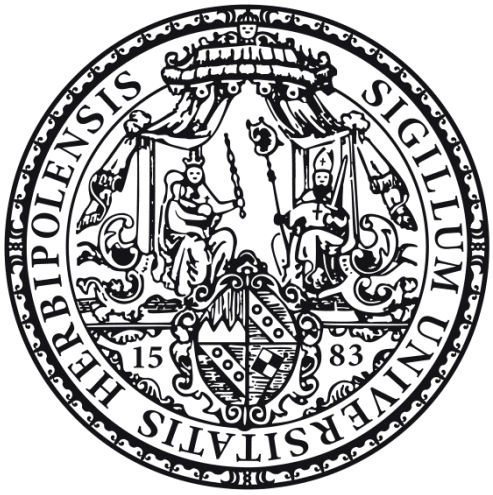
Chlamydial deubiquitinase ChlaDUB1 as regulator of host cell apoptosis and new target for anti-chlamydial therapy



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CONTENTS

SUMMARY

Chlamydia trachomatis is an obligate intracellular pathogen that replicates inside a vacuole, the so-called inclusion. During replication by a biphasic life-cycle *Chlamydia* secrete via their type 3 secretion system various effector proteins into the inclusion lumen, the inclusion membrane or the host cell cytosol to form their favored replication niche. *Chlamydia*-infected cells are highly resistant against apoptosis since the replicative form of *Chlamydia* is non-infectious and premature cell death would cause complete loss of one *Chlamydia* generation. The bacteria block apoptosis by preventing mitochondrial outer membrane permeabilization. Various proteins with anti-apoptotic function are enriched in *Chlamydia*-infected cells such as Mcl-1, cIAP2, Survivin or HIF1α. The accumulation of these proteins is a result of increased gene expression and direct protein stabilization. However, the molecular mechanisms and involved bacterial effector proteins are mostly unknown.

With this work the molecular mechanisms of Mcl-1 stabilization and the participation of chlamydial factors were investigated. Mcl-1 is a member of the Bcl-2 protein family and has an extremely short half-life causing its permanent ubiquitination and subsequent degradation by the 26S proteasome under normal homeostasis whilst Mcl-1 accumulation results in apoptosis inhibition. It was shown that during *C. trachomatis* infection Mcl-1 ubiquitination is reduced causing its stabilization albeit no cellular ubiquitin-proteasome-system components are involved in this process. However, *C. trachomatis* express the two deubiquitinases ChlaDUB1 and ChlaDUB2 which are mostly uncharacterized. With this work the expression profile, subcellular localization, substrates and function of the deubiquitinases were investigated. It was shown that ChlaDUB1 is secreted to the surface of the inclusion where it interacts with Mcl-1 which is accumulated in the proximity of this compartment. By utilization of infection experiments, heterologous expression systems and *in vitro* experiments a direct interaction of ChlaDUB1 and Mcl-1 was demonstrated. Furthermore, it was shown that Mcl-1 is deubiquitinated by ChlaDUB1 causing its stabilization. During replicative phase of infection, ChlaDUB2 seems to be accumulated in the chlamydial particles. However, ChlaDUB2 substrates could not be identified which would give an indication for the physiological role of ChlaDUB2.

Since 2011, a protocol to transform *C. trachomatis* with artificial plasmid DNA is available. As part of this work the transformation of *C. trachomatis* with plasmid DNA suitable for the permanent or inducible protein overexpression on a routinely basis was established. In addition, the first targeted homologous recombination into the chlamydial genome to replace the ChlaDUB1 gene by a modified one was performed and validated. The targeted homologous recombination was also used to create a ChlaDUB1 knock-out mutant; however deletion of ChlaDUB1 seems to be lethal for *C. trachomatis*. Due to the fact that ChlaDUB1-lacking *Chlamydia* could not be obtained an inhibitor screen was performed and identified CYN312 as a potential ChlaDUB1 inhibitor. Application of CYN312 during

infection interfered with chlamydial growth and reduced Mcl-1 quantity in infected cells. Furthermore, CYN312 treated *Ctr*-infected cells were significantly sensitized for apoptosis.

Taken together, *C. trachomatis* secretes the deubiquitinase ChlaDUB1 to the surface of the inclusion where it deubiquitinates Mcl-1 causing its accumulation in infected cells resulting in apoptosis resistance. Application of the ChlaDUB1 inhibitor CYN312 interferes with Mcl-1 stabilization sensitizing infected cells for apoptosis.

ZUSAMMENFASSUNG

Chlamydia trachomatis ist ein obligat intrazelluläres Bakterium, welches sich in einer Vakuole, der sogenannten Inclusion vermehrt. Chlamydien durchlaufen einen zweiphasigen Entwicklungszyklus während welchem sie zu bestimmten Zeitpunkten der Infektion Effektorproteine mittels ihres Typ 3 Sekretionssystems in das Inclusionslumen, die Inclusionsmembran oder das Wirtszellzytoplasma sekretieren. Durch die Aktivität der Effektorproteine schaffen die Chlamydien die für sie favorisierten Bedingungen. Zusätzlich zeigen infizierte Zellen eine hohe Resistenz gegenüber Apoptose. Ein vorzeitiger Zelltod der Wirtszelle würde zum Verlust einer vollständigen Generation an Chlamydien führen, da die replizierende Form der Chlamydien nicht infektiös ist. Chlamydien hemmen die Wirtszellapoptose indem sie die Permeabilisierung der äußeren Mitochondrienmembran verhindern. Es ist bekannt, dass mehrere anti-apoptotische Proteine wie Mcl-1, cIAP2, Survivin oder HIF1α während der Infektion mit Chlamydien zu bestimmten Zeitpunkten angereichert werden und für die Apoptoseinhibition wichtig sind. Allerdings sind die molekularen Mechanismen sowie die beteiligten bakteriellen Proteine weitestgehend unbekannt.

Mit dieser Arbeit wurden die molekularen Mechanismen der Mcl-1 Stabilisierung sowie die darin involvierten chlamydialen Proteine untersucht. Mcl-1, ein Mitglied der Bcl-2 Proteinfamilie, ist ein extrem instabiles Protein welches unter normalen Bedingungen permanent ubiquitiniert und vom 26S Proteasom abgebaut wird; eine Anreicherung von Mcl-1 hingegen führt zur Apoptoseinhibierung. In dieser Arbeit konnte gezeigt werden, dass während der Chlamydieninfektion Mcl-1 weniger ubiquitiniert wird was dessen Stabilisierung zur Folge hat. Es konnte jedoch keine Beteiligung von Komponenten des zellulären Ubiquitin-Proteasom-Systems festgestellt werden. *C. trachomatis* exprimiert zwei Deubiquitinasen welche weitestgehend uncharakterisiert sind. Ein weiteres Ziel dieser Arbeit war es das Expressionsprofil, die Lokalisierung, Substrate und die Funktion der Deubiquitinasen zu untersuchen. Es konnte gezeigt werden, dass ChlaDUB1 zur Oberfläche der Inclusion sekretiert wird und dort mit Mcl-1 interagiert, welches in diesem Kompartiment angereichert vorliegt. Unter Verwendung von Infektionsmodellen, heterologen Expressionssystemen sowie *in vitro* Experimenten konnte eine direkte Bindung beider Proteine sowie die spezifische Deubiquitinierung von Mcl-1 durch ChlaDUB1 gezeigt werden. Durch die permanente Deubiquitinierung mittels ChlaDUB1 wird Mcl-1 stabilisiert und im Bereich der Inclusionsoberfläche angereichert. Im Gegensatz zu ChlaDUB1 konnte ChlaDUB2 während der replikativen Phase der Infektion nicht im Zytoplasma sondern lediglich innerhalb der Bakterien detektiert werden. Außerdem konnten bislang keine Substrate für ChlaDUB2 identifiziert werden, welche auf die physiologische Funktion dieses Effektors schließen lassen könnten.

Seit 2011 ist ein Protokoll für die Transformation von Chlamydien mit artifizieller Plasmid-DNA verfügbar. Als Teil dieser Arbeit wurde die routinemäßige Transformation von Chlamydien mit Plasmid-DNA zur permanenten und induzierbaren Proteinüberexpression etabliert. Außerdem konnte die erste gezielte homologe Rekombination ins chlamydiale Genom durchgeführt werden. Hierbei wurde das ChlaDUB1-Gen durch eine modifizierte Form ersetzt. Die Herstellung einer ChlaDUB1-Deletionsmutante mittels homologer Rekombination war jedoch nicht erfolgreich, da ChlaDUB1 vermutlich essentiell für *C. trachomatis* ist. Da ChlaDUB1-defiziente Chlamydien nicht generiert werden konnten, wurde ein Inhibitorscreen durchgeführt und CYN312 als ChlaDUB1-Inhibitor identifiziert. Die Anwendung von CYN312 während Infektionsversuchen zeigte eine deutliche Reduktion des Chlamydienwachstums sowie eine verminderte Mcl-1 Stabilisierung. Als Folge dessen waren Chlamydien-infizierte und mit CYN312 behandelte Zellen signifikant für die Apoptoseinduktion sensibilisiert.

Mit der vorliegenden Arbeit konnte gezeigt werden, dass *C. trachomatis* die Deubiquitinase ChlaDUB1 während der Infektion an die Oberfläche der Inclusion sekretiert. Dort katalysiert ChlaDUB1 die Deubiquitinierung von Mcl-1 was dessen Anreicherung in infizierten Zellen und somit eine erhöhte Apoptoseresistenz zur Folge hat. Die Verwendung des ChlaDUB1-Inhibitors CYN312 verhindert die Mcl-1 Stabilisierung und sensibilisiert somit infizierte Zellen für Apoptose.

1 INTRODUCTION

1.1 *Chlamydia*

Chlamydia were first described by Halberstadter and von Prowazek in 1907. They found intracytoplasmic vacuoles filled with tiny particles in conjunctival epithelial cells and identified them as the causative agent of trachoma. Shortly after, the same particles were also found in cells of the uterine cervix of women and the urethral epithelium from men suffering from non-gonococcal urethritis. The newly discovered organisms were named *Chlamydozoa* (mantle viruses) and described as an intermediate form between bacteria and viruses. Just in the 1960s, *Chlamydia* were finally identified as prokaryotes since electron microscopy and molecular techniques proofed that the cell wall, ribosomes and RNA of *Chlamydia* have a gram-negative origin (Moulder, 1966).

1.1.1 Taxonomy

For a long time, the family *Chlamydiaceae* comprised just the two species *Chlamydia trachomatis* and *Chlamydia psittaci* due to their divergence in glycogen accumulation inside the inclusion (Gordon and Quan, 1965) and sulfadiazine resistance (Lin and Moulder, 1966). With the development of new DNA-based classification methods two new species were introduced: *Chlamydia pneumoniae* (Grayston et al., 1986) and *Chlamydia pecorum* (Fukushi and Hirai, 1992). The improvement of molecular diagnostic methods contributed greatly to the identification of additional chlamydial strains found in diverse tissue, host organisms and environmental niches. In the 1990s, for example, the *Chlamydia*-like bacteria *Simkania negevensis* (Kahane et al., 1995) and *Parachlamydia acanthamoebae* (Amann et al., 1997) were identified. With these new findings it was inevitable to rearrange the taxonomic classification. Based on 16S and 23S ribosomal RNA sequence analysis the former family *Chlamydiaceae* was now annotated as order *Chlamydiales* which consists of the two genera *Chlamydia* and *Chlamydophila* (Everett et al., 1999) (see Figure 1.1).

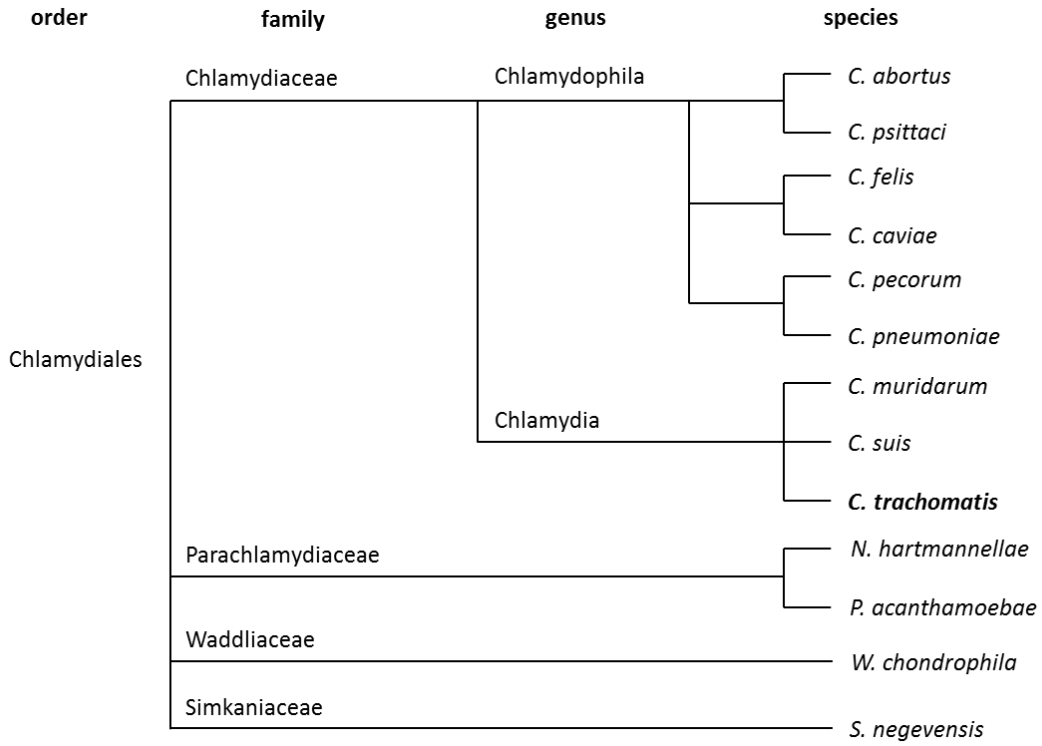

Figure 1.1: Taxonomy of the order *Chlamydiales*. According to 16S and 23S rRNA analysis, the order *Chlamydiales* comprises four families including *Chlamydiaceae*, *Parachlamydiaceae*, *Waddliaceae* and *Simkaniaceae*. The family *Chlamydiaceae* consists of the genera *Chlamydophila* and *Chlamydia*. The latter is represented by the species *C. muridarum*, *C. suis* and *C. trachomatis*. Modified after Everett at al., 1999.

1.1.2 Pathogenesis and medical relevance of *Chlamydia*

The two relevant human pathogenic *Chlamydia* species are *C. pneumoniae* and *C. trachomatis* which cause a variety of acute and chronic diseases. *C. pneumoniae* is the causative agent of community acquired pneumonia or upper respiratory tract infection (Hammerschlag, 2000) and is transmitted via aerosols. It is postulated that 90 % of *C. pneumoniae* infections are asymptomatic (Kleemola et al., 1988) and that the bacteria are able to persist up to one year in the body. This can lead to chronic diseases like asthma (Hahn et al., 1991) or lung cancer (Littman et al., 2005). Furthermore, there is increasing evidence that *C. pneumoniae* infections are associated with non-respiratory diseases like multiple sclerosis (Munger et al., 2003) and Alzheimer's disease (Balin et al., 1998).

C. trachomatis is the most frequently sexual transmitted pathogen worldwide and infects the urogenital tract and the eye. Ocular infection with *C. trachomatis* biovariant *Trachoma* serovars A to C can result in chronic conjunctivitis called trachoma which can lead to preventable blindness. The WHO estimated in 2002 that approximately 540 million people are affected by trachoma and 1.2 million people developed irreversible blindness (Resnikoff et al., 2004). The serovars D to K are mainly associated with genital infections but can also be transmitted by horizontal transfer during birth passage and cause ocular infection in new-born. In 2008, the WHO estimated about 106 million cases

of *C. trachomatis* genital tract infections worldwide which cause urethritis, prostatitis, salpingitis or pelvic inflammatory disease. Especially women with chronic *C. trachomatis* infections of the fallopian tubes suffer from increased risk of ectopic pregnancy or infertility (Bebear and de Barbeyrac, 2009). The genital tract infection with *C. trachomatis* is also assumed to be linked to cancer development (Schachter, 1999). The serovars L1, L2 and L3 belong to the *C. trachomatis* biovariant *Lymphogranuloma venereum* (LGV). These bacteria are transmitted sexually, show a tropism for lymphoid cells and have the ability to trigger systemic infections.

1.1.3 Chlamydial developmental cycle

All *Chlamydiaceae* have a unique biphasic developmental cycle. This was first discovered by Bedson and colleagues who analyzed the events during replication of psittacosis-causative agent in tissue cells (Bedson, 1932). These observations were later confirmed by electron microscopy revealing the existence of two morphologically and structural different forms of *Chlamydia* present at distinct times of the developmental cycle: elementary bodies (EBs) and reticulate bodies (RBs) (Constable, 1959; Gaylord, 1954). The developmental cycle, depicted in Figure 1.2, starts with the attachment of the EB to the surface of the host cell. The EBs are spore-like particles with a diameter of 0.3 µm and condensed chromatin (Eb et al., 1976) which led to the assumption of EBs being metabolically inactive. However, recent work performed by Omsland and colleagues showed that chlamydial EBs have metabolic and biosynthetic activity (Omsland et al., 2012). The initial attachment to the cell surface is supposed to be mediated by electrostatic interactions (Kuo et al., 1972; Kuo et al., 1973) which bring EBs and host cell receptors in close proximity. Several studies strengthen the hypothesis that the outer membrane protein OmpA on the bacterial side and heparin sulfate on the cellular side mediate this interaction (Su et al., 1996; Swanson and Kuo, 1991; Zhang and Stephens, 1992).

The most common hypothesis of EB internalization is by receptor mediated endocytosis (Hodinka et al., 1988; Wyrick et al., 1989). As host cell receptors function heparin sulfate, mannose receptor, mannose 6-phosphate receptor and the estrogen receptor (Davis et al., 2002; Kuo et al., 2002; Puolakkainen et al., 2005). Other models propose the chlamydial entry by clathrin-independent endocytosis (Jutras et al., 2003) or the involvement of lipid rafts (Stuart et al., 2003). Upon attachment of EBs to the cell the chlamydial effector protein TARP (translocated actin recruiting phosphoprotein) and other proteins are secreted into the host cell cytoplasm by a type 3 secretion system (T3SS) (Subtil et al., 2000). TARP enables actin polymerization with the involvement of small Rho GTPases (Carabeo et al., 2004; Jewett et al., 2006). In parallel, several host-tyrosine kinases such as platelet derived growth factor receptor, fibroblast growth factor receptor and ABL are activated (Birkelund et al., 1994; Elwell et al., 2008; Fawaz et al., 1997; Kim et al., 2011) and both events finally result in actin cytoskeleton rearrangements and bacterial uptake into non-phagocytic cells (Carabeo, 2011; Dautry-

Varsat et al., 2005). The internalized EBs are located in a vesicle derived from the endolysosomal pathway, the so-called inclusion. After endocytosis, the fusion with late endosomes and lysosomes is blocked and thereby enable bacterial replication inside the inclusion (Al-Younes et al., 1999; Hackstadt, 2000). The EBs differentiate within the first eight hours post infection (hpi) into RBs which represent the replicative form of *Chlamydia*. RBs are metabolic active, non-infectious and bigger in size (1 µm). After multiple replication events most of the RBs re-differentiate into EBs which are released by host cell lysis or a mechanism called extrusion and start a new infection cycle. The signals triggering the re-differentiation from RB to EB are not completely understood so far but it is supposed that physical parameters like space limitation play a role (Bavoil et al., 2000; Hackstadt et al., 1997). The life-cycle of *C. trachomatis* is completed after 48 hpi when grown in cell culture whereas *C. pneumoniae* need 72 h for completion.

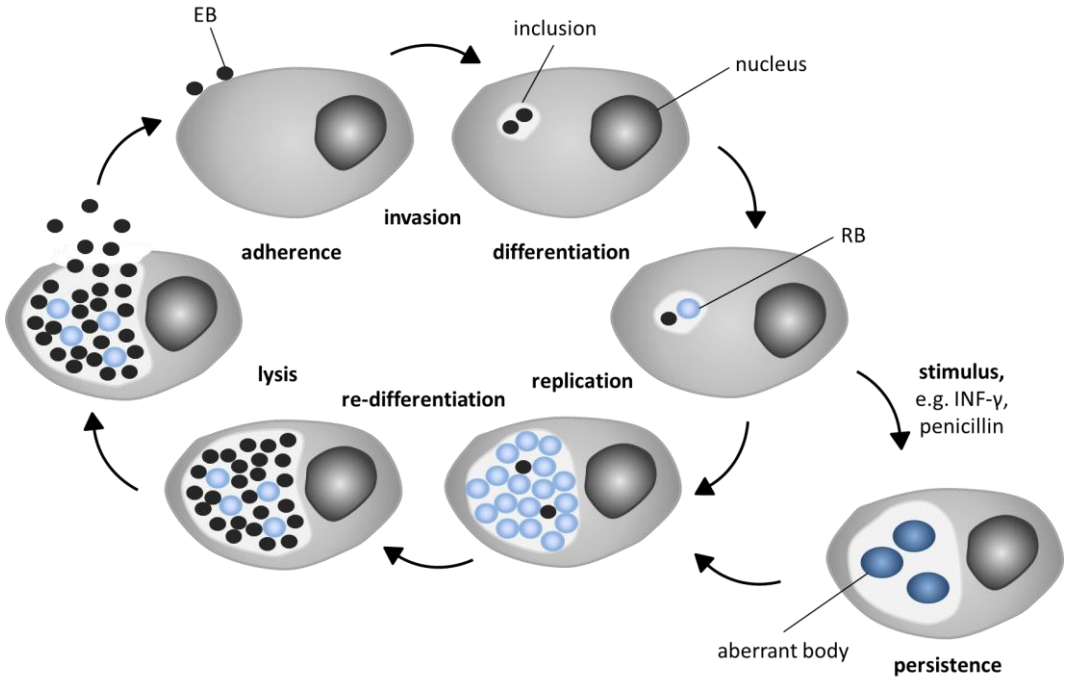

Figure 1.2: Chlamydial developmental cycle. Infectious elementary bodies (EBs) enter the host cell by endocytosis. The bacteria stay inside a vacuole, the inclusion, and prevent fusion with the endolysosomal system. The EBs differentiate into the replicative form called reticulate body (RB) and multiply by binary fission. After re-differentiation from RB to EB the host cell is lysed and new infectious EBs are released into the extracellular space. The differentiation into a persistent form of the bacteria called aberrant body (AB) can be induced by penicillin, interferon γ or nutrition starvation. Re-differentiation from AB to RB takes place if persistence inducer is removed.

In cell culture experiments several forms of treatment can lead to an incomplete, persistent infection of *Chlamydia*. After exposure to penicillin (Skilton et al., 2009), interferon gamma (IFN-γ) (Shemer and Sarov, 1985), viral co-infection (Deka et al., 2006) or tryptophan starvation (Beatty et al., 1994) the RBs differentiate into aberrant bodies (Abs) (Figure 1.2) which distinguish themselves by their enlarged size and multiple copies of their genome. The ABs are like RBs non-infectious but can be

transmitted to new cells during host cell division (Moulder et al., 1980). After the persistence inducing condition is removed, ABs can re-differentiate into RBs and complete the life cycle. So far, it is not clear if the clinically relevant persistent infection can be linked to the persistent infection occurring in cell culture characterized by the presence of ABs.

1.1.4 Type 3 secreted effector proteins

Gram-negative bacteria are able to inject effector proteins into the cell by a needle-like apparatus called type 3 secretion system (T3SS) or the injectosome (Salmond and Reeves, 1993). *Chlamydia* as intracellular gram-negative bacteria use their T3SS to secrete several proteins into the inclusion lumen, the inclusion membrane or the host cell cytoplasm (Mueller et al., 2014). Several attempts have been made to identify the secreted proteins of *Chlamydia.* In secretion assays based on the recognition of T3S signals of chlamydial proteins in *Shigella flexneri* (Subtil et al., 2005) or *Yersinia enterocolitica* (da Cunha et al., 2014a) potentially secreted proteins have been identified. Computer-aided prediction of secreted proteins complemented the secretion assay based approach (Arnold et al., 2009; Dehoux et al., 2011).

One example for secreted proteins are Inc proteins which are located in the inclusion membrane and fulfill multiple functions during chlamydial infection. Inc proteins promote interaction with host-cell proteins belonging to the Rab GTPase family. Rab GTPases are important for membrane trafficking, fusion and organelle identity (Brumell and Scidmore, 2007; Rzomp et al., 2006). The *C. trachomatis* Inc protein CT229 specifically interacts with Rab-4 (Rzomp et al., 2006) whereas the *C. pneumoniae* protein Cpn0585 selectively recruits Rab1, 10 and 11 to the inclusion (Cortes et al., 2007). Besides recruitment of Rab GTPases, Incs can bind host cell SNAREs like Syntaxin 6 and GS15 as Golgi network associated SNAREs or Vamp3, 7 and 8 belonging to the endocytic SNARE protein family (Delevoye et al., 2008; Kabeiseman et al., 2013; Moore et al., 2011). Furthermore, the Inc proteins IncA, CT813 and CT223 contain themselves SNARE-like domains mediating specific vesicle membrane fusion (Derre et al., 2011; Paumet et al., 2009). Further studies report recruitment of Src kinases by Inc proteins. Src tyrosin kinases are known to be involved in sphingomyelin acquisition and organization of the microtubule network (Mital et al., 2010).

