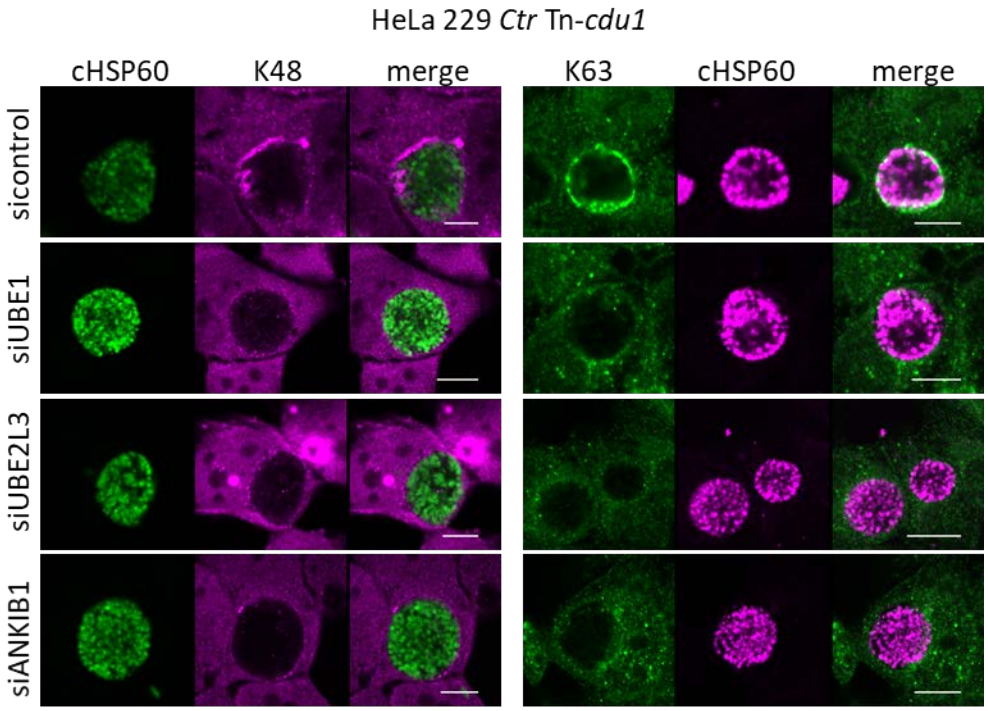
A



B



C



D

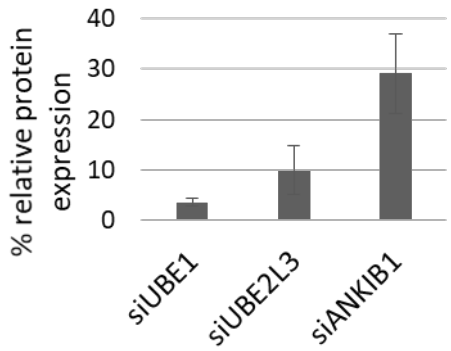


Figure 3.18. Validation of hits of the E3 ligase screen. A. SiRNA knock-down was performed in HeLa cells by transfection with siRNA targeting UBE1, UBE2L3, UBE2K, BRPF3, RNF214, Ankib1 or control and Lipofectamine RNAiMax as reagent. Cells were

infected with *Ctr* Tn-*cdu1* 24 h post transfection and fixed with 4 % PFA/sucrose 24 hpi. Cells were stained for chlamydial HSP60 (magenta) and conjugated ubiquitin (green). Scale bar, 10 µm. B. Quantification of inclusion ubiquitination in IF pictures using the FK2 antibody after siRNA knock-down and *Ctr* Tn-*cdu1* infection as described above. Mean values of three independent experiments are shown. ** p<0.01. C. HeLa cells were treated with siRNA against UBE1, UBE2L3, Ankib1 or a control gene for one day as described. Cells were infected with *Ctr* Tn-*cdu1* MOI 1 for 24 h and fixed with MetOH before IF preparation. Ubiquitin chains K48 and K63 were stained as well as chlamydial HSP60. Pictures show examples of majority. Scale bar, 10 µm. D. SiRNA knock-down of UBE1, UBE2L3 and Ankib1 in HeLa cells was performed for 48 h. Total RNA was isolated from samples and qRT-PCR was done to determine the relative protein expression of knock-down proteins. Protein amount of untreated cells was set 100 %.

Since the three identified proteins are essential for the ubiquitination of the *Ctr* Tn-*cdu1* inclusion, the localization of UBE1, UBE2L3 and Ankib1 was checked during infection. IF experiments show that Ankib1 is located in the cytoplasm, but also in close proximity to the inclusion of both *Ctr* WT and *Ctr* Tn-*cdu1* (Figure 3.19 A). UBE1 and UBE2L3 on the other hand are dispersed in the cytoplasm of the cell also during infection (Figure 3.19 B+C).

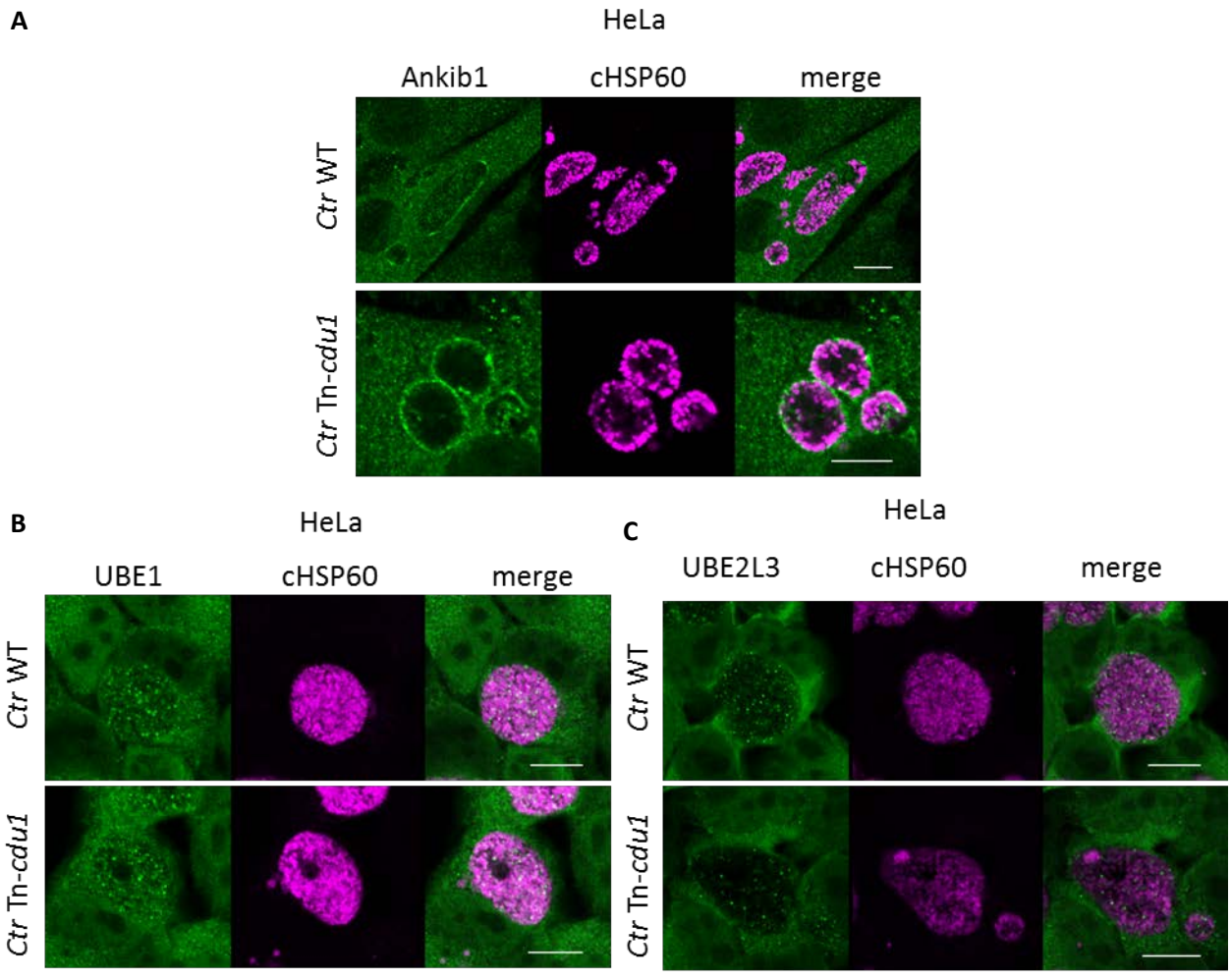


Figure 3.19. Localization of UBE1, UBE2L3 and Ankib1 in *Chlamydia* infected HeLa cells. HeLa cells were infected with *Ctr* WT and *Ctr* Tn-*cdu1* with an MOI of 1. After 24 hpi cells were fixed using 4 % PFA/sucrose and stained with antibodies targeting chlamydial HSP60 (magenta) and Ankib1 (A), UBE1 (B) or UBE2L3 (C) (green). Examples of majority are shown above. Scale bar, 10 µm.

3.4 Growth and development of *C. trachomatis* Tn-*cdu1*

3.4.1 Growth defect of *Ctr* Tn-*cdu1*

Since autophagy markers are recruited to and a LC3 positive phagophore forms around the inclusion of *Ctr* Tn-*cdu1,* the development and replication of the bacteria was investigated. A progeny assay in HeLa cells but also primary Fimb and HUVEC cells was performed to get some information about the growth of *Ctr* Tn-*cdu1*. In HeLa cells no significant growth deficit was observed for *Ctr* Tn-*cdu1*, whereas in primary cells like Fimb and HUVEC a drastic reduction of progeny formation was detected in the mutant strain (Figure 3. 20).

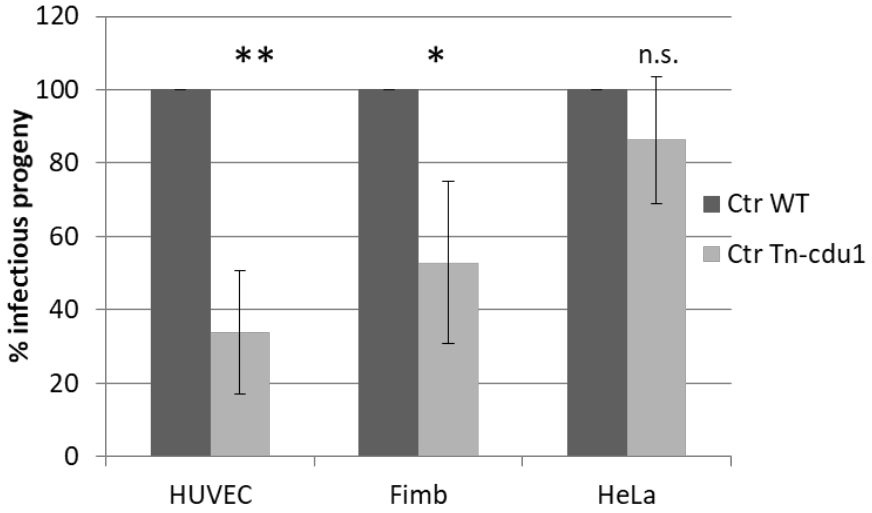
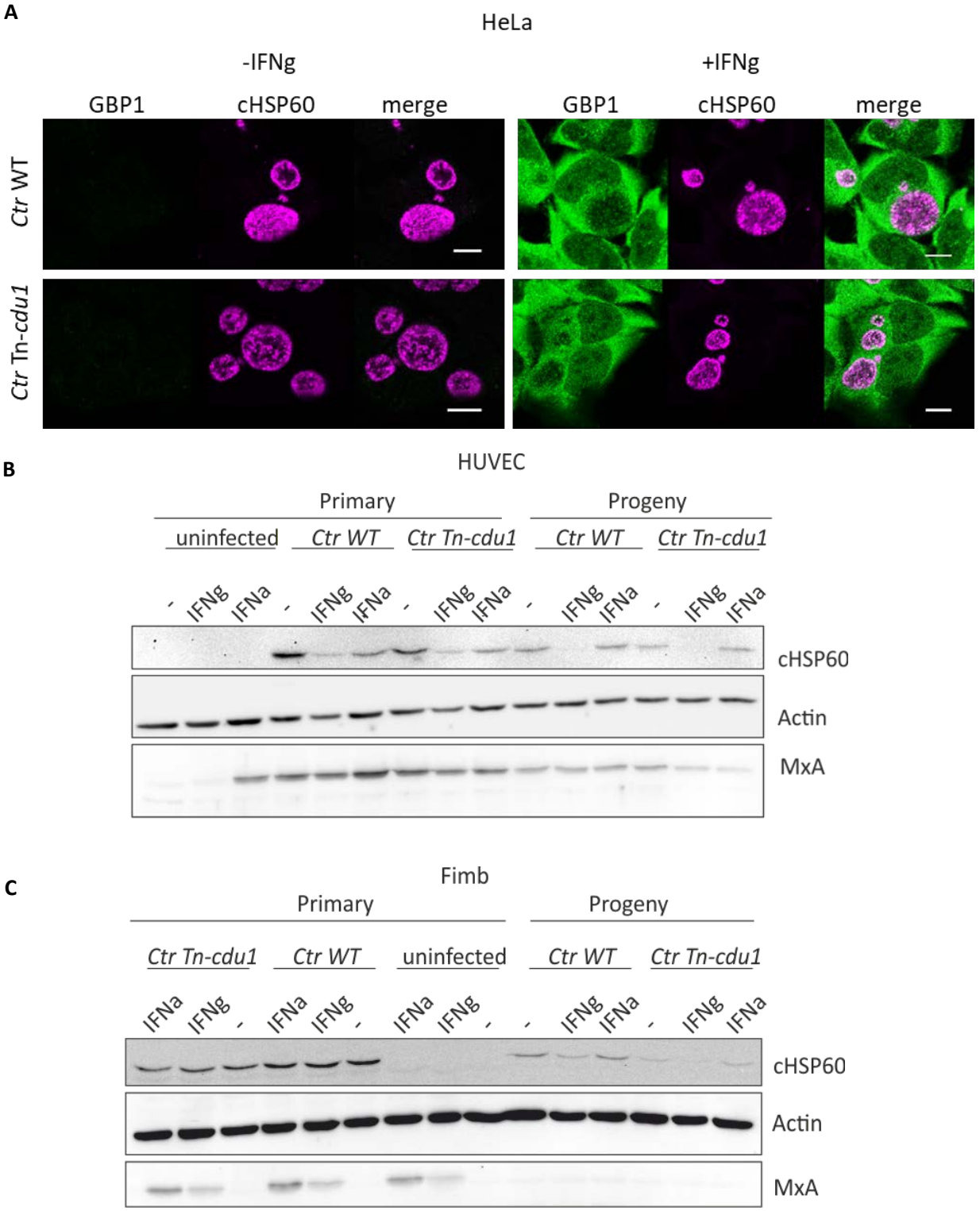


Figure 3.20. Growth of *Ctr* Tn-*cdu1* progeny is diminished in primary cells. HUVEC, Fimb or HeLa cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1,* respectively. After 48 h of infection cells were lysed and supernatant was used to re-infect fresh cells. Another 24 h later lysates were taken and analyzed in immunoblot for chlamydial HSP60. Growth of *Ctr* WT in each cell type was set 100 %. For HeLa and HUVEC cells mean values of three, for Fimb cells of five independent experiments are shown. n.s. not significant, * p< 0.05, ** p<0.01.

In natural infection the immune system challenges *C. trachomatis* and limits their spread even though it is not able to fully eradicate the bacteria. In mouse cells *C. trachomatis* infection is cleared faster, since the interferon induced genes target the inclusion, destabilize it and promote xenophagy (Haldar, et al., 2015). The defense systems of human cells, however, do not recognize the inclusion of the human pathogen even when primed with IFNγ (Haldar, et al., 2016). The mutant *Ctr* Tn-*cdu1* was challenged with INFα or IFNγ in primary cells like Fimb and HUVEC and in HeLa cell lines and chlamydial growth was analyzed by immunoblots and IF. In IF no accumulation of GBP1 at the chlamydial inclusion was observed in the absence of IFNγ, whereas treatment with IFNγ triggers the expression of GBP1 in the cytosol of HeLa cells (Figure 3.21 A). Immunoblots in HUVEC, Fimb and HeLa cells proves that treatment with IFNα or IFNγ interferes with the growth of *Ctr* WT and *Ctr* Tn-*cdu1* in the same amount (Figure 3.21 B-D). In HUVEC cells the interferon-induced GTP-binding protein Mx1 (MxA) is already expressed upon infection with *Chlamydia* (Figure 3.21 B), whereas in HeLa and Fimb cells only treatment with IFN triggers MxA expression (Figure 3.21 C+D). IFNγ treatment results in a stronger growth defect than IFNα in the progeny infection in all cell lines. However, the declined growth due to IFNγ treatment is reversible by

the addition of tryptophan (Figure 3.21 E). All in all treatment with IFNα or IFNγ affects the bacterial growth in the same way independent of the loss of *Chla*DUB1 activity in *Ctr* Tn-*cdu1*.



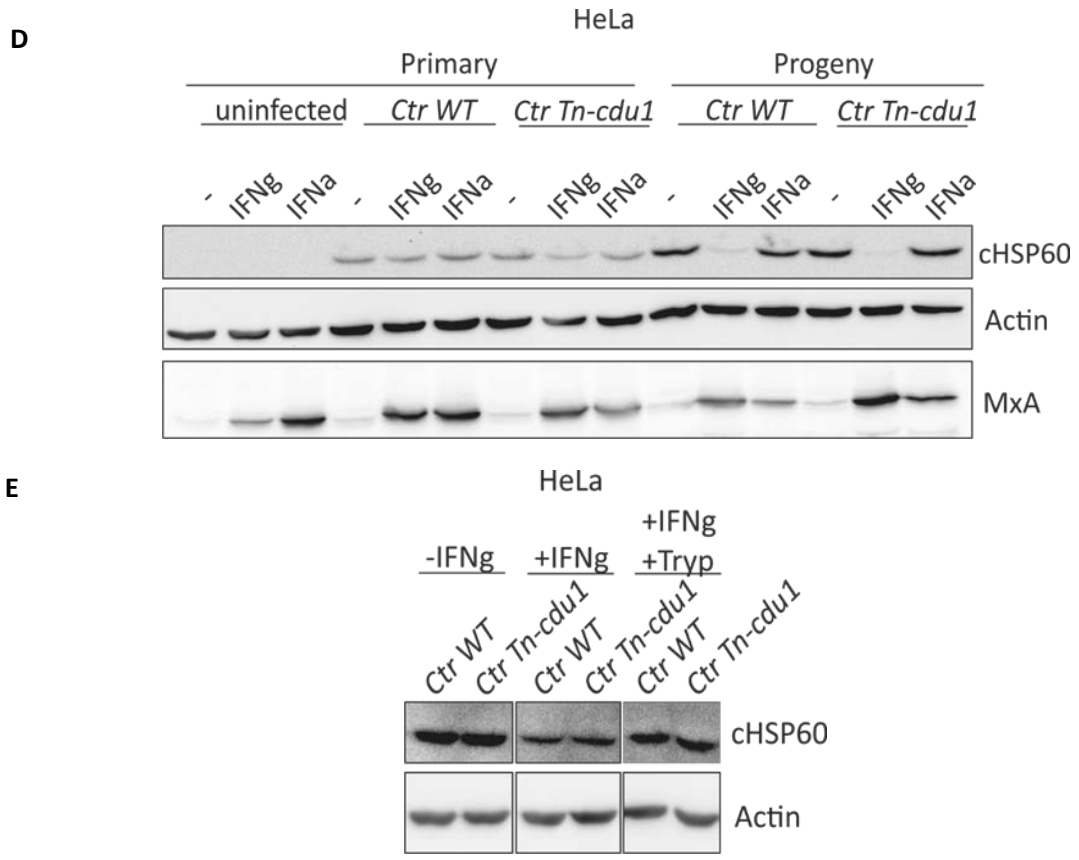


Figure 3.21. Growth defect of *Ctr* Tn-*cdu1* does not change upon IFN challenge in primary cells. A. HeLa cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* for 6 h before treatment with 5 ng/ml IFNγ (IFNg). After 24 hpi cells were fixed with 4 % PFA/sucrose and subsequent IF staining was performed. Human GBP1 is shown in green and chlamydial HSP60 in magenta. Scale bar, 10 µm. B-D. HUVEC (B), Fimb (C) or HeLa 229 (D) cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* with an MOI of 1 for HeLa and HUVEC or 0.3 for Fimb. After 6 h of infection cells were treated with 100 U/ml IFNα (IFNa) or 5 ng/ml IFNγ (IFNg) or left untreated. Lysates for primary infection were taken 24 hpi. For progeny infection cells were lysed with glass beads 48 hpi and fresh cells were infected with the supernatant. Progeny lysates were taken again 24 hpi and immunoblot was performed for chlamydial HSP60, interferon induced MxA and beta-actin. E. HeLa cell were infected with *Ctr* WT or *Ctr* Tn-*cdu1* with a MOI of 1 and treated with 5 ng/ml IFNg and 40 µg/ml tryptophan (Tryp) 6 hpi. Lysates for immunoblots were taken 24 hpi and analyzed for chlamydial HSP60 and beta-actin.

3.4.2 Development of *Ctr* Tn-*cdu1*

Chlamydial development and spread of infection depends on the completion of the developmental cycle with the formation of infectious EBs on its end. To investigate if the growth defect present in primary cells originates from a lack of EB production, inclusions of *Ctr* WT and *Ctr* Tn-*cdu1* were analyzed by electron microscopy (EM). Hereby the number of bacterial particles as well as the percentage of EBs were determined. In line with previous results, in *Ctr* Tn-*cdu1* the total amount of chlamydial particle per inclusion is with 20.6 ± 10.1 approximately 53 % lower than in *Ctr* WT inclusions (43.6 ± 23,5 % particles per inclusion) (Table 20). Moreover, the amount of infectious EBs is almost ten times lower in *Ctr* Tn-*cdu1* compared to the wildtype (Table 20).

Table 20. Chlamydial particles in Fimb cells at 24 hpi.

	Ctr WT	*Ctr* Tn-*cdu1*
Bacterial particles per inclusion	43.6 ± 23.5	20.6 ± 10.1
% EB	6.3	0.7

The whole pathway of transformation of RB to EB is not yet completely understood. However, Lee et al. showed that the size of RBs plays an essential role in transformation. During replication through division RBs get smaller in size and only after reaching a threshold in size the conversion into EBs takes place (Lee, et al., 2018). The analysis of the average diameter of RBs in *Ctr* WT and *Ctr* Tn-*cdu1* infected cells at 24 hpi revealed that wildtype *Chlamydia* are significantly smaller then RBs of the mutant strain (Figure 3.22).

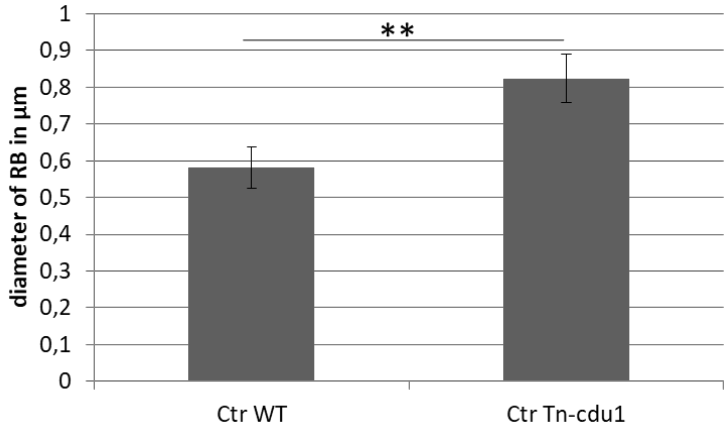


Figure 3.22. Size of chlamydial RBs of *Ctr* WT and *Ctr* Tn-*cdu1* infected cells after 24 hpi. Fimb cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* at MOI 0.3 for 24 h. Cells were fixed and prepared for electron microscopy. Five inclusions were randomly selected and the diameter of each RB was measured. Mean values of RB diameter are shown. ** p<0.01.

Replication and transformation of *Ctr* Tn-*cdu1* RBs are disturbed compared to *Ctr* WT. Reasons for that depend directly or indirectly on the inactive *Chla*DUB1 protein since sequencing of the mutant showed no other mutations (Fischer, et al., 2017). Chlamydial growth depends on many nutrients from the host cell, especially host lipids are consumed by the bacteria. *Chlamydia* synthesize some bacteria-specific lipids like PE, phosphatidylglycerol (PG) and phosphatidylserine (PS) (Hatch and McClarty, 1998). However, also host cell lipids were identified in inclusion membranes that derive form vesicles that fuse with the inclusion and provide the bacteria with lipids and nutrients (Carabeo, et al., 2003, Hackstadt, et al., 1996, Hackstadt, et al., 1995, Hatch and McClarty, 1998, Wylie, et al., 1997). Vesicles fuse with the bacterial inclusion in different ways, for example Transferrin positive vesicles from the plasma membrane can be directed to the inclusion (Elwell and Engel 2012). To test whether this pathway is still functional in *Ctr* Tn-*cdu1,* the uptake of Transferrin via the Transferrin receptor on the plasma membrane was investigated. In *Ctr* WT as well as *Ctr* Tn-*cdu1* infected cells Transferrin is visible on the

cell surface at the beginning of the experiment and gets taken up and transported to the chlamydial inclusion approximately 15 min after treatment (Figure 3.23). These findings show that the endocytotic pathway is functional in *Ctr* Tn-*cdu1* infection.

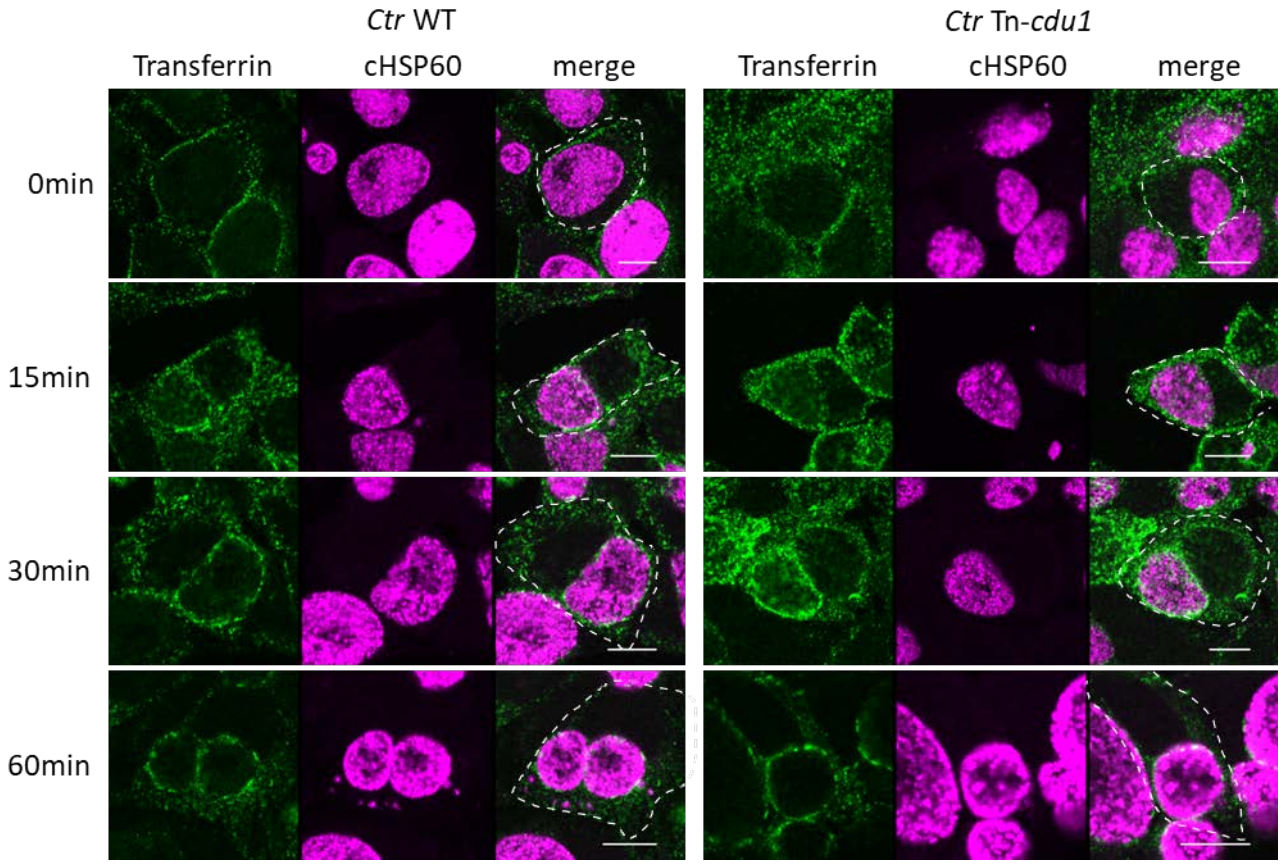


Figure 3.23. Transferrin uptake in HeLa 229 cells does not differ between *Ctr* WT and *Ctr* Tn-*cdu1* infected cells. HeLa cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* at an MOI of 1 for 24 h. Cells were washed with PBS and treated with 50 µM Transferrin-488 for 90 min at 4°C. After washing, cells were incubated at 37°C for the indicated time. Cells were fixed with 4 % PFA/sucrose and stained for chlamydial HSP60 (magenta). Contour of the cell is marked in white and transferrin is shown in green. Scale bar, 10 µm.