Additional secreted effectors involved in actin cytoskeletal reorganization are TARP (Carabeo et al., 2004; Jewett et al., 2006), CT166 as a glycosyl transferase (Thalmann et al., 2010) and CT694 which interacts with AHNAK (Hower et al., 2009). The chlamydial protein NUE (CT737) was shown to be a histon methyltransferase which is secreted into the host cell cytoplasm and subsequently transported into the nucleus (Pennini et al., 2010). Secreted effector proteins with deubiquitinating enzymatic activity have been identified, too. ChlaDUB1 and ChlaDUB2 are deubiquitinases (DUBs) of *C. trachomatis* expressed during mid and late phase of infection (Le Negrate et al., 2008; Misaghi et al.,

2006) and ChlaOTU is a DUB of *C. caviae* (Furtado et al., 2013) secreted into the host cell cytosol during infection. Within the last years many secreted effector proteins have been identified but for many proteins their physiological role during chlamydial infection is still unclear and has to be determined.

1.1.5 Chlamydial genetics

Chlamydia trachomatis has a dramatically reduced genome of approximately 1 Mb encoding for about 1000 genes which is complemented with a 7.5 kb cryptic plasmid. Due to the small number of genes and the fact that many metabolic pathways are incomplete, most of the open reading frames (ORFs) are supposed to be essential. The plasmid encodes eight ORFs named *pgp1* to *pgp8*. These genes are important for plasmid maintenance or gene expression. For instance, Pgp4 was identified to be a positive regulator for gene expression of plasmid encoded and genome encoded genes (Gong et al., 2013; Song et al., 2013a). For a long time *Chlamydia* were assumed to be incapable to genetically manipulate. *Chlamydia* mutant libraries were created by EMS (ethyl methyl sulfonate) treatment with subsequent plaque assay and whole genome sequencing to get an overview of essential genes and their function (Nguyen and Valdivia, 2012). However, in 2011 Wang and colleagues established a transformation system for DNA delivery into *Chlamydia*. A plasmid-free *C. trachomatis* strain lacking the cryptic plasmid was transformed with the cryptic 7.5 kb plasmid as well as with a modified form encoding for a GFP fusion protein and a *β-lactamase* gene for selection of the transformed bacteria. By $CaCl_2$-mediated transformation and selection with penicillin the glycogen metabolism was restored and GFP-positive *Chlamydia* were obtained (Wang et al., 2011) proving the successful delivery and maintenance of artificial DNA. In addition, *C. muridarum* was also successfully transformed with a modified plasmid containing the corresponding backbone (Song et al., 2013b). Besides the bacterial transformation via $CaCl_2$ the efficient transformation of *Chlamydia* during infection by dendrimer-complexed DNA was performed (Kannan et al., 2013). This method was also shown to be suitable for *C. pneumoniae* transformation with plasmid DNA (Gerard et al., 2013) and for the delivery of oligonucleotides encoding for antisense sequences for targeted gene knock-down in *C. trachomatis* (Mishra et al., 2012). Furthermore, the original pGFP::SW2 plasmid was modified to obtain additional fluorescence markers such as mCherry or CFP, each under control of the chlamydial IncD promoter (Agaisse and Derre, 2013). The selection of transformed *Chlamydia* was initially tested with penicillin, but modifications of the plasmid-encoded resistance markers allow also the selection with chloramphenicol (Xu et al., 2013) or blasticidin (Ding et al., 2013). The ability to use multiple selection markers opens up further possibilities for genetic manipulation of *Chlamydia* such as transposon mutagenesis. In addition, the use of inducible protein expression of a plasmid-encoded gene by tetracyclin-regulated promoters facilitates regulation of gene expression with respect to time and amount (Wickstrum et al., 2013).

Chlamydia were known to possess the ability for homologues recombination of DNA into their genome. This was shown by genome sequencing approaches of different chlamydial strains and lineages from patients isolates (Joseph and Read, 2012; Putman et al., 2013) as well as *in vitro* experiments (Jeffrey et al., 2013; Suchland et al., 2009). These findings arise the potential of targeted insertion of delivered artificial DNA into the chlamydial genome by homologues recombination depending on naturally inherent mechanisms. On the contrary, Johnson et al. used the TargeTron™ technology for site-specific insertion of a group II-intron into the *IncA* gene for targeted gene inactivation (Johnson and Fisher, 2013). Since the basis for genetic manipulation of *Chlamydia* was set, this field is dramatically developing and more and more techniques are available for targeted genetic manipulation of *Chlamydia* helping to understand the pathogenicity of this bacterium.

1.2 Programmed cell death

Programmed cell death, especially apoptosis is an important process in multicellular organisms for tissue homeostasis, during embryogenesis and for removal of dangerous cells like cancer or infected cells (Kerr et al., 1972; Reed, 2002). Furthermore, a misregulation of apoptotic processes often result in severe diseases including cancer, Huntington's disease, Alzheimer's disease, persistent infection or autoimmune disorders (Reed, 2002).

1.2.1 Types of programmed cell death

At least four different types of programmed cell death (PCD) can be distinguished by specific morphological and biochemical features (for overview see Figure 1.3). The best characterized type of PCD is apoptosis. The term apoptosis (greek: apo - off, ptosis – falling) was introduced by Kerr, Wyllie and Currie who first described the processes taking place during apoptosis in detail (Kerr et al., 1972). During apoptosis, an inherent program controls the stepwise destruction of the cell catalyzed by the activity of specific proteases, the caspases. Characteristic morphological changes accompanied with apoptosis are cell shrinkage and rounding, chromatin condensation (pyknosis), DNA-fragmentation (karyorrhexis), cell membrane blebbing and finally the collapse of the cell into multiple vesicles called apoptotic bodies. These apoptotic bodies contain cytosol, organelles and chromatin and are removed via phagocytosis by macrophages. During the whole process no intracellular material is released into the extracellular space, thereby subsequent inflammation of the surrounding tissue is prevented (Kerr et al., 1972; Wyllie et al., 1980). In contrast to apoptosis, necrosis represents an unregulated form of cell death characterized by swelling of the cytoplasm and organelles (oncosis) and finally the rupture of the plasma membrane. With the loss of plasma membrane integrity intracellular material is released into the extracellular space causing inflammation (Kerr, 1971). Autophagy, also called type II cell death,

was first described by de Duve (de Duve, 1983) and is an important tool to recycle damaged organelles or cytoplasmic material in the cell (Clarke, 1990; Deter et al., 1967). That is why autophagy is also named “self-digestion”. During autophagy cellular material is encapsulated into double-membrane vesicles, the autophagosomes, which later fuse with lysosomes for their removal (Klionsky and Emr, 2000). The autophagic cell death is characterized by the massive occurrence of autophagic vacuoles and the lack of chromatin condensation and can thereby be distinguished from apoptotic cell death (Tsujimoto and Shimizu, 2005). A special form of autophagy named xenophagy is related to innate immune response against intracellular pathogens and MHC-mediated antigen presentation (Gomes and Dikic, 2014). The fourth type of PCD is associated with microbial infection and is termed pyroptosis. During pyroptosis DNA-fragmentation, cell swelling and finally cell lysis occur causing inflammation (Cookson and Brennan, 2001). The Nomenclature Committee on Cell Death has recently given a recommendation for a death-related terminology since a variety of designations for the different types of PCD exist (Galluzzi et al., 2012; Kroemer et al., 2009).

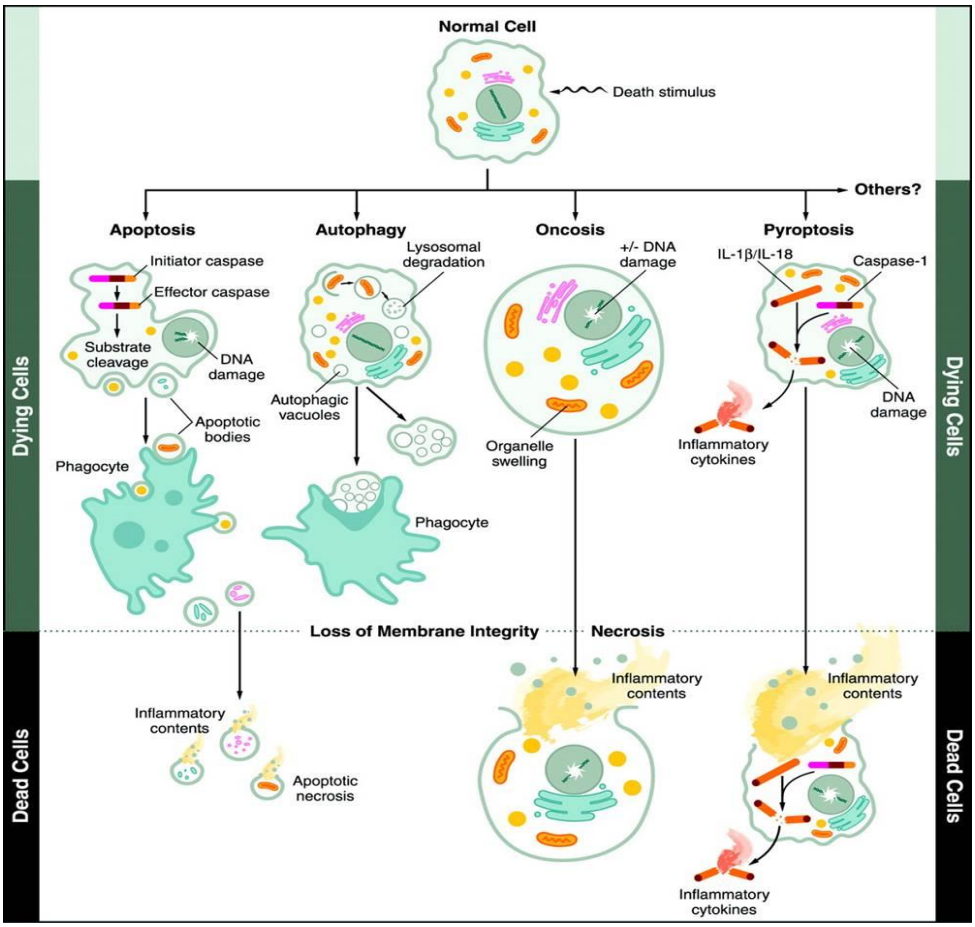

Figure 1.3: Types of programmed cell death. Depicted are the molecular pathways and morphological characteristics of the four major types of programmed cell death. Apoptosis is characterized by DNA damage, cell shrinkage and finally the breakdown of the cell into apoptotic bodies. During autophagy components of the cytosol and organelles are encapsulated into autophagosomes which are removed by the lysosomal system. Necrosis is an uncontrolled process accompanied with cell and organelle swelling leading to cell membrane rupture and inflammation. Pyroptosis is associated to infection and is characterized by DNA damage, cell swelling and the final rupture of the cell to cause inflammation (Fink and Cookson, 2005).

1.2.2 Molecular mechanisms and regulation of apoptosis

Apoptosis is a strictly regulated process which can be induced by external or several intracellular stress signals. The molecular mechanisms of apoptosis rely on the activity of evolutionary conserved cysteine-dependent aspartate specific proteases named caspases. The family of caspases and their central role in programmed cell death was first identified in *Caenorhabditis elegans ced* mutants (Ellis and Horvitz, 1986). All 14 members of the human caspase family share similarities in sequence and structure and exist under pro-survival conditions as zymogens. The zymogens can be cleaved at their pro-domain by auto-processing or by cleavage via other caspases (Boatright and Salvesen, 2003). The caspases can be grouped by their physiological role into apoptosis-related caspases (caspase-2, -3, -6, -7, -8, -9 and -10) and caspase-1 related family members (caspase-1, -4, -5, -13 and -14). The caspase-1 family members are for instance important for cytokine processing during inflammation (Creagh et al., 2003). The apoptosis-related caspases are further differentiated into initiator caspases (caspase-2, -8, -9 and -10) and effector caspases (caspase-3, -6 and -7). The initiator caspases are activated by extrinsic or intrinsic stimuli and subsequently activate the downstream acting effector caspases by cleaving their pro-domain (Boatright and Salvesen, 2003). The cleavage of target proteins by the effector caspases leads to the characteristic morphological features of apoptotic cells. Lamin cleavage causes nuclear shrinking and fragmentation (Rao et al., 1996), gelsolin and fodrin destruction provokes cell shrinkage (Kothakota et al., 1997) and apoptotic body formation is mediated by PAK2 activation (Rudel and Bokoch, 1997). The DNA fragmentation in apoptotic cells can be attributed to the activation of CAD (caspase activated DNase) by caspase-3 (Enari et al., 1998; Sakahira et al., 1998).

The activation of the caspase-cascade represents an irreversible event, therefore its activation has to be strictly controlled. The main regulators of caspases are the cellular inhibitor of apoptosis proteins (cIAPs): XIAP, cIAP-1, cIAP-2 and Survivin (Deveraux and Reed, 1999). The first cIAP was identified as the human homolog of the baculovirus IAP which shows anti-apoptotic features. The presence of a zinc binding domain, the BIR-domain (baculovirus IAP repeat), is a common characteristic of all members of the cIAP family (Crook et al., 1993). XIAP and Survivin are able to block caspase-3 and caspase-7 activation by direct binding to prevent apoptosis (Chai et al., 2001; Deveraux et al., 1997; Riedl et al., 2001; Tamm et al., 1998). A similar anti-apoptotic working mechanism was also reported for cIAP-1 and cIAP-2 (Roy et al., 1997). Especially cIAP-1 was shown to be crucial for innate immune response towards *C. pneumoniae* infections (Prakash et al., 2009).

1.2.2.1 Intrinsic and extrinsic pathway

Apoptosis induction can be triggered by several external or intracellular stress stimuli like growth factor depletion, starvation, oxidative stress, infection or DNA-damage. Figure 1.4 gives an overview of apoptosis induction in mammalian cells. In most of the cell types the apoptosis-inducing stimuli cause mitochondrial outer membrane permeabilization (MOMP) catalyzed by active Bax and Bak resulting in the release of several death-inducing factors into the cytoplasm (Bernardi et al., 1999). The death-inducing factor cytochrome c forms together with Apaf-1 and caspase-9 a protein complex called apoptosome which initiates the caspase-cascade by activation of the effector caspases (Li et al., 1997). Besides cytochrome c other factors like Smac (Verhagen et al., 2000), apoptosis inducing factor (AIF) (Susin et al., 1999) or endonuclease G (EndoG) (Li et al., 2001) gain access to the cytoplasm after MOMP. Smac interferes with cIAP-mediated inhibition of caspase activation whereas AIF and EndoG cause DNA fragmentation to trigger apoptosis progress. The Bcl-2 protein family regulates apoptosis induction at the level of MOMP and consists of either pro- or anti-apoptotic proteins. Under survival conditions Bax and Bak are kept inactive by the anti-apoptotic Bcl-2 proteins. Intracellular stress stimuli are sensed by the pro-apoptotic BH3-only subgroup of the Bcl-2 like protein family which then induce Bax and Bak activation resulting in MOMP (Narita et al., 1998; Vaux et al., 1988).

Cell death induced by extracellular stimuli is dependent on the activation of death receptors located on the cell surface. The death receptors belong to the tumor necrosis factor receptor (TNFR) superfamily and are TNFR-1, Fas/CD95 or TRAIL (Brockhaus et al., 1990; Itoh et al., 1991; Loetscher et al., 1990; Pitti et al., 1996). The death receptors possess an extracellular cysteine-rich subdomain for specific binding to their ligands which trigger trimerization and activation of the receptors. Following activation, a conserved domain called death domain (DD) located in the cytoplasmic part of the receptor interacts with adaptor molecules also containing a DD (Tartaglia et al., 1993). The receptor and the bound adaptor molecules FADD (Fas associated protein with a death domain) or TRADD (Tumor necrosis factor receptor type 1-associated death domain protein) form the death induced signaling complex (DISC) (Chinnaiyan et al., 1995; Kischkel et al., 1995). Complexed FADD recruits by its death effector domain (DED) inactive procaspase-8 which is activated at the DISC by autocatalytic cleavage (Medema et al., 1997; Muzio et al., 1998). Depending on the cell type the active caspase-8 is able to directly activate the caspase-cascade in an adequate way by caspase-3 cleavage (type I cells) or the apoptotic signal has to be amplified via the mitochondrial (intrinsic) pathway (type II cells) (Scaffidi et al., 1998). In type II cells active caspase-8 cleaves the Bcl-2 family protein Bid into the truncated form tBid. Truncated Bid translocates to the mitochondria where it induces together with Bax and Bak MOMP and cytochrome c release (Bernardi et al., 1999; Luo et al., 1998; Narita et al., 1998). Cytochrome c release further enables formation of the apoptosome and activation of the caspase-cascade as described before.

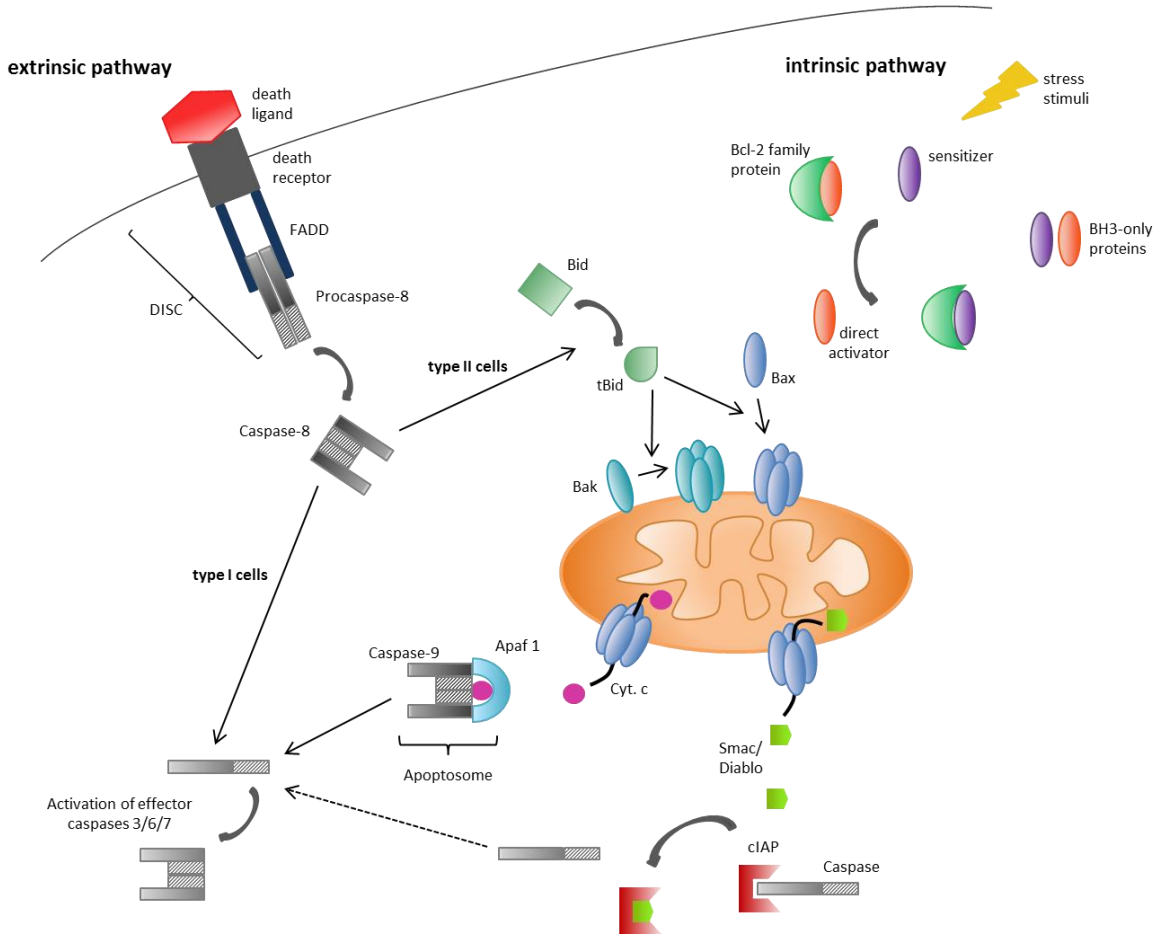

Figure 1.4: Intrinsic and extrinsic pathway of apoptosis. Extracellular death inducing signals are transmitted into the cell by binding of the death ligand to its receptor mediating the formation of the death inducing signaling complex (DISC) which triggers the activation of procaspase-8 by autocatalytic cleavage. Active caspase-8 either directly activates the effector caspases (type I cells) or the signal is amplified via the mitochondrial pathway by caspase 8-catalyzed cleavage of Bid into truncated Bid (tBid) as it is the case in type II cells. TBid or intracellular stress signals can trigger the intrinsic or mitochondrial apoptosis pathway. BH3-only proteins function as stress sensors and are able to activate Bax and Bak. Under survival conditions the pro-apoptotic BH3-only proteins as well as Bax and Bak are kept inactive by anti-apoptotic Bcl-2 proteins. Active Bax and Bak translocate and integrate into the mitochondrial outer membrane forming pores by which pro-apoptotic proteins like cytochrome c and Smac/Diablo are released into the cytoplasm. Cytochrome c, Apaf 1 and caspase 9 form together the apoptosome responsible for the activation of effector caspases. In parallel, released Smac replaces caspases complexed with cIAPs by which the caspases get receptive for activation.

1.2.3 Bcl-2 family

The Bcl-2 like protein family members contain one or more Bcl-2 homology (BH) domains and can be subdivided into three groups (Cory and Adams, 2002). The pro-survival Bcl-2 like proteins, for instance Bcl-2, Bcl-X_L, Bfl-1/A1, Bcl-w or Mcl-1 (myeloid cell leukemia 1), contain four BH domains. On the other hand, the pro-apoptotic proteins of this family possess less BH domains and are subdivided into the BH3-only proteins (Bid, Bad, Bim, Bmf, Bik, Noxa and Puma) and the Bax-group. The Bax-group is represented by Bax, Bak and Bok which have the first three BH domains (Cory and Adams, 2002). The BH3-only proteins act as stress sensors whereas the anti-apoptotic Bcl-2 proteins control the activity of the effector proteins Bax and Bak. All Bcl-2 like proteins are able to interact with each other

by their BH domain (Boyd et al., 1995; Chittenden et al., 1995). Following intracellular stress stimuli the BH3-only proteins get activated and translocate to the mitochondrial outer membrane. There they either activate Bax and Bak or inhibit the anti-apoptotic Bcl-2 family members. Active Bax and Bak then integrate via their tail-anchor into the mitochondrial outer membrane and oligomerize. Bax and Bak are supposed to form pores in the mitochondrial outer membrane by which cytochrome c can get access to the cytoplasm (Antonsson et al., 2000). Another model hypothesizes that Bax and Bak make use of already existing channels like VDAC to create larger channels sufficient for cytochrome c release out of the mitochondria (Tsujimoto and Shimizu, 2000).

The activation and oligomerization of either Bax or Bak is the crucial step in mitochondria-mediated apoptosis induction due to the fact that cells lacking Bax and Bak do not show MOMP after apoptotic stimuli (Wei et al., 2001). To answer the question how the anti-apoptotic Bcl-2 proteins regulate the effector protein activation several models have been proposed (for overview see Figure 1.5). The “derepression” or “displacement” model implies the presence of constitutively active Bax and Bak. The effectors are controlled and kept inactive by the binding of anti-apoptotic Bcl-2 family proteins. Apoptosis stimuli activate BH3-only proteins which then replace Bax and Bak in the complex with Bcl-2 proteins whereby the effectors are set free and are able to translocate to the mitochondria (Chen et al., 2005; Willis et al., 2005). The “direct activator” model classified the BH3-only proteins as direct activators (tBid, Bim and Puma) or sensitizers (Bad, Noxa, Bik and Bmf). Under survival promoting conditions the direct activators are bound by anti-apoptotic Bcl-2 family proteins. Upon stress stimuli the sensitizers are activated and replace the direct activators in the complex with Bcl-2 family proteins. The unchained direct activator proteins subsequently activate Bax and Bak to cause MOMP (Kim et al., 2006; Letai et al., 2002). In the last years both models were adapted and two additinal models were introduced: the “embedded together” and the “unified” model. These models combine principles of the displacement and the direct activator model (Llambi et al., 2011; Shamas-Din et al., 2013). The embedded together model includes the concentration and affinity towards other Bcl-2 family members as well as the integration into the mitochondrial outer membrane as major deciding reasons for MOMP. The unified model is based on the embedded together model and describes the mode of action of anti-apoptotic proteins by sequestration of activator BH3-only proteins (direct activator model) as well as sequestration of Bax and Bak (displacement model) (Shamas-Din et al., 2013).

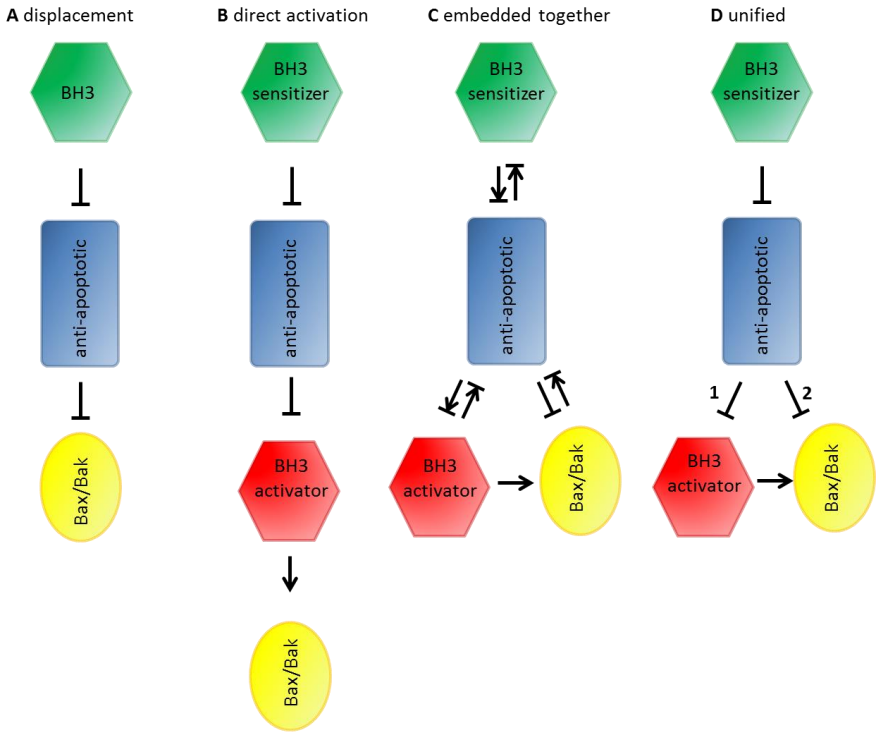

Figure 1.5: Models for the regulation of MOMP by Bcl-2 family proteins. (↑) Activation; (⊥) inhibition; (⊥↑) mutual recruitment/sequestration with explicit stress to equilibria. (A) The displacement model describes Bax and Bak as constitutively active, but held in check by anti-apoptotic members of the Bcl-2 protein family. Apoptosis inducing signals are sensed by the pro-apoptotic BH3-only proteins which than interact specifically with anti-apoptotic Bcl-2 proteins to liberate Bax and Bak. Free Bax and Bak oligomerize and cause MOMP. (B) In the direct activation model the BH3-only proteins are subdivided into "sensitizers" and "activators". The BH3 activators are sequestered by anti-apoptotic Bcl-2 proteins. Apoptotic stress signals are sensed by the BH3 sensitizers which than displace the BH3 activators in the complex with Bcl-2 proteins and free BH3 activators mediate Bax/Bak oligomerization causing MOMP. (C) The embedded together model highlights the role of protein concentration of pro- and anti-apoptotic proteins in the cell as well as their modification and localization. The pro- and anti-apoptotic proteins bind to each other with varying affinity dependent on protein modification and localization. Bax and Bak are directly activated at the mitochondrial membrane by BH3 activators and cause MOMP. This activation can be prevented by anti-apoptotic Bcl-2 proteins which sequester either BH3 proteins (activators as well as sensitizers) or Bax/Bak from binding to each other or membrane association. (D) The unified model builds on the embedded together model, but describes two modes of action. Anti-apoptotic Bcl-2 proteins sequester the BH3 activators (mode 1) and sequester Bax/Bak (mode 2). The apoptosis block by mode 1 is weaker than the one of mode 2, but both have to be overcome by BH3 sensitizers. Additionally, the unified model emphasizes the role of Bcl-2 proteins in regulation of MOMP. Modified after Shamas-Din et al., 2013.

1.2.3.1 Mcl-1

Mcl-1 is an anti-apoptotic member of the Bcl-2 protein family and was originally identified by Kozopas et al. in 1993 in differentiating myeloid cells (Kozopas et al., 1993). There exist three isoforms generated by alternative splicing. The full length protein of Mcl-1 consists of 350 amino acids and contains three BH domains and a C-terminal transmembrane domain (Kozopas et al., 1993). The N-terminal part of Mcl-1 is much longer than in other Bcl-2 family proteins and contains several PEST-domains responsible for the extreme short half-life of Mcl-1 compared to other Bcl-2 family proteins, see Figure 1.6 (Adams and Cooper, 2007; Craig, 2002; Nijhawan et al., 2003). By alternative splicing a second isoform called Mcl-1_S is expressed which lacks the BH1 and BH2 domain as well as the transmembrane domain. The structure of Mcl-1_S resembles certain BH3-only proteins and Mcl-1_S in

deed shows pro-apoptotic features (Bae et al., 2000). The third isoform Mcl-1_{ES} lacks a part of the first exon but contains all three BH domains and has pro-apoptotic characteristics, too (Kim et al., 2009).

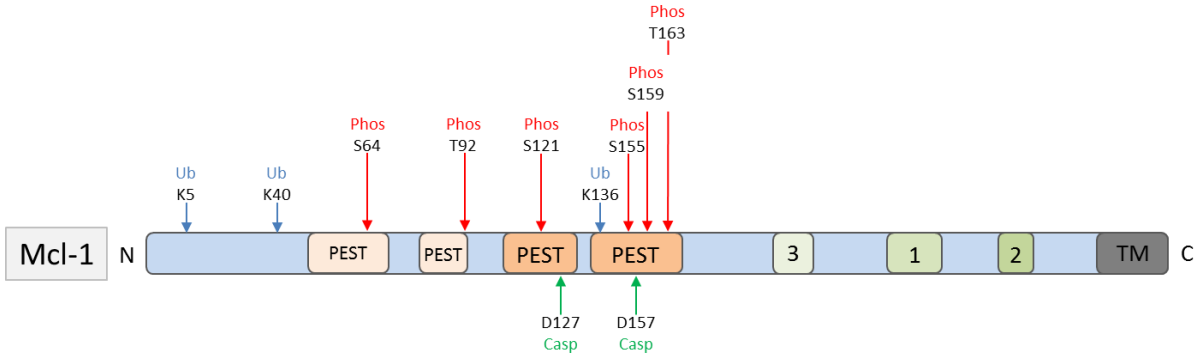

Figure 1.6: Domains and modification sites of Mcl-1. Full length Mcl-1 consists of 350 amino acids and contains three BH-domains located at the C-terminal part of the protein. The N-terminal part of Mcl-1 contains four PEST-domains responsible for the short half-life of Mcl-1. Several amino acid residues in the PEST domains can be phosphorylated altering the half-life or affinity towards interaction partners of Mcl-1. Three ubiquitination sites of Mcl-1 have been identified located in the N-terminal part of Mcl-1. Moreover, Mcl-1 can be cleaved by active caspases upon apoptosis induction at two distinct aspartate residues located in the PEST domains. Modified after Thomas et al., 2010.

Mcl-1 can be found in the cytoplasm or associated with several intracellular membranes like the mitochondrial outer membrane, the nuclear envelope or the endoplasmic reticulum (Leuenroth et al., 2000; Yang et al., 1995). Transcription of Mcl-1 is controlled by several signaling pathways (Akgul et al., 2000) but the main regulation of Mcl-1 protein level and activity is carried out by posttranslational modifications. Mcl-1 contains multiple phosphorylation sites in the PEST-domains located at the N-terminus (Figure 1.6). Phosphorylation of Mcl-1 can either alter protein stability by augmented ubiquitination and degradation or impair the anti-apoptotic function of Mcl-1 by reduced ability to bind BH3-only proteins (Thomas et al., 2010). Mcl-1 was shown to be important for development and maintenance of lymphocytes (Opferman et al., 2003), the hematopoietic system (Opferman et al., 2005), neural development (Arbour et al., 2008) as well as in regulation of cell cycle progression (Jamil et al., 2005).

The central role of Mcl-1 in apoptosis regulation is underlined by the multiple numbers of cancer cell types exhibiting increased amounts of Mcl-1. High quantity of Mcl-1 in these cells correlates with resistance towards conventional cancer therapies (Beroukhim et al., 2010; Glaser et al., 2012) whereas Mcl-1 targeting chemotherapeutics show success in sensitizing these cells for apoptotic stimuli (Akgul, 2009). Its anti-apoptotic function performs Mcl-1 by complex formation with Bax and Bak at the mitochondrial outer membrane to prevent MOMP or by binding of BH3-only proteins to prevent Bax/Bak activation (Willis et al., 2005) (see section 1.2.3). The anti-apoptotic Bcl-2 family proteins exhibit different affinities for the BH3-only proteins. For instance, Mcl-1 is known to bind Noxa, Bim and Puma with high affinity (Chen et al., 2005). After Mcl-1 is sequestered from its pro-

apoptotic binding partner upon apoptosis induction it is subsequently ubiquitinated and degraded by the proteasome. Known E3-Ubiquitin ligases specific for Mcl-1 are MULE and the SCF complexes β-TrCP and Fbw7 (Ding et al., 2007; Inuzuka et al., 2011; Zhong et al., 2005). On the contrary Mcl-1 can be rescued form proteasomal degradation by the deubiquitinating enzyme USP9X (Schwickart et al., 2010). In TRAIL-mediated apoptosis induction via caspase-8 and caspase-3 Mcl-1 is cleaved by caspase-3 at aspartic acid residue 127 and subsequently degraded resulting in a feed forward loop accelerating the apoptosis process (Snowden et al., 2003).

1.2.4 *Chlamydia trachomatis* and host cell apoptosis

Pathogenic bacteria have to prevent recognition by the innate immune system to replicate inside the host. Extracellular replicating bacteria like *Shigella, Yersinia* or *Salmonella* frequently cause cell death in macrophages or T-cells to prohibit inflammation (Hersh et al., 1999; Hilbi et al., 1997). *Chlamydia* were shown to induce TNFα-secretion in infected macrophages by which cell death in neighboring T-cells is induced (Jendro et al., 2004). Moreover, the induction of cell death by the secretion of toxins or bacterial effectors can help the bacteria to invade into deeper tissue as it was shown for *Staphylococci, Listeria, Helicobacter* or *Neisseria* (Galmiche et al., 2000; Guzman et al., 1996; Muller et al., 2000; Weinrauch and Zychlinsky, 1999).

On the contrary, *Chlamydia* or *Rickettsia* as obligate intracellular pathogens rely on the host cell for their growth and replication (Clifton et al., 1998; Fan et al., 1998; Rajalingam et al., 2001). *Chlamydia* evade recognition by the immune system by down-regulation of major histocompatibility complex (MHC) class I and II gene expression in the host cell (Zhong et al., 1999; Zhong et al., 2000). Premature host cell death occurring during early time points of *Chlamydia* infection cause a complete loss of infectious progeny since the replicating form of *Chlamydia* is non-infectious. Initial experiments showed that *Chlamydia* block apoptosis at the level of Bak and Bax activation (Fischer et al., 2004a; Xiao et al., 2004; Zhong et al., 2006) thereby preventing MOMP and cytochrome c release. By blocking MOMP but also by further mechanisms directly targeting cIAP family proteins caspase 9 and caspase 3 stay inactive (Fan et al., 1998). *Chlamydia* are solely able to block apoptosis via the mitochondrial pathway since *Chlamydia*-infected type I cells are accessible for apoptosis induction (Fischer et al., 2004a).

C. trachomatis established several mechanisms to efficiently block host cell death ranging from the activation of survival signaling pathways, the depletion of death inducing receptors from the cell surface to the alteration of protein levels of pro- and anti-apoptotic proteins (Paland et al., 2008; Rajalingam et al., 2008; Rajalingam et al., 2006). Upon *C. trachomatis* infection the PI3K/AKT and MEK/ERK survival signaling pathways are activated (Rajalingam et al., 2008; Su et al., 2004). The MEK/ERK pathway regulates nutrient acquisition and expression of pro-survival proteins whereas the

PI3K/AKT pathway promotes cell survival by blocking pro-apoptotic processes (Hess et al., 2001; Rajalingam et al., 2008; Xia et al., 2003). Furthermore, during a short period of *C. trachomatis* infection the hypoxia-induced factor 1 α (HIF1α) is massively stabilized albeit the mRNA level is not altered. The stabilization of this transcription factor between 12 to 20 hpi is dependent on MEK/ERK signaling and leads to the upregulation of Mcl-1 gene expression (Sharma et al., 2011). Additionally, PI3K signaling triggers the phosphorylation of the BH3-only protein BAD which than is sequestered to the chlamydial inclusion by interaction with 14-3-3β and the chlamydial protein IncG (Delevoye et al., 2008; Verbeke et al., 2006). The activation of the PI3K/AKT signaling pathway during infection is a common mechanism also seen during infection of *Neisseria*, *Anaplasma*, *Coxiella* or *Ehrlichia* (Rajalingam 2008).

Besides the activation of survival pathways, it was shown that *C. trachomatis*-infected cells exhibit reduced amounts of tumor necrosis factor receptor I (TNFR1) on the cell surface while the total amount of TNFR1 in the cell is even increased. The activation of the metalloproteinase TACE (TNFα converting enzyme) is responsible for the TNFR1 shedding on the cell surface by which the host cell is less susceptible for inflammatory signals (Paland et al., 2008). A third mechanism of *C. trachomatis* promoting survival of the host cell is the stabilization of anti-apoptotic proteins like Mcl-1, cIAP2 or Survivin (Rajalingam et al., 2008; Rajalingam et al., 2006). This stabilization on protein level is contributed to the upregulation of gene expression and yields high amounts of anti-apoptotic proteins in the cell. In parallel it was shown by several groups that the chlamydial protease CPAF (chlamydial protease or proteasome-like activity factor) is responsible for the degradation of the BH3-only proteins Bim, Bik and Puma (Fischer et al., 2004b; Paschen et al., 2008; Ying et al., 2005). Recent studies however disproved these results as post-lysis artefacts (Chen et al., 2012; Rajalingam et al., 2008; Snavely et al., 2014).

Taken together *C. trachomatis* makes use of several strategies to ensure host cell survival. However, the detailed mechanisms how these processes are activated and regulated on molecular basis are still unclear. The apoptosis resistance of infected cells is dependent on chlamydial effectors functioning inside the host cell but not in neighboring uninfected cells (Rajalingam et al., 2001). Furthermore, antibiotics treatment targeting bacterial transcription and translation causes loss of apoptosis resistance. This fact highlights the involvement of secreted bacterial factors in the regulation of host cell survival (Fan et al., 1998).

1.3 Ubiquitin-Proteasome-System

The ubiquitin-proteasome-system (UPS) describes the cellular processes mediating the ubiquitination and subsequent proteasomal degradation of proteins by the activity of a hierarchical enzymatic cascade consistent of the three enzymes E1, E2 and the ubiquitin ligase E3. The process of ubiquitination mediated by the enzymatic cascade can be reversed by the activity of deubiquitinases catalyzing the removal of ubiquitin from the protein (Kleiger and Mayor, 2014).

1.3.1 Ubiquitin

Ubiquitin is an 8.5 kDa protein that is highly conserved in different species ranging from yeast to humans and is ubiquitous in all tissues and cell types (Ozkaynak et al., 1984; Schlesinger et al., 1975). The protein ubiquitin was first identified in 1975 by G. Goldstein (Goldstein et al., 1975) and short after linked to the ATP-dependent protein degradation process analyzed in rabbit reticulocyte lysates. Ciechanover et al. showed that ubiquitin is essential for the proteolytic activity of reticulocyte lysates and depletion of ubiquitin blocks protein degradation even in the presence of ATP (Ciechanover et al., 1978). Further studies revealed that ubiquitin, here described as ATP-dependent proteolysis factor 1 (APF-1), was covalently linked to proteins in multiple copies thereby mediating their ATP-dependent degradation. Although ubiquitin itself showed no proteolytic activity (Ciechanover et al., 1980; Hershko et al., 1980; Wilkinson et al., 1980). Later in 1986, a large multiprotein complex called 26S proteasome was identified to be responsible for the ATP-dependent protein degradation of ubiquitinated proteins (Arrigo et al., 1988; Hough et al., 1986). To honor the work of Ciechanover, Hershko and Rose in analyzing the molecular basis of ATP-dependent protein degradation and the role of ubiquitin in this process, they were awarded with the Nobel Prize for Chemistry in 2004 (Giles, 2004).

1.3.1.1 Ubiquitination

Ubiquitination is a form of posttranslational modification that is essential for cellular processes like cell cycle, gene regulation, endocytosis, inflammatory response, stress response or apoptosis (Komander, 2009; Mani and Gelmann, 2005). Ubiquitination describes the covalent linkage of the 76 amino acids protein ubiquitin to a lysine residue of the target protein catalyzed by an enzymatic cascade under hydrolysis of ATP (see Figure 1.7). In the initial step ubiquitin is adenylated and covalently linked to the ubiquitin activating enzyme E1. The activated ubiquitin is then transferred from E1 to a conserved cysteine residue of the ubiquitin-conjugating enzyme E2. The E2-ubiquitin conjugate subsequently interacts with the substrate specific ubiquitin ligase E3 which catalyzes the transfer of ubiquitin to an internal lysine residue of the target protein (Kleiger and Mayor, 2014).

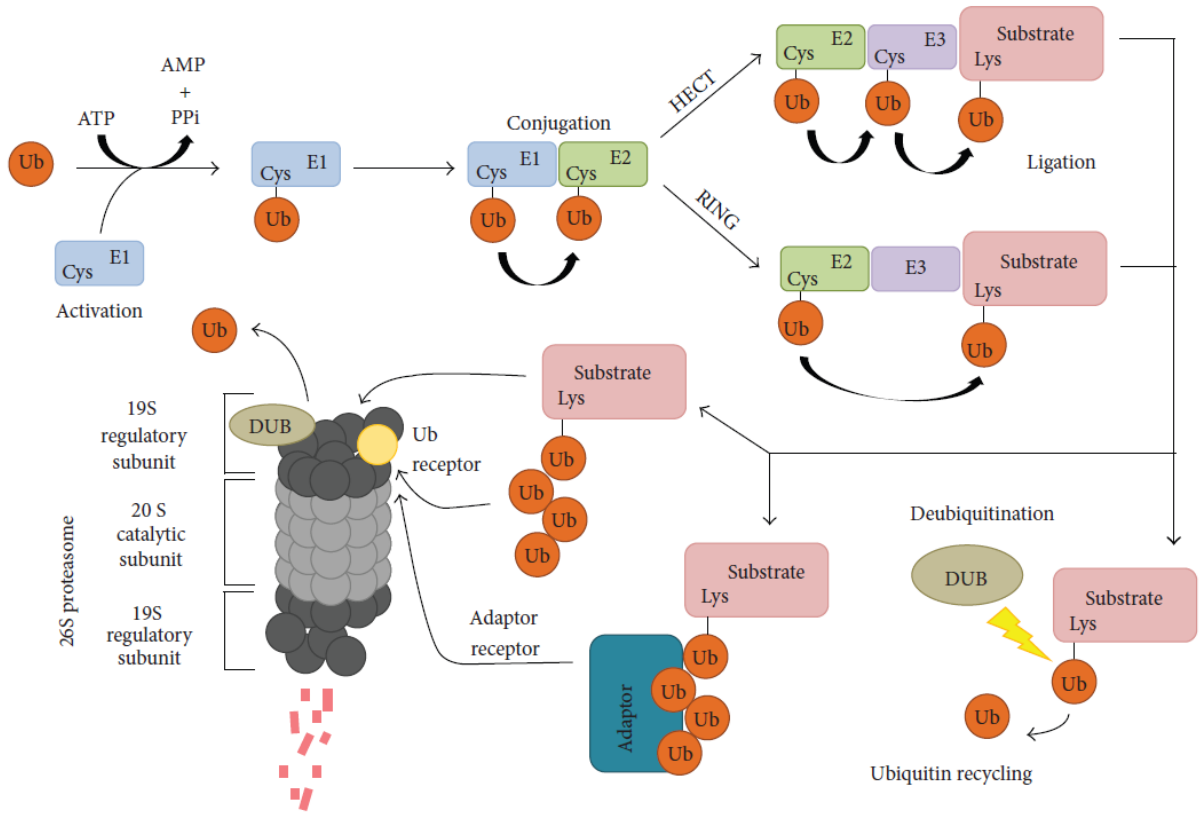

Figure 1.7: Overview of the Ubiquitin-Proteasome-System (UPS). Ubiquitin is activated by adenylation and bound to a internal cysteine residue of the E1 enzyme. Activated ubiquitin is transferred to a cysteine residue of the ubiquitin conjugating enzyme E2 before it is covalently linked to a lysine residue of the target protein. The substrate specificity of the ubiquitin transfer is mediated by the E3 ligase. HECT E3 ligases first transfer ubiquitin to an internal cysteine residue before it is linked to the target lysine of the substrate. On the contrary, RING E3 ligases simply mediate the specific and close contact between E2 and the substrate necessary for the transfer of ubiquitin. The ubiquitination process can be repeated to build poly-ubiquitin chains. Dependent on the internal lysine residue of ubiquitin used for poly-ubiquitination the protein modification has a distinct outcome, for example adaptor protein binding or protein degradation by the 26S proteasome. Reversion of ubiquitination is catalyzed by the activity of deubiquitinases (DUBs). DUBs are besides the regulation of ubiquitination responsible for the recycling of ubiquitin (Lin and Man, 2013).

The activity of the enzymatic cascade catalyzes the linkage of a single ubiquitin molecule to an internal lysine-residue of the target protein resulting in a mono-ubiquitination. Since ubiquitin contains seven lysine-residues it can also be ubiquitinated resulting in poly-ubiquitin chains linked to a protein. These poly-ubiquitin chains vary in their conformation and function depending on the lysine residue used for linkage. The nature of ubiquitination can have a completely different impact on the modified protein. Mono-ubiquitination is involved in endocytosis of cell surface receptors by the ESCRT (Endosomal Sorting Complexes Required for Transport) trafficking complexes (Haglund et al., 2003) or regulates DNA-damage response by histone modification (Wu et al., 2011). Homotypic ubiquitin chains linked by each of the individual lysine residues of ubiquitin have been identified. The most abundant form of poly-ubiquitination is the lysine (K) 48-linked form which is a signal for proteasomal degradation; this has also been shown for K11-linked poly-ubiquitination. K63-linked ubiquitin forms linear chains that are involved in DNA damage response (Hofmann and Pickart, 1999) and signaling

processes (Tokunaga et al., 2009). Other forms of ubiquitin chains linked by K27, K29 or K33 also occur but their function is mostly unknown. For a long time, it was believed that ubiquitination is only a signal for protein destruction but by the identification of the multiple variants of ubiquitination it became evident that this form of posttranslational modification is also involved in signal transduction. Ubiquitination can serve as platform for protein interaction since 20 different ubiquitin-binding domains present in multiple proteins have been identified which mediate protein-protein interaction (Hurley et al., 2006). Furthermore, ubiquitination can induce conformational changes of the modified protein important for structural stability or protein activity (Duda et al., 2008).

1.3.1.2 Ubiquitin-like proteins (UBLs)

The family of ubiquitin-like proteins (UBLs) consists of proteins that all share a three-dimensional homology with ubiquitin represented by a globular ubiquitin-fold, but exhibit only minor sequence homologies with ubiquitin (Hochstrasser, 2009). UBLs can be conjugated to proteins by an enzyme cascade which is distinct but evolutionary related to ubiquitination. The number of UBL family members is increasing but for many of them the cellular function is still unknown. The UBL with the closest sequence identity of 58% to ubiquitin is Nedd8 (neural precursor cell expressed, developmentally downregulated 8) (van der Veen and Ploegh, 2012). Neddylation is catalyzed by an enzymatic cascade consistent of E1, E2 and E3 specific for Nedd8 and can be reversed by Nedd8-specific proteases like NEDP1 or CSN5, a subunit of the COP9 signalosome (van der Veen and Ploegh, 2012). Well known targets for neddylation are the members of the cullin family. Cullins represent one subunit of the E3 SCF ubiquitin ligase complex regulating enzyme activity. Mono-neddylation of cullins results in conformational reorganization by which interaction between the E3 ligase complex and the E2 ubiquitin conjugating enzyme is enabled (Duda et al., 2008). Poly-neddylation of proteins was also observed, but the cellular function is not known yet. Depletion of Nedd8 is lethal and cellular processes supposed to be dependent on neddylation are transcription, DNA repair and replication, cell cycle progression and chromatin organization (Jones et al., 2008). In addition to the Nedd8-specific E3 ligases it was shown that some RING- and HECT-E3 ligases exhibit dual specificity towards ubiquitin and Nedd8 like IAPs or Mdm2, both important in apoptosis signaling or cell cycle regulation (Broemer et al., 2010). Neddylation pathways are frequently targeted by pathogens encoding for Nedd8-specific proteases, like *Chlamydia trachomatis* and *Plasmodium falciparum*, or effectors blocking the neddylation machinery (van der Veen and Ploegh, 2012).