Another important source for vesicles for *Chlamydia* development is the Golgi membrane system (Heuer, et al., 2009). Upon chlamydial infection the Golgi gets fragmented and recruited to the inclusion where it fuses with the inclusion membrane. However, Golgi-derived membranes were also identified inside of the inclusion indicating that at least partly the membranes have other function than expansion of the inclusion membrane (Heuer, et al., 2009). IF pictures of the inclusion and the Golgi protein GM130 were taken to investigate the vesicle transport in infected HeLa Cdu1-Flag cells. As reported, the Golgi of *Ctr* WT infected cells is fragmented and recruited to the chlamydial inclusion (Figure 3.24 A, C). The Golgi of *Ctr* Tn-*cdu1* infected cells is also fragmented, however, not located at the proximity of the inclusion but spread throughout the cell cytoplasm (Figure 3.24 A, C). The *Ctr* WT phenotype could be restored by transient expression of *Chla*DUB1 in the cytoplasm in *Ctr* Tn-*cdu1*-infected cells (Figure 3.24 A, C). This

only occurs when *Chla*DUB1 is functional as transient expression of catalytic inactive *Chla*DUB1 (Cdu1Mut) in HeLa 229 FlpIn (HeLa Cdu1Mut-Flag) does not rescue the Golgi recruitment (Figure 3.24 B).

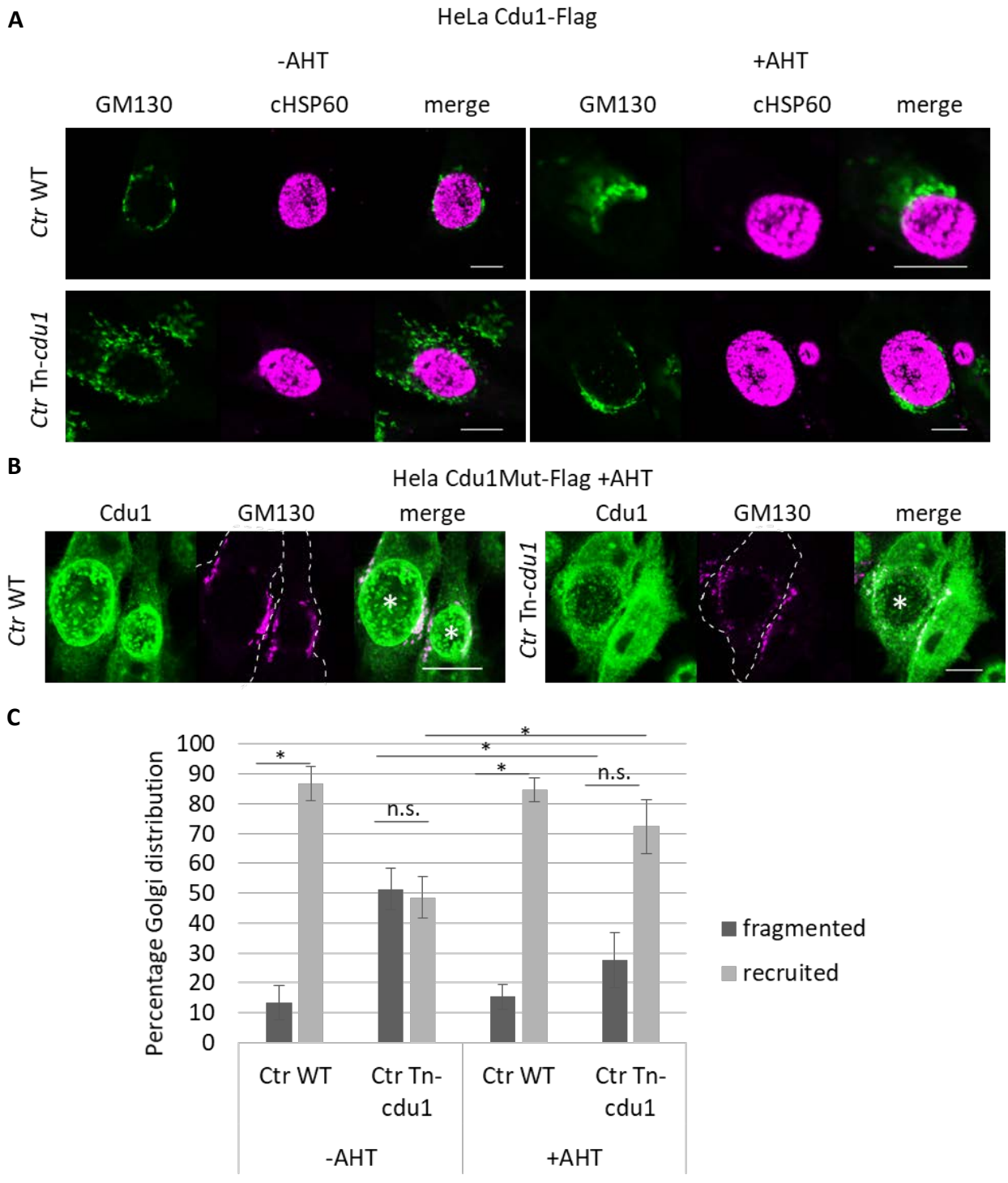


Figure 3.24. Golgi recruitment in *Ctr* Tn-*cdu1* infected cells is blocked due to *Chla*DUB1 inactivity. A. HeLa Cdu1-Flag cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* with an MOI of 1. Cells were treated with or without 50 ng/ml AHT at 22 hpi for 2 h. After 24 h of infection cells were fixed with 4 % PFA/sucrose and IF was performed. Cells were stained for chlamydial HSP60 (magenta) and the Golgi protein GM130 (green). Examples of majority are shown. Scale bar, 10 µm. B. HeLa Cdu1Mut-Flag

cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* at an MOI of 1 and treated with AHT 22 hpi for 2 h. After fixation 24 hpi with 4 % PFA/sucrose cells were stained for *Chla*DUB1 (green) and GM130 (magenta). White asterisk mark the chlamydial inclusion and the cell contour is marked in white. Pictures represent the majority. Scale bar, 10 µm. C. Quantification of IF pictures showing fragmented and recruited Golgi in *Ctr* WT or *Ctr* Tn-*cdu1* infected HeLa cells. Three independent experiments were performed. n.s. not significant, * p<0.05.

To reveal whether the altered Golgi localization is having an effect on *Ctr* Tn-*cdu1*s poor growth, the targets of *Chla*DUB1 have to be identified. Since Ankib1 was observed to be an active opponent of *Chla*DUB1, it was tested whether knock-down of Ankib1 can also rescue the Golgi phenotype in *Ctr* Tn-*cdu1*. IF shows that absent Ankib1 in the mutant chlamydial strain leads to recruitment of the Golgi to the inclusion (Figure 3.25 A). The knock-down of Ankib1 protein was validated in immunoblot (Figure 3.25 B). These results indicate that the factor which is responsible for Golgi recruitment to the chlamydial inclusion is target of both *Chla*DUB1 and Ankib1.

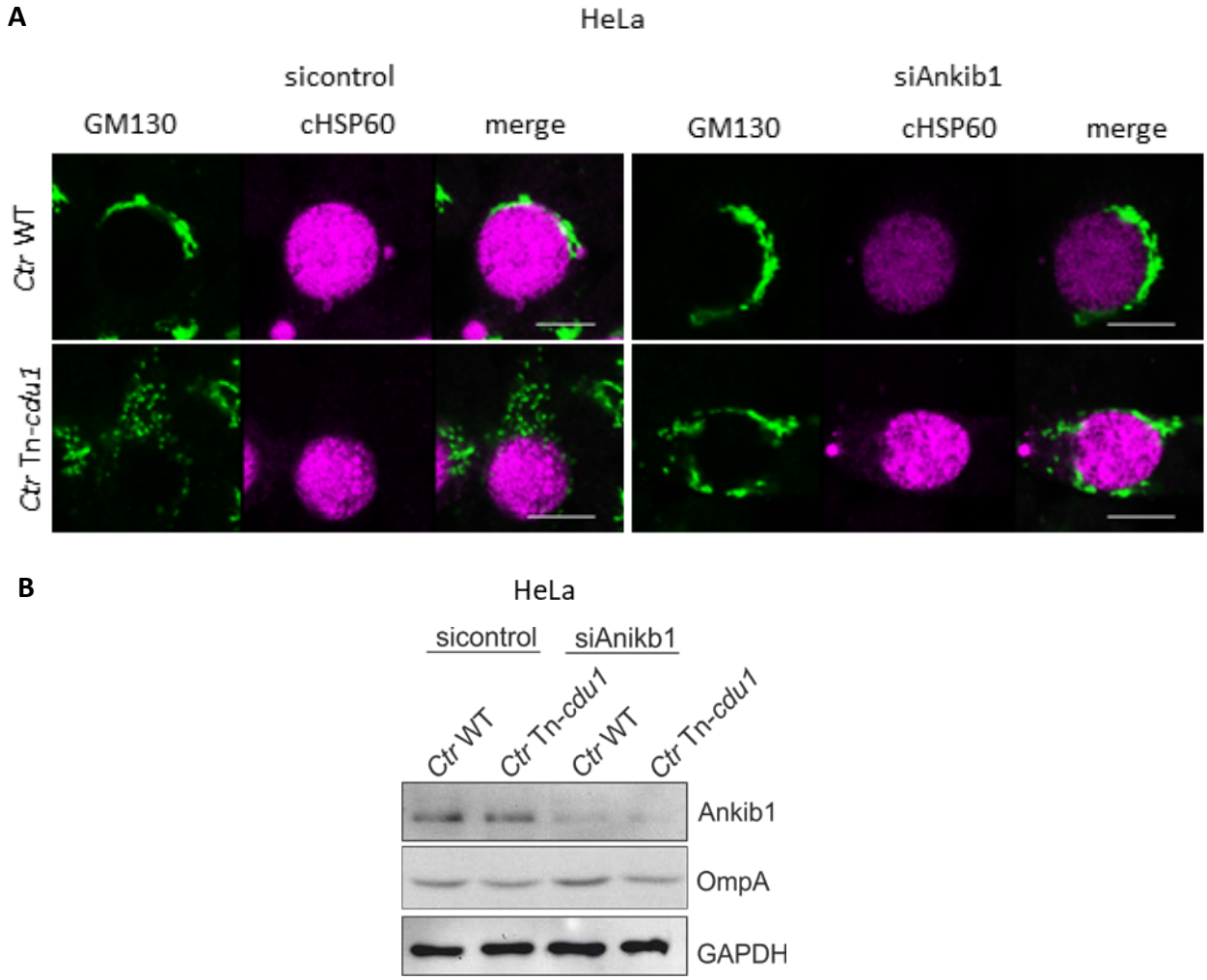


Figure 3.25. Golgi fragmentation in Ankib1 knock-down cells. A. HeLa cells were transfected with siRNA targeting Ankib1 or a control. After 24 h post transfection cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* at MOI 1 for 24 h. Fixation was performed with 4 % PFA/sucrose and cells were stained for the Golgi protein GM130 (green) and cHSP60 (magenta). Pictures are representative for the majority. Scale bar, 10 µm. B. HeLa 229 cells treated with siAnkib1 or sicontrol for 24 h were infected with *Ctr* WT or *Ctr* Tn-*cdu1* (MOI 1) for another 24 h. Lysates were taken and analyzed for knock-down of Ankib1 protein, chlamydial OmpA and GAPDH as a control.

3.4.3 Autophagy does not affect *C. trachomatis* Tn-*cdu1* growth

As shown above *Ctr* Tn-*cdu1* proliferate slower than wildtype *Chlamydia* and produce less infectious progeny in primary cells (Figure 3.20). The growth of intracellular bacteria depends on various factors that partly derive from the host cell or form the bacterium itself. The sequencing of *Ctr* Tn-*cdu1* was performed by the lab of Scott Hefty and showed no mutation in other chlamydial genes that would explain the slowed development (Fischer, et al., 2017). Additionally qRT-PCRs were performed to check for the mRNA expression level of chlamydial genes like the second deubiquitinase *Chla*DUB2 (Cdu2) and chlamydial major outer membrane protein A (OmpA) but did not show any changes. Therefore, the focus was turned towards cellular mechanisms that influence chlamydial growth. First it was checked whether *Ctr* Tn-*cdu1* inclusions get recognized by the host autonomous defense system in early time points of infection. Therefore, IF was performed that show no co-localization of ubiquitin and chlamydial HSP60 of *Ctr* WT or *Ctr* Tn-*cdu1* in the first hour of infection (Figure 3.26 A). Moreover, in HeLa cells the amount of Lamp1 positive vesicles showed no difference between *Ctr* WT and *Ctr* Tn-*cdu1* infection (Figure 3.26 B). This finding confirms that the growth defect of *Ctr* Tn-*cdu1* is not ascribed to early clearance of the inclusion but happens in later stages.

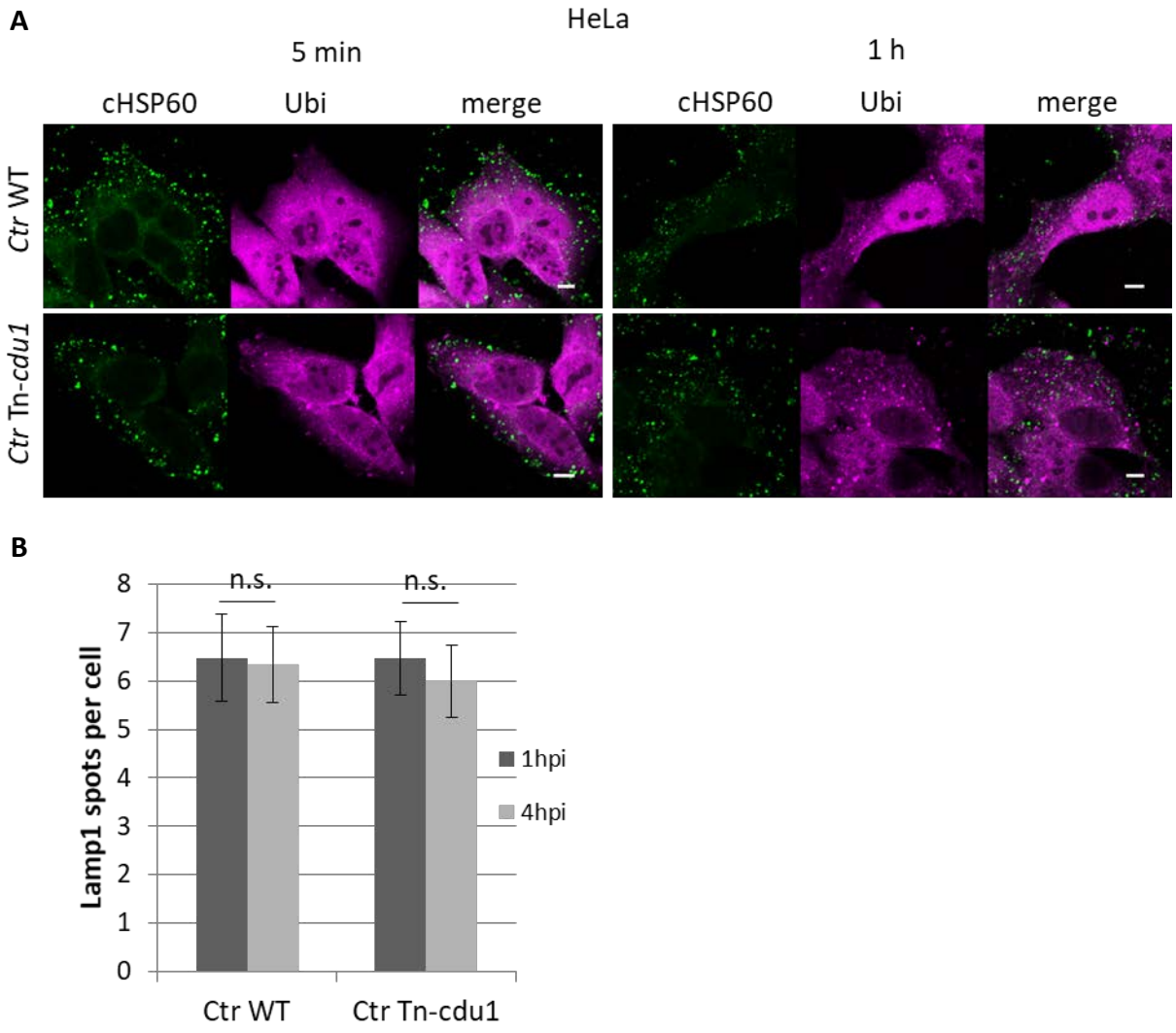


Figure 3.26. No accumulation of ubiquitin, *Chla*DUB1 and Lamp1 with the chlamydial particles in early infection. A. *Ctr* WT and *Ctr* Tn-*cdu1* were taken to infect HeLa cells. A MOI of 20 was used and cells were centrifuged in order to synchronize the infection. Cells were fixed with 4 % PFA/sucrose after 5 min or 1 h of infection. IF was performed by staining of cHSP60 (green) and ubiquitin (magenta). Scale bar, 10 µm. B. HeLa cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* with an MOI of 1 and incubated for 1 h or 4 h at 35°C. Cells were immediately fixed using 4 % PFA/sucrose, stained with antibodies targeting Lamp1, cHSP60 and Phalloidin and analyzed by the Operetta automatic microscope. For each well 12 pictures were analyzed and Lamp1 spots per cell were calculated. n.s. not significant.

The group of Weber and colleagues reports the lethal effect of ruptured chlamydial inclusions on the host cell (Weber, et al., 2017). When *Chlamydia* are forced to leave the inclusion e.g. when the inclusion membrane gets instable by Inc protein knock-out, the bacteria get easily recognized by the hosts defense system and end up in autolysosomes (Weber, et al., 2017). Moreover, the free bacteria in the cytosol trigger the autophagy pathway eventually leading to cell death (Weber, et al., 2017). Since the *Chla*DUB1 protein is like Inc proteins integrated into the inclusion membrane, it was tested whether the inclusion of *Ctr* Tn-*cdu1*, missing active *Chla*DUB1 is still stable and separating the inclusion content from the cytoplasm. Therefore, a small dextran molecule that was coupled to FITC was used, which is able to cross tiny leaks in the inclusion membrane. Infection of HeLa cells and subsequent transfection with FITC-

dextran revealed that neither in *Ctr* WT nor in *Ctr* Tn-*cdu1* infected cells the molecule was able to cross the inclusion membrane and stain the lumen (Figure 3.27).

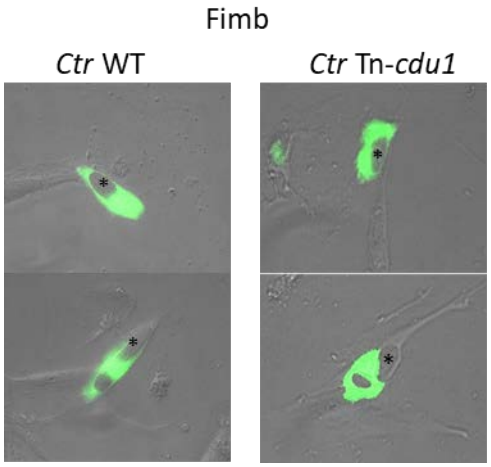


Figure 3.27. Inclusion integrity remains in *Ctr* WT and *Ctr* Tn-*cdu1* infected cells. Fimb cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* with an MOI of 0.3 for 9 h and subsequent transfected with FITC-dextran for 1 h. Distribution of FITC-dextran (green) was monitored under the microscope starting 24 hpi until lysis of the infected cell. Here pictures at 30 hpi are shown. Asterisks mark the chlamydial inclusions.

The inclusion of *Ctr* Tn-*cdu1* is not leaky, despite of the activation of the autophagy pathway in infected cells. The autophagy of bacteria - xenophagy - leads to the clearance of the intruder. In order to test whether autophagy is involved in the slowed growth of *Ctr* Tn-*cdu1*, HeLa cells were treated with Bafilomycin A1, which is an inhibitor of autophagy that works on the H+-ATPase of the lysosome. It inhibits the acidification of the compartment and consequently the degradation of targets of the autophagic system. Hence Fimb cells were treated with Bafilomycin A1 7 hpi with *Ctr* WT and *Ctr* Tn-*cdu1*. Bacteria proliferated until 26 hpi when lysates were taken and immunoblots were performed. Figure 3.28 shows the accumulation of LC3II due to lysosomal inhibition in Bafilomycin A1 treated cells (Figure 3.28 A, LC3II lower band). Moreover, the growth defect of *Ctr* Tn-*cdu1* in the primary infection is not rescued when degradation of autophagy is blocked (Figure 3.28 B).

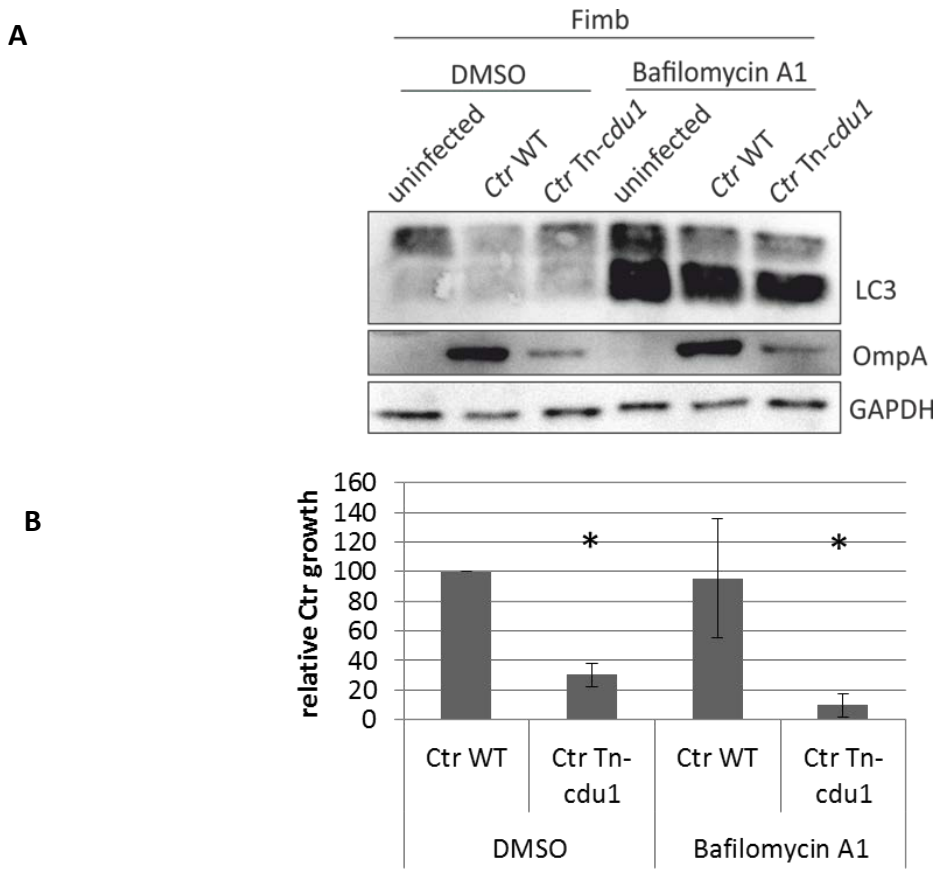


Figure 3.28. Chlamydial growth is not rescued in Bafilomycin A1 treated Fimb cells. A. Fimb cells were infected with *Ctr* Tn-*cdu1* or *Ctr* WT and incubated for 7 h until 10 nM Bafilomycin A1 was added or the same amount of DMSO as a control. After 26 hpi lysates were taken and analyzed in immunoblots for LC3, OmpA and GAPDH. B. Quantification of immunoblots showing relative chlamydial growth in Bafilomycin A1 treated Fimb cells. Growth of control treated *Ctr* WT was set 100 %. Mean values of three independent experiments are shown. * p<0.05.

In addition to block of autophagy by inhibitor treatment, knock-down of an essential autophagy marker was established and infected to check for restored growth of *Ctr* Tn-*cdu1*. P62 plays a major role in recognition of ubiquitinated proteins, acts as a receptor for other autophagy proteins and locates at the inclusion of *Ctr* Tn-*cdu1* during infection. Therefore, knock-down of p62 was established in Fimb cells before infection with *Ctr* WT or *Ctr* Tn-*cdu1*. The knock-down of p62 did not rescue the growth deficit of *Ctr* Tn-*cdu1* (Figure 3.29). This finding is in line with the inhibitor experiment that targets the late phase of autophagy whereas knock-down of p62 inhibits early stage of autophagy.

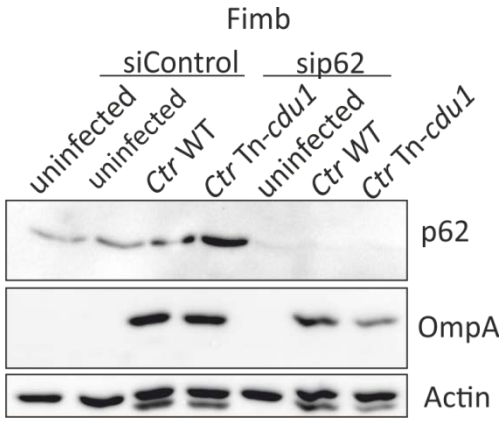


Figure 3.29. Knock-down of autophagy marker p62 does not rescue the growth defect of *Ctr* Tn-*cdu1*. Primary Fimb cells were treated with sip62 or control siRNA for 24 h before infection with *Ctr* WT or *Ctr* Tn-*cdu1* with an MOI of 0.3. After 24 hpi lysates for immunoblots were taken and analyzed for p62, chlamydial OmpA and beta-actin.

The knock-down of p62 did not lead to a rescue of the growth defect. However, multiple autophagy markers were identified to be located at the chlamydial inclusion of *Ctr* Tn-*cdu1* that could compensate the loss of p62. In order to fully exclude the autophagy machinery to be involved in the growth defect of the mutant *Chlamydia,* a progeny assay with the PentaKO cell line was performed. The PentaKO cell line is based on a HeLa cell line with knock-outs of five autophagy receptors p62, optineurin, NDP52, NBR1, TAX1BP1 as well as a deletion in the gene of the E3 ligase Parkin which is also present in the parental strain (Lazarou, et al., 2015). The growth defect of *Ctr* Tn-*cdu1,* however, could not be rescued in primary nor progeny infection of PentaKO cells (Figure 3.30). It must be noted that even though the PentaKO cell line is based on a HeLa cell the initial growth defect of *Ctr* Tn-*cdu1* is much more sensible than in the HeLa 229 cells. All in all the inhibitor experiment and the knock-down of autophagy markers excludes the participation of classic autophagy in the growth defect of *Ctr* Tn-*cdu1* in primary cell lines.