The second UBL studied in more detail is SUMO (small ubiquitin related modifier). SUMO exists in four paralogs which can be conjugated to proteins forming mixed chains and are removed by the activity of SUMO-specific proteases belonging to the SENP (Sentrin-specific proteases) family. SUMOylation affects protein conformation or acts as platform of protein-protein interaction mediated

by the SUMO-interaction motif (SIM) (Wilkinson and Henley, 2010). SUMOylation takes place in the cytoplasm as well as in the nucleus and is connected to nuclear transport and nuclear organization, transcription, chromatin remodeling, DNA repair and ribosomal biogenesis (Wilkinson and Henley, 2010). As already seen for neddylation, the SUMOylation machinery is also a target for pathogenic interference (Ribet and Cossart, 2010a). The Atg proteins are related to autophagy with the most prominent members Atg8 and Atg12 and the UBL ISG15 (interferon-stimulated gene 15) is involved in anti-viral immune response and its expression is stimulated by type one interferon (van der Veen and Ploegh, 2012).

1.3.2 E3 ubiquitin ligases

The family of E3 ligases is heterogeneous and can be divided into the two major subgroups of HECT (Homolog to E6-associated protein C-Terminus) and RING (Really Interesting New Gene) E3 ligases. Dependent on the E3 ligase subgroup ubiquitin is either directly transferred from the E2-conjugate to an internal lysine residue of the substrate or is transferred from E2 via E3 to the substrate. RING E3 ligases simply mediate the specific interaction and correct orientation of the E2-ubiquitin conjugate with the substrate during ubiquitin transfer. On the other hand, HECT E3 ligases catalyze the transfer of ubiquitin from E2 to the substrate by a thioester linked intermediate (Dye and Schulman, 2007; Pickart, 2001) (see Figure 1.7). With its C-terminal glycine ubiquitin is linked through an isopeptide bound to the ε-amino group of the substrates lysine. In mammals just one E1 enzyme and about 25 E2 enzymes have been identified. On the contrary, more than 600 genes encoding for a substrate specific E3 ligase are known.

HECT E3-ligases are single proteins which contain a HECT domain catalyzing the binding of the E2-ubiquitin conjugate and the transfer of ubiquitin to a conserved cysteine residue of the E3 ligase and subsequent transfer to the substrate (Huibregtse et al., 1995). The substrate is normally bound by further protein-protein interaction domains present in the HECT E3 ligase (Scheffner and Staub, 2007). In comparison, the heterogeneous RING-domain E3 ligase family can be categorized into the two subgroups of single- and multi-subunit proteins. The RING domain was first described in 1991 by Freemont and colleagues (Freemont et al., 1991) and is featured by conserved cysteine and histidine residues in complex with two Zn^{2+} ions (Joazeiro and Weissman, 2000). Mdm2, catalyzing the ubiquitination of p53, is an example for a single-subunit RING E3 ligase while the SCF (Skp1-cullin1-F-box protein family) E3 ligases represent multi-subunit RING E3 ligases (Deshaies, 1999; Fang et al., 2000). It is known that one E3 ligase can be specific for more than one substrate and one protein can be recruited for ubiquitination by multiple different E3 ligases (Kleiger and Mayor, 2014).

The activity of E3 ligases can be regulated either by direct modification of the enzyme regulating its activity or by altered substrate affinity. Examples are the SCF ubiquitin ligases that contain a cullin subunit which can be modified by Nedd8 for their activation (Duda et al., 2008) or the HECT E3 ligase Itch which is activated by phosphorylation (Gallagher et al., 2006). Especially phosphorylation is a prominent form of modification altering the proteins' affinity to its ubiquitin ligases as it is the case for Mcl-1. Several signaling pathways trigger phosphorylation of Mcl-1 at different threonine- and serine-residues by which the affinity of Mcl-1 to the different E3 ligases and thereby its half-life is adjusted (Thomas et al., 2010). With the usage of several E3 ligases and the possibility of individual regulation the ubiquitin-proteasome-system is a highly dynamic system which can react rapidly to altered cellular conditions.

1.3.3 Deubiquitinating enzymes (DUBs)

Almost every posttranslational protein modification can be reversed by specific enzymes. This is also applicable to ubiquitination. Deubiquitinating enzymes (DUBs) can reverse the ubiquitination catalyzed by E3 ligases. The human genome encodes for about 100 DUBs (Nijman et al., 2005) which can be categorized into 5 subgroups: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Josephins and JAB1/MPN/MOV34 metalloenzymes (JAMMs). The members of the UCH-, USP-, OTU- and Josephins-family are cysteine proteases characterized by the highly conserved amino acid residues His, Cys and Asp forming the catalytic triade (Storer and Menard, 1994) whereby the JAMMs represent metalloproteases (Nijman et al., 2005). All DUBs contain at least one ubiquitin-binding domain like the ubiquitin-interacting motif (UIM) or the ubiquitin-associated domain (UBA) and one ubiquitin-like fold (Hurley et al., 2006). UCHs are highly conserved and are relatively small in size (20-30 kDa) whereas USPs vary in size from 50 to 300 kDa. USPs share two homology regions named Cys- and His-box being located in the surrounding of the catalytic active residues. The USPs show alterations in their N-terminal region which function for protein-protein interaction and regulation of enzyme activity (Kim et al., 2003). Besides the five already mentioned DUB subfamilies two more have been identified: the Adenain family of cysteine proteases, specific for ubiquitin as well as ubiquitin-like proteins as SUMO or Nedd8, and DUBs resembling the adenovirus protease which is mostly encoded by pathogenic microorganisms (Reyes-Turcu et al., 2009).

DUBs fulfill various functions in the cell like processing of ubiquitin-precursors, recycling of inadvertently trapped ubiquitin-intermediates or poly-ubiquitin chains, proofreading and editing of protein ubiquitination, removing poly-ubiquitin chains from the proteasome or the maintenance of the concentration of free ubiquitin in the cell (Reyes-Turcu et al., 2009). One further function of DUBs is the regulation of protein stability by removal of the ubiquitin-tag from the protein which normally

mediates its destruction by the 26S proteasome or lysosomal system. DUBs also play a role in signal transduction since mono-ubiquitination is involved in DNA-damage response and cell cycle progression which has to be regulated by DUB activity, too (Komander et al., 2009). Regarding these multiple modes of action, it is not surprising that DUBs show different specificities towards ubiquitin or ubiquitin-like proteins, the nature of ubiquitin-chains and the modified substrate. Ubiquitin and UBLs share homology in their three-dimensional folding but can be distinguished by their C-terminal amino acid sequence which is used by the DUBs for recognition and specific interaction (Drag et al., 2008). Depending on the linkage type the ubiquitin chains vary in their three-dimensional conformation, as K48-chains are more compact compared to the linear K63-chains (Komander and Rape, 2012). Theses variations in chain conformation affect accessibility of the ubiquitin chain to the active center of the DUB. It was shown that some DUBs are specific for one type of ubiquitin-linkages whereas others are susceptible for various linkage-types and ubiquitin-family members (Gong et al., 2000; McGouran et al., 2013). The substrate is recognized by specific protein-protein binding domains which mediate either direct binding of the substrate or recruitment of further interaction partners mediating substrate binding (Reyes-Turcu et al., 2009).

As with ubiquitination, the process of deubiquitination has to be strictly controlled. One common mechanism to regulate cellular enzymatic activity is the adjustment of gene expression at the transcriptional level. A more precise mechanism to alter enzyme activity depends on post-translational modifications of the enzyme. Especially the phospho-signaling is closely linked to the UPS since phosphorylation can change enzyme activity or substrate recognition as already described for regulation of E3 ligases (Hunter, 2007). Allosteric regulation is a common feature of enzymes which is also applicable for DUBs. DUBs undergo conformational changes upon substrate binding by which the active center is reorganized resulting in the perfect orientation of the substrate for enzymatic reaction (Hu et al., 2002). Furthermore, by the multiple protein-protein interaction domains allosteric activators or inhibitors can bind to the DUB and regulate its activity. Additionally, by changing the subcellular localization of the DUB its accessibility for particular substrates can be modulated (Komander et al., 2009).

1.3.4 26S Proteasome

K48-linked poly-ubiquitination marks a protein for proteasomal destruction. The 26S proteasome is a huge multisubunit complex that is responsible for the ATP-dependent proteolysis of proteins into peptides (Hough et al., 1986). The proteasome consists of a 20S catalytic component (CP) and a 19S regulatory component (RP). The CP is a cylindrical particle consistent of four hetero-heptameric rings with proteolytic active sites looming into the inner space. The proteolytic active subunits are β1, β2 and β5 with caspase-like, trypsine-like and chymotrypsin-like activity, respectively

(Finley, 2009). The RP consists of two subcomplexes called base and lid. The base contains six ATPases as well as ubiquitin-binding proteins with UBL or UBA domains mediating the specific interaction with ubiquitinated proteins. The lid is formed out of nine subunits including the Poh1 (Rpn11) subunit. Poh1 is a JAMM deubiquitinase responsible for the *en block* removal of the poly-ubiquitin chains of the proteins before they are translocated into the core channel. The ubiquitin chain has to be removed for proper unfolding and threading of the protein into the CP. Additionally, it is a way to recycle ubiquitin. Taken together, the RP mediates the specific binding of ubiquitinated proteins, the removal of the ubiquitin chain and the ATP-driven unfolding of the protein (Finley, 2009). Besides Poh1, two additional deubiquitinases are associated with the 26S proteasome. Uch37 and USP14 are DUBs catalyzing removal of single ubiquitin molecules from the distal end of the chain in contrast to Poh1 which cleaves whole ubiquitin chains. By the activity of Uch37 and USP14 the ubiquitin chains can be edited or trimmed to alter affinity of the ubiquitinated protein towards the proteasome to regulate its degradation. Only proteins with the optimal chain length are efficiently threaded into the core channel and degraded into peptides (Hanna et al., 2006; Lam et al., 1997). For an overview of 26S proteasome composition and activity see Figure 1.8.

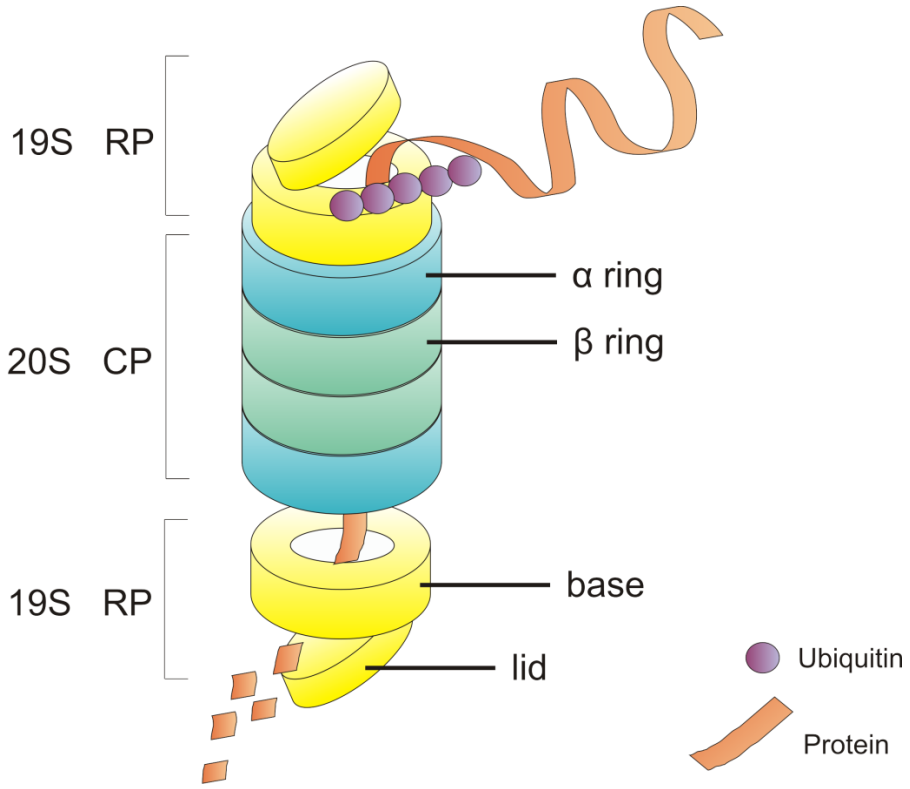

Figure 1.8: Composition of the 26S Proteasome. The 26S proteasome consists of the 19S and 20S subunit. The 19S regulator subunit (RP) consists of a base and lid. The RP base catalyzes the specific recognition and unfolding of the protein in an ATP-dependent way. The lid contains the DUB Roh1 catalyzing the *en block* removal of the ubiquitin chain. The 20S core subunit (CP) contains multiple proteolytic active residues catalyzing the destruction of the protein.

1.3.5 UPS in cancer and during infection

As the UPS regulates important cellular processes like cell cycle, apoptosis or inflammation it is not surprising that many cancer cells show defects in the UPS. Especially the amount of oncoproteins or tumor suppressor proteins like p53 is strictly regulated by ubiquitination and proteasomal degradation. The p53-specific ubiquitin ligase Mdm2 is enriched in about 7 % of all human tumors and the risk of developing breast cancer is associated to a mutation in the *BRCA1* gene encoding for a RING-domain containing E3 ubiquitin ligase. Drugs like Bortezomib targeting the activity of the 26S proteasome, which represents the final step in the UPS, were shown to selectively block growth of malignant cells and sensitize them for apoptosis (Mani and Gelmann, 2005).

Alterations of the UPS have also been observed during infection of various pathogenic bacteria and viruses. Since ubiquitination can be a signal for endocytosis some bacteria induce ubiquitination to trigger receptor internalization mediating bacterial or toxin uptake as seen for *Bacillus anthracis* toxin (Munro et al., 2007). Intracellular bacteria are opposed by the autophagy system, more precisely by a selective form of autophagy named xenophagy. Bacteria that reach the cytoplasm after vacuole escape are tagged with ubiquitin or UBLs which promote engulfment of the pathogen into the autophagosome. The autophagosomes represent double-membrane vesicles that fuse with the lysosomal system to eliminate the dangerous cargo (Gomes and Dikic, 2014). As example, *Shigella* secrete the effector protein VirG needed for intracellular motility. VirG is recognized by the protein Atg5 causing induction of autophagy. IcsB, also secreted by Shigella binds VirG and prevents its recognition by the autophagy system promoting intracellular survival of *Shigella* (Ogawa et al., 2005).

Furthermore, the activation of the inflammatory response towards pathogens is dependent on ubiquitination events. Pathogens can be recognized by toll-like receptors which recruit TRAF6 upon activation. TRAF6 is an E3 ubiquitin ligase catalyzing K63-linked ubiquitin conjugation. Receptor recruitment stimulates auto-ubiquitination of TRAF6 which subsequently ubiquitinates TAK1. TAK1 acts as a kinase activating the MAPK and NFκB signal transduction pathways resulting in inflammatory response. Especially the phosphorylation and subsequent ubiquitination of IκBα by $SCF^{\beta\text{-}TrCP}$ represents a crucial event in this signaling pathway because proteasomal destruction of IκBα leads to activation and nuclear translocation of the transcription factor NFκB activating proinflammatory host immune response (Angot et al., 2007). To prevent inflammatory response pathogenic bacteria make use of a plethora of effector proteins targeting the abovementioned pathway. Non-pathogenic bacteria were shown to induce deneddylation of cullin 1, a part of the $SCF^{\beta\text{-}TrCP}$ complex responsible for IκBα ubiquitination, to inactivate the E3 ligase complex (Collier-Hyams et al., 2005). Effector proteins secreted from pathogentic bacteria like YopJ/P from *Yersinia* or AvrA and SseL from *Salmonalla* block NFκB signaling directly by their deubiquitinating activity. Moreover, OspG secreted from *Shigella flexneri* interacts with the E2 ubiquitin conjugating enzyme UbcH5 involved in $SCF^{\beta\text{-}TrCP}$ mediated

ubiquitination processes. *Shigella* additionally secretes IpaH9.8 functioning as an E3 ligase targeting IKKγ/NEMO for degradation. Further examples for bacterial effectors with E3 ligase activity are the F-box containing proteins of *Legionella pneumophila*. F-box proteins are components of SCF E3 ligase complexes important for protein-protein-interaction. It was shown that AnkB of *Legionella* interacts with Skp1 forming an active SCF E3 ligase complex targeting ParvB responsible for actin cytoskeleton dynamics and cell survival (Perrett et al., 2011). However, the bacteria not only use the UPS to alter amount of cellular proteins but also take advantage of ubiquitination and destruction of their own effector proteins to control their activity. As example, the pore forming toxin lysteriolysin O (LLO) secreted by *Listeria monocytogenes* is important for phagosomal escape. To limit pore forming activity towards the cytoplasmic membrane after escape, which would destroy the host cell, LLO is immediately ubiquitinated and degraded by the 26S proteasome after it reaches the cytoplasm (Ribet and Cossart, 2010b).

1.4 Aim of the work

Chlamydia trachomatis as an obligate intracellular pathogen is an expert in manipulating its host cell in terms of intracellular trafficking and nutrition acquisition, signaling and cell death. By a RNA interference screen several host cell factors were identified to be crucial for *Chlamydia*-mediated apoptosis inhibition of the host cell. Among others, Mcl-1 is dramatically enriched during *Ctr* infection. In this work the molecular mechanism resulting in Mcl-1 stabilization during *Ctr* infection should be revealed. Since protein turnover is performed by the UPS and many pathogens are known to actively manipulate the UPS, a participation of host cell UPS factors in *Ctr*-mediated Mcl-1 stabilization was investigated. Furthermore, the two chlamydial deubiquitinases ChlaDUB1 and ChlaDUB2 should be characterized and their participation in apoptosis inhibition determined.

In 2011 a method to transform *Chlamydia trachomatis* was published which should be established in our department, too. Moreover, this method was ought to be modified for targeted recombination into the chlamydial genome, targeted knock-out or knock-down of any gene of interest and inducible protein expression. These methods to genetically manipulate *Ctr* were established and adapted to be used for analysis of the expression and function of the two chlamydial deubiquitinases ChlaDUB1 and ChlaDUB2.

In collaboration with the department of pharmacy (University of Mainz) a library of potential cysteine protease inhibitors was provided which should be tested for their ability to block ChlaDUB1 and ChlaDUB2 activity. Furthermore, the effect of these inhibitors during cell culture infection experiments should be tested.

2 MATERIAL AND METHODS

2.1 Materials

2.1.1 Cell lines

Table 2-1: Cell lines

Cell line	Properties	Media/supplements	Source
HeLa229	Human cervix adenocarcinoma cells	RPMI 1640	ATCC® CCL-2.1™
HEp-2	HeLa contaminant, larynx epithelium	RPMI 1640	ATCC® CCL-23™
293T	Human embryonic kidney epithelial cells	DMEM	ATCC® CRL-3216™
McCoy	Mouse fibroblast cells	RPMI 1640	ATCC® CRL-1696™
293T/pcDNA4TO	293T cells stably transfected with pcDNA4TO plasmid	DMEM 5 µg/ml Blasticidin, 300 µg/ml Zeocin™	This work
293T/TRex Cdu1-FLAG	293T cells stably transfected with pcDNA6TR plasmid and pcDNA4TO/Cdu1-FLAG plasmid	DMEM 5 µg/ml Blasticidin, 300 µg/ml Zeocin™	This work
293T/TRex Cdu1(C234A)-FLAG	293T cells stably transfected with pcDNA6TR plasmid and pcDNA4TO/Cdu1(C345A)-FLAG plasmid	DMEM 5 µg/ml Blasticidin, 300 µg/ml Zeocin™	This work

HeLa229, HEp-2 and McCoy cells were cultured in RPMI 1640 supplemented with 10 % heat inactivated FCS and 293T cells were cultured in DMEM supplemented with 10% heat inactivated FCS.

2.1.2 Bacterial strains

Table 2-2: *E. coli* strains

Bacterial strain	Properties/usage
E. coli DH5α	cloning
E. coli XL1blue	cloning
E. coli BL21star Codon plus	recombinant protein overexpression
E. coli JM110	deficient for Dam and Dcm methylases

E. coli strains were grown on LB agar plates or in LB medium.

Table 2-3: Chlamydial strains

Species	Serovar	Properties	Source
C. trachomatis	LGV L2 (434)		ATCC ® VR-902B™
C. trachomatis	D		ATCC ® VR-885™
C. trachomatis pGFP::SW2	LGV L2 (434)	*C. trachomatis* transformed with pGFP::SW2 plasmid. Selection with 100 U PenG/ml	Dr. A. Mehlitz
C. trachomatis pCdu1(C345A)-FLAG::SW2	LGV L2 (434)	*C. trachomatis* transformed with pCdu1(C345A)-FLAG::SW2 plasmid. Selection with 10 U PenG/ml	This work
C. trachomatis pTet/Cdu1-FLAG::SW2	LGV L2 (434)	*C. trachomatis* transformed with pTet/Cdu1-FLAG::SW2 plasmid. Selection with 10 U PenG/ml. Induction with 10 ng/ml AHT.	This work
C. trachomatis pTet/Cdu1(C345A)-FLAG::SW2	LGV L2 (434)	*C. trachomatis* transformed with pTet/Cdu1(C345A)-FLAG::SW2 plasmid. Selection with 10 U PenG/ml. Induction with 10 ng/ml AHT.	This work
C. trachomatis pTet/Cdu1ΔCD-FLAG::SW2	LGV L2 (434)	*C. trachomatis* transformed with pTet/Cdu1ΔCD-FLAG::SW2 plasmid. Selection with 10 U PenG/ml. Induction with 10 ng/ml AHT.	This work
C. trachomatis pTet/Cdu2(C282A)-FLAG::SW2	LGV L2 (434)	*C. trachomatis* transformed with pTet/Cdu2(C282A)-FLAG::SW2 plasmid. Selection with 10 U PenG/ml. Induction with 10 ng/ml AHT.	D. Auer (master thesis) (Auer, 2014)
C. trachomatis pIncA-FLAG::SW2	LGV L2 (434)	*C. trachomatis* transformed with pIncA-FLAG::SW2 plasmid. Selection with 100 U PenG/ml	Prema Subbarayal
C. pneumoniae	CWL-029		ATCC® VR-1310™

2.1.3 Plasmids

All plasmids designed and used in this work are listed in Table 2-4 and Table 2-5. Oligonucleotides and restriction sites used for cloning of the plasmids are indicated.

Table 2-4: Plasmids

Plasmid	Comment	Source
pcDNA3	expression vector for eukaryotic cells	Invitrogen
pcDNA3-FLAG	pcDNA3 expression vector for introducing a C-terminal FLAG-tag	(Kozjak-Pavlovic et al., 2010)
pEGFP-C1	expression vector of N-terminal GFP-fusion proteins for eukaryotic cells	Clontech
pET28a	IPTG-inducible prokaryotic expression vector for expression of N-terminal HIS-tagged fusion proteins	Novagen®
pGEX-4t-3	IPTG-inducible prokaryotic expression vector for expression of N-terminal GST-tagged fusion proteins	GE Healthcare
pcDNA6TR	Regulatory vector that expresses the tetracycline (Tet) repressor	Invitrogen
pcDNA4TO	Tetracycline-regulated expression plasmid for mammalian cells	Invitrogen
pTet::SW2	chlamydial expression plasmid for tetracycline-regulated protein expression	D. Auer (master thesis) (Auer, 2014)

Table 2-5: Constructs

Plasmid	Vector	Comment	Source
pcDNA3.1/hMcl1	pcDNA3.1	plasmid #25375	Addgene; (Morel et al., 2009)
pcDNA3/Cdu1-FLAG	pcDNA3-FLAG	Full length Cdu1 was amplified from gDNA of *C. trachomatis* 434 with Cdu1 frw and Cdu1 rev and cloned into the expression vector pCDNA3-FLAG (HindIII/BamHI; DiTriSec)	This work
pcDNA3/Cdu1(C345A)-FLAG	pcDNA3-FLAG	Cdu1(C345A)-FLAG was generated by Quick change site directed mutagenesis resulting in the amino acid exchange of cysteine 345 to alanine in Cdu1-FLAG.	This work
pcDNA3/Cdu1ΔCD-FLAG	pcDNA3-FLAG	Cdu1ΔCD-FLAG was amplified from gDNA of C. trachomatis 434 with Cdu1 EcoRI frw and Cdu1ΔCD-FLAG XhoI rev and cloned into the expression vector pCDNA3 (EcoRI/XhoI)	This work
pEGFP-C1/Cdu1	pEGFP-C1	Full length Cdu1 was amplified from gDNA of *C. trachomatis* 434 with Cdu1 BglII frw and Cdu1 PstI rev and cloned into the expression vector pEGFP-C1 (BglII/PstI)	This work

Plasmid	Vector	Comment	Source
pEGFP-C1/Cdu1ΔCD	pEGFP-C1	Cdu1ΔCD was amplified from gDNA of *C. trachomatis* 434 with Cdu1 BglII frw and Cdu1ΔCD PstI rev and cloned into the expression vector pEGFP-C1 (BglII/PstI)	This work
pET28a/Cdu1	pET28a	Full length Cdu1 was amplified from gDNA of *C. trachomatis* 434 with Cdu1 frw II and Cdu1 rev II and cloned into the expression vector pET28a (BamHI/HindIII; DiTriSec)	This work
pET28a/Cdu2	pET28a	Full length Cdu2 was amplified from gDNA of *C. trachomatis* 434 with Cdu2 frw II and Cdu2 rev II and cloned into the expression vector pET28a (BamHI/HindIII; DiTriSec)	This work
pET28a/GroEL	pET28a	Truncated GroEL ranging from amino acid 1 to 300 was amplified from gDNA of *C. trachomatis* 434 with GroEL frw and GroEL rev and cloned into the expression vector pET28a (BamHI/HindIII; DiTriSec)	S. Tirier (Bachelor student)
pQE30/Cdu1	pQE30	Full length Cdu1 was amplified from gDNA of *C. trachomatis* 434 with Cdu1 XmaI frw and Cdu1 PstI rev and cloned into the expression vector pQE30 (XmaI/PstI)	This work
pGEX-4t-3/Mcl1	pGEX4t3	Human Mcl-1 was amplified from the pcDNA3.1/hMcl1 construct with Mcl-1 BamHI frw and Mcl-1 SalI rev and cloned into the expression vector pGEX4t3 (BamHI/SalI)	This work
pcDNA4TO/Cdu1-FLAG	pcDNA4TO	Full length Cdu1-FLAG was amplified from gDNA of *C. trachomatis* 434 with Cdu1 EcoRI frw and Cdu1-FLAG NotI rev and cloned into the expression vector pcDNA4TO (EcoRI/NotI)	This work
pcDNA4TO/Cdu1(C345A)-FLAG	pcDNA4TO	Full length Cdu1(C345A)-FLAG was amplified from pcDNA3/Cdu1(C345A)-FLAG with Cdu1 EcoRI frw and Cdu1-FLAG NotI rev and cloned into the expression vector pcDNA4TO (EcoRI/NotI)	This work
pcDNA3/myc-Mcl1	pcDNA3	Full length Mcl-1 was amplified from pcDNA3.1/hMcl1 with Mcl-1-myc HindIII frw and Mcl-1 BamHI rev and cloned into the expression vector pcDNA3 (HindIII/BamHI)	This work
pcDNA3.1/HA-Ubiquitin		eukaryotic expression of HA-ubiquitin	D. Bohmann
GST-ΔN-HectH9		Bacterial overexpression of GST-tagged HectH9ΔN protein	(Adhikary et al., 2005)
pGFP::SW2		Chlamydial expression plasmid encoding for a GFP-CAT fusion protein	(Wang et al., 2011)
pAH1		Suicidal plasmid for the homologues recombination of ChlaDUB1-FLAG into the chlamydial genome	This work
pAH3		Suicidal plasmid for the creation of a ChlaDUB1 knock-out *Ctr* strain	This work

Plasmid	Vector	Comment	Source
pCdu1(C345A)-FLAG::SW2		Chlamydial expression plasmid encoding for ChlaDUB1(C345A)-FLAG	This work
pdCas9-bacteria		plasmid #44249	Addgene; (Qi et al., 2013)
pTet/Cdu1-FLAG::SW2	pTet::SW2	Chlamydial expression plasmid for tetracycline-inducible expression of ChlaDUB1-FLAG	This work
pTet/Cdu1(C345A)-FLAG::SW2	pTet::SW2	Chlamydial expression plasmid for tetracycline-inducible expression of ChlaDUB1(C345A)-FLAG	This work
pTet/Cdu1ΔCD-FLAG::SW2	pTet::SW2	Chlamydial expression plasmid for tetracycline-inducible expression of ChlaDUB1ΔCD-FLAG	This work
pTet/Cdu2(C282A)-FLAG::SW2	pTet::SW2	Chlamydial expression plasmid for tetracycline-inducible expression of ChlaDUB2(C282A)-FLAG	D. Auer (Auer, 2014)

2.1.4 Oligonucleotides

Table 2-6: Oligonucleotides used for cloning

Name	Sequence (5' → 3')	comment
Cdu1 frw	CTGATGTTATCTCCCACCAACTCA	DiTriSec
Cdu1 rev	TCGTGCTTCAGGCCAAGAAAGCT	DiTriSec
Cdu1 Mut frw	GATCCAGCGCCGGCGCTT	Site directed mutagenesis
Cdu1 Mut rev	AAGCGCCGGCGCTGGATC	Site directed mutagenesis
Cdu2 frw	TCGGAACCAATTCATAATCCTCCCC	DiTriSec
Cdu2 rev	CTGTTAATCCGTAGTTGGCCAGCT	DiTriSec
Cdu2 Mut frw	GAATTCACCGCTGGAGCTTG	Site directed mutagenesis
Cdu2 Mut rev	CAAGCTCCAGCGGTGAATTC	Site directed mutagenesis
Cdu1 frw II	TCGTTATCTCCCACCAACTCAACTT	DiTriSec
Cdu1 rev II	CTGTTATGCTTCAGGCCAAGAAAGC	DiTriSec
Cdu2 frw II	TCGGAACCAATTCATAATCCTCCCC	DiTriSec
Cdu2 rev II	CTGTTAATCCGTAGTTGGCCAGCT	DiTriSec
Mcl-1 BamHI frw	GATCGGATCCTTTGGCCTCAAAAGAAACGCG	BamHI
Mcl-1 SalI rev	GATCGTCGACCTATCTTATTAGATATGCCAAAC	SalI
GroEL frw	TCGGTCGCTAAAAACATTAAATACAAC	DiTriSec
GroEL rev	CTGTTAGAGTTGACCGCCAGTTAAGA	DiTriSec
Cdu1 EcoRI frw	GATCGAATTCATGTTATCTCCCACCAACTCAACT	EcoRI
Cdu1-FLAG NotI rev	GATCGCGGCCGCTTACTTATCGTCGTCATCCTTGTAATC TGCTTCAGGCCAAGAAAGCT	C-term. FLAG; NotI
Cdu1 XmaI frw	GATCCCCGGGTTATCTCCCACCAACTCAACT	XmaI

Name	Sequence (5' → 3')	comment
Cdu1 PstI rev	GATCCTGCAGTTATGCTTCAGGCCAAGAAAGCT	PstI
Cdu1 BglII frw	GATCAGATCTTTATCTCCCACCAACTCAACT	BglII
Cdu1ΔCD-FLAG XhoI rev	GATCCTCGAGTTACTTATCGTCGTCATCCTTGTAATCTA GCTGTTTCATAGTCTGCATG	C-term. FLAG; XhoI rev
Cdu1ΔCD PstI rev	GATCCTGCAGTTATAGCTGTTTCATAGTCTGCATG	PstI
Mcl-1-myc HindIII frw	GATCAAGCTTATGGAGCAGAAACTCATCTCTGAAGAGG ATCTGTTTGGCCTCAAAAGAAACGCG	N-term. myc; HindIII
Mcl-1 BamHI rev	GATCGGATCCCTATCTTATTAGATATGCCAAAC	BamHI
pAH1 P1	GATCACTAGTATGTTATCTCCCAC	SpeI and Cdu1
pAH1 P2	ATGCAGCTCCCGGAATTCTCACCAATAA	14 bp overlap selection cassette and terminator
pAH1 P3	TTATTGGTGAGAATTCCGGGAGCTGCAT	14 bp overlap terminator and selection cassette
pAH1 P4	GATTAGAAAAGAGTTTACGCCCCGCCCT	14 bp overlap 3' region and selection cassette
pAH1 P5	AGGGCGGGGCGTAAACTCTTTTCTAATC	14 bp overlap selection cassette and 3' region
pAH1 P6	GATCACTAGTTCTTCTTGATCGGA	SpeI and 3' region
pAH1 P7	GATCACGCGTGACTCCTGTTGATAGATCCAG	MluI and terminator
pAH1 P8	GATCACGCGTTTACTTATCGTCGTCATCCTTGTAATCTG CTTCAGGCCAAGAAAGCT	MluI-FLAG-Cdu1
pAH3 P1	GATGCTGTTTTACGAGGGGAGC	DiTriSec
pAH3 P2	CGTCTAAAATCGAGATGATTTTCTGT	DiTriSec
pAH3 P3	GATCATCTGATAATGGTTTCTTAGA	ClaI and *bla*
pAH3 P4	CATAGGCTCCGCCCCTGACGCTCAGTGG	14 bp overlap *bla* and GFP-CAT
pAH3 P5	CCACTGAGCGTCAGGCGCCCAATACGCA	14 bp overlap GFP-CAT and *bla*
pAH3 P6	GATCGTCGACCATTAATCTAGATA	SalI and GFP-CAT
pCdu1::SW2 P1	CAAAAGGAAGCCGATATGTTATCTCCCAC	14 bp overlap Pnm and Cdu1
pCdu1::SW2 P2	GATCGTCGACTTACTTATCGTCGTCATCCTTG	SalI-FLAG
pCdu1::SW2 P3	GATCGTCGACTCTAGAGGATCCGTTTGTTCT	SalI and SW2 part
pCdu1::SW2 P4	GTGGGAGATAACATATCGGCTTCCTTTTG	14 bp overlap Cdu1 and Pnm
pTet P1	GATCACGCGTCGGAAATTTGGTTATCTACTTTATC	MluI-selection cassette
pTet P2	CTTGCATGCCTGCATTAAGACCCACTTT	14 bp overlap with Tet-promoter

Name	Sequence (5' → 3')	comment
pTet P3	CTTGCATGCCTGCATTAAGACCCACTTT	14 bp overlap with selection cassette
pTet P4	GATCGTCGACGATCACTAGTTTTCTCCTCTTTAGATCTTTTGA	SalI-Spacer-SpeI cloning site and Tet-promoter
pTet GFP frw	AGCATGGTGGATCCCCGGGTAC	DiTriSec
pTet GFP rev	GACTTACGCCCCGCCCTGCC	DiTriSec
pTet Cdu1 frw	AGCATGTTATCTCCCACCAACTCA	DiTriSec
pTet FLAG rev	GACTTACTTATCGTCGTCATCCTTGTAATC	DiTriSec
pTet Cdu2 frw	AGCATGGAACCAATTCATAATCCTCCCCC	DiTriSec

Table 2-7: Oligonucleotides used for sequencing

Name	Sequence (5'→3')	Sequencing of plasmid
T7 frw	TAATACGACTCACTATAGG	pcDNA3/pET28a
SP6 rev	CTATAGTGTCACCTAAAT	pcDNA3
pEGFP-C1 frw	CCACTACCTGAGCACCCAGT	pEGFP-C1
pEGFP-C1 rev	GACAAACCACCACTAGAATGCAG	pEGFP-C1
CMV frw	CGCAAATGGGCGGTAGGCGTG	pcDNA4TO
BGH rev	TAGAAGGCACAGTCGAGG	pcDNA4TO
pGEX frw	GGGCTGGCAAGCCAGGTTTGGTG	pGEX-4t-3
pGEX rev	CCGGGAGCTGCATGTGTCAGAGG	pGEX-4t-3
T7 Term. rev	GCTAGTTATTGCTCAGCGG	pET28a
pQE30 frw	CCCGAAAAGTGCCACCTG	pQE30
pQE30 rev	GTTCTGAGGTCATTACTGG	pQE30
Seq. Pnm frw	GCTATTGTTTTATATCAAAATATAATCATTTTTA	pCdu1::SW2 plasmids
Seq. SW2 rev	CTTCAAAGAAGCTGGCTCTAATAT	pCdu1::SW2 and pTet::SW2
pTet Seq. frw	ATTCCGACCTCATTAAGCAGCTCT	pTet::SW2

Table 2-8: siRNA oligonucleotides

target	Sequence	source
USP9X	siGENOME SMARTpool siRNA; pool of four sequences	Dharmacon, M-006099
Mcl-1	siGENOME SMARTpool siRNA; pool of four sequences	Dharmacon, M-004501
control	siGENOME control Pool	Dharmacon, D-001206-13-20

2.1.5 Antibodies

Table 2-9: Primary antibodies

Antibody	Origin	Dilution	Company
Actin	Mouse monoclonal	1:3000 WB	Sigma Aldrich (A5441)
AKT	rabbit polyclonal	1:1000 WB	Cell Signaling (#4685)
Bcl-2	rabbit monoclonal	1:1000 WB	Epitomics (1017-1)
Bim	rabbit monoclonal		Epitomics (1036-1)
Cdu1	Rabbit polyclonal	1:500 WB; 1:50 IF	Self-made; CnBr affinity purified
Cdu2	Rabbit polyclonal	1:25 IF	Self-made, (Auer, 2014)
CUL1	Rabbit monoclonal	1:1000 WB	Epitomics (2436-1)
ERK	rabbit polyclonal	1:1000 WB	Cell Signaling (#9108)
Fbw7	mouse	1:500	Martin Eilers
FLAG	Mouse monoclonal	1:1000 WB; 1:100 IP 1:400 IF	Sigma Aldrich (F3165, clone M2) Sigma Aldrich (F1804, clone M2)
GFP	mouse monoclonal	1:1000 WB	Santa Cruz Biotech. (sc-9996)
His	Mouse monoclonal	1: 1000 WB	Santa Cruz Biotech. (sc-8036)
HSP60 (bacterial)	Mouse monoclonal	1:1000 WB; 1:400 IF	Santa Cruz Biotech. (sc-57840)
HSP60 (*C.tr*)	Rabbit polyclonal	1:300 IF	Self-made, CnBr affinity purified
Lasu1/Ureb1/MULE	rabbit polyclonal	1:1000 WB	Bethyl lab. (A300-486A)
Mcl-1	Rabbit monoclonal	1:1000 WB; 1:400 IF 1:100 IP	Epitomics (1239-1)
Myc-tag	Mouse monoclonal	1:1000 WB; 1:100 IP	Santa Cruz Biotech. (sc-40)
Nedd8	Rabbit monoclonal	1:1000 WB	Cell Signaling (#2754)
pAKT (Thr308)	rabbit polyclonal	1:1000 WB	Cell Signaling (#2965)
PARP1/2	rabbit polyclonal	1:1000 WB	Santa Cruz Biotech. (sc-7150)
pERK (Thr202/Tyr204)	mouse monoclonal	1:1000 WB	Cell Signaling (#9106)
Survivin	mouse monoclonal	1500 WB	Santa Cruz Biotech. (sc-17779)
Ubiquitin	Mouse monoclonal	1:500 WB	Santa Cruz Biotech. (sc-8017)
USP9	mouse monoclonal	1:1000 WB	Santa Cruz Biotech. (sc-100628)

(WB: western blot; IF: immunofluorescence staining; IP: immunoprecipitation)

Table 2-10: Secondary antibodies

Antibody	Origin	Dilution	Company
ECL™ anti-mouse IgG HRP linked	goat	1:2500 WB	Santa Cruz Biotech. (sc-2005)
ECL™ anti-rabbit IgG HRP linked	goat	1:2500 WB	Santa Cruz Biotech. (sc-2004)
Anti-mouse IgG Cy2™-linked	goat	1:200 IF	Dianova
Anti-rabbit IgG Cy2™-linked	goat	1:200 IF	Dianova
Anti-mouse IgG Cy3™-linked	goat	1:200 IF	Dianova
Anti-rabbit IgG Cy3™-linked	goat	1:200 IF	Dianova
Anti-mouse IgG Cy5™-linked	goat	1:200 IF	Dianova
Anti-rabbit IgG Cy5™-linked	goat	1:200 IF	Dianova

2.1.6 Kits

Table 2-11: Commercial Kits

Kit	Supplier
GeneJET™ Gel Extraction Kit	Thermo Scientific
NucleoBond®PC100	Machery Nagel
AxyPrep™ Plasmid Miniprep Kit	Axygen Biosciences
Ubiquitin thioester/conjugation initiation kit	Boston Biochem

2.1.7 Markers

DNA and protein markers used in this work were GeneRuler™ 1kb DNA ladder (Thermo Scientific), PageRuler™ Prestained Protein ladder (Thermo Scientific) and PageRuler™ Plus Prestained Protein ladder (Thermo Scientific).

2.1.8 Buffers, solutions and media

Table 2-12: Media and solutions for cell culture

Medium	Supplier
RPMI 1640	GIBCO
DMEM	Sigma Aldrich
Opti-MEM® Reduced Serum Medium	GIBCO
DPBS	GIBCO
TrypLE™ Express	GIBCO
Fetal calf serum (FCS)	PAA
stocking media	FCS + 10 % (v/v) DMSO

Table 2-13: Bacterial culture media and solutions

Medium	Ingredients
LB medium	10 g tryptone, 5 g yeast extract, 10 g NaCl (ad 1l dH_2O)
LB Agar	10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar (ad 1l dH_2O)
LB medium salt-free	10 g tryptone, 5 g yeast extract (ad 1l dH_2O)
SOC	2 % (w/v) bacto-tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose
SPG buffer	75 g sucrose, 0.52 g KH_2PO_4, 1.22 g Na_2HPO_4, 0.72 g L-glutamic acid (ad 1l dH_2O), adjust to pH 7.4 and sterile filter
$CaCl_2$ buffer	10 mM Tris HCl pH 7.4, 50 mM $CaCl_2$
TfB1	30 mM $C_2H_3KO_2$, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$, 15 % (v/v) glycerin, pH 5.8
TfB2	10 mM MOPS, 75 mM $CaCl_2$, 10 mM KCl, 15 % (v/v) glycerin, pH 7.0

Table 2-14: Buffers for agarose gel electrophoresis, SDS-PAGE and immunoblotting

Buffer	Ingredients
50x TAE	242 g Tris, 57.1 ml acetic acid, 37.2 g EDTA (ad 1l dH_2O)
SDS upper buffer	0.5 M 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
6 % SDS lower gel solution	for 10 ml: 2.5 ml SDS lower buffer, 2.1 ml 30 % acrylamide, 5.4 ml H_2O, 75 µl 10 % APS, 7.5 µl TEMED
10 % SDS lower gel solution	for 10 ml: 2.5 ml SDS lower buffer, 3.4 ml 30 % acrylamide, 4.1 ml H_2O, 75 µl 10 % APS, 7.5 µl TEMED
12 % SDS lower gel solution	for 10 ml: 2.5 ml SDS lower buffer, 4.0 ml 30 % acrylamide, 3.5 ml H_2O, 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 H_2O, 100 µl 10 % APS, 20 µl TEMED
Laemmli 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
10x SDS-PAGE running buffer	30.3 g Tris, 144.1 g glycine, 10 g SDS
1x Semi Dry Transfer buffer	192 mM glycine, 0.1 % (w/v) SDS, 25 mM Tris, 20 % (v/v) methanol
1x Wet blot Transfer buffer	2.9 g glycine, 5.8 g Tris, 0.37 g SDS, 200 ml Methanol (ad 1l dH_2O)
10x TBS-T	60.5 g Tris, 87.5 g NaCl, 5 ml Tween20, adjust to pH 7.5 with HCl
Coomassie staining solution	44 % methanol, 11% acetic acid, 0.2 % (w/v) coomassie R-250
Coomassie destaining solution	20 % methanol, 7 % acetic acid
blocking solution	5 % (w/v) dry milk powder or BSA in TBS-T
ECL solution 1	100 mM Tris HCl pH 8.6, 2.5 mM Luminol, 0.4 mM p-coumaric acid
ECL solution 2	100 mM Tris HCl pH 8.6, 0.02 % H_2O_2

Table 2-15: Buffers for immunofluorescence staining and immunoprecipitations

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 % Triton-X-100 in 1x PBS
Blocking solution	2 % FCS in 1x PBS
Mowiol mounting medium	2.4 g Mowiol 4-88, 6 g glycerol, 6 ml H_2O, 12 ml 0.2 M Tris HCl pH 8.5
denaturing lysis buffer	50 mM Tris-HCl pH 7.5, 1 % SDS
RIPA buffer	50 mM Tris HCl pH7.5, 150 mM NaCl, 1 % NP40, 1 % Triton-X-100, 0.1 % SDS
Co-IP lysis buffer	10 mM Tris HCl pH 7.5, 200 mM NaCl, 0.5 mM EDTA, 0.2 % (v/v) Triton-X-100, 0.3 % (v/v) NP40,
IP wash buffer	10 mM TrisHCl pH 7.5, 200 mM NaCl, 0.5 mM EDTA

Table 2-16: Buffers for protein purification, binding assay and serum purification

Buffer	Ingredients
NiNTA lysis buffer	50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 % (v/v) Triton-X-100, 1 % (v/v) NP-40
NiNTA wash buffer	50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole
NiNTA stringent wash buffer	50 mM NaH_2PO_4, 300 mM NaCl, 50 mM/150 mM imidazole
NiNTA elution buffer	50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole
STE buffer	10 mM Tris HCl pH 8, 150 mM NaCl, 1mM EDTA
Sarcosyl stock	10 % (v/v) Sarcosyl in STE
Triton stock	10 % (v/v) Triton-X-100 in STE
Elution buffer	50 mM Tris HCl pH 8, 10 mM reduced glutathione
Binding assay buffer	10 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, 0.5 % (v/v) Glycerol, 1 % (v/v) NP-40
0.2 M carbonate buffer	0.2 ml 1 M Na_2CO_3, 1.8 ml 1 M $NaHCO_3$ (ad 10 ml dH_2O), pH 9

Table 2-17: Buffers for *in vitro* ubiquitination and deubiquitination

Buffer	Ingredients
DUB buffer	50 mM Tris HCl pH 7.6, 150 mM NaCl, 2 mM EDTA, 2 mM DTT
Ub-AMC buffer	50 mM HEPES, 0.5 mM EDTA, 1 mM DTT

2.1.9 Enzymes

Polymerases used in this work were MolTaq DNA polymerase (Molzym), iProof™ High-Fidelity DNA polymerase (BIO-RAD), ReproFast Polymerase (Genaxxon) and Phusion Polymerase (Thermo Scientific). For cloning Klenow Fragment, T4 DNA Polymerase, T4 DNA Ligase and restriction enzymes were obtained from Thermo Scientific. UBE1 (E1), UbcH5a (E2) and UCH-L3 enzymes to study protein ubiquitination were purchased from Boston Biochem.

2.1.10 Chemicals

Table 2-18: Inhibitors, inductors and fine chemicals

Compound	Supplier
Acrylamid Rotiphorese Gel 30 (37.5:1)	Roth
Albumin Fraktion V (BSA)	Roth
AMC standard	Boston Biochem
Anhydrotetracycline	Acros Organics
Blasticidine	Fluka
Bradford reagent	Sigma
Complete protease inhibitor EDTA free	Roche
Cyanogen bromide (CnBr) activated sepharose 4B	Sigma
Cycloheximide (CHX)	Sigma
Doxycycline (Dox)	Sigma
DRAQ5	Alexis
Dynabeads® Protein G	Invitrogen
Geneticin G418	Gibco
Glutathione Sepharose™ 4B	GE Healthcare
LY 29004	Cell Signaling
MG-132 in solution	Calbiochem
N-Ethylmaleimide (NEM)	Sigma
PerfectPro Ni-NTA Agarose	5 PRIME
Polyethylenimine (PEI)	Polyscience
Restore™ Plus Western Blot Stripping buffer	Thermo Scientific
TNFα	Cell Signaling
Ub-AMC	Boston Biochem
UO126	Cell Signaling
Zeocin	Invitrogen

Chemicals not listed separately were purchased from Sigma Aldrich, Roth, Serva or Merck Chemicals if not stated otherwise.

2.1.11 Technical equipment

The following technical devices were used in this study: Hera Cell 240i incubator (Thermo), Hera Safe sterile bench (Thermo), Megafuge 1.0R centrifuge (Heraeus), cold centrifuge CT15RE (Himac), PerfectBlue™ 'Semi-Dry'-Elektroblotter (Peqlab Biotechnology), PerfectBlue™ Tank Electroblotter (Peqlab Biotechnology), PerfectBlue™ Dual Gel Twin PAGE chambers (Peqlab Biotechnology), TCS SPE confocal microscope (Leica), TCS SP5 confocal microscope (Leica), DMIL light microscope (Leica), Plate reader infinite 200 (TECAN), Thermal cycler 2720 (Applied Biosystems), pH Electrode SenTix (WTW), Scanjet G4010 (HP), Shaker TR125 (Infors HT), Sonifier 250 (Branson), Thermo mixer comfort (Eppendorf), Spectrophotometer Ultrospec 3100 pro (Amersham Bioscience), NanoDrop 1000

spectrophotometer (Peqlab Biotechnology), FACSaria III (BD), Chemiluminescence camera system (Intas), electric balance ABS-80-4 (Kern), Gene Pulser™ electroporator (Biorad).