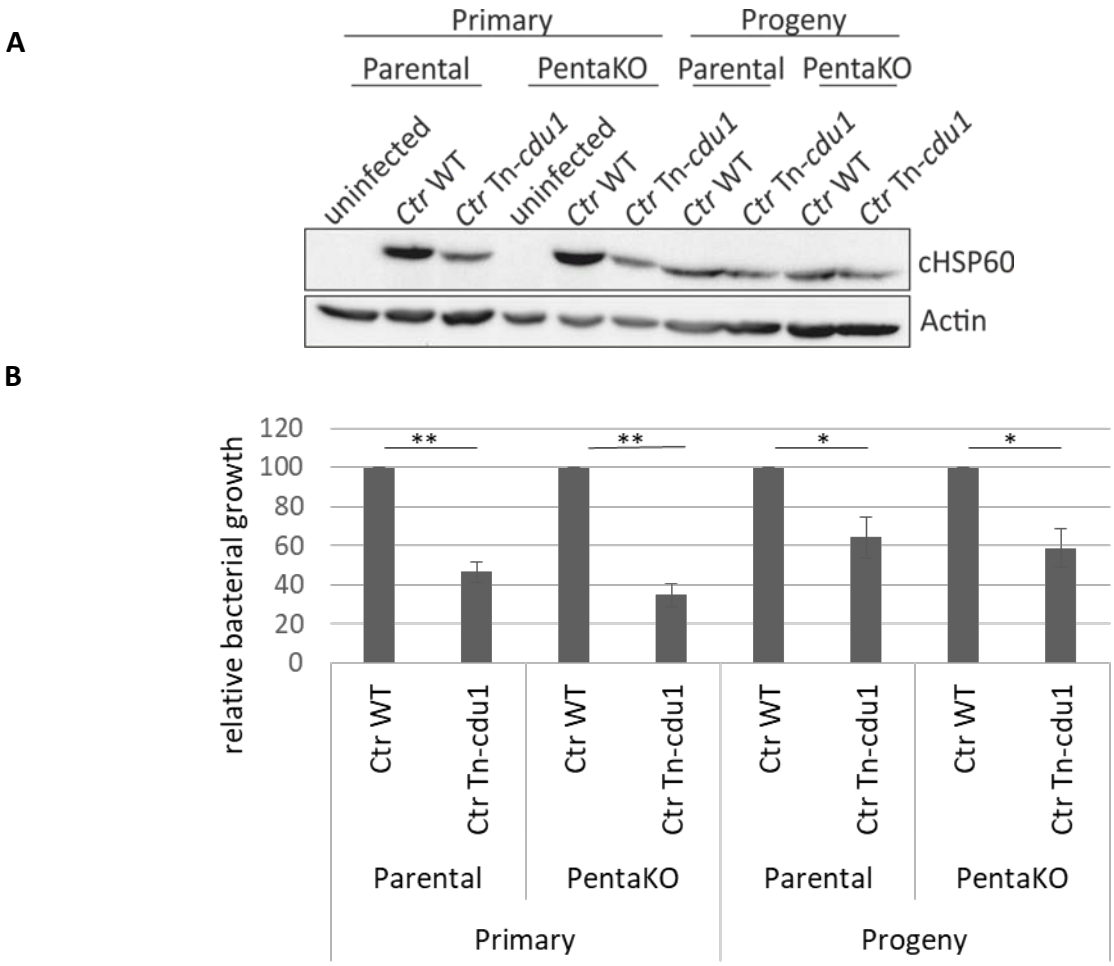


Figure 3.30. Growth defect of *Ctr* Tn-*cdu1* is not rescued in PentaKO cell line. A. PentaKO cells were obtained by the group of Lazarou and are characterized by deletions in five autophagy receptor genes NDP52, optineurin, p62, NBR1 and TAX1BP1 as well as the lack of Parkin that is also missing in the parental strain. PentaKO and Parental cells were seeded and infected with *Ctr* WT or *Ctr* Tn-*cdu1* (MOI 1) for 24 h before lysates for primary infection were obtained. Cells infected for 48 h were lysed with glass beads and infectious supernatant was used to re-infect fresh cells for 24 h. Lysates were taken for progeny infection and immunoblot was performed and analyzed for cHSP60 and beta-actin. B. Quantification of immunoblots showing relative bacterial growth in PentaKO and Parental cell lines. Growth of *Ctr* WT was set 100 %. Results of three independent experiments are shown.* p<0.05, ** p<0.01.

3.5 Conservation in other *Chlamydia* species and host cells

3.5.1 *Chla*DUB1 in different *Chlamydia* species

The previous experiments were all performed in *C. trachomatis* L2 strains. However, also other serovars of *C. trachomatis*, specialized to different cell types and even diverse *Chlamydia* species that target separate hosts encode for the deubiquitinase 1. The serovars *C. trachomatis* A and D as well as the mouse pathogen *C. muridarum* encode for the deubiquitinase. *C. pneumoniae* on the other hand does not encode for *Chla*DUB1 and is used here as a negative control. Analysis of the nucleotide sequence shows the similarities within the species of *C. trachomatis* serovar L2, A and D (appendix). The nucleotide sequence of *Chla*DUB1 from *C. muridarum* shows with 37.6 % a higher variety of mismatches compared

to the sequence of the *C. trachomatis* L2 strain (appendix).

In order to check for the expression of the deubiquitinase in different *Chlamydia* strains and species an immunoblot was performed. The antibody raised against the *Chla*DUB1 protein of *C. trachomatis* L2 recognizes the DUB of the other *C. trachomatis* strains A and D (Figure 3.31 A+B). The DUB of *C. muridarum* on the other hand cannot be detected using this antibody, probably due to the divergent sequences and subsequent different protein folding (Figure 3.31 A). As expected *C. pneumoniae* does not express the DUB, since it does not encode for it. The expression level of the DUB does not influence the expression level of host Ankib1 in any chlamydial strain, only in *C. pneumoniae* samples the level of Ankib1 is increased (Figure 3.31 A). Next it was examined whether all C*hlamydia* strains that express *Chla*DUB1 also show the same localization of the DUB around the chlamydial inclusion. In *C. trachomatis* serovar A the *Chla*DUB1 protein can be clearly localized to the chlamydial inclusion whereas in serovar D only a slight signal for *Chla*DUB1 is visible around the inclusion (Figure 3.31 B). This finding goes hand in hand with the immunoblot data that *C. trachomatis* D does not express as much amount of *Chla*DUB1 protein as the strains A or L2. The inclusions of all *Chlamydia* are clean from ubiquitin signal independent of the expression of *Chla*DUB1, since the inclusion of *C. pneumoniae* is also free of ubiquitin (Figure 3.31 C). Moreover, it was tested where Ankib1 localizes during infection of these bacteria. The IF pictures show the presence of Ankib1 around the inclusion of *C. trachomatis* serovar A and D, but not for *C. muridarum* or *C. pneumoniae* (Figure 3.31 D).

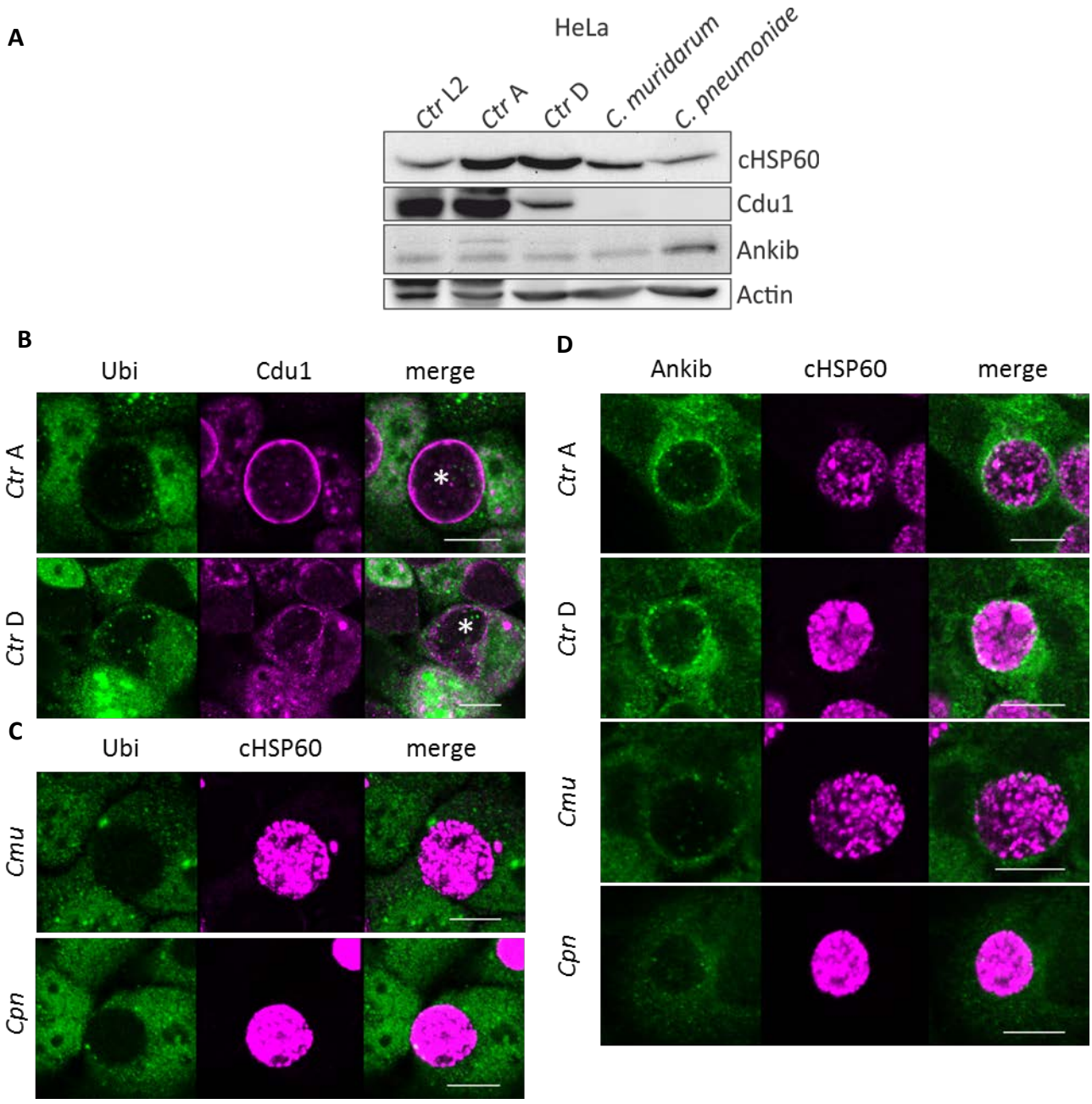


Figure 3.31 Expression and localization of *Chla*DUB1 and Ankib1 in different *Chlamydia* species. A. Immunoblot of HeLa cells infected with *C. trachomatis* serovar L2, A or D, *C. muridarum* for 24 h or *C. pneumoniae* for 48 h. Infection was performed with an MOI of 1 for all bacteria. Lysates were taken and analyzed in immunoblot for chlamydial HSP60 and *Chla*DUB1 expression. Beta-actin was used as a loading control. B-D. Immunofluorescence of HeLa cells infected with *C. trachomatis* serovar L2, A or D, *C. muridarum* and *C. pneumoniae*. HeLa cells were infected with the chlamydial strains with an MOI of 1 for 24 h and 48 h for *C. pneumoniae* before fixation with 4 % PFA/sucrose. Cells were stained for ubiquitin (B+C, green), Cdu1 (B, magenta), cHSP60 (C+D, magenta) and Ankib1 (D, green). Pictures are examples representing the majority. Scale bar, 10 µm.

3.5.2 *Ctr* Tn-*cdu1* in mouse cells

The response to bacterial invasion in mouse cells differs from the human host defense mechanisms. The mouse encodes for 13 GBP proteins that are transcribed upon bacteria-induced IFNɣ secretion and enable the activation of the inflammasome (Man, et al., 2017). Moreover, upon *C. trachomatis* infection and IFNɣ challenge mouse cells express IRGs that bind to the inclusion membrane, destabilize it and induce apoptosis (MacMicking, 2012, Meunier and Broz, 2015). The mouse pathogen *C. muridarum* on the other hand has adapted to its host and prevents IFNɣ induced clearance (Haldar, et al., 2016). In order to analyze what role *Chla*DUB1 plays during cell autonomous defense in mouse cells, murine cells were infected with the *C. trachomatis* mutant *Ctr* Tn-*cdu1*. *C. trachomatis* infection gets cleared in murine cells only in the presence of IFNɣ that is secreted by immune cells (Haldar, et al., 2015). Since *Ctr* Tn-*cdu1* shows a strong growth defect in human primary cells the growth of the mutant in mouse Mef cells was tested in the absence of IFNɣ. The immunoblot reveals reduced growth for *Ctr* Tn-*cdu1* in primary infection that amplifies in progeny infection (Figure 3.32 A). Therefore, the lack of *Chla*DUB1 is also leading to a developmental diminution of the bacteria in mouse cells. In the next step the presence of autophagy markers were checked in mouse epithelial cells. As for human cells in mouse cells the inclusion of *Ctr* Tn-*cdu1* gets ubiquitinated and decorated by p62 autophagy receptor, whereas the wildtype stays clear of this posttranslational modification (Figure 3.32 B). *Ctr* Tn-*cdu1* inclusions are ubiquitinated in mouse cells, therefore experiments were performed to test whether Ankib1 is also the E3 ligase responsible for the posttranslational modification. Unfortunately the human Ankib1 antibody did not stain for the mouse Ankib1 protein as the antigen sequence identity for mouse was only 77 %. However, the knock-down of Ankib1 by siRNA and subsequent IF staining reveals the necessity of Ankib1 for ubiquitination in *Ctr* Tn-*cdu1* infected cells (Figure 3.32 C). Analysis of IF data shows that more than 70 % of *Ctr* Tn-*cdu1* inclusions in mouse McCoy cells with control knock-down are decorated with ubiquitin, while only 28 % of cells treated with Ankib1 siRNA show the modification (Figure 3.32 D). Taken together Ankib1 is not only essential for ubiquitination of *Ctr* Tn-*cdu1* inclusion in human but also in murine cells.

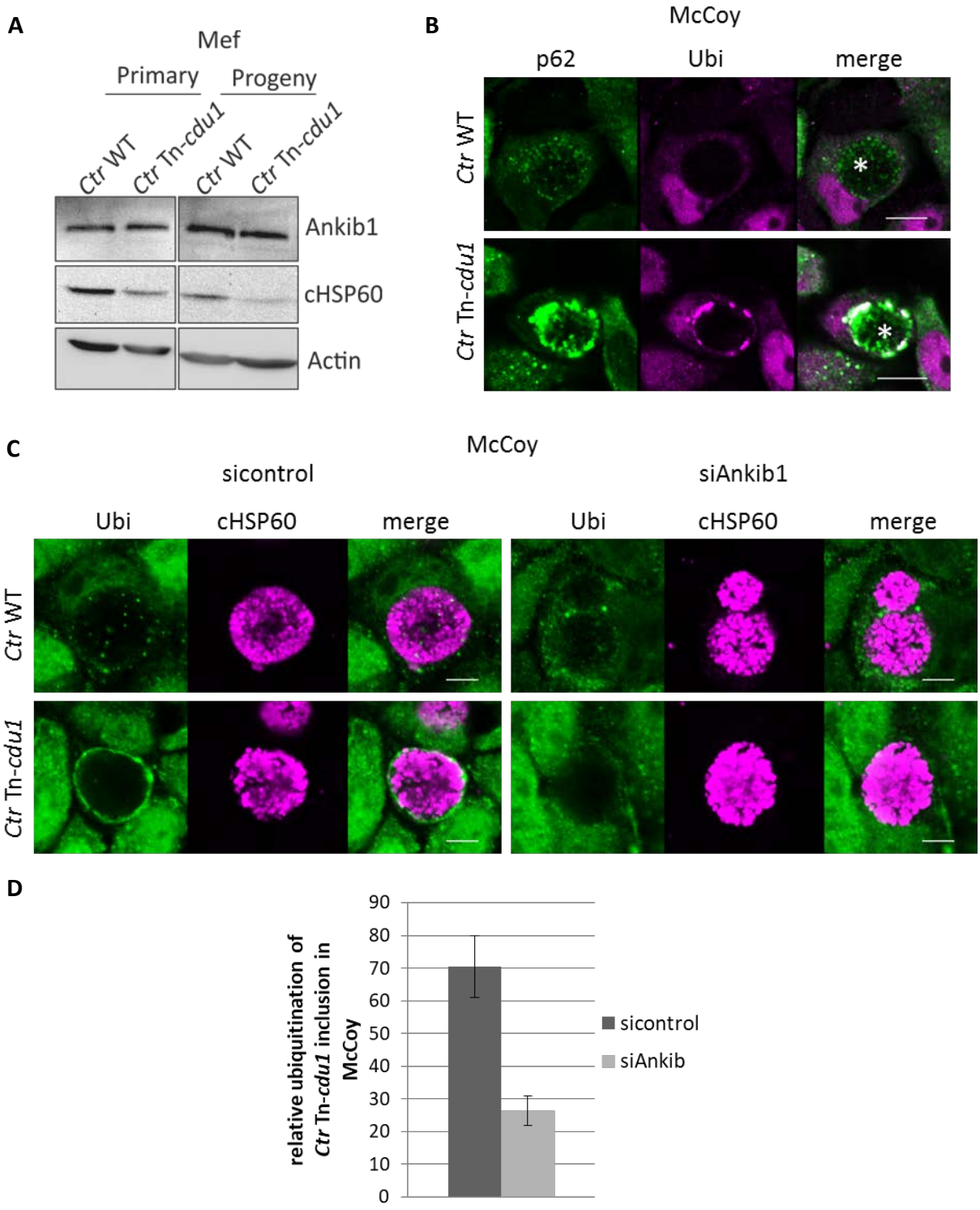


Figure 3.32. Infection of murine cells with *Ctr* WT and *Ctr* Tn-*cdu1*. A. Mef cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* at an MOI of 1 for 24 h, before primary lysates were taken. For progeny infection cells were lysed with glass beads 48 hpi and fresh Mef cells were infected with the *Chlamydia* containing supernatant. Progeny lysates were taken 24 hpi and immunoblots were performed to check for HSP60 levels. B. McCoy cells were infected with *Ctr* WT or *Ctr* Tn-*cdu1* with an MOI of 1 and incubated for 24 h. Cells were fixed with 4 % PFA/sucrose and stained for p62 (green) and ubiquitin (magenta). The asterisk indicates the chlamydial inclusion. Examples of the majority are shown. Scale bar, 10 µm. C. McCoy cells were treated with siRNA targeting Ankib1 or a control sequence for 24 h, before infection with *Ctr* WT or *Ctr* Tn-*cdu1* at MOI 1. 24 hpi cells were

fixed using 4 % PFA/sucrose and stained for chlamydial HSP60 (magenta) or ubiquitin (green). Pictures are representative for the majority. Scale bar, 10 µm. D. Quantification of ubiquitination of *Ctr* Tn-*cdu1* inclusions in infected McCoy cells treated with siAnkib1 or sicontrol. IF pictures were analyzed according to the ubiquitin signal around the inclusion of *Ctr* Tn-*cdu1*. Mean values of two independent experiments are shown.

3.5.3 *Ctr* Tn-*cdu1* infection in neutrophils

Chlamydia are able to infect epithelial, endothelial and immune cells. During infection of the host immune cells migrate to the side of infection and get in contact with emerging bacteria. Immune cells like macrophages phagocytose *Chlamydia* and the bacteria are active until they are directed to the lysosome and degraded (Beagley, et al., 2009). Neutrophils can induce neutrophil extracellular trap (NET) formation upon bacterial contact and thus capture extracellular pathogens limiting their spread (Brinkmann, et al., 2004). However, it is also known that *Chlamydia* can induce cell death in infected immune cells in order to restrain the number of potential opponents and to evade the host immune system (Jendro, et al., 2004, Olivares-Zavaleta, et al., 2011). Moreover, the serovar L2 of *C. trachomatis* uses macrophages and monocytes to invade submucosal tissues and to migrate to lymph nodes where the infection establishes. In order to test how the truncated *Chla*DUB1 protein alters the infection in immune cells, freshly isolated human neutrophils were infected with *Ctr* WT and *Ctr* Tn-*cdu1* for 4 h before lysis of the cells and subsequent re-infection of HeLa cells using the infectious supernatant. Figure 3.33 A and B clearly show that *Ctr* WT can persist in the cell for a couple of hours without complete degradation and is able to re-infect HeLa cells. The re-infection of *Ctr* Tn-*cdu1* on the other hand results in slight number of inclusion and consequently a weak cHSP60 signal (Figure 3.33 A+B). Survival or re-infection of *Ctr* Tn-*cdu1* is diminished in neutrophils. It is known that the secreted chlamydial protease-like activating factor (CPAF) is essential for avoidance of neutrophil activation. CPAF is accumulated in the chlamydial inclusion and gets released into the extracellular matrix together with the bacteria by cell lysis where it cleaves the formyl peptide receptor 2 (FPR2) and consequently prevents neutrophil NET formation (Rajeeve, et al., 2018). *Ctr* Tn-*cdu1* infected HeLa cells were analyzed for CPAF expression in order to rule out that missing CPAF causes activation of neutrophils and consequently the decline in re-infection. The immunoblot clearly shows that the protein level of CPAF is not altered in *Ctr* Tn-*cdu1* compared to the wildtype (Figure 3.33 C). These findings indicate that *Chla*DUB1 has a direct or indirect impact on survival of *Chlamydia* in neutrophils.

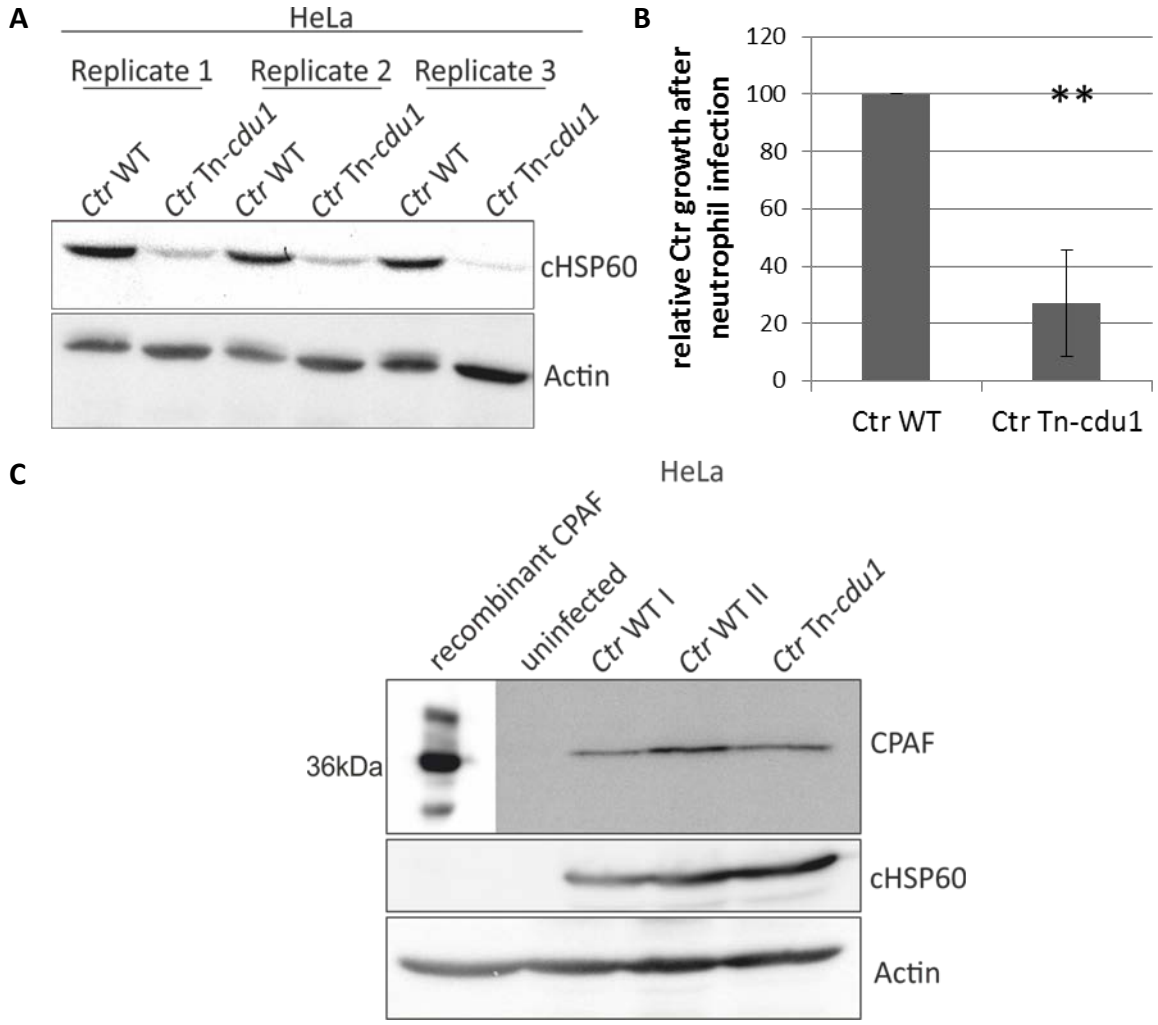


Figure 3.33. Survival of *Ctr* Tn-*cdu1* in neutrophils decreases. A. Freshly isolated human neutrophils were infected in triplicates with *Ctr* WT or *Ctr* Tn-*cdu1* with an MOI of 10 for 4 h before washing and lysis of the cells using glass beads. Supernatant was used to re-infect HeLa cells and cells were incubated for 24 h until lysates were taken and immunoblot was performed. Blots were probed with antibody against cHSP60 and beta-actin. B. Quantification of immunoblots of chlamydial growth in HeLa cells after neutrophil infection. Growth of *Ctr* WT was set 100 %. Mean values of four independent experiments are shown. ** p< 0.01. C. HeLa cells were infected with two wildtype strains from different origins or *Ctr* Tn-*cdu1* with an MOI of 1 for 36 h. Lysates were taken and immunoblot was performed to check for the presence of CPAF (36 kDa). Recombinant CPAF was used as a positive control. Two wildtype strains and the *Ctr* Tn-*cdu1* strain were tested for CPAF expression.

4 Discussion

Genetic manipulation is a common tool to investigate bacteria and the infection pathways. For *Chlamydia* some transformation methods to integrate or adapt genes are known, nevertheless, time consuming and laborious. Moreover, not all chlamydial genes can be knocked out since genes became essential due to the reduced genome size. The establishment of the inducible knock-down of genes using the CRISPRi system, shown here, can be of great help to further study chlamydial gene products and their impact on pathogenesis.

The human pathogen *C. trachomatis* has perfectly adapted to its host in order to survive and proliferate. As an obligate intracellular bacteria *Chlamydia* has reduced its genome over time and compensates missing genes and synthesis pathways by recruitment and consumption of host nutrients (Stephens, et al., 1998). Along these lines *C. trachomatis* mobilizes lipids like sphingosine, cholesterol, PE and PS from the host cell to the inclusion where it fuses and enables further growth of bacteria and the inclusion membrane (Carabeo, et al., 2003, Hackstadt, et al., 1995, Hatch and McClarty, 1998, Wylie, et al., 1997). However, *Chlamydia* not only adapted to the host cell, but to the whole host organism. Since the life cycle of the bacteria comprises a rather long period of non-infectious RB, where host cell death would lead to the loss of all progeny, it is essential for the spread of the bacteria to keep the host cell alive and undetected by the immune system. In fact adaptation to the host is so adapted that the human pathogen is capable to develop in human cells, whereas infection of mice leads to a rather fast clearance of infection (Haldar, et al., 2015). Also vice versa, the infection of the mouse pathogen *C. muridarum* gets cleared in human cells, while it stays undetected in mouse cells (Haldar, et al., 2016). The host cell has evolved strategies, the so called cell autonomous immunity to get rid of the bacterial intruder. Recognition of the bacteria by ubiquitination and subsequent engulfment in a phagosome, which destroys the cargo during xenophagy is as much part of it as the recruitment of immune cells to the site of infection (Dikic, 2017). With this work the role of the deubiquitinase *Chla*DUB1 in avoidance of the host cell defense mechanism should be revealed.

4.1 CRISPRi system in *C. trachomatis*

The CRISPRi system is widely used in eukaryotic and in prokaryotic cell systems to reduce gene transcription. Knock-down of proteins is often used when knock-out mutants are not viable. As it comes to *C. trachomatis* the reduced genome hardy leaves space for feasible knock-outs. In order to investigate chlamydial genes further we planned to establish a CRISPRi system in *C. trachomatis*.

The plasmid pCRISPRi:gCdu1III encodes for a gRNA which targets *Chla*DUB1 shortly after the start codon, preventing transcription of the gene (Figure 3.1 B). In *E. coli* the size and functionality of the dCas9 protein originating from *S. pyogenes* was confirmed by a gRNA targeting likewise transfected GFP (Figure 3.2). However, the size of dCas9 in *C. trachomatis* decreases after sorting for single clones (Figure 3.3). No single clone was found that expresses the correct size of the dCas9 protein. Repeated investigation on the *Ctr* pCRISPRi:gCdu1III A8 pool that initially showed traces of full size dCas9 revealed vanishing full size dCas9 over time. It seems that *C. trachomatis* is not able to express a stable protein in full size and

processes it to a smaller protein. Sequencing of the pCRISPRi:gCdu1III plasmid out of transformed *Chlamydia* showed no mutations or deletions in the dCas9 sequence. However, microscopy showed that single clones A8.2 and A8.3 after two rounds of isolation still harbor bacteria that do not express GFP, which is also encoded on the plasmid. Since the selection pressure with PenG is strictly depleting wildtype bacteria, this means that the plasmid is not stable in *C. trachomatis*, which was also observed by Ouellette (Ouellette, 2018). RT-PCR data and the immunoblot results do not show dCas9 RNA or protein in infected cells in the absence of AHT, meaning the inducible promoter is tightly regulated and not leaky (Figure 3.7, 3.3 B, 3.6 A). Though the dCas9 protein is not expressed in the right size the knock-down of *Chla*DUB1 works for up to 50 % of total DUB protein and can be induced to various time points (Figure 3.6). Important for knock-down of proteins is also the half-life and stability of the already expressed protein. Proteins with a short half-live period will lead faster to a stronger knock-down than proteins that are stable for a long time. The investigation on cell survival revealed that the CRISPRi dependent reduction of *Chla*DUB1 is not enough to overcome apoptosis resistance provided by *C. trachomatis* infection (Figure 3.8). Likely the remaining *Chla*DUB1 enzyme on the chlamydial inclusion is active and still stabilizes Mcl-1, which was stable on protein level also during *Chla*DUB1 knock-down (Figure 3.8 A). Furthermore, the bacteria not only regulate Mcl-1 levels by stabilization of the protein but also through increased transcription (Rajalingam, et al., 2008). CRISPR systems often face the problem of unwanted side effects on other genes. Here a strong decrease of GFP signal is observed when infected cells are treated with AHT (Figure 3.4). In fact up to 9 nucleotides of the gRNA against *Chla*DUB1 also match with the GFP sequence. Publications of Fu and Pattanayak show that mismatches of up to 5 nucleotides between gRNA and target can still lead to the binding and subsequent down regulation of transcription (Fu, et al., 2013, Pattanayak, et al., 2013). The knock-down hereby can be as strong as for the real target or even exceed it (Pattanayak, et al., 2013). For GFP the number of mismatches with the gRNA is higher but might be an explanation for the diminished signal in immunoblot and flow cytometry (Figure 3.4). AHT treatment is not negatively influencing GFP since *Ctr* pTet:GFP express the fluorophore in the presence of AHT. *Ctr* pCRISPRi:gCdu1III at first seemed to grow normal in cells, since number and morphology of inclusions was not conspicuously changed. However, knock-down induction negatively influences the growth of bacteria, as microscopy showed (Figure 3.5). This growth deficit is not due to AHT treatment as wildtype *C. trachomatis* grow normally even in the presence of the inducer. It is not totally clear what leads to the growth deficiency in *Ctr* pCRISPRi:gCdu1III. One possibility is that the expression of the dCas9 protein, that is 3-4 times the size of usual chlamydial proteins, creates a deficiency of components in the bacteria that cannot be compensated and are consequently missing for bacterial protein synthesis. Additionally the knock-down of the DUB, even when not so strong, can influence the fitness of the bacteria negatively. Further investigations with different gRNAs will help to clarify this issue. Since the CRISPRi system did not show a very strong effect on *Chla*DUB1 protein levels and in the meantime a chlamydial mutant lacking the active protease became available, further investigations on *Chla*DUB1 and its role during infection were performed with the *Ctr* Tn-*cdu1* mutant.

4.2 Activation of autophagic system in *Ctr* Tn-*cdu1* infected cells

During xenophagy the bacteria are recognized via receptors like NOD or TLR which leads to the onset of various pathways (Akira, et al., 2006). E3 ligases modify the pathogen or the surrounding membrane with ubiquitin that serves as a signal for autophagy receptors like p62 to assemble and consequently to the

formation of an autophagophore and destruction (Dikic, 2017). *C. trachomatis* in an infected human cell is recognized by PRRs, nevertheless, the secreted bacterial effectors in the host cell suppress the response to these signals in order to keep the cell alive and silent (Fischer and Rudel, 2016). The deubiquitinase *Chla*DUB1 is one of these chlamydial effectors that is secreted to the inclusion membrane and faces the cytosol with the active site (Fischer, et al., 2017, Wang, et al., 2018). The DUB is known to inhibit NF-κB activation by deubiquitination of the inhibitory unit IκBα, which protects it from proteasomal degradation (Le Negrate, et al., 2008). Upon activation, NF-κB translocates into the nucleus and enables expression of several hundred genes that are also involved in inflammatory and immune response (Bonizzi and Karin, 2004). Another substrate of *Chla*DUB1 is the anti-apoptotic protein Mcl-1 that is stabilized on protein level during infection (Fischer, et al., 2017). Inhibition of the NF-κB pathway and stabilization of anti-apoptotic Mcl-1 facilitates survival for *Chlamydia.*

However, the inactive DUB is not able to remove ubiquitin any more from the inclusion surface (Figure 3.9, 3.10) (Fischer, et al., 2017). It is currently not known what proteins, human or bacterial, are getting ubiquitinated at the inclusion of *Ctr* Tn-*cdu1*. Autophagy markers bind to ubiquitin and the typical receptors for xenophagy p62, NDP52 and NBR1 (Randow and Youle, 2014) were identified to be at the inclusion in different cell lines (Figure 3.11, 3.13 and 3.32 B). Only optineurin, also a typical maker for xenophagy (Randow and Youle, 2014) was missing at the inclusion of the mutant. NDP52 and p62 are adaptor proteins that bind both ubiquitin and LC3, facilitating the formation of a phagosome (Moscat and Diaz-Meco, 2009, Thurston, et al., 2009). During infection with *Ctr* Tn-*cdu1* the ubiquitin signal as well as p62 and LC3 increases at the inclusion with time of infection (Figure 3.11 C). The re-complementation with active *Chla*DUB1 protein in the host cell cytoplasm, however, results in the loss of ubiquitin and autophagic markers from the mutant inclusion (Figure 3.15). This means it is likely that during infection also the wildtype chlamydial inclusion is recognized by one or more E3 ligases, but the DUB activity on the surface of the inclusion removes the modification immediately.

During selective autophagy the autophagosome forms around the cargo and eventually fuses with endosomes and lysosomes that leads to the acidification of the compartment and degradation of cargo (Randow and Youle, 2014). Bacteria are usually detected in early stages of infection when they escape into the cytosol or interact with the host cell inside their vacuole. During *Ctr* Tn-*cdu1* infection the LC3 containing phagophore is observed to form around the inclusion starting rather late 20 hpi when the inclusion is already established (Figure 3.11 and 3.12). Enzymes for membrane formation were detected 24 hpi and 30 hpi the double membrane forming around the *Ctr* Tn-*cdu1* inclusion was visualized (Figure 3.13 E, F, 3.12 A). However, the complete closure of the phagophore was never observed. Formation of the phagosome around *Ctr* Tn-*cdu1* inclusion was observed in mid phase of infection and number of LC3 positive inclusions increase during infection. Nevertheless, fusion with the lysosome was never observed, since no co-localization with Lamp1 occurred (Figure 3.14). While the phagophore grows the inclusion size increases as well until it fills the majority of the cell cytoplasm and the cell ruptures. The average size of autophagosomes encompasses single organelles or few bacteria in early stages. Enclosure of a structure the size of the chlamydial inclusion has never been observed. In early infection the inclusion of *C. pneumoniae* is positive for Rab5 and Rab7, lysosomal markers, that diminish with progressing infection, but also Rab11 and Rab14 are present at the inclusion, which stay throughout the infection and mark it as recycling endosome protected against degradation (Mölleken and Hegemann, 2017). Also *Mycobacterium bovis* engulfed in a phagosome prevents lysosomal degradation by removal of Rab7 from

the compartment (Via, et al., 1997). It was published that the inclusion of *C. trachomatis* is positive for Rab11 which protects it from fusion with lysosomes (Rzomp, et al., 2003). Other studies revealed that lysosomal markers like Rab5 and Rab7 are missing on the inclusion of *C. trachomatis* (Hügelschäffer, 2018, Rzomp, et al., 2003). Investigation of Rab proteins at the inclusion of *Ctr* Tn-cdu1 will clarify how the inclusion is protected from lysosomal fusion. Moreover, Rab proteins are essential for the correct trafficking of vesicles between the plasma membrane, ER and Golgi network. Rab6 and Rab11 are known to be recruited to the chlamydial inclusion facilitating the tethering and fusion with specific membranes (Rzomp, et al., 2003).

4.3 Growth defect in *Ctr* Tn-*cdu1*

Successful growth of *Chlamydia* faces various obstacles and depends on nutrient supply from the host cell. The bacteria rely on host metabolites and lipids to thrive, nevertheless, limitation of nutrients as well as excess of special amino acids leads to persistency or strongly reduced inclusion size (Al-Younes, et al., 2004). Bacteria and the host cell compete for the same resources, it is essential for the bacteria to gain enough components from the cell, but also that the cell is healthy enough to provide them. *Ctr* Tn-*cdu1* develops slower than wildtype *Chlamydia* since infection of primary cells with the same amount of bacteria results in less progeny for the mutant (Figure 3.20). Also in mouse cells the growth defect of *Ctr* Tn-*cdu1* could be shown (Figure 3.32 A). In the *Ctr* Tn-*cdu1* strain the RBs take more time to replicate and transform into EBs as the RB size is bigger compared with the wildtype (Figure 3.22), which prevents differentiation (Lee, et al., 2018). The growth defect of *Ctr* Tn-*cdu1* in primary cells is strong whereas in HeLa 229 cells only a slight difference between wildtype and mutant growth can be observed (Figure 3.20). Nonetheless, infection of the PentaKO and Parental cell line also shows a strong growth deficit in *Ctr* Tn-*cdu1* (Figure 3.30). Cancer cells and primary cells distinguish from gene expression and metabolic activity. The differences between cells seem to influence chlamydial growth. Also in mice experiments the bacterial load of *Ctr* Tn-*cdu1* was less after five days of infection emphasizing the importance of *Chla*DUB1 during mammalian infection (Fischer, et al., 2017). The growth defect can be explained by either slower replication of the bacteria lacking the DUB or increased clearance of the pathogen through xenophagy and cell autonomous immunity. Xenophagy can be excluded here, since no fusion with late endosomes and lysosomes with the inclusion were observed at any time of infection. Also the rescue of growth through inhibition of autophagy by knock-down and inhibitor treatment was not successful (Figure 3.28, 3.29 and 3.30). However, *Chlamydia* and autophagy are linked since the interference with autophagy regulators in infected cells negatively controls the bacterial development (Al-Younes, et al., 2004). Mechanisms and benefit behind this finding remain to be elucidated but are currently speculated to be involved in provision of nutrients. *Ctr* Tn-*cdu1* seem to grow and replicate slower than the wildtype (Figure 3.22) the mechanism behind that needs further investigation. On the one hand engulfment of the inclusion by LC3 positive phagophore (Figure 3.12) might be a steric obstacle for vesicles that fuse with the inclusion bringing lipids and nutrients. In contrast to that, experiments showed that the endocytic uptake of Transferrin and transport to the inclusion is not slowed down in *Ctr* Tn-*cdu1* infection though (Figure 3.23) and also ceramide transport is normal in mutant strains (Hügelschäffer, 2018). On the other hand the Golgi, a very important source of nutrients for *Chlamydia,* is not recruited to the inclusion surface in bacteria lacking the DUB, but can be recruited again when exogenous *Chla*DUB1 is provided

(Figure 3.24). This result was also verified by the group of David Komander that observed less Golgi membranes around the chlamydial inclusion of *Chlamydia* lacking the active DUB. However, in contrast to this study they did not detect Golgi fragmentation in cells infected with *Ctr* Tn-*cdu1* (Pruneda, et al., 2018). All in all the finding strongly indicates that *Chla*DUB1 activity is directly or indirectly involved in Golgi recruitment during chlamydial infection. The mechanism is unknown but could act via stabilization of certain proteins or via change in signaling. The access of Golgi vesicles is essential for chlamydial growth. Destabilization of the Golgi by knock-out of golgin-84 supports chlamydial growth whereas prevention of fragmentation by Rab11 knock-out inhibits it (Lipinski, et al., 2009). In macrophages the growth of *C. trachomatis* is delayed since Golgi disruption and consequently lipid acquisition is prevented. Moreover, bacteria are recognized by the autophagic machinery and targeted toward lysosomes (Sun, et al., 2012). Therefore, the fragmentation and localization of the Golgi plays a vital role in chlamydial development.

In the more complex mouse system *Ctr* Tn-*cdu1* are less abundant than wildtype *Chlamydia* (Fischer, et al., 2017). Here immune cells play an important role, apart from the before mentioned points. Immune cells like neutrophils get recruited to sites of infection and capture extracellular bacteria by NET formation (Brinkmann, et al., 2004). It was published that immune cells can also be infected by *Chlamydia,* which does not necessarily end in bacterial degradation and cell death (Beagley, et al., 2009). The reason why *Chlamydia* can prevent NET formation and captivity is the secretion of the chlamydial protease CPAF into the inclusion lumen, that pours into the extracellular space along with the bacteria and cleaves FRP2 receptor inhibiting NET activation (Rajeeve, et al., 2018). *Ctr* Tn-*cdu1* as well as *Ctr* WT both express CPAF protease, nevertheless, only the wildtype bacteria survives efficiently in the immune cell and re-infects HeLa cells (Figure 3.33).

The DUB is actively expressed in mid to late phase of infection, nevertheless, lesser amounts of protein are present in early time points too. Whether *Chla*DUB1 directly is important for uptake and survival in neutrophils or whether a secondary protein, influenced by the DUB, causes the effect remains to be elucidated. In epithelial cells no difference between *Ctr* WT and *Ctr* Tn-*cdu1* regarding ubiquitination of the inclusion in early time points can be observed (Figure 3.26). Chlamydial inclusions do not co-localize with the ubiquitin signal and also the number of Lamp1 positive vacuoles, the lysosomes, does not increase in cells infected with bacteria lacking the DUB. The growth defect of *Ctr* Tn-*cdu1* is not due to clearance of the inclusion in early time points of infection. It is pivotal for prevention of detection and onset of the immune response that the inclusion stays intact throughout the development cycle. Weber and colleagues showed that missing Inc proteins in the chlamydial inclusion destabilize it, activate STING and trigger intrinsic apoptosis (Weber, et al., 2017). Since *Chla*DUB1 is also integrated into the chlamydial inclusion the integrity was tested in the bacterial mutant but gives no sign of leakage or enriched cell death (Figure 3.27). This was also confirmed by the master thesis of Sophie Hügelschäffer where the integrity of the inclusion was verified by IF (Hügelschäffer, 2018). All in all the growth defect of *Ctr* Tn-*cdu1* is a direct or indirect cause of *Chla*DUB1 inactivity.

The cell autonomous immunity differs between species as can be seen in human and murine cells. In mouse cells more interferon stimulated genes are active that target bacterial structures. For example the GTPases GBP1 and GBP2 are recruited to *C. trachomatis* inclusions in mouse cells upon IFNγ induction that facilitates the destabilization of the inclusion and clearance through inflammasome activation (Haldar, et al., 2014, Haldar, et al., 2013). Moreover, the inclusions are positive for Irga6, Irgb6 and

Irgb10 which promote bacterial clearance through onset of intracellular host response (Al-Zeer, et al., 2009, Bernstein-Hanley, et al., 2006, Coers, et al., 2008). *C. trachomatis* inclusion gets ubiquitinated in mouse cells which recruits p62 to the surface of the inclusion. Recruitment of the E3 ligase TRIM21 and TRAF6 amplifies the ubiquitin modification emphasizing the signal (Haldar, et al., 2015). These mechanisms of host cell defense are only triggered during infection with the human pathogen. The infection of murine cells with the adapted mouse pathogen *C. muridarum* does not activate IRG or GBP binding to the inclusion surface in the first place, which is probably due to an unknown bacterial factor secreted by the mouse specialist (Finethy, et al., 2015, Nelson, et al., 2005).

Even when challenged with IFNγ no GBP1 could be visualized at the chlamydial inclusion in wildtype or mutant strains in infected human cells (Figure 3.21 A). Also growth of bacteria was enhanced in the same amount when the growth deficit, induced by IDO, was counteracted by tryptophan supply (Figure 3.21 D). Immunoblots confirm that the growth defect in *Ctr* Tn-*cdu1* compared to *Ctr* WT is not changed in presence of IFNα or IFNγ (Figure 3.21). Of course *in vivo* the secretion of IFNs and the associated recruitment of immune cells plays a pivotal role in containment of the infection. Here only two important types of IFN were tested, other types as well as IFN-stimulated genes remain to be elucidated. In Figure 3.21 the progeny infection is much lower in IFNγ treated cells for all *Chlamydia* strains than in IFNα or untreated cells, because IFNγ induces IDO expression leading to tryptophan depletion, which is an essential amino acid for chlamydial growth (Beatty, et al., 1994). IFNα on the other side only increases IDO expression very slightly, hardly affecting tryptophan levels (Wichers and Maes, 2004). Persistency was not observed in IFNγ treated cells, since low concentrations were used in order to not directly interfere with the development of the bacteria. Interestingly in immunoblot it was observed that HUVEC cells express the IFN-stimulated protein MxA when infected with *Chlamydia*, whereas Fimb and HeLa cells need exogenously added IFN for MxA induction (Figure 3.21). This finding suggests that HUVEC cells express IFN upon *Chlamydia* infection. Since only immune cells can secrete IFNγ, HUVEC cells are likely to express IFN type I upon bacterial infection. During the experiments also pre-incubation of cells with IFNγ was tested but strongly interfered with the bacterial growth, even in primary infection. Therefore, addition of all types of IFN was postponed to 6 hpi for the shown experiments.

4.4 Ankib1 as E3 ligase of chlamydial inclusion

Eukaryotic cells have evolved mechanisms to defeat bacterial intruders. One way is the recognition of the bacterium and the mark for degradation, the ubiquitin modification. The ubiquitination of bacteria happens either on the bacterial membrane, for those that escape into the cytosol, or on the vacuole, in which bacteria grow (Mitchell and Isberg, 2017). Special ubiquitin E3 ligases are activated and conjugate ubiquitin to the substrate. For the pathogen *Salmonella typhimurium* for example three E3 ligases LRSAM1, HOIP and ARIH1 were identified to ubiquitinate the bacteria upon escape into the cytosol (Huett, et al., 2012, Noad, et al., 2017, Polajnar, et al., 2017). The intracellular pathogen *Mycobacterium tuberculosis* was found to be K63 ubiquitinated by the RBR E3 ligase Parkin, but also K48 ubiquitin by an yet unknown E3 ligase was described (Manzanillo, et al., 2013). In *C. trachomatis* infected mouse cells challenged with IFNγ it was found that GKS proteins are getting ubiquitinated by TRAF6 and Trim21, which leads to the recruitment of p62 and GBPs that destabilize the inclusion membrane (Haldar, et al., 2015). Human and mouse cells show some fundamental differences in ubiquitination of the inclusion

since human cells lack some IRGs that are involved in membrane targeting in mice (Bekpen, et al., 2005, Haldar, et al., 2015)

4.4.1 SiRNA knock-down screen revealed Ankib1 as E3 ligase of *C. trachomatis* inclusion

In order to identify the E3 ligase that is responsible for the K48 and K63 ubiquitination of the *Ctr* Tn-*cdu1* inclusion, a siRNA knock-down screen was performed. The 589 targets included E1 and E2 enzymes as well as E3 ligases and proteins of E3 complexes like F-box proteins (appendix). For the readout the ubiquitin antibody was not used, since staining was not always reliable. Staining with p62 antibody proved to give good results and was used here instead. Therefore, only targets that are involved in pathways that consequently recruit p62 to the inclusion were considered. In fact p62 preferentially binds to K63 ubiquitin chains, but is also able to bind K48 chains (Cemma, et al., 2011), which are both present at the inclusion of *Ctr* Tn-*cdu1* (Figure 3.10). The screen revealed the involvement of the E1 enzyme UBE1 in ubiquitination of the mutant inclusion, which serves here as a good control for a functional experiment, since only two E1 enzymes are currently known to be involved in ubiquitin-protein conjugation (Jin, et al., 2007). The E2 conjugating enzyme identified was UBE2L3 (Figure 3.16 B and 3.18) that is known to work together with RBR E3 ligases like Ankib1 (Smit and Sixma, 2014) that was also found in the screen (Figure 3.16 B and 3.18). The actual proof that UBE2L3 and Ankib1 are working together to ubiquitinate a substrate on the chlamydial inclusion remains to be given.

The neddylation of cullin in CRLs is necessary for activation and happens via a cascade similar to ubiquitination. The activating enzyme NAE needs ATP to bind Nedd8 and subsequent delivers it to a conjugating enzyme. The inhibitor MLN4924 is able to block activation of NAE by covalent binding to the enzyme (Petroski, 2010). Consequently activation of E3 ligases of the CRL family are inhibited by usage of MLN4924 inhibitor. Using the MLN4924 inhibitor it was excluded that CRL E3 ligases were involved in the ubiquitination of *Ctr* Tn-*cdu1* (Figure 3.17). Since all cullins are neddylated the same way this result is true not only for cullin1, as shown, but all cullins in the cell. Interestingly cullin1 is enriched in cells infected with either *Chlamydia* strain, though the neddylation status of cullin1 is not changed upon infection (Figure 3.17 A). It is known that infection with non-pathogenic bacteria can prevent the neddylation of cullin1 in order to cope with the inflammatory response (Collier-Hyams, et al., 2005). In addition pathogenic *E. coli* strains interfere with the de-neddylation of cullin1, which leads to accumulation of the protein and inactivation of CRLs. Therefore, interference with cullin1 activity is a way that different bacteria and likely *C. trachomatis* use to establish a beneficial environment in the cell.

Only Ankib1 of the RBR family was identified to ubiquitinate the *Ctr* Tn-*cdu1* inclusion (Figure 3.18). Other pathogens like *Salmonella typhimurium* are known to get ubiquitinated by several ligases that are specific for different linkage types (Huett, et al., 2012, Noad, et al., 2017, Polajnar, et al., 2017). HOIP and ARIH1, two of the E3 ligases targeting *Salmonella,* are also members of the RBR family (Polajnar, et al., 2017) as well as Parkin, a E3 ligase recognizing *Mycobacterium tuberculosis* (Manzanillo, et al., 2013). The knock-down of one E3 ligase detecting *Salmonella* always leaves an ubiquitin signal at the bacterium, indicating that more ligases are involved (Polajnar, et al., 2017). The knock-down of Ankib1 in *Chlamydia* infected cells removed all ubiquitin molecules, namely K48 as well as K63 ubiquitin chains from the inclusion of the mutant (Figure 3.18 C), which emphasis Ankib1 to be the only E3 ligase involved. This finding also means that Ankib1 is involved in the synthesis of K48 and K63 ubiquitin chains. Also in mouse

cells knock-down of Ankib1 leads to loss of ubiquitination around the inclusion of the mutant *Ctr* Tn-*cdu1* (Figure 3.32 C), indicating that also in mouse cells Ankib1 is the E3 ligase targeting the *C. trachomatis* inclusion. Complementation with active *Chla*DUB1 or knock-down of Ankib1 is capable to remove the ubiquitin conjugate from the inclusion of the mutant *Chlamydia* in mammalian cells (Figure 3.15 and 3.32 C, D). Unfortunately, no targets of Ankib1 are known so far and remain to be elucidated.
Moreover, the knock-down of Ankib1 as well as the complementation with active *Chla*DUB1 restores the Golgi recruitment to the inclusion of the mutant (Figure 3.24 and 3.25). Therefore, Ankib1 and *Chla*DUB1 are likely to have the same unknown target that is ubiquitinated by Ankib1 and deubiquitinated by the chlamydial DUB. The removal of the ubiquitin modification is hereby essential for the successful localization of Golgi stacks to the proximity of the inclusion. Since microtubules support the structure of the Golgi and mediate the recruitment of the stacks to the inclusion (Al-Zeer, et al., 2014), it is likely that Ankib1 and *Chla*DUB1 interfere with organization of microtubules. The targets of Ankib1 and *Chla*DUB1 still need further investigation.

4.4.2 Ankib1 conservation in different host cells and during *Chlamydia* species infection

Several studies revealed that the immune response between *Chlamydia* species and even *C. trachomatis* serovars vary widely (Dessus-Babus, et al., 2002, Haldar, et al., 2016). While infection of the mouse pathogen *C. muridarum* is cleared in human cells, the human pathogen can prevent detection and completes its life cycle (Haldar, et al., 2016). Even between serovar E and L2 of *C. trachomatis* differences in TNFα secretion have been observed (Dessus-Babus, et al., 2002). The human pathogen *C. pneumoniae* does not encode for *Chla*DUB1, but is capable to establish infection in lung tissue. Therefore, different *C. trachomatis* serovars, representing the three biovars as well as the mouse pathogen *C. muridarum* and the lung infecting bacteria *C. pneumoniae* were checked regarding the expression of *Chla*DUB1. For *C. pneumoniae* and *C. muridarum* it was not possible to receive *Chla*DUB1 protein bands likely for different reasons (Figure 3.31 A). *C. pneumoniae* does not encode for the DUB and was used here as a control (Kalman, et al., 1999, Read, et al., 2000). The sequence of the deubiquitinase between the mouse and human strain differ too much from each other, so that the antibody raised against *C. trachomatis Chla*DUB1 does not recognize the murine protease. Interestingly *C. trachomatis* serovar D, the cause of genital tract infections, expresses less *Chla*DUB1 than the biovars for LGV (serovar L2) and ocular infections (serovar A) (Figure 3.31 A+B), while sequence identity is similar (appendix). These differences indicate the importance of the deubiquitinase for immune evasion in LGV and ocular strains, but more insignificance for genital serovars. Moreover, all chlamydial strains and serovars showed no ubiquitin at the inclusion surface regardless of the expression of the DUB (Figure 3.31 B), claiming that *C. pneumoniae* evolved other mechanisms to evade recognition of the inclusion. Here it should be noted that also *C. trachomatis* combines several strategies to suppress ubiquitination, since in early time points when *Chla*DUB1 is not expressed no co-localization of the bacteria with ubiquitin appears (Figure 3.26). The E3 ligase Ankib1 was identified at the inclusion of *C. trachomatis* serovar L2, A and D, nevertheless not with *C. muridarum* and *C. pneumoniae* infection (Figure 3.19 and 3.31 C), stating that Ankib1 is specific for *C. trachomatis* infection.