2.1.12 Software

Windows 7, Office 2010, EndNote X2, Photoshop CS4, Corel Draw X5, LAS AF confocal microscopy software, ImageJ, LabImage Chemostar (Intas), Codon Code Aligner 3.7.1, Serial Cloner 2.5, GraFit (Version 5.0.13, Erithacus Software Ltd.)

2.2 Methods

2.2.1 Bacterial culture techniques

Growth of *E. coli*

E. coli were grown on LB agar plates supplemented with the appropriate antibiotics over night at 37 °C or in antibiotics containing LB media at 37 °C and 190 rpm.

Stocking of *E. coli*

750 µl of an *E. coli* overnight culture were mixed with 150 µl glycerol and stored at -80 °C.

Preparation of chemo-competent *E. coli*

To obtain chemo-competent *E. coli* 1 ml of overnight culture was diluted 1:100 in fresh LB media and grown at 37 °C and 190 rpm to an optical density (OD_{600}) of 0.5. The bacteria were pelleted at 1500 g for 5 min at 4 °C. The pellet was resuspended in 30 ml of pre-cooled TfB I-buffer and incubated for 15 min on ice. After additional centrifugation the bacterial pellet was suspended in 8 ml pre-cooled TfB II-buffer and incubated for 15 min on ice. Aliquots of 200 µl were prepared and stored at -80 °C.

Transformation of chemo-competent *E. coli*

A 200 µl aliquot of frozen chemo-competent *E. coli* were thawed on ice, mixed with 0.68 µl β-mercaptoethanol and incubated for 10 min. Recombinant vector DNA was added to the bacteria and incubated for further 30 minutes on ice. The bacteria were heat-shocked for 90 sec at 42 °C in a water bath and directly incubated on ice for 2 min. 800 µl of pre-warmed SOC-media was added to the mixture and the transformed bacteria were incubated for 2 h at 37 °C and 190 rpm. Bacteria were plated on antibiotics-containing LB agar plates.

Preparation of electro-competent *E. coli*

A liquid overnight culture of *E. coli* was diluted 1:20 in 500 ml salt-free LB media and grown to the OD_{600} of 0.8. The cells were washed with 500 ml ice cold diH_2O and resuspended in 2.5 ml cold 10 % glycerol. Aliquots of 100 µl were prepared and stored at -80 °C.

Transformation of electro-competent *E. coli*

An aliquot of frozen electro-competent *E. coli* was thawed on ice. The bacteria were mixed with the recombinant vector DNA and transferred into a pre-cooled electroporation cuvette. The conditions for electroporation were set to 200 Ω, 2.4 kV and 25 µF. The electroporation was performed with the Gene Pulser™ electroporator. After electroporation the bacteria were mixed with 800 µl pre-warmed SOC media and incubated for 2 h at 37 °C and 190 rpm. The bacteria were plated on LB agar plates containing the appropriate antibiotic for selection (see Table 2-19).

Table 2-19: Antibiotics working concentrations

Antibiotics	working concentration
Ampicillin	100 µg/ml
Kanamycin	50 µg/ml
Chloramphenicol	30 µg/ml
Tetracycline	10 µg/ml
Anhydrotetracycline	10-100 ng/ml

IPTG-induced protein overexpression

A liquid *E. coli* BL21 overnight culture containing 0.25 % Glucose was diluted 1:20 in fresh LB media supplemented with the appropriate antibiotics. The culture was grown at 37 °C and 190 rpm until an OD_{600} of 0.5 was reached. The protein expression under control of a lac-repressor was induced by addition of isopropyl-β-D-thiogalactopyranosid (IPTG) to a final concentration of 0.5 mM to the bacterial culture. Upon IPTG addition the bacteria were incubated at 30 °C and 190 rpm for 3 h. The bacteria were collected by centrifugation for 10 min at 4000 g and 4 °C and stored at -20 °C.

2.2.2 Cell culture methods and infections

Cell line cultivation

Cells were cultured in 75 cm^2 flasks at 37 °C and 5 % CO_2 and passaged every two to three days. For passaging cells were washed once with PBS and incubated in 1 ml Trypsin at 37 °C and 5 % CO_2 until all cells detached. Fresh cell culture medium supplemented with 10 % FCS was added to the suspension and cells were split into a new flask or seeded in multi-well plates or cell culture dishes for experiments. Media and supplements used for the cultivation of the cell lines are listed in Table 2-1.

Preparation of cell culture stocks

For preparation of cell stocks an 80 % confluent 75 cm^2 flask was washed once with PBS and cells were detached with 1 ml of trypsin. Cells were mixed with 5 ml of cell culture media and transferred to a 15 ml tube to pellet them by centrifugation at 800 g for 5 min. Cell pellet was resuspended in 5 ml stocking media and portioned into 1 ml aliquots which were gradually cooled to -80 °C in isopropanol.

Transfection of plasmid DNA

Plasmid DNA-transfection of HeLa229 and 293T cells was performed with polyethylenimine (PEI). A 1 mg/ml stock solution of PEI in H_2O neutralized with HCl was prepared and sterile filtered. Aliquots were stored at -20 °C. Cells were grown to a confluency of 60-70% in 12 well plates (about $3x10^5$ cells) or 10cm Ø cell culture dishes (about $4.2x10^6$ cells). For transfection of $3x10^5$ cells mixtures of 50 µl Opti-MEM® with 1 µg DNA and 50 µl Opti-MEM® with 3 µl PEI were prepared. After an incubation of 5 min at room temperature (RT) the two mixtures were combined and incubated for further 25 min at RT. For transfection of cells grown in cell culture dishes the amount of PEI and plasmid DNA was adjusted corresponding to the cell number. The cell culture media was replaced by 400 µl fresh 5 % FCS containing media in 12 well plates or 5 ml in 10 cm Ø dishes. The mixture was added dropwise to the cells and solution was mixed gently. Cells were incubated at 37 °C and 5 % CO_2 and medium was replaced by fresh 5 % FCS containing medium 5-7 h post transfection.

Selection of stable cell lines

Cells transfected with plasmids encoding for antibiotics resistance could be used to create stable cell lines. Cells were transfected with plasmid DNA using PEI as described before. 24 h post transfection media was replaced by 10 % FCS containing cell culture medium supplemented with the appropriate antibiotic. Cells transfected with a pcDNA3 plasmid were selected with 500 µg/ml G418 to obtain stable overexpression cell lines. To create stable inducible overexpression cell lines the pcDNA6TR and pcDNA4TO plasmids of the TRex system (Invitrogen) were used. Cells were first transfected with the tetracycline repressor encoding plasmid pcDNA6TR and were selected with 5 µg/ml Blasticidin. Afterwards, the pcDNA6TR cell line was transfected with the pcDNA4TO plasmid encoding for the protein of interest under control of a tetracycline-regulated promoter and selection was performed with 300 µg/ml Zeocin™. Stable cell lines were cultivated in presence of the appropriate antibiotic as indicated in Table 2-1. Protein expression in TRex cell lines could be induced by addition of 1 µg/ml Doxycycline or 50 µg/ml anhydrotetracycline.

Transfection of siRNA

HeLa229 cells grown to a confluency of 60 % were transfected with siRNA in a final concentration of 120 nM using PEI. The transfection protocol is equivalent to the plasmid DNA transfection. Cells transfected with siRNA targeting USP9X were sub-cultured 24 h post transfection and used for infection experiments 48 h post transfection. HeLa229 cells transfected with Mcl-1-targeting siRNA were infected 24 h post transfection.

***C. trachomatis* infection**

Cells were infected with *C. trachomatis* at a confluency of 70 %. The appropriate amount of chlamydial EBs to reach the desired multiplicity of infection (MOI) was mixed in 5 % FCS containing cell culture media and added to the cells. The cell culture media was replaced by the infection media and incubated at 35 °C and 5 % CO_2. After 2.5 h of infection the infection media was replaced by fresh 5 % FCS containing media for further cultivation.

Infection with *C. pneumoniae*

For infection of adherent epithelial cells grown to a confluency of 70 % the cell culture media was replaced by the infection media consisting of 5 % FCS cell culture media and the appropriate amount of *C. pneumoniae* EBs. The EB particles were centrifuged onto the cells by centrifugation at 910 g and 37 °C for 30 min and further incubated at 35 °C and 5 % CO_2. 2.5 h post infection (hpi) the infection media was replaced by cell culture media containing 2 µg/ml cycloheximide (CHX).

Preparation of *Chlamydia* stocks

Chlamydia can only be cultivated in cell culture due to their obligate intracellular life style. Therefore, the infectious EBs have to be isolated out of *Chlamydia*-infected cells and are stored in aliquots at -80 °C. For *C. trachomatis* or *C. pneumoniae* stock preparation a 75 cm² flask of HeLa229 cells was infected at MOI 1 and incubated at 35 °C and 5 % CO_2 for 48 h or 72 h, respectively. The infected cells were collected with a rubber scraper and transferred to a flacon tube with sterile glass beads. Cells were mechanically ruptured by vortexing in presence of glass beads and 1 ml of the lysate was used to infect one fresh 150 cm^2 dish of HeLa229 cells. After further incubation for the duration of one developmental cycle of *Chlamydia* cells were again collected and lysed by vortexing with sterile glass beads. The cell debris was removed by 10 min centrifugation at 1500 g and 4 °C and supernatant containing chlamydial EBs was further centrifuged at 35,000 g and 4 °C for 30 min. The chlamydial pellet was washed once with ice cold SPG buffer and finally resuspended in 1 ml of SPG buffer per infected 150 cm^2 dish. To disrupt clumps of chlamydial EBs the suspension was passed through a 20G and 18G needle. Aliquots were prepared and stored at -80 °C. For each infection experiment a freshly thawed aliquot was used and diluted in RPMI1640 + 5 % FCS to reach the desired MOI.

After each stock preparation the required volume of chlamydial EB suspension to reach a MOI of 1 in infection experiments had to be determined. To define the volume necessary to get an infection of every cell deriving from one inclusion forming unit (IFU), HeLa229 cells were seeded in 12-well plates and infected with increasing volumes of *C. trachomatis* or *C. pneumoniae* stocks, as described before. Degree of infection was monitored by bright field microscopy or immunofluorescence staining and concentration of chlamydial stocks was calculated.

Infectivity assay

To investigate whether the expression of the FLAG-tagged form of ChlaDUB1 as well as the integration of the selection cassette into the chlamydial genome alters the progression of the chlamydial developmental cycle and the ability to form infectious progeny an infectivity assay was performed. HeLa229 cells were infected with *Chlamydia* at a MOI of one and incubated for 48 h at 35 °C and 5 % CO_2. The infected cells were ruptured with sterile glass beads and different volumes of the lysate were transferred to uninfected HeLa229 cells for a second round of infection. Lysates of the primary as well as progeny infection 24 hpi were prepared and analyzed by immunoblot.

Inhibitor treatment

The impact of different compounds on chlamydial growth and ability to block host cell apoptosis was tested during infection. For inhibition of ERK activation and signaling, cells were treated with 15 and 30 µM UO126 (Cell Signaling) in DMSO upon 10 hpi. The activation of PI3K and signaling was blocked by LY29004 at concentrations of 15 and 30 µM in DMSO added to the cells 10 hpi. Control cells were treated with solvent alone. To investigate the ability of inhibitor treated infected cells to stabilize Mcl-1 lysates were prepared 24 hpi and analyzed by immunoblot. The ChlaDUB1 inhibitor CYN312 was dissolved in DMSO and added at the indicated time point to the infected cells at concentrations ranging from 10 to 20 µM. Control cells were treated with DMSO or control compound 7. Chlamydial growth was monitored by bright field microscopy or GFP fluorescence signal before lysates were prepared. Effect of CYN312 treatment on chlamydial growth, *Chlamydia*-mediated Mcl-1 stabilization and apoptosis resistance was studied by immunoblot.

Apoptosis induction

To induce apoptosis cells were treated with 50 ng/ml tumor necrosis factor α (TNFα) in presence of 5 µg/ml CHX. The addition of CHX is required to block translation of anti-apoptotic proteins and to provoke apoptosis. Apoptosis was induced 20 hpi by addition of TNFα and CHX containing cell culture medium for the indicated time points. The degree of apoptosis induction was monitored by bright field microscopy and subsequently analyzed by anti-PARP immunoblot.

Transformation of *C. trachomatis*

C. trachomatis can be transformed with artificial plasmid DNA via calciumchloride. For transformation of *Chlamydia* $2x10^7$ IFU were mixed with 12 µg of plasmid DNA in 200 µl sterile $CaCl_2$ buffer and incubated for 30 min at RT. In the meantime $1x10^7$ McCoy cells were trypsinized and washed twice with PBS by centrifugation at 800 g for 5 min. The cell pellet was finally resuspended in 200 µl sterile $CaCl_2$ buffer. The bacterial and cell suspension was combined and incubated at RT for 20 min with

occasional mixing. Finally, the mixture was added to 10 ml of RPMI + 10 % FCS and cultivated in a 75 cm^2 flask at 35 °C and 5 % CO_2 for 48 h.

The plasmids used for transformation of *Chlamydia* contain a gene encoding for β-lactamase mediating resistance towards penicillin. To select the *Chlamydia*, 2 units (U)/ml of Penicillin G (Sigma Aldrich) were added to the medium after infection. Untransformed *Chlamydia* turn persistent in presence of penicillin and are not able to replicate and complete their developmental cycle to form infectious progeny. On the contrary, transformed *Chlamydia* encode for the β-lactamase and are able to overcome the persistence pressure and grow in presence of penicillin. The *Chlamydia* were passaged every 48 h post infection by mechanical rupture of the host cell with glass beads and infection of fresh McCoy cells with the lysate. 5 h post infection the media was replaced by fresh RPMI + 10 % FCS supplemented with 2 U/ml Penicillin G. The selection with 2 U/ml Penicillin G was continued until active inclusions were visible. Afterwards, a more stringent selection with increasing concentrations of Penicillin G was performed. The selection of replicating plasmids was performed with 30 U/ml of Penicillin G whereas *Chlamydia* transformed with the suicidal plasmid forced to perform homologues recombination of the selection cassette into the chlamydial genome were selected with up to 10 U/ml of Penicillin G.

***C. trachomatis* single cell clone sorting**

To obtain single cell clones of plasmid-transformed *C. trachomatis* infected cells were sorted by flow cytometry. A confluent HeLa229 T75 flask was infected with the *C. trachomatis* pool with a MOI of 0.25. 30 hpi the cells were trypsinized and resuspended in media. If the transformed *Chlamydia* expressed GFP, infected cells were sorted by their GFP-positive signal. Cells infected with transformed *Chlamydia* that did not express GFP were gated by their increased granularity compared to uninfected cells. Single cells infected with *Chlamydia* were sorted into 96 Well plates containing 50 µl media. After single cells attached to the bottom the number of cells in the well as well as infection was checked by bright field microscopy. Afterwards, $3x10^4$ HeLa229 cells were added to the well and media was supplemented with 2 U/ml PenG. As soon as actively replicating *Chlamydia* were observed EBs were transferred to fresh HeLa229 cells and multiplied.

2.2.3 DNA methods

Genomic DNA isolation

Genomic DNA of *C. trachomatis* was isolated with the illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare) according to the manufacturers' instructions. One 50 µl aliquot of a *Chlamydia*-stock was thawed on ice and used for genomic DNA isolation. Genomic DNA was finally eluted with 50 µl dH_2O and concentration was measured.

Plasmid isolation

Isolation of plasmid DNA out of *E. coli* was performed using the AxyPrep™ Plasmid Miniprep Kit (Axygen) or NucleoBond®PC100 Midiprep Kit (Machery Nagel). For a miniprep plasmid isolation 3-5 ml of liquid bacterial overnight culture was used whereas midiprep plasmid isolation was performed from 40 ml of overnight culture. The plasmid isolation was executed following the manual instructions.

Polymerase chain reaction (PCR)

PCR reactions for cloning were performed using the ReproFast polymerase (Genaxxon) or iProof™ polymerase (BIO-RAD) in the following 50 µl reaction mixes:

x µg	template DNA	x µg	template DNA
1 µl	frw primer	1 µl	frw primer
1 µl	rev primer	1 µl	rev primer
1 µl	dNTPs	1 µl	dNTPs
5 µl	10X ReproFast buffer	10 µl	5X iProof™ buffer
0.25 µl	ReproFast polymerase	0.5 µl	iProof™ polymerase
add 50 µl	dH_2O	add 50 µl	dH_2O

After an initial denaturation step at 94 °C for 5 min, 35 cycles at 94 °C for 30 sec for denaturation, annealing at 50-60 °C (depending on the melting temperature of the oligonucleotide primers) for 30 sec and elongation at 72 °C were conducted. The elongation time was calculated according to the length of template and used polymerase (ReproFast: 1 min/kb; iProof™: 30 sec/kb). After a final elongation step at 72 °C for 5 min the PCR reaction was gradually cooled down to 4 °C. PCR products were analyzed on agarose gels containing Intas HD Green (Intas) and visualized under UV light.

Colony PCR

In order to screen for *E. coli* transformed with the desired plasmid, a colony-PCR was performed. Bacterial material from *E. coli* single colonies grown on LB agar plates was resuspended in 20 µl of dH_2O and boiled for 10 min at 94 °C. Cellular debris was pelleted for 2 min at 15,000 g and 2.5 µl of the supernatant was used as template for the PCR reaction using a MolTaq polymerase in the following mixture:

2.5 µl	template DNA
0.5 µl	frw primer
0.5 µl	rev primer
0.5 µl	dNTPs
2.5 µl	10X MolTaq buffer
0.25 µl	MolTaq polymerase
add 25 µl	dH_2O

Initial denaturation was performed at 94 °C for 5 min followed by 25 cycles at 94 °C for 30 sec, 50-60 °C for 30 sec for annealing (temperature dependent on melting temperature of oligonucleotide primers) and 1 min/kb elongation at 72 °C dependent on template length. After a final elongation step at 72 °C for 5 min the PCR reaction was cooled down to 4 °C. Presence of PCR product was analyzed by agarose gel electrophoresis and visualization using Intas HD Green (Intas) under UV light.

Overlap-PCR

To fuse two DNA fragments without digestion with endonucleases and subsequent ligation the overlap-PCR method was used. Initially, each part of the construct was amplified separately from its respective template using the corresponding primer pair and purified, see Figure 2.1 . The primers consist of 14 nucleotides of each fragment which should be fused to each other.

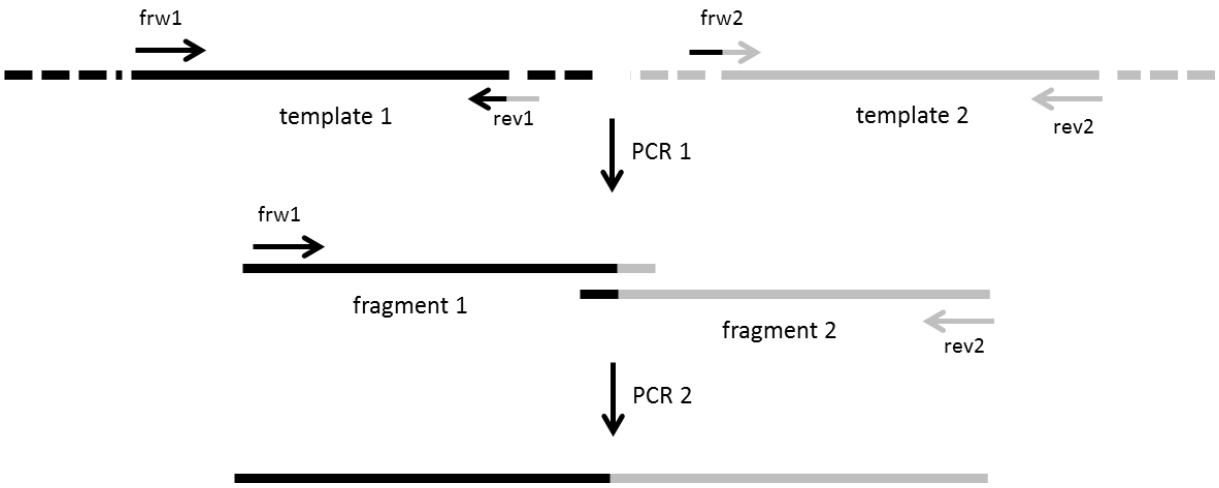

Figure 2.1: Schematic overview of the overlap-PCR method. DNA fragments were amplified separately by PCR with indicated corresponding primer pairs. The rev1 and frw2 primers consist of 14 extra nucleotides corresponding to the fragment which should be fused to the template DNA. After PCR reaction 1 the fragments were purified and used as template for PCR reaction 2. The fragments can anneal to each other by their 14 nucleotide overlap in PCR reaction 2 and are finally amplified using the primer pair frw1 and rev2.

The PCR reaction 1 to generate the single fragments was performed as described before using iProof™ or ReproFast polymerase dependent on the fragment length. Equimolar amounts of all fragments were mixed for PCR reaction 2 which was performed using Phusion polymerase.

x µl	fragment 1
x µl	fragment 2
1 µl	dNTPs
10 µl	5X Phusion buffer
0.5 µl	Phusion polymerase
add 50 µl	dH_2O

After an initial denaturation at 94 °C for 5 min 20 cycles of 30 sec denaturation at 94 °C, 30 sec of annealing at 50 °C and elongation at 72 °C followed. The elongation time was calculated with 20 sec/kb template length. After the initial 20 cycles 1 µl of the frw1 and rev2 was added to the reaction and 15 additional cycles were performed. The PCR 2 product was separated by agarose gel electrophoresis and the correct fusion product was isolated out of the gel and used for further cloning steps.

Site-directed mutagenesis

The QuickChange site-directed mutagenesis was used to generate defined point mutations in plasmid constructs. Oligonucleotide primers were designed with a nucleotide exchange in the middle of the sequence, each complementary to opposite strands of the plasmid and with an annealing temperature of 60 °C. The primers were extended in a PCR reaction using Phusion polymerase by which a new mutated plasmid with staggered nicks was generated. The Phusion polymerase was inactivated by 5 min incubation at -80 °C. The original template DNA was digested using 2 units DpnI which was added directly into the PCR reaction and incubated for 1 h at 37 °C. DpnI endonuclease is specific for methylated DNA but does not cut newly synthesized DNA in the PCR reaction mixture. DpnI was inactivated by a 20 min incubation step at 80 °C. The newly synthesized nicked vector DNA containing the desired mutations was directly used to transform chemo-competent *E. coli* XL1blue and site-directed mutagenesis was confirmed by sequencing (SeqLab, Göttingen, Germany).

Agarose gel electrophoresis

DNA samples were mixed with 6x loading dye and separated on a 1 % agarose gel (TAE buffer) containing Intas HD Green (Intas) by applying 120 V. DNA separation was visualized under UV light.

Gel extraction and PCR purification

PCR products or restricted DNA were purified using the GeneJet™ Gel Extraction Kit (Thermo Scientific). In order to make sure that only one desired DNA fragment was purified the PCR reaction or DNA digestion was separated in an agarose gel and the correct band was excised under UV light. The DNA was extracted out of the agarose gel using the GeneJet™ Gel Extraction Kit (Thermo Scientific).

Restriction and ligation

Amplified DNA fragments or vector DNA were digested using restriction endonucleases from Thermo Scientific. If two restriction enzymes were used the buffer compatibility was checked using the online tool Double digest (http://www.thermoscientificbio.com/webtools/doubledigest). Ligation of vector and insert was performed using T4 DNA ligase (Thermo Scientific). The vector/insert ratio was set to 1:5. To obtain optimal ligation efficiency equimolar amounts of insert and vector were calculated with the Cranenburgh's formula (Cranenburgh, 2004).

DiSecTriSec cloning

The DiSecTriSec cloning method relies on the generation of complementary di- or trinucleotide sticky ends at the linearized vector and PCR-amplified insert. The vector is digested with two suitable restriction enzymes and the sticky end overhangs were altered by nucleotide fill in using Klenow fragment. The sticky end nucleotide overhangs of the DNA insert were generated using the 3'-5' exonuclease activity of the T4 DNA polymerase. The resulting di- or trinucleotide overhangs are not complementary to each other by which a self-ligation of the vector is avoided. The vector DNA (1µg) was digested with the restriction enzymes BamHI and HindIII and purified. The cleaved vector was incubated with 1 mM dATP and dGTP together with 4 U Klenow fragment for 15 min at RT followed by heat inactivation at 75 °C for 15 min. The DNA fragment amplified by PCR was incubated with 2 U T4 DNA polymerase in presence of 2 mM dCTP for 30 min at 12 °C and heat inactivated for 10 min at 75 °C. Modified vector and insert were purified and ligation was performed according to Cranenburgh's formula.