4.5 Outlook

The CRISPRi system works also in *C. trachomatis*, which was shown in this work as well as in the work of Ouellette (Ouellette, 2018). However, some inconsistencies are still present. Control plasmids were cloned that only express the dCas9 protein or the gRNA as well as plasmids with gRNAs targeting GFP that could not be transformed into *C. trachomatis*. One reason could be the big size of the plasmid that complicates transformation, which could be reduced by using a different dCas9 protein for example of *S. aureus* which is approximately 1 kb smaller and the removal of genes like GFP from the backbone. Moreover, a two plasmid system with separated dCas9 and gRNA can in future lead to smaller plasmids and faster transformant selection. Theoretically, it is also possible to integrate the dCas9 sequence into the chlamydial genome, so that only plasmids harboring the gRNA have to be transformed. However, two different antibiotics have to be used in these approaches, but selection with chloramphenicol only decreases growth of bacteria and does not prevent it, making selection of transformants more time consuming. Methods like TageTron® by Sigma Aldrich could be used to facilitate transformation of *Chlamydia*, since the $CaCl_2$ method does not work reliably. The knock-down of *Chla*DUB1 using the gRNA gCdu1III showed reduced protein amount of maximum 50 %. Protein levels might diminish even stronger by simultaneous usage of multiple gRNAs, targeting the promoter and the coding region at different positions. Mcl-1 is a target of *Chla*DUB1, though the Mcl-1 level did not show any change upon incomplete *Chla*DUB1 knock-down. However, the ubiquitination level of Mcl-1 was not investigated yet. The pulldown of Mcl-1 and subsequent analysis of the ubiquitination pattern could give some indication of the activity of remaining *Chla*DUB1 protease on the inclusion surface. Further investigations will help to evolve a reliable strategy to knock-down chlamydial proteins during infection in order to study *Chlamydia* survival in cells.

In this thesis it was shown that the autophagic machinery is activated in *C. trachomatis* infected cells when *Chla*DUB1 is inactive. Moreover, the E3 ligase Ankib1 was identified to ubiquitinate the inclusion of *Ctr* Tn-*cdu1* in human and mouse cells, as well as the involvement of UBE1 and UBE2L3. Upon inactivation of *Chla*DUB1 the Golgi is fragmented but recruitment to the inclusion is inhibited, which is reversed by *Chla*DUB1 addition or Ankib1 knock-down. Still, the substrates of Ankib1 and *Chla*DUB1 remain to be elucidated. Using a Bio-ID approach proteins in close proximity of the E3 ligase and the DUB can be tagged with biotin by fusion with BirA*, pulled down using streptavidin and identified by mass spectrometry. The data carries direct targets of Ankib1 and *Chla*DUB1 as well as indirect proteins involved in recruitment or scaffolding. When Ankib1 and *Chla*DUB1 are tagged with a Flag tag, they can also be pulled down by immunoprecipitation and direct interacting partners can be identified by mass spectrometry. Data shown above suggests that the E3 ligase and the DUB have the same target at the surface of the chlamydial inclusion, which can be of bacterial or eukaryotic origin. Moreover, one target of Ankib1 and *Chla*DUB1 is involved in the recruitment of the Golgi stacks to the inclusion. In order to identify all changes in protein set up at the inclusion the purification of wildtype and mutant inclusions can be performed and proteins identified by mass spectrometry. The composition of lipids of the inclusion or bacteria can be analyzed by lipidomics and would give information whether the stalled bacterial development is due to altered lipid acquisition. Since Ankib1 was not the focus of any study before it would be interesting to get a better understanding into its mechanism and tasks in the cell. Therefore, crystallography of Ankib1 can give insights into binding and regulation of the E3 ligase.

Moreover, the currently identified inhibitor of *Chla*DUB1 (Ramirez, et al., 2018) can be tested during infection to see whether inactivation of the DUB also activates the autophagic machinery and decreases bacterial development.

Since Ankib1 is ubiquitinating the *Ctr* Tn-*cdu1* inclusion in mouse and human cells it is worth investigating if other cells use Ankib1 as a defense against *Chlamydia* and if other bacteria are also targeted by the E3 ligase. The host cell defense in mouse and human cells differs, nevertheless, both show the same reaction to *Ctr* Tn-*cdu1*. Further experiments can answer how *Chla*DUB1 is involved in each host cell species also under influence of cytokines like IFNγ. Mice lacking Ankib1 gene expression are supposed to overcome chlamydial development problems and show the same bacterial burden as the wildtype.

The growth defect of *Chlamydia* with inactive *Chla*DUB1 should be rescued by re-complementation with active DUB. Experiments using the HeLa Cdu1-Flag cell line did not survive *Chla*DUB1 overexpression for the duration of the *Chlamydia* to complete the developmental cycle. Alternatively the re-complementation of *Ctr* Tn-*cdu1* with a plasmid inducible expressing active *Chla*DUB1 would be a great advantage. Also the growth defect of the mutant strain should be reversed by knock-down of Ankib1 for the duration of chlamydial development. *Chlamydia* are known to infect immune cells and to survive despite antimicrobial mechanisms in the cell (Beagley, et al., 2009). It is very interesting that the mutant strain does not survive in neutrophils as good as the wildtype. The reason for it remains to be elucidated. Since *Chla*DUB1 is not expressed in EB stage it has to be examined whether DUB protein expressed in RB stage is present in this phase or whether targets of the DUB are the cause for this result.

5 References

Abdul-Sater, A. A., E. Koo, G. Häcker and D. M. Ojcius. (2009), 'Inflammasome-Dependent Caspase-1 Activation in Cervical Epithelial Cells Stimulates Growth of the Intracellular Pathogen Chlamydia Trachomatis', *Journal of Biological Chemistry* Vol. 284, No. 39, pp. 26789-26796.

Abdul-Sater, A. A., N. Saïd-Sadier, E. V. Padilla and D. M. Ojcius. (2010), 'Chlamydial Infection of Monocytes Stimulates Il-1β Secretion through Activation of the Nlrp3 Inflammasome', *Microbes and infection* Vol. 12, No. 8-9, pp. 652-661.

Agaisse, H. and I. Derré. (2013), 'A C. Trachomatis Cloning Vector and the Generation of C. Trachomatis Strains Expressing Fluorescent Proteins under the Control of a C. Trachomatis Promoter', *PLoS One* Vol. 8, No. 2, pp. e57090.

Akira, S., S. Uematsu and O. Takeuchi. (2006), 'Pathogen Recognition and Innate Immunity', *Cell* Vol. 124, No. 4, pp. 783-801.

Al-Younes, H. M., M. A. Al-Zeer, H. Khalil, J. Gussmann, A. Karlas, N. Machuy, V. Brinkmann, P. R. Braun and T. F. Meyer. (2011), 'Autophagy-Independent Function of Map-Lc3 During Intracellular Propagation of Chlamydia Trachomatis', *Autophagy* Vol. 7, No. 8, pp. 814-828.

Al-Younes, H. M., V. Brinkmann and T. F. Meyer. (2004), 'Interaction of Chlamydia Trachomatis Serovar L2 with the Host Autophagic Pathway', *Infection and immunity* Vol. 72, No. 8, pp. 4751-4762.

Al-Zeer, M. A., H. M. Al-Younes, P. R. Braun, J. Zerrahn and T. F. Meyer. (2009), 'Ifn-Γ-Inducible Irga6 Mediates Host Resistance against Chlamydia Trachomatis Via Autophagy', *PLoS One* Vol. 4, No. 2, pp. e4588.

Al-Zeer, M. A., H. M. Al-Younes, M. Kerr, M. Abu-Lubad, E. Gonzalez, V. Brinkmann and T. F. Meyer. (2014), 'C Hlamydia Trachomatis Remodels Stable Microtubules to Coordinate G Olgi Stack Recruitment to the Chlamydial Inclusion Surface', *Molecular microbiology* Vol. 94, No. 6, pp. 1285-1297.

Amann, R., N. Springer, W. Schönhuber, W. Ludwig, E. N. Schmid, K. Müller and R. Michel. (1997), 'Obligate Intracellular Bacterial Parasites of Acanthamoebae Related to Chlamydia Spp', *Applied and environmental microbiology* Vol. 63, No. 1, pp. 115-121.

Auer, D. (2014), 'Characterization of the Chlamyidal Deubiquitinating Enzyme Chladub2 and Its Impact on Host Protein Ubiquitination'.

Balin, B. J., H. C. Gérard, E. J. Arking, D. M. Appelt, P. J. Branigan, J. T. Abrams, J. A. Whittum-Hudson and A. P. Hudson. (1998), 'Identification and Localization of Chlamydia Pneumoniae in the Alzheimer's Brain', *Medical microbiology and immunology* Vol. 187, No. 1, pp. 23-42.

Barrangou, R., C. Fremaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, D. A. Romero and P. Horvath. (2007), 'Crispr Provides Acquired Resistance against Viruses in Prokaryotes', *Science* Vol. 315, No. 5819, pp. 1709-1712.

Beagley, K., W. M. Huston, P. M. Hansbro and P. Timms. (2009), 'Chlamydial Infection of Immune Cells: Altered Function and Implications for Disease', *Critical Reviews™ in Immunology* Vol. 29, No. 4.

Beatty, W. L. (2006), 'Trafficking from Cd63-Positive Late Endocytic Multivesicular Bodies Is Essential for Intracellular Development of Chlamydia Trachomatis', *Journal of cell science* Vol. 119, No. 2, pp. 350-359.

Beatty, W. L., T. A. Belanger, A. A. Desai, R. P. Morrison and G. I. Byrne. (1994), 'Tryptophan Depletion as a Mechanism of Gamma Interferon-Mediated Chlamydial Persistence', *Infection and immunity* Vol. 62, No. 9, pp. 3705-3711.

Bedson, S. and J. Bland. (1932), 'A Morphological Study of Psittacosis Virus, with the Description of a Developmental Cycle', *British journal of experimental pathology* Vol. 13, No. 5, pp. 461.

Bekpen, C., J. P. Hunn, C. Rohde, I. Parvanova, L. Guethlein, D. M. Dunn, E. Glowalla, M. Leptin and J. C. Howard. (2005), 'The Interferon-Inducible P47 (Irg) Gtpases in Vertebrates: Loss of the Cell

Autonomous Resistance Mechanism in the Human Lineage', *Genome biology* Vol. 6, No. 11, pp. R92.

Belland, R. J., G. Zhong, D. D. Crane, D. Hogan, D. Sturdevant, J. Sharma, W. L. Beatty and H. D. Caldwell. (2003), 'Genomic Transcriptional Profiling of the Developmental Cycle of Chlamydia Trachomatis', *Proc Natl Acad Sci U S A* Vol. 100, No. 14, pp. 8478-83.

Bernstein-Hanley, I., J. Coers, Z. R. Balsara, G. A. Taylor, M. N. Starnbach and W. F. Dietrich. (2006), 'The P47 Gtpases Igtp and Irgb10 Map to the Chlamydia Trachomatis Susceptibility Locus Ctrq-3 and Mediate Cellular Resistance in Mice', *Proceedings of the National Academy of Sciences* Vol. 103, No. 38, pp. 14092-14097.

Bertelli, C., O. H. Cisse, B. Rusconi, C. Kebbi-Beghdadi, A. Croxatto, A. Goesmann, F. Collyn and G. Greub. (2016), 'Crispr System Acquisition and Evolution of an Obligate Intracellular Chlamydia-Related Bacterium', *Genome biology and evolution* Vol. 8, No. 8, pp. 2376-2386.

Bikard, D., W. Jiang, P. Samai, A. Hochschild, F. Zhang and L. A. Marraffini. (2013), 'Programmable Repression and Activation of Bacterial Gene Expression Using an Engineered Crispr-Cas System', *Nucleic acids research* Vol. 41, No. 15, pp. 7429-7437.

Bonizzi, G. and M. Karin. (2004), 'The Two Nf-Kb Activation Pathways and Their Role in Innate and Adaptive Immunity', *Trends in immunology* Vol. 25, No. 6, pp. 280-288.

Borodovsky, A., B. M. Kessler, R. Casagrande, H. S. Overkleeft, K. D. Wilkinson and H. L. Ploegh. (2001), 'A Novel Active Site-Directed Probe Specific for Deubiquitylating Enzymes Reveals Proteasome Association of Usp14', *The EMBO journal* Vol. 20, No. 18, pp. 5187-5196.

Brinkmann, V., U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch and A. Zychlinsky. (2004), 'Neutrophil Extracellular Traps Kill Bacteria', *Science* Vol. 303, No. 5663, pp. 1532-1535.

Buchholz, K. R. and R. S. Stephens. (2008), 'The Cytosolic Pattern Recognition Receptor Nod1 Induces Inflammatory Interleukin-8 During Chlamydia Trachomatis Infection', *Infection and immunity* Vol. 76, No. 7, pp. 3150-3155.

Byrne, G. I. and D. M. Ojcius. (2004), 'Chlamydia and Apoptosis: Life and Death Decisions of an Intracellular Pathogen', *Nature Reviews Microbiology* Vol. 2, No. 10, pp. 802.

Caldwell, H. D., H. Wood, D. Crane, R. Bailey, R. B. Jones, D. Mabey, I. Maclean, Z. Mohammed, R. Peeling, C. Roshick, J. Schachter, A. W. Solomon, W. E. Stamm, R. J. Suchland, L. Taylor, S. K. West, T. C. Quinn, R. J. Belland and G. McClarty. (2003), 'Polymorphisms in Chlamydia Trachomatis Tryptophan Synthase Genes Differentiate between Genital and Ocular Isolates', *J Clin Invest* Vol. 111, No. 11, pp. 1757-69.

Carabeo, R. A., D. J. Mead and T. Hackstadt. (2003), 'Golgi-Dependent Transport of Cholesterol to the Chlamydia Trachomatis Inclusion', *Proceedings of the National Academy of Sciences* Vol. 100, No. 11, pp. 6771-6776.

Cemma, M., P. K. Kim and J. H. Brumell. (2011), 'The Ubiquitin-Binding Adaptor Proteins P62/Sqstm1 and Ndp52 Are Recruited Independently to Bacteria-Associated Microdomains to Target Salmonella to the Autophagy Pathway', *Autophagy* Vol. 7, No. 3, pp. 341-345.

Chau, V., J. W. Tobias, A. Bachmair, D. Marriott, D. J. Ecker, D. K. Gonda and A. Varshavsky. (1989), 'A Multiubiquitin Chain Is Confined to Specific Lysine in a Targeted Short-Lived Protein', *Science* Vol. 243, No. 4898, pp. 1576-83.

Cheng, A. W., H. Wang, H. Yang, L. Shi, Y. Katz, T. W. Theunissen, S. Rangarajan, C. S. Shivalila, D. B. Dadon and R. Jaenisch. (2013), 'Multiplexed Activation of Endogenous Genes by Crispr-on, an Rna-Guided Transcriptional Activator System', *Cell research* Vol. 23, No. 10, pp. 1163.

Chumduri, C., R. K. Gurumurthy, P. K. Zadora, Y. Mi and T. F. Meyer. (2013), 'Chlamydia Infection Promotes Host DNA Damage and Proliferation but Impairs the DNA Damage Response', *Cell host & microbe* Vol. 13, No. 6, pp. 746-758.

Ciehanover, A., Y. Hod and A. Hershko. (1978), 'A Heat-Stable Polypeptide Component of an Atp-Dependent Proteolytic System from Reticulocytes', *Biochemical and biophysical research communications* Vol. 81, No. 4, pp. 1100-1105.

Clifton, D., K. Fields, S. Grieshaber, C. Dooley, E. Fischer, D. Mead, R. Carabeo and T. Hackstadt. (2004), 'A Chlamydial Type Iii Translocated Protein Is Tyrosine-Phosphorylated at the Site of Entry and Associated with Recruitment of Actin', *Proceedings of the National Academy of Sciences* Vol. 101, No. 27, pp. 10166-10171.

Coers, J., I. Bernstein-Hanley, D. Grotsky, I. Parvanova, J. C. Howard, G. A. Taylor, W. F. Dietrich and M. N. Starnbach. (2008), 'Chlamydia Muridarum Evades Growth Restriction by the Ifn-Γ-Inducible Host Resistance Factor Irgb10', *The Journal of Immunology* Vol. 180, No. 9, pp. 6237-6245.

Collier-Hyams, L. S., V. Sloane, B. C. Batten and A. S. Neish. (2005), 'Cutting Edge: Bacterial Modulation of Epithelial Signaling Via Changes in Neddylation of Cullin-1', *The Journal of Immunology* Vol. 175, No. 7, pp. 4194-4198.

Comanducci, M., R. Manetti, L. Bini, A. Santucci, V. Pallini, R. Cevenini, J. Sueur, J. Orfila and G. Ratti. (1994), 'Humoral Immune Response to Plasmid Protein Pgp3 in Patients with Chlamydia Trachomatis Infection', *Infection and immunity* Vol. 62, No. 12, pp. 5491-5497.

Damiani, M. T., J. Gambarte Tudela and A. Capmany. (2014), 'Targeting Eukaryotic Rab Proteins: A Smart Strategy for Chlamydial Survival and Replication', *Cellular microbiology* Vol. 16, No. 9, pp. 1329-1338.

Darville, T. and T. J. Hiltke. (2010), 'Pathogenesis of Genital Tract Disease Due to Chlamydia Trachomatis', *The Journal of infectious diseases* Vol. 201, No. Supplement_2, pp. S114-S125.

Dean, D., J. Rothschild, A. Ruettger, R. P. Kandel and K. Sachse. (2013), 'Zoonotic Chlamydiaceae Species Associated with Trachoma, Nepal', *Emerging infectious diseases* Vol. 19, No. 12, pp. 1948.

Debonneville, C., S. Y. Flores, E. Kamynina, P. J. Plant, C. Tauxe, M. A. Thomas, C. Münster, A. Chraïbi, J. H. Pratt and J. D. Horisberger. (2001), 'Phosphorylation of Nedd4-2 by Sgk1 Regulates Epithelial Na+ Channel Cell Surface Expression', *The EMBO journal* Vol. 20, No. 24, pp. 7052-7059.

Deltcheva, E., K. Chylinski, C. M. Sharma, K. Gonzales, Y. Chao, Z. A. Pirzada, M. R. Eckert, J. Vogel and E. Charpentier. (2011), 'Crispr Rna Maturation by Trans-Encoded Small Rna and Host Factor Rnase Iii', *Nature* Vol. 471, No. 7340, pp. 602.

DeMars, R., J. Weinfurter, E. Guex, J. Lin and Y. Potucek. (2007), 'Lateral Gene Transfer in Vitro in the Intracellular Pathogen Chlamydia Trachomatis', *Journal of bacteriology* Vol. 189, No. 3, pp. 991-1003.

Dessus-Babus, S., T. L. Darville, F. P. Cuozzo, K. Ferguson and P. B. Wyrick. (2002), 'Differences in Innate Immune Responses (in Vitro) to Hela Cells Infected with Nondisseminating Serovar E and Disseminating Serovar L2 of Chlamydia Trachomatis', *Infection and immunity* Vol. 70, No. 6, pp. 3234-3248.

Dikic, I. (2017), 'Proteasomal and Autophagic Degradation Systems', *Annual review of biochemistry* Vol. 86, pp. 193-224.

Ding, H., S. Gong, Y. Tian, Z. Yang, R. Brunham and G. Zhong. (2013), 'Transformation of Sexually Transmitted Infection-Causing Serovars of Chlamydia Trachomatis Using Blasticidin for Selection', *PLoS One* Vol. 8, No. 11, pp. e80534.

Duda, D. M., L. A. Borg, D. C. Scott, H. W. Hunt, M. Hammel and B. A. Schulman. (2008), 'Structural Insights into Nedd8 Activation of Cullin-Ring Ligases: Conformational Control of Conjugation', *Cell* Vol. 134, No. 6, pp. 995-1006.

Dunphy, W. G. and J. E. Rothman. (1985), 'Compartmental Organization of the Golgi Stack', *Cell* Vol. 42, No. 1, pp. 13-21.

Eissenberg, L. G. and P. B. Wyrick. (1981), 'Inhibition of Phagolysosome Fusion Is Localized to Chlamydia Psittaci-Laden Vacuoles', *Infection and immunity* Vol. 32, No. 2, pp. 889-896.

Elwell, C., K. Mirrashidi and J. Engel. (2016), 'Chlamydia Cell Biology and Pathogenesis', *Nature Reviews Microbiology* Vol. 14, No. 6, pp. 385.

Engel, J. (2004), 'Tarp and Arp: How Chlamydia Induces Its Own Entry', *Proceedings of the National Academy of Sciences* Vol. 101, No. 27, pp. 9947-9948.

Everett, K. D., R. M. Bush and A. A. Andersen. (1999), 'Emended Description of the Order Chlamydiales, Proposal of Parachlamydiaceae Fam. Nov. And Simkaniaceae Fam. Nov., Each Containing One Monotypic Genus, Revised Taxonomy of the Family Chlamydiaceae, Including a New Genus and Five New Species, and Standards for the Identification of Organisms', *International Journal of Systematic and Evolutionary Microbiology* Vol. 49, No. 2, pp. 415-440.

Farencena, A., M. Comanducci, M. Donati, G. Ratti and R. Cevenini. (1997), 'Characterization of a New Isolate of Chlamydia Trachomatis Which Lacks the Common Plasmid and Has Properties of Biovar Trachoma', *Infection and immunity* Vol. 65, No. 7, pp. 2965-2969.

Finethy, R. and J. Coers. (2016), 'Sensing the Enemy, Containing the Threat: Cell-Autonomous Immunity to Chlamydia Trachomatis', *FEMS microbiology reviews* Vol. 40, No. 6, pp. 875-893.

Finethy, R., I. Jorgensen, A. K. Haldar, M. R. De Zoete, T. Strowig, R. A. Flavell, M. Yamamoto, U. M. Nagarajan, E. A. Miao and J. Coers. (2015), 'Guanylate Binding Proteins Enable Rapid Activation of Canonical and Noncanonical Inflammasomes in Chlamydia-Infected Macrophages', *Infection and immunity*, pp. IAI. 00856-15.

Fischer, A., K. S. Harrison, Y. Ramirez, D. Auer, S. R. Chowdhury, B. K. Prusty, F. Sauer, Z. Dimond, C. Kisker, P. S. Hefty and T. Rudel. (2017), 'Chlamydia Trachomatis-Containing Vacuole Serves as Deubiquitination Platform to Stabilize Mcl-1 and to Interfere with Host Defense', *Elife* Vol. 6.

Fischer, A. and T. Rudel. (2016), 'Subversion of Cell-Autonomous Host Defense by Chlamydia Infection'.

Friis, R. R. (1972), 'Interaction of L Cells and Chlamydia Psittaci: Entry of the Parasite and Host Responses to Its Development', *Journal of bacteriology* Vol. 110, No. 2, pp. 706-721.

Fu, Y., J. A. Foden, C. Khayter, M. L. Maeder, D. Reyon, J. K. Joung and J. D. Sander. (2013), 'High-Frequency Off-Target Mutagenesis Induced by Crispr-Cas Nucleases in Human Cells', *Nature biotechnology* Vol. 31, No. 9, pp. 822.

Fukushi, H. and K. Hirai. (1992), 'Proposal of Chlamydia Pecorum Sp. Nov. For Chlamydia Strains Derived from Ruminants', *International Journal of Systematic and Evolutionary Microbiology* Vol. 42, No. 2, pp. 306-308.

Furtado, A. R., M. Essid, S. Perrinet, M. E. Balañá, N. Yoder, P. Dehoux and A. Subtil. (2013), 'The Chlamydial Otu Domain-Containing Protein Chlaotu Is an Early Type Iii Secretion Effector Targeting Ubiquitin and Ndp 52', *Cellular microbiology* Vol. 15, No. 12, pp. 2064-2079.

Gallagher, E., M. Gao, Y.-C. Liu and M. Karin. (2006), 'Activation of the E3 Ubiquitin Ligase Itch through a Phosphorylation-Induced Conformational Change', *Proceedings of the National Academy of Sciences* Vol. 103, No. 6, pp. 1717-1722.

Gérard, H. C., M. K. Mishra, G. Mao, S. Wang, M. Hali, J. A. Whittum-Hudson, R. M. Kannan and A. P. Hudson. (2013), 'Dendrimer-Enabled DNA Delivery and Transformation of Chlamydia Pneumoniae', *Nanomedicine: Nanotechnology, Biology and Medicine* Vol. 9, No. 7, pp. 996-1008.

Gomes, L. C. and I. Dikic. (2014), 'Autophagy in Antimicrobial Immunity', *Molecular cell* Vol. 54, No. 2, pp. 224-233.

Gong, L., T. Kamitani, S. Millas and E. T. Yeh. (2000), 'Identification of a Novel Isopeptidase with Dual Specificity for Ubiquitin-and Nedd8-Conjugated Proteins', *Journal of Biological Chemistry* Vol. 275, No. 19, pp. 14212-14216.

Gong, S., Z. Yang, L. Lei, L. Shen and G. Zhong. (2013), 'Characterization of Chlamydia Trachomatis Plasmid-Encoded Open Reading Frames', *Journal of bacteriology*, pp. JB. 00511-13.

Grayston, J. T., C.-C. Kuo, S.-p. Wang and J. Altman. (1986), 'A New Chlamydia Psittaci Strain, Twar, Isolated in Acute Respiratory Tract Infections', *New England Journal of Medicine* Vol. 315, No. 3, pp. 161-168.

Groettrup, M., C. Pelzer, G. Schmidtke and K. Hofmann. (2008), 'Activating the Ubiquitin Family: Uba6 Challenges the Field', *Trends in biochemical sciences* Vol. 33, No. 5, pp. 230-237.

Hackstadt, T., D. Rockey, R. Heinzen and M. Scidmore. (1996), 'Chlamydia Trachomatis Interrupts an Exocytic Pathway to Acquire Endogenously Synthesized Sphingomyelin in Transit from the Golgi Apparatus to the Plasma Membrane', *The EMBO journal* Vol. 15, No. 5, pp. 964-977.

Hackstadt, T., M. A. Scidmore and D. D. Rockey. (1995), 'Lipid Metabolism in Chlamydia Trachomatis-Infected Cells: Directed Trafficking of Golgi-Derived Sphingolipids to the Chlamydial Inclusion', *Proceedings of the National Academy of Sciences* Vol. 92, No. 11, pp. 4877-4881.

Halberstaedter, L. and S. Von Prowazek. (1907), 'Untersuchungen Über Die Malariaparasiten Der Affen', *Arb K GesundhAmt* Vol. 26, pp. 37-43.

Haldar, A. K., C. Foltz, R. Finethy, A. S. Piro, E. M. Feeley, D. M. Pilla-Moffett, M. Komatsu, E.-M. Frickel and J. Coers. (2015), 'Ubiquitin Systems Mark Pathogen-Containing Vacuoles as Targets for Host Defense by Guanylate Binding Proteins', *Proceedings of the National Academy of Sciences* Vol. 112, No. 41, pp. E5628-E5637.

Haldar, A. K., A. S. Piro, R. Finethy, S. T. Espenschied, H. E. Brown, A. M. Giebel, E.-M. Frickel, D. E. Nelson and J. Coers. (2016), 'Chlamydia Trachomatis Is Resistant to Inclusion Ubiquitination and Associated Host Defense in Gamma Interferon-Primed Human Epithelial Cells', *MBio* Vol. 7, No. 6, pp. e01417-16.

Haldar, A. K., A. S. Piro, D. M. Pilla, M. Yamamoto and J. Coers. (2014), 'The E2-Like Conjugation Enzyme Atg3 Promotes Binding of Irg and Gbp Proteins to Chlamydia-and Toxoplasma-Containing Vacuoles and Host Resistance', *PLoS One* Vol. 9, No. 1, pp. e86684.

Haldar, A. K., H. A. Saka, A. S. Piro, J. D. Dunn, S. C. Henry, G. A. Taylor, E. M. Frickel, R. H. Valdivia and J. Coers. (2013), 'Irg and Gbp Host Resistance Factors Target Aberrant,“Non-Self” Vacuoles Characterized by the Missing of “Self” Irgm Proteins', *PLoS pathogens* Vol. 9, No. 6, pp. e1003414.

Hammerschlag, M. R. (2000), 'Chlamydia Pneumoniae and the Lung', *European Respiratory Journal* Vol. 16, No. 5, pp. 1001-1007.

Hatakeyama, S., M. Yada, M. Matsumoto, N. Ishida and K.-I. Nakayama. (2001), 'U Box Proteins as a New Family of Ubiquitin-Protein Ligases', *Journal of Biological Chemistry* Vol. 276, No. 35, pp. 33111-33120.

Hatch, G. M. and G. McClarty. (1998), 'Phospholipid Composition of Purifiedchlamydia Trachomatis Mimics That of the Eucaryotic Host Cell', *Infection and immunity* Vol. 66, No. 8, pp. 3727-3735.

Hatch, T., M. Miceli and J. Silverman. (1985), 'Synthesis of Protein in Host-Free Reticulate Bodies of Chlamydia Psittaci and Chlamydia Trachomatis', *Journal of bacteriology* Vol. 162, No. 3, pp. 938-942.

Hershko, A., A. Ciechanover, H. Heller, A. L. Haas and I. A. Rose. (1980), 'Proposed Role of Atp in Protein Breakdown: Conjugation of Protein with Multiple Chains of the Polypeptide of Atp-Dependent Proteolysis', *Proceedings of the National Academy of Sciences* Vol. 77, No. 4, pp. 1783-1786.

Heuer, D., A. R. Lipinski, N. Machuy, A. Karlas, A. Wehrens, F. Siedler, V. Brinkmann and T. F. Meyer. (2009), 'Chlamydia Causes Fragmentation of the Golgi Compartment to Ensure Reproduction', *Nature* Vol. 457, No. 7230, pp. 731.

Hofmann, R. M. and C. M. Pickart. (1999), 'Noncanonical Mms2-Encoded Ubiquitin-Conjugating Enzyme Functions in Assembly of Novel Polyubiquitin Chains for DNA Repair', *Cell* Vol. 96, No. 5, pp. 645-653.

Huang, L., E. Kinnucan, G. Wang, S. Beaudenon, P. M. Howley, J. M. Huibregtse and N. P. Pavletich. (1999), 'Structure of an E6ap-Ubch7 Complex: Insights into Ubiquitination by the E2-E3 Enzyme Cascade', *Science* Vol. 286, No. 5443, pp. 1321-1326.

Huber, A. (2014), 'Chlamydial Deubiquitinase Chladub1 as a Regulator of Host Cell Apoptosis and a New Target for Anti-Chlamydial Therapy'.

Huett, A., R. J. Heath, J. Begun, S. O. Sassi, L. A. Baxt, J. M. Vyas, M. B. Goldberg and R. J. Xavier. (2012), 'The Lrr and Ring Domain Protein Lrsam1 Is an E3 Ligase Crucial for Ubiquitin-Dependent Autophagy of Intracellular Salmonella Typhimurium', *Cell host & microbe* Vol. 12, No. 6, pp. 778-790.

Hügelschäffer, S. (2018), 'The Role of Chlamydial Deubiquitinase 1 for Chlamydial Intracellular Replication'.

Huibregtse, J. M., M. Scheffner, S. Beaudenon and P. M. Howley. (1995), 'A Family of Proteins Structurally and Functionally Related to the E6-Ap Ubiquitin-Protein Ligase', *Proceedings of the National Academy of Sciences* Vol. 92, No. 7, pp. 2563-2567.

Hybiske, K. and R. S. Stephens. (2007), 'Mechanisms of Host Cell Exit by the Intracellular Bacterium Chlamydia', *Proceedings of the National Academy of Sciences* Vol. 104, No. 27, pp. 11430-11435.

Iliffe-Lee, E. R. and G. McClarty. (1999), 'Glucose Metabolism in Chlamydia Trachomatis: The 'Energy Parasite'hypothesis Revisited', *Molecular microbiology* Vol. 33, No. 1, pp. 177-187.

Jendro, M. C., F. Fingerle, T. Deutsch, A. Liese, L. Köhler, J. G. Kuipers, E. Raum, M. Martin and H. Zeidler. (2004), 'Chlamydia Trachomatis-Infected Macrophages Induce Apoptosis of Activated T Cells by Secretion of Tumor Necrosis Factor-A in Vitro', *Medical microbiology and immunology* Vol. 193, No. 1, pp. 45-52.

Jin, J., X. Li, S. P. Gygi and J. W. Harper. (2007), 'Dual E1 Activation Systems for Ubiquitin Differentially Regulate E2 Enzyme Charging', *Nature* Vol. 447, No. 7148, pp. 1135-8.

Jin, L., A. Williamson, S. Banerjee, I. Philipp and M. Rape. (2008), 'Mechanism of Ubiquitin-Chain Formation by the Human Anaphase-Promoting Complex', *Cell* Vol. 133, No. 4, pp. 653-665.

Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna and E. Charpentier. (2012), 'A Programmable Dual-Rna–Guided DNA Endonuclease in Adaptive Bacterial Immunity', *Science*, pp. 1225829.

Johnson, C. M. and D. J. Fisher. (2013), 'Site-Specific, Insertional Inactivation of Inca in Chlamydia Trachomatis Using a Group Ii Intron', *PLoS One* Vol. 8, No. 12, pp. e83989.

Kabeiseman, E. J., K. Cichos, T. Hackstadt, A. Lucas and E. R. Moore. (2013), 'Vamp4 and Syntaxin 6 Interactions at the Chlamydial Inclusion', *Infection and immunity*, pp. IAI. 00584-13.

Kahane, S., E. Metzer and M. G. Friedman. (1995), 'Evidence That the Novel Microorganism 'Z'may Belong to a New Genus in the Family Chlamydiaceae', *FEMS microbiology letters* Vol. 126, No. 2, pp. 203-207.

Kalman, S., W. Mitchell, R. Marathe, C. Lammel, J. Fan, R. W. Hyman, L. Olinger, J. Grimwood, R. W. Davis and R. Stephens. (1999), 'Comparative Genomes of Chlamydia Pneumoniae and C. Trachomatis', *Nature genetics* Vol. 21, No. 4, pp. 385.

Kannan, R. M., H. C. Gérard, M. K. Mishra, G. Mao, S. Wang, M. Hali, J. A. Whittum-Hudson and A. P. Hudson. (2013), 'Dendrimer-Enabled Transformation of Chlamydia Trachomatis', *Microbial pathogenesis* Vol. 65, pp. 29-35.

Kim, H. C. and J. M. Huibregtse. (2009), 'Polyubiquitination by Hect E3s and the Determinants of Chain Type Specificity', *Molecular and cellular biology* Vol. 29, No. 12, pp. 3307-3318.

Kirisako, T., K. Kamei, S. Murata, M. Kato, H. Fukumoto, M. Kanie, S. Sano, F. Tokunaga, K. Tanaka and K. Iwai. (2006), 'A Ubiquitin Ligase Complex Assembles Linear Polyubiquitin Chains', *The EMBO journal* Vol. 25, No. 20, pp. 4877-4887.

Kirkin, V., T. Lamark, T. Johansen and I. Dikic. (2009), 'Nbr1 Co-Operates with P62 in Selective Autophagy of Ubiquitinated Targets', *Autophagy* Vol. 5, No. 5, pp. 732-733.

Koegl, M., T. Hoppe, S. Schlenker, H. D. Ulrich, T. U. Mayer and S. Jentsch. (1999), 'A Novel Ubiquitination Factor, E4, Is Involved in Multiubiquitin Chain Assembly', *Cell* Vol. 96, No. 5, pp. 635-644.

Kokes, M., J. D. Dunn, J. A. Granek, B. D. Nguyen, J. R. Barker, R. H. Valdivia and R. J. Bastidas. (2015), 'Integrating Chemical Mutagenesis and Whole-Genome Sequencing as a Platform for Forward and Reverse Genetic Analysis of Chlamydia', *Cell host & microbe* Vol. 17, No. 5, pp. 716-725.

Komander, D., M. J. Clague and S. Urbé. (2009), 'Breaking the Chains: Structure and Function of the Deubiquitinases', *Nature reviews Molecular cell biology* Vol. 10, No. 8, pp. 550.

Komander, D. and M. Rape. (2012), 'The Ubiquitin Code', *Annual review of biochemistry* Vol. 81, pp. 203-229.

Kumar, S., W. H. Kao and P. M. Howley. (1997), 'Physical Interaction between Specific E2 and Hect E3 Enzymes Determines Functional Cooperativity', *Journal of Biological Chemistry* Vol. 272, No. 21, pp. 13548-13554.

Kunin, V., R. Sorek and P. Hugenholtz. (2007), 'Evolutionary Conservation of Sequence and Secondary Structures in Crispr Repeats', *Genome biology* Vol. 8, No. 4, pp. R61.

Kuo, C.-C., L. A. Jackson, L. A. Campbell and J. T. Grayston. (1995), 'Chlamydia Pneumoniae (Twar)', *Clinical microbiology reviews* Vol. 8, No. 4, pp. 451-461.

Kuo, C., A. M. Gown, E. P. Benditt and J. T. Grayston. (1993), 'Detection of Chlamydia Pneumoniae in Aortic Lesions of Atherosclerosis by Immunocytochemical Stain', *Arteriosclerosis and thrombosis: a journal of vascular biology* Vol. 13, No. 10, pp. 1501-1504.

Lazarou, M., D. A. Sliter, L. A. Kane, S. A. Sarraf, C. Wang, J. L. Burman, D. P. Sideris, A. I. Fogel and R. J. Youle. (2015), 'The Ubiquitin Kinase Pink1 Recruits Autophagy Receptors to Induce Mitophagy', *Nature* Vol. 524, No. 7565, pp. 309.

Le Negrate, G., A. Krieg, B. Faustin, M. Loeffler, A. Godzik, S. Krajewski and J. C. Reed. (2008), 'Chladub1 of Chlamydia Trachomatis Suppresses Nf-Kappab Activation and Inhibits Ikappabalpha Ubiquitination and Degradation', *Cell Microbiol* Vol. 10, No. 9, pp. 1879-92.

Lee, J. K., G. A. Enciso, D. Boassa, C. N. Chander, T. H. Lou, S. S. Pairawan, M. C. Guo, F. Y. Wan, M. H. Ellisman and C. Sütterlin. (2018), 'Replication-Dependent Size Reduction Precedes Differentiation in Chlamydia Trachomatis', *Nature communications* Vol. 9, No. 1, pp. 45.

Lei, W., Q. Li, S. Su, J. Bu, Q. Huang and Z. Li. (2017), 'Chlamydia Trachomatis Plasmid-Encoded Protein Porf5 Protects Mitochondrial Function by Inducing Mitophagy and Increasing Hmgb1 Expression', *Pathogens and disease* Vol. 75, No. 9, pp. ftx111.

Leznicki, P. and Y. Kulathu. (2017), 'Mechanisms of Regulation and Diversification of Deubiquitylating Enzyme Function', *J Cell Sci*, pp. jcs. 201855.

Li, Z., C. Chen, D. Chen, Y. Wu, Y. Zhong and G. Zhong. (2008), 'Characterization of Fifty Putative Inclusion Membrane Proteins Encoded in the Chlamydia Trachomatis Genome', *Infection and immunity* Vol. 76, No. 6, pp. 2746-2757.

Lillestøl, R., P. Redder, R. A. Garrett and K. Brügger. (2006), 'A Putative Viral Defence Mechanism in Archaeal Cells', *Archaea* Vol. 2, No. 1, pp. 59-72.

Lipinski, A. R., J. Heymann, C. Meissner, A. Karlas, V. Brinkmann, T. F. Meyer and D. Heuer. (2009), 'Rab6 and Rab11 Regulate Chlamydia Trachomatis Development and Golgin-84-Dependent Golgi Fragmentation', *PLoS pathogens* Vol. 5, No. 10, pp. e1000615.

Lippai, M. and P. Lőw. (2014), 'The Role of the Selective Adaptor P62 and Ubiquitin-Like Proteins in Autophagy', *BioMed Research International* Vol. 2014.

Lucas, A. L., S. P. Ouellette, E. J. Kabeiseman, K. H. Cichos and E. A. Rucks. (2015), 'The Trans-Golgi Snare Syntaxin 10 Is Required for Optimal Development of Chlamydia Trachomatis', *Frontiers in cellular and infection microbiology* Vol. 5, pp. 68.

Maass, M., C. Bartels, P. M. Engel, U. Mamat and H.-H. Sievers. (1998), 'Endovascular Presence of Viable Chlamydia Pneumoniaeis a Common Phenomenon in Coronary Artery Disease', *Journal of the American College of Cardiology* Vol. 31, No. 4, pp. 827-832.

MacMicking, J. D. (2012), 'Interferon-Inducible Effector Mechanisms in Cell-Autonomous Immunity', *Nature Reviews Immunology* Vol. 12, No. 5, pp. 367.

Malhotra, M., S. Sood, A. Mukherjee, S. Muralidhar and M. Bala. (2013), 'Genital Chlamydia Trachomatis: An Update', *The Indian journal of medical research* Vol. 138, No. 3, pp. 303.

Man, S. M., D. E. Place, T. Kuriakose and T. D. Kanneganti. (2017), 'Interferon-Inducible Guanylate-Binding Proteins at the Interface of Cell-Autonomous Immunity and Inflammasome Activation', *Journal of leukocyte biology* Vol. 101, No. 1, pp. 143-150.

Manavi, K. (2006), 'A Review on Infection with Chlamydia Trachomatis', *Best Practice & Research Clinical Obstetrics & Gynaecology* Vol. 20, No. 6, pp. 941-951.

Manzanillo, P. S., J. S. Ayres, R. O. Watson, A. C. Collins, G. Souza, C. S. Rae, D. S. Schneider, K. Nakamura, M. U. Shiloh and J. S. Cox. (2013), 'The Ubiquitin Ligase Parkin Mediates Resistance to Intracellular Pathogens', *Nature* Vol. 501, No. 7468, pp. 512.

Martinon, F., K. Burns and J. Tschopp. (2002), 'The Inflammasome: A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of Proil-B', *Molecular cell* Vol. 10, No. 2, pp. 417-426.

McGouran, J. F., S. R. Gaertner, M. Altun, H. B. Kramer and B. M. Kessler. (2013), 'Deubiquitinating Enzyme Specificity for Ubiquitin Chain Topology Profiled by Di-Ubiquitin Activity Probes', *Chemistry & biology* Vol. 20, No. 12, pp. 1447-1455.

Metzger, M. B., V. A. Hristova and A. M. Weissman. (2012), 'Hect and Ring Finger Families of E3 Ubiquitin Ligases at a Glance', *J Cell Sci* Vol. 125, No. 3, pp. 531-537.

Meunier, E. and P. Broz. (2015), 'Interferon-Induced Guanylate-Binding Proteins Promote Cytosolic Lipopolysaccharide Detection by Caspase-11', *DNA and cell biology* Vol. 34, No. 1, pp. 1-5.

Miller, S. L., E. Malotky and J. P. O'Bryan. (2004), 'Analysis of the Role of Ubiquitin-Interacting Motifs (Uims) in Ubiquitin Binding and Ubiquitylation', *Journal of Biological Chemistry*.

Misaghi, S., Z. R. Balsara, A. Catic, E. Spooner, H. L. Ploegh and M. N. Starnbach. (2006), 'Chlamydia Trachomatis-Derived Deubiquitinating Enzymes in Mammalian Cells During Infection', *Mol Microbiol* Vol. 61, No. 1, pp. 142-50.

Mitchell, G. and R. R. Isberg. (2017), 'Innate Immunity to Intracellular Pathogens: Balancing Microbial Elimination and Inflammation', *Cell host & microbe* Vol. 22, No. 2, pp. 166-175.

Mizushima, N., T. Yoshimori and Y. Ohsumi. (2011), 'The Role of Atg Proteins in Autophagosome Formation', *Annual review of cell and developmental biology* Vol. 27, pp. 107-132.

Mölleken, K. and J. H. Hegemann. (2017), 'Acquisition of Rab11 and Rab11-Fip2—a Novel Strategy for Chlamydia Pneumoniae Early Survival', *PLoS pathogens* Vol. 13, No. 8, pp. e1006556.

Moscat, J. and M. T. Diaz-Meco. (2009), 'P62 at the Crossroads of Autophagy, Apoptosis, and Cancer', *Cell* Vol. 137, No. 6, pp. 1001-1004.

Moulder, J. W. (1966), 'The Relation of the Psittacosis Group (Chlamydiae) to Bacteria and Viruses', *Annual Reviews in Microbiology* Vol. 20, No. 1, pp. 107-130.

Moulder, J. (1991), 'Interaction of Chlamydiae and Host Cells in Vitro', *Microbiological reviews* Vol. 55, No. 1, pp. 143-190.

Muscarella, L. A., V. Guarnieri, M. Coco, S. Belli, P. Parrella, G. Pulcrano, D. Catapano, V. A. D'Angelo, L. Zelante and L. D'Agruma. (2010), 'Small Deletion at the 7q21. 2 Locus in a Ccm Family Detected by Real-Time Quantitative Pcr', *BioMed Research International* Vol. 2010.

Narendra, D., A. Tanaka, D.-F. Suen and R. J. Youle. (2008), 'Parkin Is Recruited Selectively to Impaired Mitochondria and Promotes Their Autophagy', *The Journal of cell biology* Vol. 183, No. 5, pp. 795-803.

Nelson, D. E., D. P. Virok, H. Wood, C. Roshick, R. M. Johnson, W. M. Whitmire, D. D. Crane, O. Steele-Mortimer, L. Kari and G. McClarty. (2005), 'Chlamydial Ifn-Γ Immune Evasion Is Linked to Host Infection Tropism', *Proceedings of the National Academy of Sciences* Vol. 102, No. 30, pp. 10658-10663.

Nijman, S. M., M. P. Luna-Vargas, A. Velds, T. R. Brummelkamp, A. M. Dirac, T. K. Sixma and R. Bernards. (2005), 'A Genomic and Functional Inventory of Deubiquitinating Enzymes', *Cell* Vol. 123, No. 5, pp. 773-786.

Noad, J., A. von der Malsburg, C. Pathe, M. A. Michel, D. Komander and F. Randow. (2017), 'Lubac-Synthesized Linear Ubiquitin Chains Restrict Cytosol-Invading Bacteria by Activating Autophagy and Nf-Kb', *Nature microbiology* Vol. 2, No. 7, pp. 17063.

Nozawa, K., C. A. Casiano, J. C. Hamel, C. Molinaro, M. J. Fritzler and E. K. Chan. (2002), 'Fragmentation of Golgi Complex and Golgi Autoantigens During Apoptosis and Necrosis', *Arthritis Research & Therapy* Vol. 4, No. 4, pp. R3.

Nuñez, J. K., A. S. Lee, A. Engelman and J. A. Doudna. (2015), 'Integrase-Mediated Spacer Acquisition During Crispr–Cas Adaptive Immunity', *Nature* Vol. 519, No. 7542, pp. 193.

Olivares-Zavaleta, N., A. Carmody, R. Messer, W. M. Whitmire and H. D. Caldwell. (2011), 'Chlamydia Pneumoniae Inhibits Activated Human T Lymphocyte Proliferation by the Induction of Apoptotic and Pyroptotic Pathways', *The Journal of Immunology*, pp. 1100393.

Olzmann, J. A. and L.-S. Chin. (2008), 'Parkin-Mediated K63-Linked Polyubiquitination: A Signal for Targeting Misfolded Proteins to the Aggresome-Autophagy Pathway', *Autophagy* Vol. 4, No. 1, pp. 85-87.

Omsland, A., J. Sager, V. Nair, D. E. Sturdevant and T. Hackstadt. (2012), 'Developmental Stage-Specific Metabolic and Transcriptional Activity of Chlamydia Trachomatis in an Axenic Medium', *Proceedings of the National Academy of Sciences* Vol. 109, No. 48, pp. 19781-19785.

Ouellette, S. P. (2018), 'Feasibility of a Conditional Knockout System for Chlamydia Based on Crispr Interference', *Frontiers in cellular and infection microbiology* Vol. 8, pp. 59.

Pachikara, N., H. Zhang, Z. Pan, S. Jin and H. Fan. (2009), 'Productive Chlamydia Trachomatis Lymphogranuloma Venereum 434 Infection in Cells with Augmented or Inactivated Autophagic Activities', *FEMS microbiology letters* Vol. 292, No. 2, pp. 240-249.

Pattanayak, V., S. Lin, J. P. Guilinger, E. Ma, J. A. Doudna and D. R. Liu. (2013), 'High-Throughput Profiling of Off-Target DNA Cleavage Reveals Rna-Programmed Cas9 Nuclease Specificity', *Nature biotechnology* Vol. 31, No. 9, pp. 839.

Petroski, M. D. (2010), 'Mechanism-Based Neddylation Inhibitor', *Chemistry & biology* Vol. 17, No. 1, pp. 6-8.

Pickart, C. M. (2001), 'Mechanisms Underlying Ubiquitination', *Annual review of biochemistry* Vol. 70, No. 1, pp. 503-533.

Pickart, C. M. and D. Fushman. (2004), 'Polyubiquitin Chains: Polymeric Protein Signals', *Current opinion in chemical biology* Vol. 8, No. 6, pp. 610-616.

Pickett, M. A., J. S. Everson, P. J. Pead and I. N. Clarke. (2005), 'The Plasmids of Chlamydia Trachomatis and Chlamydophila Pneumoniae (N16): Accurate Determination of Copy Number and the Paradoxical Effect of Plasmid-Curing Agents', *Microbiology* Vol. 151, No. 3, pp. 893-903.

Pokrovskaya, I., J. Szwedo, A. Goodwin, T. Lupashina, U. Nagarajan and V. Lupashin. (2012), 'Chlamydia Trachomatis Hijacks Intra-Golgi Cog Complex-Dependent Vesicle Trafficking Pathway', *Cellular microbiology* Vol. 14, No. 5, pp. 656-668.

Polajnar, M., M. S. Dietz, M. Heilemann and C. Behrends. (2017), 'Expanding the Host Cell Ubiquitylation Machinery Targeting Cytosolic Salmonella', *EMBO reports* Vol. 18, No. 9, pp. 1572-1585.

Pruneda, J. N., R. J. Bastidas, E. Bertsoulaki, K. N. Swatek, B. Santhanam, M. J. Clague, R. H. Valdivia, S. Urbé and D. Komander. (2018), 'A Chlamydia Effector Combining Deubiquitination and Acetylation Activities Induces Golgi Fragmentation', *Nature microbiology*, pp. 1.

Pruneda, J. N., C. H. Durkin, P. P. Geurink, H. Ovaa, B. Santhanam, D. W. Holden and D. Komander. (2016), 'The Molecular Basis for Ubiquitin and Ubiquitin-Like Specificities in Bacterial Effector Proteases', *Molecular cell* Vol. 63, No. 2, pp. 261-276.

Rajalingam, K., M. Sharma, C. Lohmann, M. Oswald, O. Thieck, C. J. Froelich and T. Rudel. (2008), 'Mcl-1 Is a Key Regulator of Apoptosis Resistance in Chlamydia Trachomatis-Infected Cells', *PLoS One* Vol. 3, No. 9, pp. e3102.

Rajeeve, K., S. Das, B. K. Prusty and T. Rudel. (2018), 'Chlamydia Trachomatis Paralyses Neutrophils to Evade the Host Innate Immune Response', *Nature microbiology* Vol. 3, No. 7, pp. 824.

Ramirez, Y. A., T. Adler, E. Altmann, C. Tiesmeyer, T. Klemm, F. Sauer, S. Kathman, A. Statsyuk, C. Sotriffer and C. Kisker. (2018), 'Structural Basis of Substrate Recognition and Covalent Inhibition of Cdu1 from Chlamydia Trachomatis', *ChemMedChem*.

Ramsey, K., J. Schripsema, B. Smith, Y. Wang, B. Jham, K. O'Hagan, N. Thomson, A. Murthy, R. Skilton and P. Chu. (2014), 'Plasmid Coding Sequence-Five Influences Infectivity and Virulence in a Mouse Model of Chlamydia Trachomatis Urogenital Infection', *Infection and immunity*, pp. IAI. 01795-14.

Randow, F. and R. J. Youle. (2014), 'Self and Nonself: How Autophagy Targets Mitochondria and Bacteria', *Cell host & microbe* Vol. 15, No. 4, pp. 403-411.

Rath, D., L. Amlinger, A. Rath and M. Lundgren. (2015), 'The Crispr-Cas Immune System: Biology, Mechanisms and Applications', *Biochimie* Vol. 117, pp. 119-128.

Read, T. D., R. Brunham, C. Shen, S. Gill, J. Heidelberg, O. White, E. Hickey, J. Peterson, T. Utterback and K. Berry. (2000), 'Genome Sequences of Chlamydia Trachomatis Mopn and Chlamydia Pneumoniae Ar39', *Nucleic acids research* Vol. 28, No. 6, pp. 1397-1406.

Rehman, S. A. A., Y. A. Kristariyanto, S.-Y. Choi, P. J. Nkosi, S. Weidlich, K. Labib, K. Hofmann and Y. Kulathu. (2016), 'Mindy-1 Is a Member of an Evolutionarily Conserved and Structurally Distinct New Family of Deubiquitinating Enzymes', *Molecular cell* Vol. 63, No. 1, pp. 146-155.

Ripa, T. and P. Nilsson. (2006), 'A Variant of Chlamydia Trachomatis with Deletion in Cryptic Plasmid: Implications for Use of Pcr Diagnostic Tests', *Weekly releases (1997–2007)* Vol. 11, No. 45, pp. 3076.

Rockey, D. D. (2011), 'Unraveling the Basic Biology and Clinical Significance of the Chlamydial Plasmid', *Journal of Experimental Medicine* Vol. 208, No. 11, pp. 2159-2162.

Ronzone, E. (2014), 'Insight into the Mechanism of Inca, a Type Iii Secreted Effector Protein in Chlamydia Trachomatis', Thomas Jefferson University.

Russell, M., T. Darville, K. Chandra-Kuntal, B. Smith, C. W. Andrews and C. M. O'Connell. (2011), 'Infectivity Acts as in Vivo Selection for Maintenance of the Chlamydial Cryptic Plasmid', *Infection and immunity* Vol. 79, No. 1, pp. 98-107.

Rzomp, K. A., L. D. Scholtes, B. J. Briggs, G. R. Whittaker and M. A. Scidmore. (2003), 'Rab Gtpases Are Recruited to Chlamydial Inclusions in Both a Species-Dependent and Species-Independent Manner', *Infection and immunity* Vol. 71, No. 10, pp. 5855-5870.

Schlesinger, D. H., G. Goldstein and H. D. Niall. (1975), 'Complete Amino Acid Sequence of Ubiquitin, an Adenylate Cyclase Stimulating Polypeptide Probably Universal in Living Cells', *Biochemistry* Vol. 14, No. 10, pp. 2214-2218.