2.2.3.1 Cloning of pCdu1(C345A)-FLAG::SW2

The pGFP::SW2 plasmid is able to replicate inside *E. coli* as well as in *C. trachomatis*. The strong neisserial promoter drives the expression of the GFP-CAT fusion protein in *E. coli* and *C. trachomatis* and can be used to overexpress any gene of interest in *Chlamydia*. To create a dominant-negative mutant of ChlaDUB1 in *C. trachomatis* a plasmid mediating the overexpression of ChlaDUB1(C345A)-FLAG mutant enzyme was cloned. In order not to change the distance between the TATA-box of the promoter and the start codon the ChlaDUB1(C345A)-FLAG gene was cloned into the plasmid using overlap PCR. The ChlaDUB1(C345A)-FLAG gene was amplified from the pcDNA3/Cdu1(C345A)-FLAG

construct using the primers pCdu1::SW2 P1 and P2 generating a DNA fragment with a 14 bp overhang complementary to the neisserial promoter region and a SalI cutting site. With the primer pair pCdu1::SW2 P3 and P4 the pGFP::SW2 plasmid was amplified lacking the GFP-CAT fusion gene, but with a 14 bp overhang complementary to the ChlaDUB1 sequence. The two fragments were fused by overlap PCR and the plasmid was circularized by SalI digestion and ligation, see Figure 2.2. The correct sequence was confirmed by sequencing and the expression of ChlaDUB1(C345A)-FLAG in *E. coli* was tested by immunoblot analysis before the construct was used to transform *C. trachomatis*.

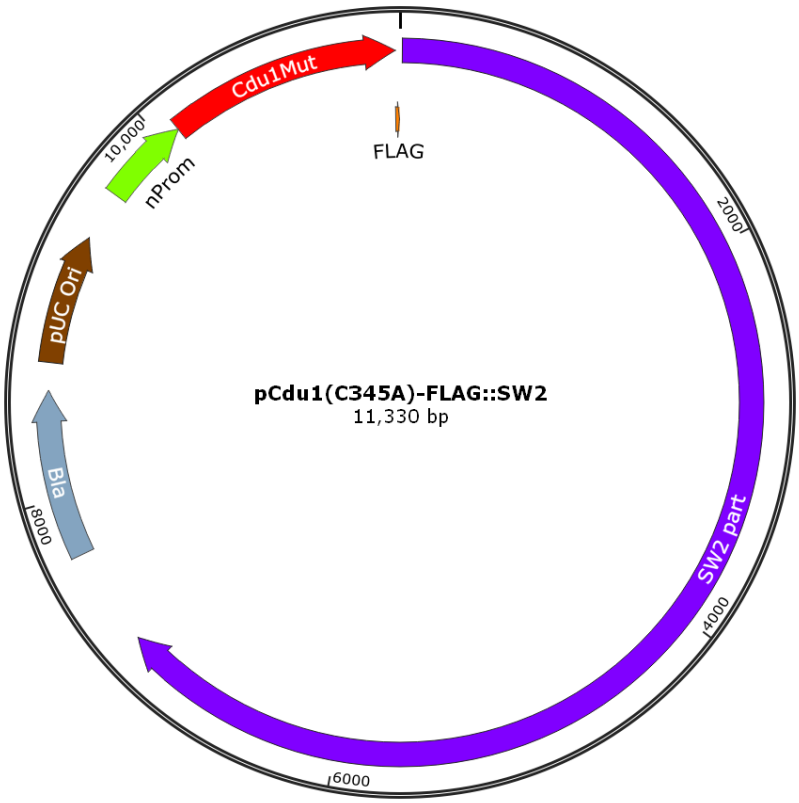

Figure 2.2: Vector map of the pCdu1(C345A)-FLAG::SW2 plasmid. The plasmid was constructed by overlap PCR. The Cdu1(C345A)-FLAG fragment with an overlap to the neisserial promoter was amplified from pcDNA3/Cdu1(C345A)-FLAG plasmid and the SW2-backbone together with the bla gene and the neisserial promoter with an overlap complementary to the Cdu1 gene was amplified from the pGFP::SW2 vector. Both fragments were fused by overlap PCR to obtain the pCdu1(C345A)-FLAG::SW2 plasmid.

2.2.3.2 Cloning of pTet::SW2 constructs

The pTet::SW2 plasmid was cloned by Daniela Auer, a master student who was supervised by me (Auer, 2014). The theoretical work and construct design was performed by me. For construction of the pTet::SW2 plasmid the constitutively active neisserial promoter was replaced by a tetracycline-inducible promoter. The selection cassette of the pGFP::SW2 plasmid was amplified with the primer pair pTet P1+P2 to create a 14 bp overhang complementary to the Tet-inducible promoter sequence. The Tet-inducible promoter was amplified from the plasmid pdCas9-bacterial (pdCas9-bacteria was a gift from Stanley Qi (Addgene plasmid # 44249)) with the primer pTet P3 and P4 with a 14 bp overhang

of the selection cassette. The two fragments were fused by overlap-PCR. The newly synthesized DNA-fragment and the pGFP::SW2 vector were digested with MluI and SalI. The SW2-backbone part of pGFP::SW2 was eluted from the gel and ligated with the Tet-promoter PCR product to obtain the pTet::SW2 vector (Figure 2.3).

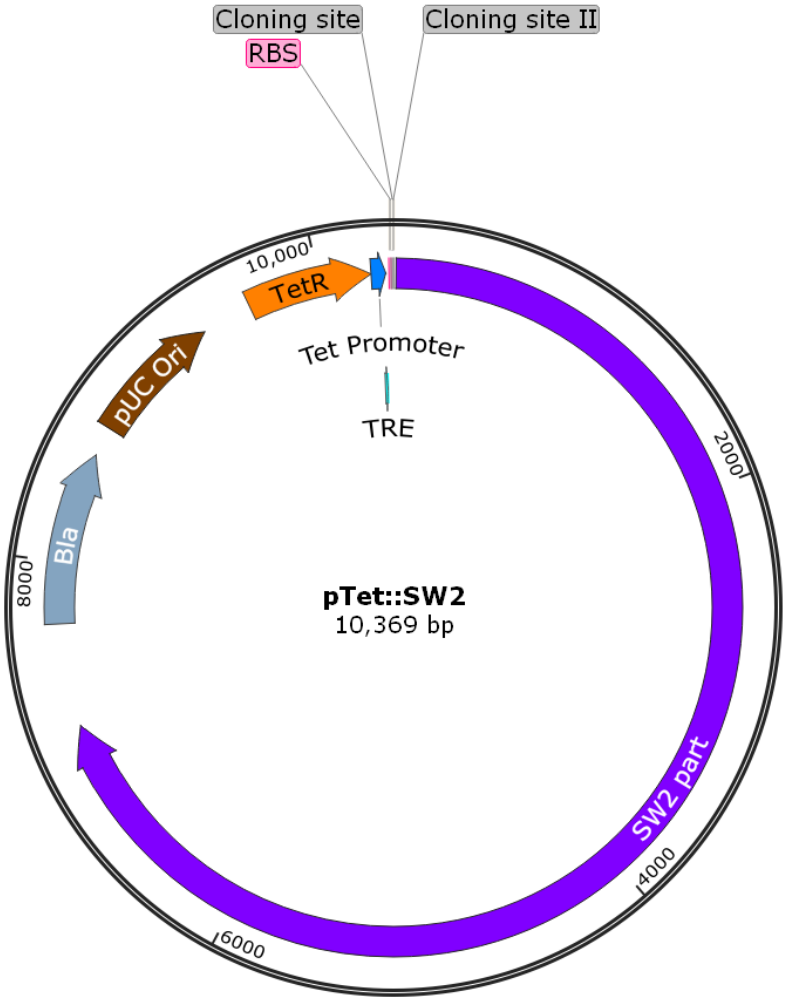

Figure 2.3: Vector map of the pTet::SW2 plasmid. The pTet::SW2 plasmid contains a tetracycline-inducible promoter combined with a DiSecTriSec compatible cloning site for integration of any gene of interest under control of the tet-inducible promoter. The vector encodes for the *bla* gene for selection. The vector contains the pUC ori for replication in *E. coli* and the SW2 backbone with the chlamydial ori for replication in *Chlamydia*.

Any DNA-fragment can be cloned into the pTet::SW2 plasmid by DiSecTriSec-cloning using the SpeI/SalI endonuclease cutting sites. With this method constructs suitable for the tetracyclin-inducible overexpression of GFP-CAT fusion protein (pTet GFP frw/pTet GFP rev), ChlaDUB1-FLAG (pTet Cdu1 frw/pTet FLAG rev), ChlaDUB1(C345A)-FLAG (pTet Cdu1 frw/pTet FLAG rev), ChlaDUB1ΔCD-FLAG (pTet Cdu1 frw/pTet FLAG rev) and ChlaDUB2(C282A)-FLAG (pTet Cdu2 frw/pTet FLAG rev) were generated using the pcDNA3 constructs as templates. After sequencing the plasmids were transformed into *E. coli* JM110 and used to transform *C. trachomatis*.

2.2.4 Protein biochemical methods

SDS-PAGE

Protein samples were prepared in 2x Laemmli buffer and denatured by boiling at 94 °C for 10 min. Sodium dodecyl sulphate (SDS) complexes with the denatured proteins and negatively charges them proportional to their mass. The protein samples were separated under denaturing conditions in presence of SDS in 6-12 % polyacrylamide gels by gel electrophoresis (SDS-PAGE).

Western blotting and immunoblot

Proteins separated by SDS-PAGE were transferred to a PVDF membrane for immunoblotting. PVDF membranes were activated in Methanol for 1 min and directly transferred to Semi-dry transfer buffer. Sandwiches consistent of Whatman paper, PVDF membrane, acrylamide gel and Whatman paper were prepared and proteins were blotted in a semi-dry apparatus for 2 h at 1 mA/cm^2. For transfer of high molecular weight proteins blotting was performed in a wet blot apparatus at 200 mA overnight in wet blot transfer buffer.

For immunoblotting membranes were incubated in milk or BSA blocking solution for 1 h at RT. Subsequently membranes were incubated with primary antibodies diluted in blocking solution over night at 4 °C. Blots were washed three times for 10 min with TBS-T prior to incubation with horseradish-peroxidase (HRP)-coupled secondary antibody. After one hour incubation time the membranes were washed three times for 10 min with TBS-T and ECL-mix was added. Chemiluminescece was detected by the photo-sensitive Intas imager system. Quantification of proteins was performed by ImageJ software.

Coomassie staining

Proteins separated by SDS-PAGE were stained by Coomassie blue in acrylamide gels. After gel electrophoresis gels were incubated for 30 min in Coomassie staining solution. For destaining, gels were washed with Coomassie destaining solution with repeated buffer exchange until protein bands were visible.

Indirect immunofluorescence staining

Cells were grown on cover slips and fixed with 4 % PFA/Sucrose for 30 min at RT. Cells were permeabilized by incubation in permeabilization solution for 40 min at RT and subsequently incubated in blocking solution for 1 h at RT. Primary antibodies were diluted in blocking solution and incubated with cover slips for 1 h at RT. Cover slips were washed three times with PBS and incubated with fluorescence dye-conjugated secondary antibodies diluted in blocking solution for 1 h at RT in the dark. After three times washing with PBS DNA was stained with DAPI diluted 1:3000 in PBS for 30 min at RT in the dark. Cover slips were again washed three times with PBS and embedded in

Mowiol mounting media on glass slides. After drying overnight slides were analyzed by confocal microscopy.

Cytotoxicity assay

Cell viability was analyzed by AnnexinV/7AAD staining analyzed by flow cytometry. *C. trachomatis*-infected cells treated with CYN312 or control compounds were trypsinized and pelleted by centrifugation. Cells were resuspended in Annexin binding buffer supplemented with AnnexinV-APC and 7AAD and stained for 15 min in the dark at room temperature. Cells were immediately analyzed by flow cytometry.

Binding assay

The direct interaction of ChlaDUB1 and Mcl-1 was confirmed by an *in vitro* binding assay with purified recombinant proteins. 5 µg GST-Mcl-1 was bound to Glutathione sepharose by incubation in binding buffer at 4 °C for 2 h. Sepharose was washed with binding buffer and 5 µg of His-tagged ChlaDUB1, ChlaDUB2 or GroEL were added to the samples, respectively. After 1 h incubation at 4 °C the probes were washed extensively and proteins were eluted by boiling in 2x Laemmli at 94 °C for 7 min. Interaction of Mcl-1 with ChlaDUB1 or control proteins was analyzed by SDS-PAGE and immunoblot.

Antibody serum affinity purification

To remove serum components and unspecific antibodies from the antibody serum obtained from immunoglobe® after immunization we performed affinity purification using antigen columns. The buffer of the antigen was exchanged to 0.2 M carbonate buffer. 0.4 g cyanobromide (CnBr) beads were swelled for 10 min in 1mM HCl followed by one washing step with 0.2 M carbonate buffer. Activated CnBr beads were immediately mixed with 300 µg antigen and incubated for 1 h at RT followed by 4 °C overnight. Beads were washed twice with 0.2 M carbonate buffer and remaining coupling sites were blocked with 100 mM Ethanolamine for 1 h at RT. After three more washing steps with 0.2 M carbonate buffer beads were equilibrated in 500 mM NaCl in PBS and mixed with 10-20 ml serum. Affinity matrix and antibody serum was incubated at 4 °C overnight on a rotary shaker. Beads were harvested by centrifugation at 500 g for 5 min and 4 °C and washed twice with 20 ml 500 mM NaCl in PBS. The beads were transferred into a column and washing was continued with 150 ml 500 mM NaCl in PBS. Antigen-specific antibodies were eluted under low pH with 0.2 M Glycine pH 2.5 and immediately neutralized by addition of 200 µl/ml 1 M Tris HCl/pH 7.5. Fractions of antibody elution were combined and concentrated using Amicon ultra centrifugation filter units with a molecular weight cut off of 10 kDa. Antibody fraction was mixed with one volume of 87 % glycerol and stored at -20 °C.

2.2.4.1 Analyzing ubiquitination/deubiquitination

Ub-AMC assay

ChlaDUB1 deubiquitinating enzyme activity was visualized by cleavage of the fluorogenic ubiquitin derivate Ub-7-amido-4-methylcoumarin (Ub-AMC). Release of the fluorophore AMC by deubiquitinaing enzymes can be monitored at Ex_{380}/Em_{460nm}. Purified recombinant enzymes ChlaDUB1 and UCH-L3 were pre-incubated in Ub-AMC buffer for 30 min to obtain complete enzyme activity. 15 nM of ChlaDUB1 and 0.4 nM of UCH-L3 were incubated with inhibitors in the indicated concentrations for 30 min at 37 °C. Enzyme activity was displayed by addition of 200 nM Ub-AMC in Corning 96 black well plates and immediate analysis at TECAN infinite M200 plate reader. Definition of Michaelis constant K_m was conducted with 5 nM ChlaDUB1 and CYN312 (15 µM)-treated ChlaDUB1 or 0.4 nM UCH-L3 in 96 black well plates with increasing substrate concentration ranging from 200 nM to 4000 nM Ub-AMC. Experimental procedure was same as described before. Data analysis for IC_{50} and K_m value calculation was performed with the program GraFit (Version 5.0.13, Erithacus Software Ltd.).

***In vitro* ubiquitination**

To obtain poly-ubiquitinated Mcl-1 suitable as substrate for an *in vitro* DUB assay purified recombinant Mcl-1 was incubated with enzymes belonging to the ubiquitin ligation cascade and the Mcl-1 specific E3 ubiquitin ligase HectH9. 2.5 mg GST-Mcl-1 was mixed with 200 ng Ube1 (E1), 150 ng UbcH5c (E2), 500 ng HectH9ΔN (E3) (Adhikary et al., 2005) and 50 µg Ubiquitin in ubiquitin conjugation initiation buffer (Boston Biochem). Reaction was started by addition of Mg-ATP solution and incubated for 4 h at 37 °C. Successful poly-ubiquitination of Mcl-1 was visualized by SDS-PAGE and immunoblot using anti-ubiquitin or anti-Mcl-1 antibody. Poly-ubiquitinated Mcl-1 was immediately used as substrate in the *in vitro* DUB assay.

***In vitro* DUB assay**

Equal amounts of poly-ubiquitinated Mcl-1 were mixed with recombinant His-ChlaDUB1 (20 nM), His-ChlaDUB1(C345A) (20 nM), UCH-L3 (0.4 nM) or by NEM chemically inactivated His-ChlaDUB1 (20 nM) in DUB buffer. Samples were incubated 2 h at 37 °C and enzymatic reaction was stopped by adding 2x Laemmli buffer and boiling at 94 °C for 7 min. Ubiquitination status of Mcl-1 was visualized by SDS-PAGE and immunoblot.

2.2.4.2 Immunoprecipitation

Immunoprecipitation of Mcl-1 under stringent conditions

Immunoprecipitation under stringent conditions limits the possibility of protein-protein interaction and precipitation of protein complexes. For immunoprecipitation of Mcl-1, cells were pre-incubated with 20 µM MG-132 before lysis. *C. trachomatis* infected cells and uninfected control cells induced for

apoptosis were lysed in RIPA buffer containing cOmplete™ protease inhibitor cocktail (Roche), 30 mM NEM and 30 µM MG-132 proteasome inhibitor. $7x10^6$ cells were lysed for 30 min at 4 °C and debris was removed by centrifugation at maximum speed for 5 min. Lysates were incubated with anti-Mcl-1 rabbit monoclonal antibody in a 1:100 dilution at 4 °C on a rotary shaker. After 2 h of incubation equilibrated Protein G magnetic beads were added to the samples and incubated at 4 °C on a rotary shaker for further 2 h. Samples were washed several times with RIPA buffer and eluted by heating at 94 °C for 7 min in 2x Laemmli buffer. Precipitated Mcl-1 was analyzed by SDS-PAGE and immunoblotting.

Immunoprecipitation of Mcl-1 under denaturing conditions

To analyze the ubiquitination pattern of Mcl-1 it is important that no binding partners of Mcl-1 are co-precipitated which can manipulate the ubiquitination signal. To free Mcl-1 of any protein-protein complexes and prohibit any further interaction cells were lysed in denaturing lysis buffer containing SDS and subsequently boiled at 94 °C for 10 min. Lysates were diluted ten fold with RIPA buffer containing cOmplete™ protease inhibitor cocktail (Roche) and 30 µM MG-132 and incubated on ice for 15 minutes. Cellular debris was removed by 5 min centrifugation at maximum speed and supernatant was supplemented with 1:100 anti-Mcl-1 rabbit monoclonal antibody. Primary antibody was incubated for 2 h at 4 °C on a rotary shaker. After Protein G magnetic beads were added the samples were incubated for two more hours. Samples were washed several times with RIPA buffer and precipitated Mcl-1 was eluted by 2x Laemmi buffer and boiling at 94 °C for 7 min. Ubiquitination pattern of precipitated Mcl-1 was analyzed by SDS-PAGE and immunoblot.

Co-immunoprecipitation of FLAG-tagged proteins and Mcl-1

Lysates for co-immunoprecipitation of ChlaDUB1-FLAG or Mcl-1 were prepared in Co-IP lysis buffer containing cOmplete™ protease inhibitor cocktail (Roche), 30 µM MG-132 proteasome inhibitor and 30 mM NEM. Lysates from $7x10^6$ cells either infected with *C. trachomatis* expressing FLAG-tagged proteins or 293T cells transfected with FLAG-tagged constructs were prepared by incubation in Co-IP lysis buffer on ice for 30 min with occasional vortexing. Debris was removed by centrifugation and samples were incubated with 3 µg anti-FLAG mouse monoclonal antibody for 2 h at 4 °C followed by incubation with protein G magnetic beads for 2 h at 4 °C. For co-IP of ChlaDUB1-FLAG and Mcl-1 samples were washed briefly with IP wash buffer and incubated with fresh cell lysate of uninfected cells for 30 min at 4 °C. Co-IP of ChlaDUB1-FLAG and Mcl-1 out of cells overexpressing both proteins was performed as describe before but without the additional incubation step with fresh cell lysate. Samples were washed several times with IP wash buffer and co-precipitates were eluted by addition of 2x Laemmli buffer and heating to 94 °C. Precipitated proteins were separated by SDS-PAGE and visualized by immunoblotting.

Co-Immunoprecipitation of Mcl-1 and Bim

Interaction of Mcl-1 and Bim was proved by co-immunoprecipitation in uninfected and *C. trachomatis*-infected Hela229 cells. 4 h prior to lysis the proteasome inhibitor MG132 was added to the cells to block protein degradation. $7x10^6$ cells were lysed in Co-IP lysis buffer supplemented with cOmplete™ protease inhibitor cocktail (Roche), 30 µM MG-132 proteasome inhibitor and 30 mM NEM and incubated for 30 min on ice with occasional vortexing. Lysate was cleared by centrifugation and supernatant was incubated with 1:100 anti-Mcl-1 rabbit monoclonal antibody or 1:100 anti-myc-tag rabbit monoclonal antibody for 2 h at 4 °C. Protein G magnetic beads were added and samples were incubated at 4 °C for 2 more hours. After repeated washing with IP wash buffer proteins were eluted by addition of 2x Laemmli and boiling at 94 °C for 7 min. Samples were analyzed by SDS-PAGE and immunoblot.

2.2.4.3 Purification of recombinant proteins out of *E. coli*

His-tag purification using NiNTA agarose

Cell pellet of 500 ml bacteria liquid culture after IPTG-induced protein overexpression was thawed on ice and resuspended in 10 ml NiNTA lysis buffer supplemented with 0.5 mg/ml lysozyme. After 30 min incubation on ice the lysate was sonified for 5 min (50% duration, Output 4) under permanent cooling. Cellular debris was removed by centrifugation at 10,000 g and 4 °C for 25 min. Cleared supernatant was load onto an equilibrated NiNTA agarose column and incubated at 4 °C on a rotary shaker for 2 h. Column was emptied by gravity and beads were washed with ice cold NiNTA wash buffer. A second washing step with NiNTA stringent wash buffer was performed. For purification of His-ChlaDUB1 and His-ChlaDUB1(C345A) the NiNTA stringent wash buffer contained 150 mM imidazole whereas imidazole concentrations of 50 mM were used for His-ChlaDUB2 and His-GroEL purification. After stringent washing the His-tagged proteins were eluted by addition of 5 ml NiNTA elution buffer. Buffer exchange and concentration was performed with Amicon Ultra centrifugation filter units with a 10 kDa molecular weight cut off. Purified proteins were stored at 4 °C, protein concentration was defined by Bradford assay following the manufacturers' instructions and purity was tested by SDS-PAGE and coomassie staining.

GST-tag purification using Glutathione sepharose

Bacterial pellet collected from 200 ml liquid culture after IPTG-induced protein overexpression was thawed on ice and resuspended in 3.4 ml STE buffer supplemented with 100 µg/ml lysozyme. After incubation on ice for 15 min, 600 µl sarcosyl stock was added and lysate was sonified for 5 min (50 % duration/ Output 4). Lysate was centrifuged for 5 min at 14,000 rpm and 4 °C and supernatant was mixed with 1 ml Triton stock. 240 µl Glutathione sepharose (GS) beads were equilibrated in STE, combined with the lysate and incubated for 1 h at 4 °C on a rotary shaker. GS beads were collected by

5 min centrifugation at 500 g and washed three times with PBS. Proteins were eluted by addition of 200 µl elution buffer and incubation at 25 °C and 700 rpm for 20 min. Protein-containing supernatant was separated from GS beads by centrifugation and buffer exchange was performed using Amicon Ultra centrifugation filter units with a 10 kDa molecular weight cut off. The protein concentration was defined by Bradford following the manufacturers' instructions and purity was checked by SDS-PAGE and coomassie staining.

3 Results

3.1 Mcl-1 stabilization during chlamydial infection is based on its deubiquitination

It is known that the protein set up of a cell is adjusted by transcriptional and translational control as well as by protein degradation via the 26S proteasome. Since the manipulation of the host UPS is a common strategy of pathogenic bacteria (Angot et al., 2007) the ubiquitome of uninfected and *Ctr*-infected HeLa229 cells 24 hpi was investigated by western blot analysis (Figure 3.1 A). No major differences between the two samples could be observed as this was shown for example during *Listeria monocytogenes* infection impairing host cell SUMOylation (Ribet et al., 2010). Mcl-1 expression and half-life is regulated by the MEK/ERK and PI3K/AKT survival signaling pathways. Especially in the initial phase of infection the activation of these pathways is essential for replication of *Chlamydia* (Rajalingam et al., 2008). However, during mid and late phase of infection the inactivation of these pathways by inhibitor treatment results in no loss of replicating *Chlamydia* and the ability to stabilize Mcl-1 is reduced but not completely gone (Figure 3.1 B). These results indicate that further mechanisms than the activation of signaling pathways might be involved in Mcl-1 stabilization in the mid and late phase of infection.