Schneider-Schaulies, S., J. Schneider-Schaulies, A. Schuster, M. Bayer, J. Pavlovic and V. Ter Meulen. (1994), 'Cell Type-Specific Mxa-Mediated Inhibition of Measles Virus Transcription in Human Brain Cells', *Journal of virology* Vol. 68, No. 11, pp. 6910-6917.

Sharma, M. and T. Rudel. (2009), 'Apoptosis Resistance in Chlamydia-Infected Cells: A Fate Worse Than Death?', *FEMS Immunology & Medical Microbiology* Vol. 55, No. 2, pp. 154-161.

Shemer, Y. and I. Sarov. (1985), 'Inhibition of Growth of Chlamydia Trachomatis by Human Gamma Interferon', *Infection and immunity* Vol. 48, No. 2, pp. 592-596.

Shimura, H., N. Hattori, S.-i. Kubo, Y. Mizuno, S. Asakawa, S. Minoshima, N. Shimizu, K. Iwai, T. Chiba and K. Tanaka. (2000), 'Familial Parkinson Disease Gene Product, Parkin, Is a Ubiquitin-Protein Ligase', *Nature genetics* Vol. 25, No. 3, pp. 302.

Short, B., A. Haas and F. A. Barr. (2005), 'Golgins and Gtpases, Giving Identity and Structure to the Golgi Apparatus', *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* Vol. 1744, No. 3, pp. 383-395.

Shorter, J. and G. Warren. (2002), 'Golgi Architecture and Inheritance', *Annual review of cell and developmental biology* Vol. 18, No. 1, pp. 379-420.

Sinai, A. P. and K. A. Joiner. (1997), 'Safe Haven: The Cell Biology of Nonfusogenic Pathogen Vacuoles', *Annual Reviews in Microbiology* Vol. 51, No. 1, pp. 415-462.

Skilton, R. J., L. T. Cutcliffe, D. Barlow, Y. Wang, O. Salim, P. R. Lambden and I. N. Clarke. (2009), 'Penicillin Induced Persistence in Chlamydia Trachomatis: High Quality Time Lapse Video Analysis of the Developmental Cycle', *PLoS One* Vol. 4, No. 11, pp. e7723.

Smit, J. J. and T. K. Sixma. (2014), '"Ubiquitylation: Mechanism and Functions" Review Series: Rbr E3-Ligases at Work', *EMBO reports* Vol. 15, No. 2, pp. 142-154.

Song, L., J. H. Carlson, W. M. Whitmire, L. Kari, K. Virtaneva, D. E. Sturdevant, H. Watkins, B. Zhou, G. L. Sturdevant and S. F. Porcella. (2013), 'The Chlamydia Trachomatis Plasmid-Encoded Pgp4 Is a Transcriptional Regulator of Virulence Associated Genes', *Infection and immunity*, pp. IAI. 01305-12.

Song, L., J. H. Carlson, B. Zhou, K. Virtaneva, W. M. Whitmire, G. L. Sturdevant, S. F. Porcella, G. McClarty and H. D. Caldwell. (2014), 'Plasmid-Mediated Transformation Tropism of Chlamydial Biovars', *Pathogens and disease* Vol. 70, No. 2, pp. 189-193.

Staub, O. and D. Rotin. (2006), 'Role of Ubiquitylation in Cellular Membrane Transport', *Physiological reviews* Vol. 86, No. 2, pp. 669-707.

Stephens, R. S. (1999), *Chlamydia: Intracellular Biology, Pathogenesis, and Immunity*, Zondervan.

Stephens, R. S., S. Kalman, C. Lammel, J. Fan, R. Marathe, L. Aravind, W. Mitchell, L. Olinger, R. L. Tatusov and Q. Zhao. (1998), 'Genome Sequence of an Obligate Intracellular Pathogen of Humans: Chlamydia Trachomatis', *Science* Vol. 282, No. 5389, pp. 754-759.

Su, H., G. McClarty, F. Dong, G. M. Hatch, Z. K. Pan and G. Zhong. (2004), 'Activation of Raf/Mek/Erk/Cpla2 Signaling Pathway Is Essential for Chlamydial Acquisition of Host Glycerophospholipids', *Journal of Biological Chemistry* Vol. 279, No. 10, pp. 9409-9416.

Suchland, R., K. M. Sandoz, B. Jeffrey, W. Stamm and D. Rockey. (2009), 'Horizontal Transfer of Tetracycline Resistance among Chlamydia Spp. In Vitro', *Antimicrobial agents and chemotherapy* Vol. 53, No. 11, pp. 4604-4611.

Sun, H. S., E. W. Eng, S. Jeganathan, A. T. W. Sin, P. C. Patel, E. Gracey, R. D. Inman, M. R. Terebiznik and R. E. Harrison. (2012), 'Chlamydia Trachomatis Vacuole Maturation in Infected Macrophages', *Journal of leukocyte biology* Vol. 92, No. 4, pp. 815-827.

Sütterlin, C., P. Hsu, A. Mallabiabarrena and V. Malhotra. (2002), 'Fragmentation and Dispersal of the Pericentriolar Golgi Complex Is Required for Entry into Mitosis in Mammalian Cells', *Cell* Vol. 109, No. 3, pp. 359-369.

Svenning, S. and T. Johansen. (2013), 'Selective Autophagy', *Essays in biochemistry* Vol. 55, pp. 79-92.

Swatek, K. N. and D. Komander. (2016), 'Ubiquitin Modifications', *Cell research* Vol. 26, No. 4, pp. 399.

T'ang, F.-F., H.-L. Chang and Y.-. Huang. (1957), 'Studies on the Etiology of Trachoma with Special Reference to Isolation of the Virus in Chick Embryo', *Chinese medical journal* Vol. 75, No. 6, pp. 429-47.

Tam, J. E., C. H. Davis and P. B. Wyrick. (1994), 'Expression of Recombinant DNA Introduced into Chlamydia Trachomatis by Electroporation', *Canadian journal of microbiology* Vol. 40, No. 7, pp. 583-591.

Terns, R. M. and M. P. Terns. (2014), 'Crispr-Based Technologies: Prokaryotic Defense Weapons Repurposed', *Trends in Genetics* Vol. 30, No. 3, pp. 111-118.

Thurston, T. L., G. Ryzhakov, S. Bloor, N. Von Muhlinen and F. Randow. (2009), 'The Tbk1 Adaptor and Autophagy Receptor Ndp52 Restricts the Proliferation of Ubiquitin-Coated Bacteria', *Nature immunology* Vol. 10, No. 11, pp. 1215.

Thurston, T. L., M. P. Wandel, N. von Muhlinen, Á. Foeglein and F. Randow. (2012), 'Galectin 8 Targets Damaged Vesicles for Autophagy to Defend Cells against Bacterial Invasion', *Nature* Vol. 482, No. 7385, pp. 414.

Todi, S. V., B. J. Winborn, K. M. Scaglione, J. R. Blount, S. M. Travis and H. L. Paulson. (2009), 'Ubiquitination Directly Enhances Activity of the Deubiquitinating Enzyme Ataxin-3', *Embo j* Vol. 28, No. 4, pp. 372-82.

Tokgöz, Z., T. J. Siepmann, F. Streich, B. Kumar, J. M. Klein and A. L. Haas. (2012), 'E1-E2 Interactions in Ubiquitin and Nedd8 Ligation Pathways', *Journal of Biological Chemistry* Vol. 287, No. 1, pp. 311-321.

Tokunaga, F., S.-i. Sakata, Y. Saeki, Y. Satomi, T. Kirisako, K. Kamei, T. Nakagawa, M. Kato, S. Murata and S. Yamaoka. (2009), 'Involvement of Linear Polyubiquitylation of Nemo in Nf-Kb Activation', *Nature cell biology* Vol. 11, No. 2, pp. 123.

Uchida, C. and M. Kitagawa. (2016), 'Ring-, Hect-, and Rbr-Type E3 Ubiquitin Ligases: Involvement in Human Cancer', *Current cancer drug targets* Vol. 16, No. 2, pp. 157-174.

Udeshi, N. D., T. Svinkina, P. Mertins, E. Kuhn, D. Mani, J. W. Qiao and S. A. Carr. (2013), 'Refined Preparation and Use of Anti-Diglycine Remnant (K-E-Gg) Antibody Enables Routine Quantification of 10,000 S of Ubiquitination Sites in Single Proteomics Experiments', *Molecular & Cellular Proteomics* Vol. 12, No. 3, pp. 825-831.

Via, L. E., D. Deretic, R. J. Ulmer, N. S. Hibler, L. A. Huber and V. Deretic. (1997), 'Arrest of Mycobacterial Phagosome Maturation Is Caused by a Block in Vesicle Fusion between Stages Controlled by Rab5 and Rab7', *Journal of Biological Chemistry* Vol. 272, No. 20, pp. 13326-13331.

Wang, F., J. Li, Q. Li, R. Liu, M. Zheng, Q. Wang, J. Wen and G. Zhao. (2017), 'Changes of Host DNA Methylation in Domestic Chickens Infected with Salmonella Enterica', *Journal of genetics* Vol. 96, No. 4, pp. 545-550.

Wang, H., H. Yang, C. S. Shivalila, M. M. Dawlaty, A. W. Cheng, F. Zhang and R. Jaenisch. (2013), 'One-Step Generation of Mice Carrying Mutations in Multiple Genes by Crispr/Cas-Mediated Genome Engineering', *Cell* Vol. 153, No. 4, pp. 910-918.

Wang, X., K. Hybiske and R. S. Stephens. (2018), 'Direct Visualization of the Expression and Localization of Chlamydial Effector Proteins within Infected Host Cells', *Pathogens and disease* Vol. 76, No. 2, pp. fty011.

Wang, Y., S. Kahane, L. T. Cutcliffe, R. J. Skilton, P. R. Lambden and I. N. Clarke. (2011), 'Development of a Transformation System for Chlamydia Trachomatis: Restoration of Glycogen Biosynthesis by Acquisition of a Plasmid Shuttle Vector', *PLoS pathogens* Vol. 7, No. 9, pp. e1002258.

Watson, R. O., P. S. Manzanillo and J. S. Cox. (2012), 'Extracellular M. Tuberculosis DNA Targets Bacteria for Autophagy by Activating the Host DNA-Sensing Pathway', *Cell* Vol. 150, No. 4, pp. 803-815.

Weber, M. M., J. L. Lam, C. A. Dooley, N. F. Noriea, B. T. Hansen, F. H. Hoyt, A. B. Carmody, G. L. Sturdevant and T. Hackstadt. (2017), 'Absence of Specific Chlamydia Trachomatis Inclusion Membrane Proteins Triggers Premature Inclusion Membrane Lysis and Host Cell Death', *Cell reports* Vol. 19, No. 7, pp. 1406-1417.

Weidberg, H., E. Shvets and Z. Elazar. (2011), 'Biogenesis and Cargo Selectivity of Autophagosomes', *Annual review of biochemistry* Vol. 80, pp. 125-156.

Wichers, M. C. and M. Maes. (2004), 'The Role of Indoleamine 2, 3-Dioxygenase (Ido) in the Pathophysiology of Interferon-A-Induced Depression', *Journal of Psychiatry and Neuroscience* Vol. 29, No. 1, pp. 11.

Wickstrum, J., L. R. Sammons, K. N. Restivo and P. S. Hefty. (2013), 'Conditional Gene Expression in Chlamydia Trachomatis Using the Tet System', *PLoS One* Vol. 8, No. 10, pp. e76743.

Wu, X., L. Yen, L. Irwin, C. Sweeney and K. L. Carraway. (2004), 'Stabilization of the E3 Ubiquitin Ligase Nrdp1 by the Deubiquitinating Enzyme Usp8', *Molecular and cellular biology* Vol. 24, No. 17, pp. 7748-7757.

Wylie, J. L., G. M. Hatch and G. McClarty. (1997), 'Host Cell Phospholipids Are Trafficked to and Then Modified by Chlamydia Trachomatis', *Journal of bacteriology* Vol. 179, No. 23, pp. 7233-7242.

Xu, S., L. Battaglia, X. Bao and H. Fan. (2013), 'Chloramphenicol Acetyltransferase as a Selection Marker for Chlamydial Transformation', *BMC research notes* Vol. 6, No. 1, pp. 377.

Zhang, L., F. Zhou, Y. Drabsch, R. Gao, B. E. Snaar-Jagalska, C. Mickanin, H. Huang, K.-A. Sheppard, J. A. Porter and C. X. Lu. (2012), 'Usp4 Is Regulated by Akt Phosphorylation and Directly Deubiquitylates Tgf-B Type I Receptor', *Nature cell biology* Vol. 14, No. 7, pp. 717.

Zhang, Y., L. Yeruva, A. Marinov, D. Prantner, P. B. Wyrick, V. Lupashin and U. M. Nagarajan. (2014), 'The DNA Sensor, Cyclic Gmp–Amp Synthase, Is Essential for Induction of Ifn-B During Chlamydia Trachomatis Infection', *The Journal of Immunology*, pp. 1302718.

Zhong, J., M. Karberg and A. M. Lambowitz. (2003), 'Targeted and Random Bacterial Gene Disruption Using a Group Ii Intron (Targetron) Vector Containing a Retrotransposition-Activated Selectable Marker', *Nucleic acids research* Vol. 31, No. 6, pp. 1656-1664.

Zhou, W., X. Wang and M. G. Rosenfeld. (2009), 'Histone H2a Ubiquitination in Transcriptional Regulation and DNA Damage Repair', *The international journal of biochemistry & cell biology* Vol. 41, No. 1, pp. 12-15.

6 Appendix

6.1 Abreviations

µ	micro
AB	Abberant body
AHT	Anhydrotetracycline
Ankib1	Ankyrin repeat and IBR-domain containing protein 1
APS	Ammonium persulfate
Atg	Autophagy related gene
ATP	Adenosine triphosphate
Bcl-2	B cell lymphoma 2
BRPF3	Bromodomain and PHD-finger containing protein 3
BSA	Bovine serum albumin
C. muridarum	*Chlamydia muridarum*
C. pneumoniae	*Chlamydia pneumoniae*
C. trachomatis	*Chlamydia trachomatis*
CAT	Chloramphenicol acetyltransferase
CCM	Cerebral cavernous malformation
Cdu1	Chlamydial deubiquitinase 1
Cdu2	Chlamydial deubiquitinase 2
CHX	Chloramphenicol
*Chla*DUB1	Chlamydial deubiquitinase 1
*Chla*DUB2	Chlamydial deubiquitinase 2
Cmu	*Chlamydia muridarum*
CPAF	Chlamydial protease-like activating factor
Cpn	*Chlamydia pneumoniae*
CRISPR	clustered regularly interspaced short palindromic repeats
CRISPRi	CRISPR interference
CRL	Cullin RING E3 ligase
crRNA	CRIPSR RNA
C-terminal	Carboxyl-terminal
Ctr	*Chlamydia trachomatis*
DAMP	Damage-associated molecular pattern
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleosid triphosphate
DTT	Dithiothreitol
DUB	Deubiquitinase
E. coli	*Escherichia coli*
EB	Elementary body
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetetraacetic acid
FCS	Fetal calf serum
Fimb	Cells of human fimbriae
FPR2	Formyl peptide receptor 2
GAPDH	Glycerinaldehyde 3-dehydrogenase
GBP	Guanylate-binding protein

GFP	Green fluorescent protein
gRNA	Guide RNA
h	Hour(s)
HDR	Homology directed repair
HECT	Homologous to the E6-AP carboxyl terminus
HIP2	Ubiquitin conjugating enzyme E2 K
hpi	Hours post infection
HRP	Horseradish peroxidase
HSP60	Heat shock protein 60
IDO	Indole-2,3-dioxygenase
IF	Immunofluorescence
IFNγ	Interferon gamma
IPTG	Isopropyl-β-D-thiogalactopyranosid
IRG	Immunity related genes
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
JAMM	JAB1/MPN/Mov34
K	Lysine
kb	Kilobases
kDa	Kilodalton
l	liter
LAP	LC3-assisted phagocytosis
LB	Lysogeny broth
LPS	Lipopolysaccharide
M	Molar
Mb	Megabases
Mcl-1	Myeloid cell leukemia 1
min	Minute (s)
MINDY	MUI-containing novel DUB family
MJD	Machado-Joseph disease protease
MOI	Multiplicity of infection
MOMP	mitochondrial outer membrane permeabilization
mRNA	Messenger RNA
ms	Mouse
MxA	interferon-induced GTP-binding protein Mx1
NBR1	Neighbor of BRCA1 gene
NDP52	Calcium-binding and coiled-coil domain-containing protein 2
Nedd8	Neural precursor cell expressed, developmentally downregulated 8
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non homologous end joining
nm	Nanometer
NOD	Nucleotide binding oligomerization domain
N-terminal	Amino-terminal
OD600	Optical density measured at a wavelength of 600 nm
OmpA	Major outer membrane protein A
OUT	Ovarian tumor protease
P62	Sequestosome
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PEI	Polyethylenimine
PenG	Penicillin G
PFA	Paraformaldehyde
PG	Phosphatidylglycerol
PI3K	Phosphoinositide 3-kinase
PRR	Pattern recognition receptor
PS	Phosphatidylserine
RB	Reticulate body
rb	Rabbit
RBR	ring between ring
RING	Really interesting new gene
RNA	Ribonucleic acid
RNAi	RNA interference
RNF214	RING finger protein 214
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Skp1, cullin, F-box protein
SDS	Sodium dodecyl sulphate
sec	Second (s)
siRNA	Small interfering RNA
SOC	Super optimal broth with catabolite repression
STING	Stimulator of interferon genes
T3SS	Type 3 secretion system
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
TLR	Toll like receptor
TNFα	Tumor necrosis factor alpha
tracrRNA	Trans activating crRNA
UBD	Ubiquitin binding domain
UBE1	Ubiquitin-like modifier-activating enzyme 1
UBE2L3	Ubiquitin conjugating enzyme E2 L3
Ubi	Ubiquitin
UCH	Ubiquitin C-terminal hydrolase
USP	Ubiquitin specific protease
WT	Wildtype

6.2 Publication and presentation

Publication

Fischer A, Harrison KS, Ramirez Y, Auer D, Chowdhury SR, Prusty BK, Sauer F, Dimond Z, Kisker C, Scott Hefty P, Rudel T (eLife) 2017 *Chlamydia trachomatis*-containing vacuole serves as deubiquitination platform to stabilize Mcl-1 ant to interfere with host defense

Oral and poster presentation

Auer D, Fischer A, Rudel T (2017) How chlamydial deubiquitinase Cdu1 interferes with cell autonomous defense. Annual Retreat of GRK2243, Eibelstadt (poster)

Auer D (2018) Function of chlamydial DUBs during infection. Annual Retreat of GRK2243, Muggendorf (oral presentation)

6.3 Danksagung

An dieser Stelle möchte ich mich bei allen bedanken, die mir das Gelingen meiner Doktorarbeit ermöglicht haben und deren Hilfe ich sehr schätze.
Ich möchte mich zu allererst bei Herrn Prof. Dr. Thomas Rudel bedanken, der es mir ermöglicht hat meine Arbeit im Lehrstuhl für Mikrobiologie anfertigen. Des Weiteren trugen unsere gemeinsamen Besprechungen und Ideen zum Gelingen dieser Arbeit maßgeblich bei.
Bedanken möchte ich mich auch bei Prof. Dr. Alexander Buchberger für die Übernahme der Zweitkorrektur, sowie die Ideen und Anregungen, die ich bei meinen Vorträgen bekommen habe.
Ein ganz besonderer Dank geht an meine Betreuerin Dr. Annette Fischer, die von Anfang bis Ende dieser Arbeit stets mit Rat und Tat an meiner Seite stand. Danke für die Zeit, die du dir für meine Fragen und Diskussionen genommen hast. Es war bestimmt nicht immer leicht alles unter einen Hut zu bekommen.
Ich danke auch allen Kooperationspartnern, vor allem Prof. Scott Hefty für die Bereitstellung der Mutante. Den Mitgliedern des GRK 2243 möchte ich für den Austausch von Informationen und Laborbedarf danken.
Den ganzen ehemaligen und heutigen Mitarbeitern des Lehrstuhls möchte ich für die angenehme Arbeitsatmosphäre danken. Der Zusammenhalt aller Kollegen, die Hilfe und Unterstützung bei großen und kleinen Problemen war großartig. Dabei geht ein großes Dankeschön an alle Mitarbeiter und Studenten im Labor C238 für die tägliche Unterstützung. Ganz besonders möchte ich mich bei Maximilian Klepsch für die Hilfe bei technischen Fragen und statistischer Auswertung bedanken. Kathrin Stelzner danke ich für die Unterstützung beim FACS und offenen Ohren für Diskussionen auch außerhalb der Arbeit. Ein Dank geht an Jessica Horn für die Tipps im Umgang mit RNA und für das Teilen gehorteter Enzyme in knappen Zeiten. Dagmar Beier hat mich stets kompetent bei Fragen zu Klonierungsstrategien beraten, dafür ein Danke. Ich danke auch Franziska Solger für die Bereitstellung isolierter Neutrophilen und all den Spendern für ihr Opfer. Auch den ehemaligen Doktoranden Franziska Hagen und Anastasija Reimer möchte ich für die gemeinsame Zeit und den Austausch von Ideen danken, der auch über ihre eigene Zeit am Lehrstuhl hinausging.
Abschließend geht ein großes Danke an meine Eltern und meine Schwester, für die nicht enden wollende Unterstützung, Motivation und guten Worte. Dafür, dass sie Verständnis gezeigt haben und sich alles angehört und nachgefragt haben.

6.4 Selbständigkeitserklärung

Eidesstattliche Erklärungen nach §7 Abs. 2 Satz 3, 4, 5 der Promotionsordnung der Fakultät für Biologie

Affidavit

I hereby declare that my thesis entitled: „Impact of the chlamydial deubiquitinase *Chla*DUB1 on host cell defense" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore I verify that the thesis has not been submitted as part of another examination process neither in identical nor in similar form.

Besides I declare that if I do not hold the copyright for figures and paragraphs, I obtained it from the rights holder and that paragraphs and figures have been marked according to law or for figures taken from the internet the hyperlink has been added accordingly.

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation: „Impact of the chlamydial deubiquitinase *Chla*DUB1 on host cell defense", eigenständig, d. h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen, als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Weiterhin erkläre ich, dass bei allen Abbildungen und Texten bei denen die Verwertungsrechte (Copyright) nicht bei mir liegen, diese von den Rechtsinhabern eingeholt wurden und die Textstellen bzw. Abbildungen entsprechend den rechtlichen Vorgaben gekennzeichnet sind sowie bei Abbbildungen, die dem Internet entnommen wurden, der entsprechende Hypertextlink angegeben wurde.

Würzburg, den

Appendix 1: siRNA based E3 ligase screen. List of all siRNAs as well as mean and standard deviation (StDv) of p62 spots forming around *Ctr* Tn-*cdu1* inclusion of three biological replicates.

siRNA	Mean p62 around inclusion	StDv	siRNA	Mean p62 around inclusion	StDv
Ctr Tn-*cdu1*	100	0	FBXL7	76,0246908	19,095562
Ctr WT	14,4828647	4,2454585	FBXL11	70,5839469	13,6638371
sip62	13,2591843	11,469451	WSB2	88,3039294	15,1383759
sicontrol	109,548412	9,2055008	FBXO16	106,50651	12,8913599
FBXL15	86,1780773	7,07582208	ASB14	97,8998242	15,741558
FBXW10	98,632379	16,5091219	FBXO5	79,954949	11,6577565
FBXO42	81,4948966	29,9963618	FBXL17	117,523608	11,0042104
ASB13	101,089957	17,0010818	ASB5	110,216471	12,3491222
SPSB1	110,284303	12,512872	CISH	92,0437479	11,3606354
SPSB3	75,623759	0,30359177	BTRC	104,542637	18,5450578
FBXO31	87,5535141	11,8439993	ASB18	87,0081241	12,5411906
SOCS3	104,880746	18,384365	FBXL20	83,6481871	6,34954922
FBXO15	85,180038	17,6404276	SOCS2	97,6470083	3,39663586
NLRC5	109,090426	1,86210254	FBXW11	113,532936	15,2009611
FBXO18	89,8974489	16,420631	ASB6	123,768417	8,97140652
FBXO21	101,25547	15,5548909	FBXO28	124,759637	9,5155424
FBXO40	107,369706	8,02538187	FBXO46	105,131452	13,8137992
SOCS6	95,0837642	7,45223639	ASB8	124,127565	7,49554166
FBXO6	95,4915018	13,1454266	FBXW9	89,1174107	15,7045754
FBXO9	89,1454698	8,3177786	FBXL12	127,707832	17,5911558
FBXL3P	101,456503	14,5541994	FBXL16	68,9679712	10,7822909
FBXO3	89,7068918	8,47880156	FBXO27	79,4384041	11,0178396
FBXO22	110,808436	12,8639463	FBXO43	84,5844146	18,2655894
CCNF	95,5983302	5,66963985	LOC200933	141,470238	21,8585649
LOC554251	89,8902514	17,9033398	RAB40C	106,934863	11,4641971
FBXO30	109,260095	11,3109392	FBXW2	107,554488	12,9622528
FBXO17	94,3430988	11,3108605	LOC342897	116,322154	19,5531231
FBXL8	93,8874174	9,61756422	FBXL18	112,105938	14,3960696
ASB16	92,159622	3,03203997	LOC652759	119,109785	29,5754253
SPSB2	105,110161	1,23348009	TULP4	123,157079	19,5223169
WDR71	105,250063	12,5682588	UBE2C	86,2547657	28,9528912
FBXO11	80,5289188	12,2107781	SMURF1	92,3656685	3,54637134
FBXL14	107,571247	6,17122008	EDD1	92,4445081	8,73214889
LL0XNC01-237H1.1	111,081569	13,2833515	HIP2	61,7295495	8,37052074
FBXL19	98,990158	18,2203897	HECTD1	105,227939	2,8888596
FBXL10	113,303864	8,04065649	UBE2T	96,9721529	8,28373383
SKP2	105,140764	15,7182531	CDC34	98,35516	14,886853
LRRC29	108,056116	1,59414277	FLJ34154	98,0022795	3,94705491
FBXW8	104,547624	10,2130062	DCUN1D1	119,00467	9,61493767
FBXO41	101,549173	10,7682416	CUL2	135,460032	7,1666867
SOCS5	112,359358	4,00290621	HERC3	79,9135267	27,1915358
FBXO7	86,8189225	1,97194612	UBE2W	85,9444617	9,07546704
FBXO25	102,82632	4,76074837	UBE2V2	71,3121859	13,3524229
FBXO24	85,7572135	7,17316876	DCUN1D5	83,9518232	7,23269752
WSB1	109,569398	15,6192831	HERC2	85,9977021	9,58433928
SOCS7	108,899939	11,9716139	UBE2N	93,7870716	6,93321206
ASB12	109,024185	10,5870147	UBE2Z	115,465376	10,3635691
ASB9	93,5122188	6,38717548	UBE2L3	64,6846242	10,5522508
FBXO4	105,23734	7,77137041	HERC5	108,251234	14,784473
FBXL13	104,954876	15,4799446	UBE2NL	109,692159	10,6212648
ASB11	84,4607183	9,12388585	DCUN1D3	81,8221756	29,3965132

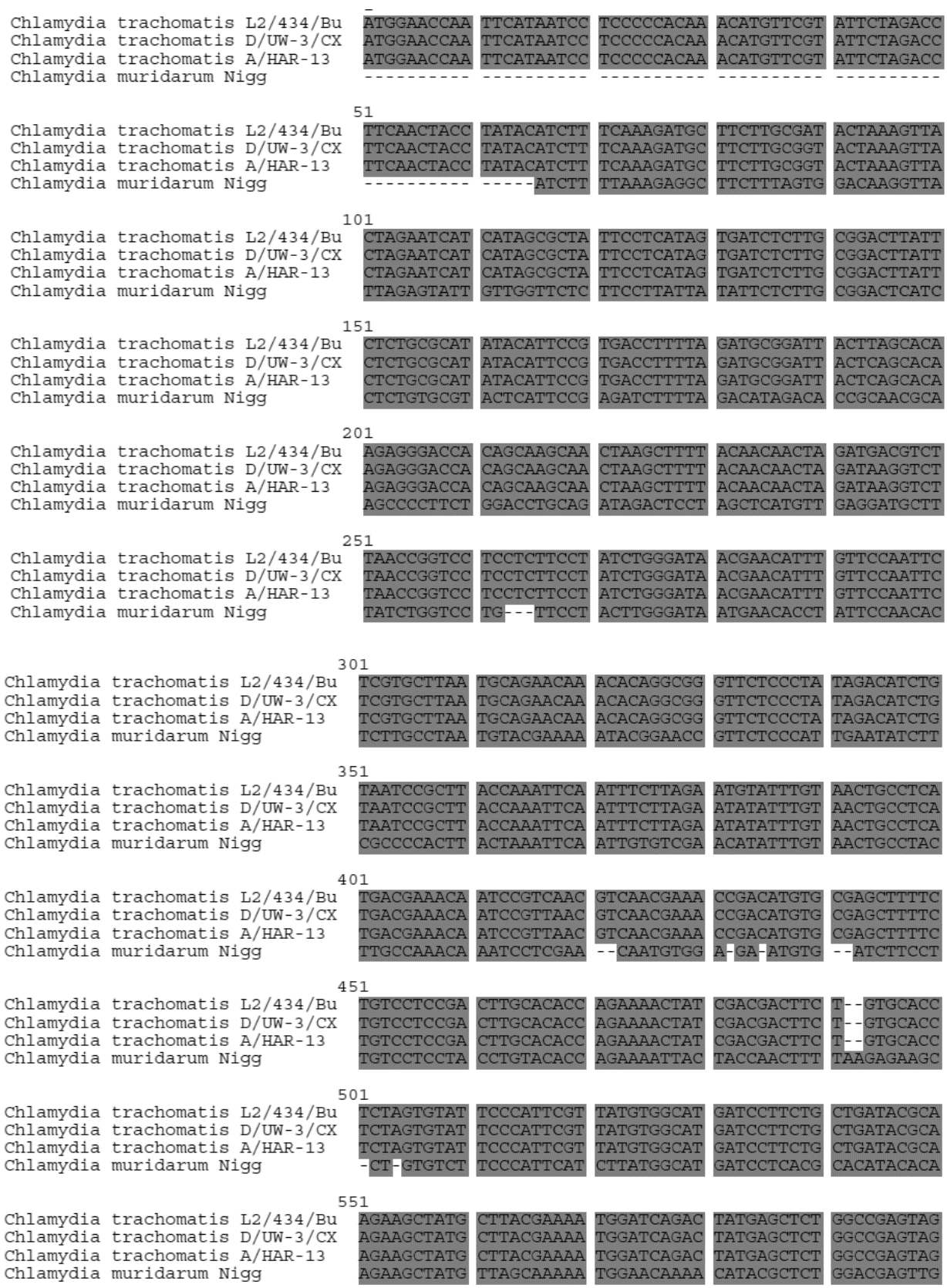
RAB40A	116,776892	11,8305074	BIRC6	81,8362605	29,4423178
FBXL2	102,052243	13,760198	UBE2J2	98,1911696	14,024061
FBXO2	98,3865235	9,47910693	HECW1	75,3518653	11,3140919
FBXO8	92,8591118	17,3855401	UBE1L	106,729376	9,42992245
FBXO38	110,187578	23,9178978	UBE1	43,4464155	5,83823257
LGR6	94,8738229	5,34966005	HERC1	111,487234	8,18281012
FBXW12	110,465419	9,28870749	HACE1	99,6211078	21,1409592
FBXL6	102,130519	8,83319456	CUL7	99,9439251	20,4448098
FBXL4	102,821664	17,5002146	UBE2S	78,9552733	13,0952185
SHFM3	111,787719	11,7135212	CUL3	57,3136508	26,8066921
FBXO47	99,8749809	1,41948824	ITCH	85,5138452	23,2568483
ASB17	111,415732	8,39142826	HUWE1	89,3755035	24,2728874
ASB3	104,522229	8,99457233	CAND2	98,698175	16,1726294
FBXO44	104,175259	19,0969048	UBE2D3	72,2071215	15,3934511
ASB7	101,678728	26,5790216	NEDD4	106,894477	4,09398531
NEURL2	94,2435085	12,853647	UBE1DC1	101,873382	25,3258526
FBXO32	109,549828	8,24526049	UBE2M	102,993813	25,1543671
ASB15	103,391617	3,51562706	UEVLD	100,28693	22,1713886
SPSB4	91,2613619	15,5946517	UBE2F	82,9748079	31,5119512
FBXL5	111,351279	14,6195537	DCUN1D2	72,9613946	33,3887699
FBXO34	33,1540784	41,7763369	CUL1	80,790009	15,550874
ASB1	110,82621	10,3644772	UBE3B	91,6016691	21,8373057
FBXL22	98,7570574	4,57252931	UBE2A	104,633695	16,3052071
ASB2	97,0759639	18,8803213	UBE2E2	86,0128688	9,70893588
FBXL3A	86,3287756	18,3333741	HECTD3	92,7941201	18,4384984
FBXO36	106,410566	23,0972595	UBE2I	105,93811	35,5201883
FBXW7	86,8257342	5,04616158	UBE2Q2	83,8848365	25,3878622
FBXW5	91,445859	4,15034119	HERC6	73,9831562	15,3211159
FBXO39	92,8583078	12,2822209	WWP1	97,3083941	37,1919273
FBXO10	105,692764	11,7418437	MDM2	88,1123371	18,6940551
SOCS4	100,804603	5,79004139	PHF7	101,975735	8,35613733
ASB10	101,251599	8,69120097	TRIM39	104,497787	11,4487802
RAB40B	108,091818	8,49781918	TRIM41	94,2664825	17,5924349
LOC440456	108,053228	24,0874125	RNF133	105,507444	8,01505807
ASB4	78,2966628	8,35724362	KIAA1718	103,269767	11,4283961
SOCS1	105,439226	24,1858841	RNF32	104,823002	10,7547809
FBXO33	103,072689	2,7546407	INTS12	103,160926	4,98098048
FLJ10916	87,251539	22,9753417	ZNF592	98,5736896	7,5441796
TRIM67	116,315298	2,39110679	LOC92312	110,572423	13,074801
LOC644006	113,182319	4,71030293	PRPF19	97,1584828	29,1348271
PHF6	93,2892263	3,64432423	RBX1	98,4074716	12,4915325
TRIM63	120,748661	13,6718503	LOC653111	103,531509	6,98020508
RFPL4B	100,616132	7,40450784	HRC	100,273538	7,29475739
PDZRN3	92,6504814	12,5469941	MYCBP2	106,626266	15,1258819
C6ORF49	98,7778386	6,73213029	PRICKLE1	112,376258	14,8033574
PHF5A	75,8734569	12,0554109	CBL	92,9399745	8,31545932
C20ORF18	95,7163943	4,11333655	CHD5	98,8216745	8,12009563
TRIM75	107,607859	8,98000167	RNF180	105,03973	10,209257
PHF17	110,808066	4,43140852	PHF21A	110,23768	14,0446273
TRIM42	120,785783	10,6878347	TRIM6-TRIM34	97,2293269	35,5428767
BAHD1	108,026761	9,02969966	LOC642678	90,5141061	20,6029945
WDR24	127,936149	2,69921047	MARCH8	99,2911238	14,273343
DTX4	111,671027	13,0652423	PHF20L1	96,0341058	4,55485506
TRIM26	96,6505697	14,5646477	RNF152	93,5830395	8,90878589
PHF21B	81,0393083	22,4820594	RFWD2	105,696592	7,50944199
MARCH4	86,2784943	23,8131155	RSPRY1	73,5926565	5,9226307
ANKIB1	52,2802814	10,4554075	MID2	87,5220349	4,71985617
OIT3	100,777222	11,9395173	MLLT6	92,8147158	9,66819605

MGRN1	104,982566	3,19951283	ZMYND11	108,024681	21,1775907
RNF7	106,655669	3,75933782	BIRC3	92,5496286	38,4928366
WDSUB1	108,711839	4,73468444	MIB2	82,6481849	9,52803018
PCGF1	116,491373	8,47100547	TRIM62	98,1985739	6,21126637
TRIM3	95,4122328	13,2515159	RNF125	114,9965	10,5200948
ZNF645	104,643289	21,5856671	BMI1	100,622592	11,7706948
RNF5	78,6158257	12,3610212	PHF20	108,907192	6,33888178
PHF15	83,321193	15,9229215	ZNF313	95,0883215	10,7129233
BRCA1	112,499915	3,90731217	RNF185	102,067779	16,5052342
ZFAND6	110,594499	9,19050753	ZNRF3	82,6499807	11,785941
SH3RF2	141,954445	14,3623923	JARID1B	87,9892376	24,6038801
RNF150	117,39449	8,82313639	RNF123	66,1435521	24,0065147
PCGF3	104,266508	7,67696753	PHF11	106,584615	15,3867644
RNF8	97,2732457	9,09835736	TRIM60	108,99871	1,64467471
RNF38	80,694857	19,2980356	TRIM8	98,0644493	5,3432122
TRIM15	81,1656141	12,8149576	RNF122	80,1151487	6,54919637
IRF2BP1	94,5822034	22,7589486	RNF39	112,17525	24,0369165
MLL2	86,2120216	22,4446164	RNF12	99,1796232	28,8196125
PARC	96,3301205	10,8746287	RNF135	92,5614185	33,0851432
HR	84,9749178	17,0926945	ZNRF2	66,1526464	22,5248551
PHF23	116,668629	5,79949727	RKHD1	81,600939	30,1192625
RNF25	119,41734	7,05514433	ZNF330	101,043997	11,6177596
PHF13	109,502055	7,82837408	RNF148	100,351016	7,12351682
DTX3L	114,011577	14,1618799	VPS41	115,517579	10,9314862
TNFAIP3	100,636633	12,5700349	UBE4B	92,2075003	20,6896312
MKRN3	93,9757848	20,0216699	CCL20	86,7829793	7,66980589
PHF12	109,0563	28,4295623	RNF141	105,065338	6,84625163
MID1	78,0197041	20,2582491	WHSC1L1	84,8236783	14,0519843
TRIM14	87,8884978	21,4208528	MARCH2	78,552971	17,2131479
RUFY1	76,8867806	17,2981955	NDP52	110,903673	8,37881249
RFFL	108,465013	14,5675648	UBR2	103,758152	6,89399386
LRSAM1	106,057163	7,84277513	TRIM2	85,2747757	5,48841148
RNF139	100,902934	10,9299428	RNF103	97,8025036	6,15239696
RAPSN	100,274794	18,8924376	TRIML1	88,4712078	4,15407426
PJA1	92,2702907	5,90810671	TOPORS	94,6804366	7,10577565
RKHD2	94,1335637	5,1711117	WHSC1	89,3147382	4,93940812
DPF1	88,0482135	11,0617201	TRIM17	88,9934858	8,46079812
UBE4A	84,7482534	5,34165393	TRIM33	90,7009622	10,3615977
DTX3	88,7952678	5,07581433	RC3H2	98,459277	7,78578872
AMFR	97,945645	7,20794418	LOC643904	97,9538488	11,0929197
SCEL	109,349895	5,82227353	TRIM40	110,418342	13,1840657
MARCH5	106,588223	7,08733362	RFPL2	92,1941224	10,5087949
LOC652591	99,5841523	5,85881929	PHF16	91,2603109	5,97318211
MAP3K1	99,238898	9,099488	TRIM22	98,5891266	11,4775011
C1orf164	99,794619	7,73915645	RNF31	95,8688174	8,809921
LOC120824	98,6532086	1,50861053	ZNRF4	86,7753371	10,7226496
LOC441061	82,2215571	5,24153935	RNF166	96,4648075	5,03359307
M96	104,109718	10,5144741	LOC649055	80,9243022	20,9924906
RNF40	90,7743429	6,67051055	RNF168	92,2836981	9,38791086
RNF20	113,169619	17,3524934	TRIM43	108,5951	7,28332318
PHF14	82,4214479	8,66341778	ZNF179	88,4198365	6,39400171
LOC729974	91,3481887	10,6330012	RAD18	81,5299014	3,16528891
RNF157	108,665492	2,05918976	STUB1	84,6782811	5,51805319
LOC51255	103,326736	12,535983	MARCH6	97,4735264	2,49207817
ZNF547	102,110849	6,24582918	BARD1	92,5340057	1,16147681
SHPRH	103,216973	1,774609	RCHY1	99,2991879	7,57182549
KIAA1542	100,554113	11,3887906	TRIP	91,0281144	6,3388424
UHRF2	90,0670738	4,85875094	LMO6	87,9280085	10,2505026

TRIM35	113,482534	6,76821441	TRIM52	103,245525	5,26436216
CNOT4	97,2727197	15,0242358	CBLC	99,6335625	8,84219753
RFPL3	107,698283	10,3383027	RNF44	84,4632088	17,2800556
CHD4	107,385126	4,84298669	MLL3	81,7259764	7,67062431
RNF34	97,1102574	11,0590436	RNF149	90,7585773	7,85076849
TRIM64	102,464285	6,39601033	TRIM37	112,475698	4,78084914
ZNF185	99,1403835	9,24943278	BIRC7	85,2596849	7,07921077
SMARCA3	101,238452	10,2974813	CBLB	95,8860924	8,11299258
DTX2	92,0251012	16,4108551	RNF6	93,8096418	15,7626588
MIB1	97,9332235	10,5836963	RNF144	93,4509269	16,1608655
TRIM56	115,733283	4,74811436	LONRF1	98,3491925	3,88845161
RNF167	98,2351976	19,8586574	TRIM55	93,763594	1,04277976
LONRF2	88,8998588	3,55561547	BRPF3	58,6323907	4,26313311
LOC642446	76,368894	10,876418	TRIM73	74,0829711	22,9399144
RFWD3	107,90436	6,42058326	IBRDC2	124,48635	15,4005856
LOC642219	115,730901	10,0020086	PPIL2	121,250224	18,2262599
RNF151	105,593177	9,09252716	ARIH2	98,6713905	5,07396966
WDR59	105,463485	5,2790463	TIF1	109,833843	11,7971799
VPS11	80,7056498	7,98389792	TRAF3	104,20885	5,07598808
ZNF278	104,08838	5,85253517	TRIM4	100,962351	18,1114674
BRAP	102,791502	8,49666718	PCGF6	104,619039	14,8155764
C13ORF7	100,08731	10,2046807	TRIM31	83,2812756	16,3950327
TRIM23	128,245879	5,30495458	LOC652433	110,680028	8,29972524
RNF10	102,087927	4,30210925	ZNF650	87,7544832	36,2018543
DPF3	95,5606203	12,4911186	ISL1	120,823082	26,1240494
RC3H1	88,7289919	20,7299719	VPS18	106,953268	10,1338782
RNF128	94,8800268	8,44684111	PDZRN4	100,015489	10,9276045
NSD1	104,985854	5,5332795	RNF215	97,437643	16,7684835
RNF41	103,2049	5,40867664	TRIM69	94,8894691	16,9569913
RNF190	96,7154707	3,88427753	AIRE	81,4848255	15,3575208
CXXC1	106,671393	15,8936013	PARK2	90,7618349	26,6534252
LOC196346	85,9096652	9,5941458	LMTK3	104,27569	17,0395134
TRIM28	112,378488	7,03152924	LOC390231	98,9930474	3,70617983
HBXAP	94,5593096	11,6770154	LOC283116	96,701754	31,6653855
TRIM6	114,466316	19,2318008	TRAF6	127,498628	26,8254358
ANAPC11	100,702826	15,4143489	RNF2	122,636504	26,7899839
MARCH9	99,5179905	15,5171928	PXMP3	121,615864	7,27924652
MKRN2	91,6357279	7,16209355	TRIM25	120,075699	10,6747783
LOC51136	97,2652328	8,19406834	BIRC2	120,099886	13,2779929
TRIM72	83,4113059	12,81491	BAZ2B	105,262192	10,5633468
TRIAD3	92,8747573	1,98663006	RNF130	122,92236	14,5642673
KIAA0644	90,6879609	12,3523568	TRAF5	92,5147972	9,58117486
LOC652859	106,057069	7,66245523	PRICKLE2	100,434924	8,64684939
ZNF294	105,455906	2,19111117	LOC653978	115,276376	17,3807861
FLJ31951	103,642187	3,90549649	TRIM71	106,684422	17,7008055
SYTL3	108,262994	16,2915472	SH3MD4	117,139778	21,5393991
JARID1D	89,0167601	7,19730824	TTC3	112,308302	14,7916996
LOC653192	102,508298	9,05456338	LONRF3	117,446636	15,33776
TRIM46	104,249688	4,14625335	DZIP3	115,635796	24,3331726
PHF3	80,9179679	0,75753802	TNFRSF25	105,58045	10,8070772
RFPL1	99,4649365	8,34911902	SPRYD5	109,988687	14,7323088
RNF183	92,4195638	10,3491175	UBR1	137,121092	17,8573912
ZSWIM2	109,223133	5,02642239	MLL4	95,3110803	19,0920326
ZNF216	122,070748	8,07339558	RNF113B	126,798411	6,861035
PHF19	100,785064	2,85733797	PHF1	94,7125167	29,6226296
PDC	99,0232743	4,47736215	SH3MD2	109,560582	24,2037286
DTX1	97,6460648	10,9451623	RING1	111,136705	16,6860917
PHF10	83,2336544	7,13923023	FSD1L	117,916596	18,8403513

TRIM45	115,373723	12,602438	RAG1	120,559531	16,4772311
TRIM32	91,8007408	11,487495	TRAF4	124,688876	12,4440583
RNF121	73,5064986	2,78065455	CGRRF1	113,792197	15,3882003
RNF26	122,086701	10,2748396	MSL2L1	102,909576	10,3324935
KRTAP5-9	96,1191381	14,6262991	RNF138	112,213073	10,9160945
RNF13	113,805338	3,10760752	LOC646754	105,239186	5,1695699
IBRDC3	103,675317	4,44800765	PCGF2	97,9202495	4,46529443
VPS8	102,006922	0,01882063	C1orf166	101,800498	8,81342545
TRIM49	107,560626	6,02875377	TRIM59	97,5266727	1,69527317
SYTL4	95,063991	4,45025933	SIAH2	107,923486	7,54436656
RNF4	96,8806555	12,1375104	RNF43	87,6660824	9,86871062
LMO7	122,703361	12,8322514	IBRDC1	83,9137073	5,68109781
CHFR	115,587711	7,11888469	RNF186	79,9675415	23,8629635
MNAT1	81,7184234	10,8368792	MARCH7	76,8758211	4,91760484
JARID1C	83,6176747	11,6382487	ZA20D1	82,0618746	20,5139832
BAZ1B	82,0781618	5,9870622	TRIM54	100,108935	4,19452733
AOF1	105,20683	17,1304187	SYVN1	89,0776265	3,410951
RNF113A	107,436553	8,46773245	RKHD3	97,4462525	5,78377248
PCGF5	100,35113	3,07342233	RNF14	91,7180533	14,0220857
MDM4	93,0449413	7,08242665	LPXN	107,644073	6,72395961
SSA1	105,208353	18,7378248	RNF11	86,8845114	7,39953004
KIAA1333	95,41186	5,30557774	RFP2	82,7420747	17,8539713
LOC399940	113,027773	13,2906564	TRIM58	80,2737702	9,47805474
TRIM50A	110,847287	3,77812672	BFAR	95,6785474	11,425635
MEFV	97,4321615	6,46971011	RNF19	84,477241	14,2335739
RBBP6	119,073704	4,70777196	BRPF1	103,58018	7,29927589
UNK	100,157466	4,02656881	RNF126	88,0491268	7,71730242
RNF111	99,9554175	5,48045834	TRIM38	106,549508	4,41275245
TRIM68	108,392898	4,86018919	RNF169	87,8495288	5,79091307
TRIM47	92,6592797	4,34091768	CBLL1	85,6495992	8,44917066
BIRC4	99,4611423	4,72141424	LNX1	94,7999687	11,9560781
RNF208	98,0861481	10,2935317	ARIH1	85,6901319	21,0435062
NHLRC1	93,9313353	10,4591176	RNF175	98,8140862	27,6952221
BIRC8	95,7906561	3,6653704	NEURL	107,674173	16,3268186
TRIM48	83,2198883	16,4091788	LNX2	73,6947665	6,98185978
MARCH1	102,543318	3,36333902	RNF17	103,313094	3,08237182
MLLT10	110,0264	9,1802855	RNF24	95,7396545	3,32201469
ZNF364	89,3064119	14,9302898	RNF146	92,7994764	3,93275234
MKRN1	107,649572	13,7497701	PHF2	95,9881447	4,6713603
FLJ14627	102,71322	17,4984857	PEX10	91,8237873	10,7975223
RNF207	90,9407263	20,1084165	MYLIP	95,7645155	9,52079966
MARCH3	95,4192174	14,2916069	ZNRF1	79,3642621	11,6637315
TRIM74	98,9105647	5,43123736	TRIM11	87,1122332	2,48470587
RNF165	78,8288876	11,7461485	TRIM5	92,3359333	10,7370892
TRIM10	132,628945	17,9183363	TRAF7	70,3502731	15,1991577
EEA1	104,037087	21,0485213	PHF8	94,3299481	10,6188362
RFP	115,636664	16,6907243	RNF170	104,649431	2,13061971
C17ORF27	112,649309	14,9762217	RNF182	101,762777	3,1002072
TRIM61	98,5672374	19,255038	UHRF1	109,75797	4,99177836
TRIM29	109,747531	18,4532456	DKFZP547C195	62,0561433	3,68147272
UBOX5	92,4382895	5,36478492	PJA2	115,215121	7,24125266
TRIM65	107,6961	3,36713765	PML	85,0967946	16,443612
DPF2	88,5134444	10,8201409			

Appendix 2: Alignment of *Chla*DBU1 gene sequence of *Chlamydia trachomatis* serovar L2 434 Bu, D UW-3 CX, A HAR-13 and *Chlamydia muridarum* Nigg. Alignment performed by SeaView 4.7.



```
                                601
Chlamydia trachomatis L2/434/Bu  GAAATAGTCA CTGGGTACTG GTTATCGTAG ATAT----CG AGTATCGATG
Chlamydia trachomatis D/UW-3/CX  GAAATAGTCA CTGGGTACTG GTTATCGTAG ATAT----CG AGCATCGATG
Chlamydia trachomatis A/HAR-13   GAAATAGTCA CTGGGTACTG GTTATCGTAG ATAT----CG AGCATCGATG
Chlamydia muridarum Nigg         GAAATAGCCA CTGGGCATTA ATCATTGTAG ATATATGCCG AA----GATG

                                651
Chlamydia trachomatis L2/434/Bu  TGTCACATTC TTCGACAGTT TGTGCGACTA TGTAGCCTCT CCACAACAAA
Chlamydia trachomatis D/UW-3/CX  TGTCACATTC TTCGACAGTT TTTACGACTA TATAGCCTCT CCACAACAAA
Chlamydia trachomatis A/HAR-13   TGTCACATTC TTCGACAGTT TTTACAACTA TATAGCCTCT CCACAACAAA
Chlamydia muridarum Nigg         TGTCACATAT TTTGATAGCC TTTACGACTT TGTATGGCAT CCTGAACAAA

                                701
Chlamydia trachomatis L2/434/Bu  TGCGGGAACA ATTAGAAGGA CTTGCCGTCT CC-CTTGGAG CTATCTATCC
Chlamydia trachomatis D/UW-3/CX  TGCGGGAACA ATTAGAAGGA CTTGCCGCCT CC-CTTGGAG CTATCTATCC
Chlamydia trachomatis A/HAR-13   TGCGGGAACA ATTAGAAGGA CTTGCCGCCT CC-CTTGGAG CTATCTATCC
Chlamydia muridarum Nigg         CCCAAGCACA ACTAAACGAG CTAGCT-TCT GCTCTCGGGA ATATCTATCC

                                751
Chlamydia trachomatis L2/434/Bu  CAAAGAAGGT GGAGCAGACT CCGATCAAGA AGAATTACTT TCTCCTTTCC
Chlamydia trachomatis D/UW-3/CX  CAAAGAAGGT GGAGCAGACT CCGATCAAGA AGAATTACTT TCTCCTTTCC
Chlamydia trachomatis A/HAR-13   CAAAGAAGGT GGAGCAGACT CCGATCAAGA AGAATTACTT TCTCCTTTCC
Chlamydia muridarum Nigg         ---AGAAGG- ---------- ---------- AGAATCTAAT ACTCCTTTCC

                                801
Chlamydia trachomatis L2/434/Bu  AGGTACGCAT CGGATCGACA GTAAAAGTCC AATCTCCTGG AGAATTCACC
Chlamydia trachomatis D/UW-3/CX  AGGTACGCAT CGGATCGACA GTAAAAGTCC AATCTCCTGG AGAATTCACC
Chlamydia trachomatis A/HAR-13   AGGTACGCAT CGGATCGACA GTAAAAGTCC AATCTCCTGG AGAATTCACC
Chlamydia muridarum Nigg         AAACCTGCAT CGGATCAACA TTCCAAGTAC AACCCCTAGG AGAATTAAGC

                                851
Chlamydia trachomatis L2/434/Bu  TGTGGAGCTT GGTGCTGTCA ATTCTTGGCG TGGTACCTAG AAAATCCTGA
Chlamydia trachomatis D/UW-3/CX  TGTGGAGCTT GGTGCTGTCA ATTCTTGGCG TGGTACCTAG AAAATCCTGA
Chlamydia trachomatis A/HAR-13   TGTGGAGCTT GGTGCTGTCA ATTCTTGGCG TGGTACCTAG AAAATCCTGA
Chlamydia muridarum Nigg         TGTGGTGTTT GGTGCTGCCA ATTCTTAGAG TGGTACCTTG AAAATCCGGA


                                901
Chlamydia trachomatis L2/434/Bu  CTTTGATCTT GAAGAGAAAG TACCTACAAA CCCATCTGAG AGAAGAGCTC
Chlamydia trachomatis D/UW-3/CX  CTTTGATCTT GAAGAGAAAG TACCTACAAA CCCATCTGAG AGAAGAGCTC
Chlamydia trachomatis A/HAR-13   CTTTGATCTT GAAGAGAAAG TACCTACAAA CCCATCTGAG AGAAGAGCTC
Chlamydia muridarum Nigg         TTTCTCTCTC GAAGATAAAG TCCCCGTAAG CGTATGCGAT AGAAAATCTA

                                951
Chlamydia trachomatis L2/434/Bu  TACTCGCT-G ATTTTATCTC TACAACGGAA CAAGCTATGT CTAGATACTC
Chlamydia trachomatis D/UW-3/CX  TACTCGCT-G ATTTTATCTC TACAACGGAA CAAGCTATGT CTAGATACTC
Chlamydia trachomatis A/HAR-13   TACTCGCT-G ATTTTATCTC TACAACGGAA CAAGCTATGT CTAGATACTC
Chlamydia muridarum Nigg         TGCTCACTAG ATTTT-GCGC TGCATCCCAA GCATCCATGT CCAAATATTC

                               1001
Chlamydia trachomatis L2/434/Bu  ATCTTTGAGC TGGCCAACTA CGGATTAG
Chlamydia trachomatis D/UW-3/CX  ATCTTTGAGC TGGCCAACTA CGGATTAG
Chlamydia trachomatis A/HAR-13   ATCTTTGAGC TGGCCAACTA CGGATTAG
Chlamydia muridarum Nigg         CGCTTTAAAC TGGCCA---- --------
```