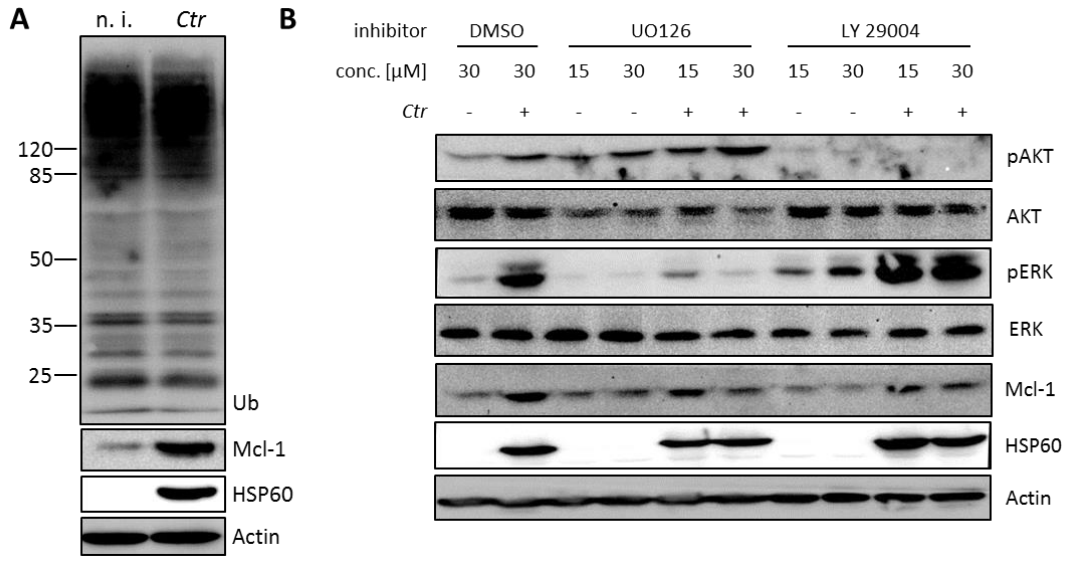

Figure 3.1: Analysis of ubiquitination pattern and Mcl-1 stabilization during *Ctr* infection. (A) HeLa229 cells were infected with *Ctr* (MOI1) and 24 hpi lysates were prepared, separated by SDS-PAGE and ubiquitination was analyzed by immunoblot using anti-Ubiquitin antibody. Mcl-1 stabilization was detected using anti-Mcl-1 antibody. Immunoblot for HSP60 and Actin served as controls. (B) HeLa229 cells were infected with *Ctr* (MOI1) and inhibitors LY29004 and UO126 were added 10 hpi in the indicated concentration. UO126 is an inhibitor of the MEK/ERK pathway whereas LY29004 blocks PI3K signaling. Lysates were prepared 24 hpi and analyzed by immunoblot. Phospho-signals indicate active signaling pathways. HSP60 and Actin signal represent infection and loading control.

The presence of Mcl-1 is not mandatory for chlamydial replication but is important for apoptosis resistance of the infected cell (Rajalingam et al., 2008). To gain a more detailed view of the posttranslational modifications, especially the ubiquitination of Mcl-1, Mcl-1 was immunoprecipitated from infected cells induced for apoptosis by TNFα/CHX and control cells. To prevent direct destruction of ubiquitinated proteins the proteasome was blocked prior to cell lysis by MG132 treatment. The precipitated Mcl-1 was separated by SDS-PAGE and stained for ubiquitin in immunoblot (Figure 3.2 A, right panel). The immunoblot analysis reveals that *Ctr* infection results in reduced ubiquitination of Mcl-1 compared to uninfected control cells, even after apoptosis induction. These results indicate that the reduced ubiquitination of Mcl-1 is responsible for its stabilization and is linked to apoptosis resistance of *Ctr*-infected cells. However, the molecular mechanism for reduced ubiquitination and stabilization of Mcl-1 and whether bacterial factors are involved in this process had to be investigated in detail.

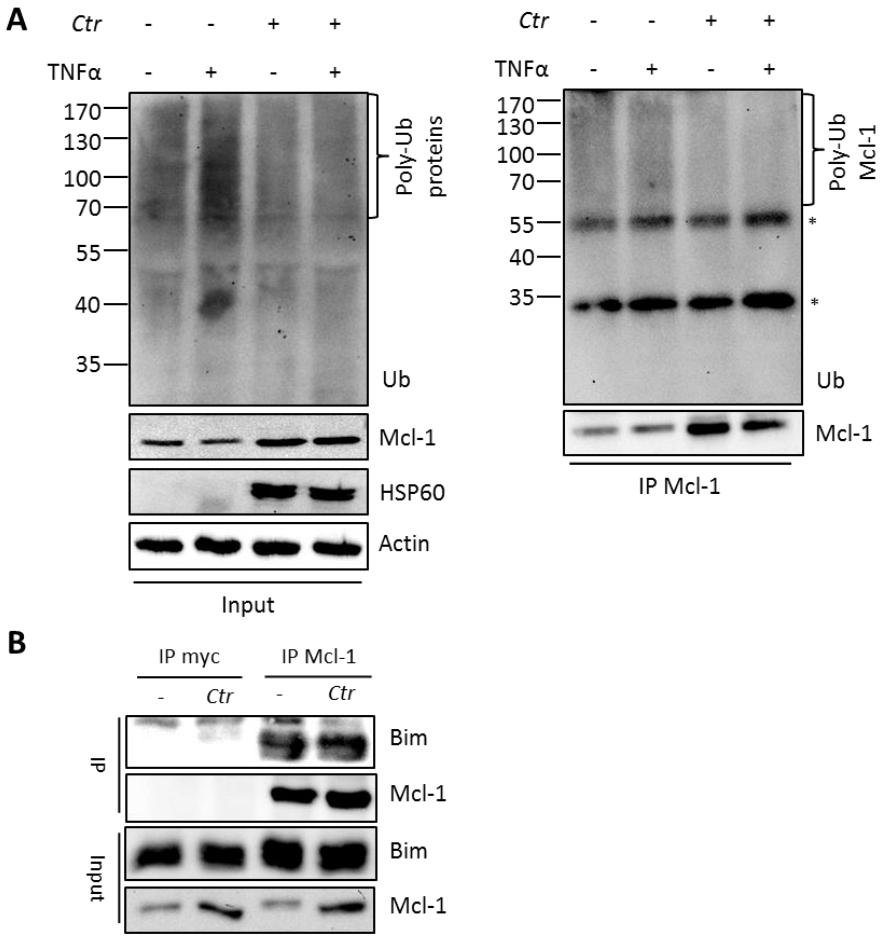

Figure 3.2: Analysis of molecular features of Mcl-1 during *Ctr* infection. (A) *Ctr*-infected HeLa229 cells (MOI1) were induced for apoptosis by TNFα/CHX 20 hpi and proteasomal protein degradation was blocked by MG132 treatment 1 h before cells were lysed in RIPA-buffer. Mcl-1 was immunoprecipitated using anti-Mcl-1 rabbit monoclonal antibody and analyzed by immunoblot. Ubiquitination of precipitated Mcl-1 was detected using anti-Ubiquitin antibody in immunoblot. * mark heavy and light chain of IgG used for immunoprecipitation. (B) HeLa229 cells were infected with *Ctr* (MOI1) and proteasome inhibitor MG132 was added upon 20 h of infection. 24 hpi cells were lysed in Co-IP lysis buffer and Mcl-1 was immunoprecipitated using anti-Mcl-1 antibody. Immunoprecipitation with unspecific anti-myc antibody served as control. Precipitated Mcl-1 and co-precipitated Bim was detected by immunoblot.

The anti-apoptotic function of Mcl-1 relies on its ability to interact with pro-apoptotic Bcl-2 protein family members and to block mitochondrial outer membrane permeabilization. One prominent interaction partner of Mcl-1 is the BH3-only protein Bim. Co-immunoprecipitation experiments of Mcl-1 and Bim showed that this interaction is not altered during *Ctr* infection (Figure 3.2 B). In addition, the hypothesis of *Ctr*-induced degradation of BH3-only proteins by the chlamydial protease CPAF could be disproved. In conformity with our results, several groups recently showed that the degradation of BH3-only proteins by CPAF were post-lysis artefacts (Chen et al., 2012; Snavely et al., 2014).

3.2 Cellular components of the host UPS system play minor role in Mcl-1 stabilization during infection

3.2.1 Expression pattern of cellular E3 Ub-ligases and deubiquitinase

Various cancer cell lines show dysfunction of the UPS or altered gene regulation resulting in increased amount of Mcl-1 which stays in conflict with the mode of action of multiple cancer therapeutics operating by apoptosis induction in malignant cells (Azmi et al., 2011). Since Mcl-1 is a strong anti-apoptotic protein it can counteract the effect of the chemotherapeutics and cause resistance. To overcome this resistance combined medication with conventional cancer therapeutics and drugs targeting Mcl-1 are used (Akgul, 2009). Compared to malignant cells, *Ctr*-infected cells exhibit similar properties with increased amounts of Mcl-1 mediating apoptosis resistance. To reveal the molecular mechanisms leading to reduced ubiquitination and thereby stabilization of Mcl-1 in the infected cell, we investigated the role of the host UPS factors regulating Mcl-1 turnover.

Known enzymes catalyzing Mcl-1 turnover are the HECT E3 ubiquitin ligase MULE (Mcl-1 ubiquitin ligase E3) (Zhong et al., 2005), the SCF^{Fbw7} (Inuzuka et al., 2011) and the $SCF^{\beta TrCP}$ E3 ligase complexes (Ding et al., 2007). The two F-box proteins Fbw7 and βTrCP mediate Mcl-1 binding to the SCF multi-subunit complex whereas MULE directly interacts with Mcl-1 by its BH3 domain. On the contrary, the deubiquitinase USP9X is known to deubiquitinate Mcl-1 prolonging its half-life (Schwickart et al., 2010). By immunoblot analysis of uninfected and *Ctr*-infected HeLa229 cells 24 hpi the amount of the mentioned Mcl-1 interacting proteins was investigated. The results depicted in Figure 3.3 A and B show no alterations in protein amount or infection induced degradation of UPS factors regulating Mcl-1 quantity in the cell.

Furthermore, the SCF E3 ligase complexes can be regulated in their activity by neddylation of the cullin 1 subunit. *C. trachomatis* is known to express two effectors with deneddylating activity, ChlaDUB1 and ChlaDUB2. A deneddylation of cullin 1 would result in inactivation of the respective E3 ligase leading to reduced ubiquitination of Mcl-1 as it was shown during *Chlamydia* infection. Therefore the neddylation status of cullin 1 24 hpi was examined by immunoblot and the results are depicted in Figure 3.3 B. The major band of unmodified cullin 1 is detected at the molecular size of 90 kDa and an additional band at approximately 100 kDa represents the neddylated form of cullin 1. However, no change in protein level or neddylation of cullin 1 during *C. trachomatis* infection was observed.

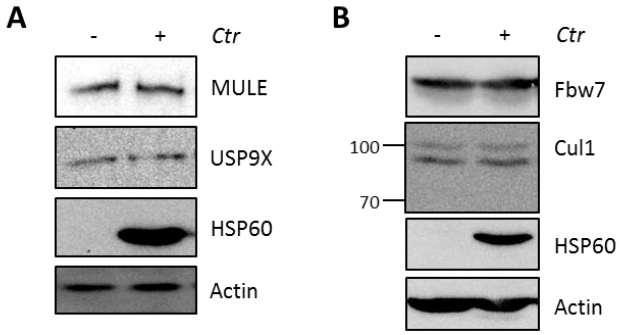

Figure 3.3: Expression and abundance of host UPS components regulating Mcl-1 turnover. (A, B) Whole cell lysates of uninfected or *Ctr*-infected HeLa229 cells (MOI1) 24 hpi were separated by SDS-PAGE. Samples for analysis of MULE and USP9X were transferred to PVDF by wet blot (A) whereas other samples were prepared by semi-dry western blotting (B). Cellular UPS proteins were detected by immunoblot. Immunoblot with HSP60 and Actin served as controls for infection and equal loading.

3.2.2 RNAi against USP9X

Not only the amount of UPS enzymes but also their activity or accessibility can have an impact on substrate ubiquitination. To rule out any manipulation of USP9X as the known deubiquitinase of Mcl-1 at the regulatory level by *Chlamydia* an USP9X knock-down by RNA interference was performed. HeLa229 cells were transfected with a pool of siRNAs targeting USP9X or control siRNAs for 48 h prior to infection with *Ctr* (MOI1). After 20 h of infection apoptosis was induced by TNFα/CHX and cells were lysed for immunoblot analysis. Apoptosis induction can be monitored and quantified by cleavage of the caspase-3 substrate PARP (poly (ADP-ribose) polymerase) in immunoblot. The ratio of full length PARP to cleaved PARP (cPARP) indicates the degree of apoptosis induction. In addition to PARP cleavage, the successful knock-down of USP9X and amount of Mcl-1 in the samples were analyzed (Figure 3.4 A). Relative Mcl-1 values from three individual experiments are depicted in Figure 3.4 B and show no significant reduction of Mcl-1 stabilization in *Ctr*-infected USP9X knock-down cells compared to cells transfected with control siRNA.

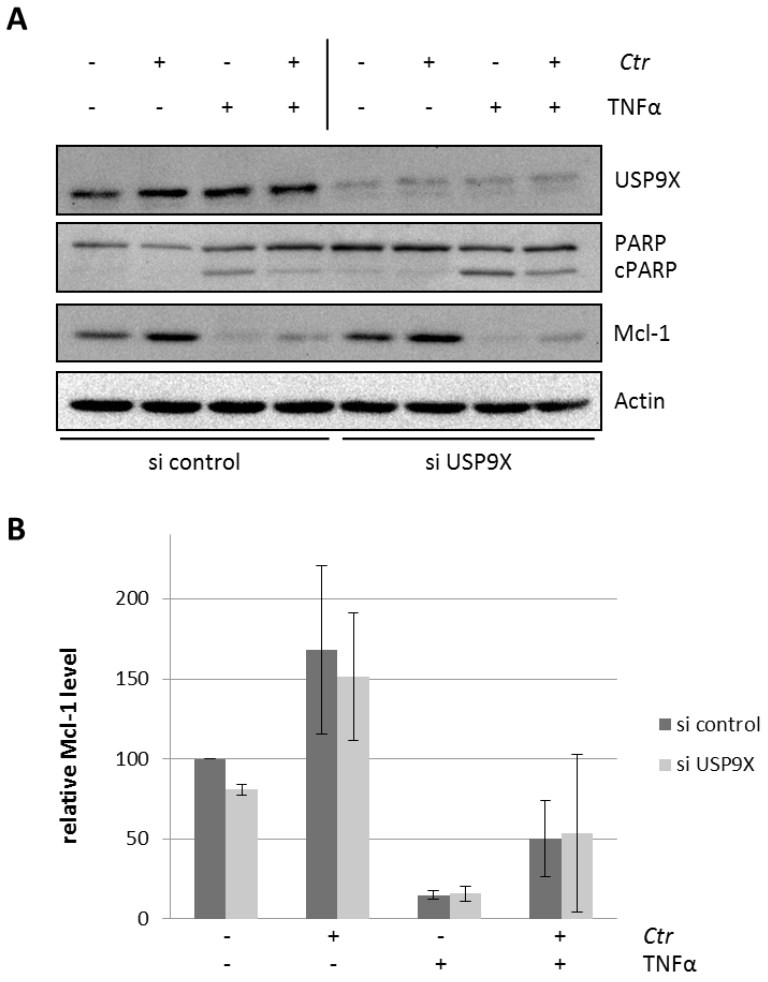

Figure 3.4: USP9X knock-down by RNAi during infection and apoptosis induction. (A) HeLa229 cells were transfected with siRNA pools targeting USP9X or with control siRNAs for 48h and then infected with *Ctr* (MOI1). 20 hpi apoptosis was induced by TNFα/CHX, lysates were separated by SDS-PAGE and analyzed by immunoblot. USP9X knock-down was controlled by anti-USP9X immunoblot and apoptosis induction was detected by PARP cleavage using anti-PARP antibody. (B) Relative amount of Mcl-1 quantified by immunoblot and calculated from three individual experiments. Shown are mean values ±SD.

3.3 Chlamydial deubiquitinating enzymes

3.3.1 Expression pattern of ChlaDUB1 and ChlaDUB2

The initial experiments approved no major participation of the host UPS factors in *Ctr*-mediated Mcl-1 stabilization. In 2006, Misaghi and colleagues used activity-based probes to isolate DUBs out of *Ctr*-infected or uninfected control cells (Misaghi et al., 2006). The ubiquitin derivate HA-ubiquitin-vinylmethylester (HA-UbVME) specifically reacts with deubiquitinases as a suicide inhibitor and can further be isolated by its HA-tag. With this activity-based approach two DUBs encoded by *C. trachomatis* were isolated out of infected cells 24 hpi and identified by mass spectrometry as ChlaDUB1 (CTL0247) and ChlaDUB2 (CTL0246). Both enzymes are supposed to be cysteine proteases and exhibit *in vitro* deubiqutinating and deneddylating activity (Misaghi et al., 2006). Figure 3.5 shows the composition of the two enzymes with their conserved domains.

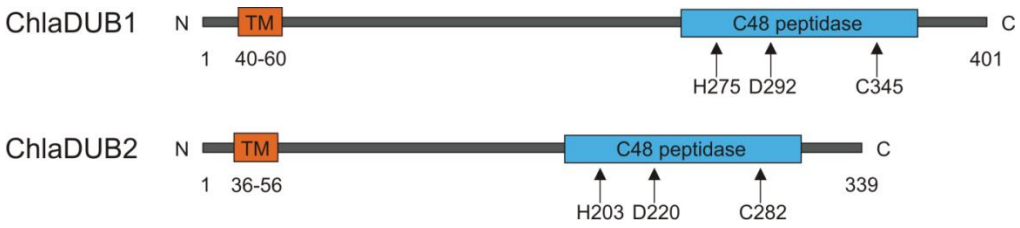

Figure 3.5: Domains of the two chlamydial proteins ChlaDUB1 (CTL0247) and ChlaDUB2 (CTL0246). ChlaDUB1 is encoded by the gene CTL0247 and consists of 401 amino acids. ChlaDUB1 contains a N-terminal single pass transmembrane domain (TM) and a C-terminal peptidase domain with the conserved amino acid residues H275, D292 and C345. The gene CTL0246 encodes for the 339 amino acid protein ChlaDUB2 which possesses a N-terminal transmembrane domain and the conserved amino acid residues H203, D220 and C282 represent the active residues of the peptidase domain located at the C-terminus.

Further work demonstrated that ChlaDUB1 is supposed to be secreted into the host cell cytoplasm and catalyze the deubiquitination of IκBα to prevent NFκB signaling and inflammation (Le Negrate et al., 2008). For some bacterial effector proteins multiple enzymatic activities as well as substrates have been identified. For instance YopJ from Yersinia acts as a DUB for TRAF6 and IκBα but is also an acetyltransferase interfering in MAPK signaling (Perrett et al., 2011). For ChlaDUB2 no substrate has been identified so far and it is very likely that ChlaDUB1 has additional host cell targets besides IκBα, especially depending on the bi-functional enzymatic activity of the protein (Misaghi et al., 2006). ChlaDUB1 is supposed to be expressed upon 16 hpi till the end of the life cycle shown by RT-PCR (Le Negrate et al., 2008). Mcl-1 is also known to be stabilized upon 16 hpi (Rajalingam et al., 2008) and initial results depicted in Figure 3.2 A confirmed a reduced ubiquitination of Mcl-1 in infected cells. This correlation asked for further characterization of expression and function of the two chlamydial DUBs.

Since no antibodies targeting ChlaDUB1 or ChlaDUB2 are commercially available purified recombinant proteins were sent for immunization to immunoGlobe®. Protein expression was induced by IPTG in *E. coli* containing the pET28a/ChlaDUB1 plasmid and His-tagged full length ChlaDUB1 was purified under native conditions using NiNTA agarose beads and sent for immunization. It was not possible to overexpress and purify full length ChlaDUB2 out of *E. coli* containing the pET28a/ChlaDUB2 plasmid in sufficient amounts. Therefore a truncated form of ChlaDUB2 was overexpressed in *E. coli* containing the pET28a/ChlaDUB2-fragment plasmid and isolated out of the formed inclusion bodies by the master student Daniela Auer, who was supervised by me (Auer, 2014). The purified ChlaDUB2 fragment was dialyzed and sent for immunization. The delivered immune sera were further purified by affinity columns consistent of Cdu1-CnBr-Sepharose or Cdu2-CnBr-Sepharose beads. By this purification step serum components and unspecific antibodies were removed and the antigen-specific antibody was enriched.

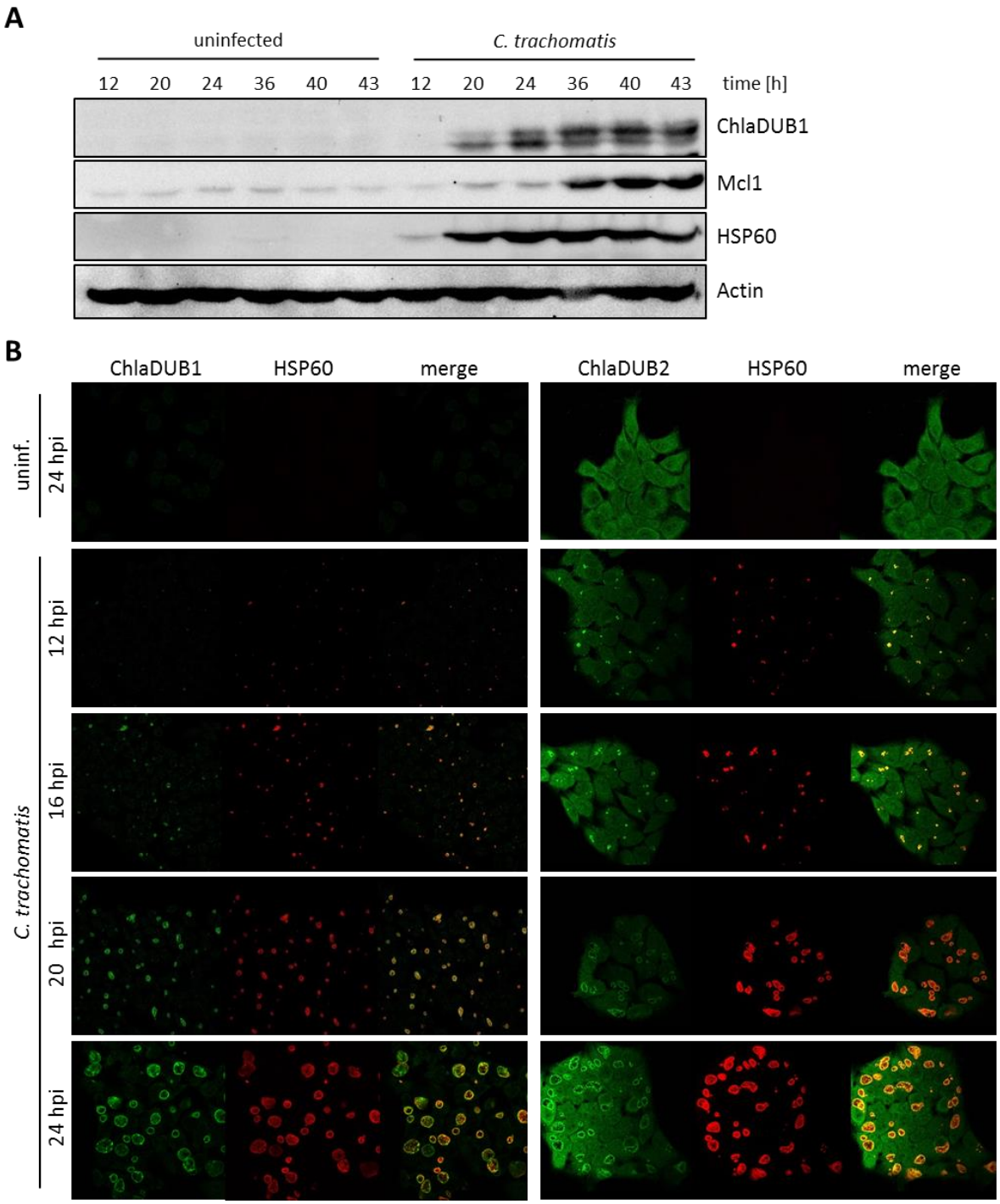

Figure 3.6: Expression pattern of the chlamydial enzymes ChlaDUB1 and ChlaDUB2. (A) HeLa229 cells were infected with *Ctr* (MOI1) and lysates were harvested at the indicated time point post infection. Proteins were separated by SDS-PAGE and chlamydial protein expression (ChlaDUB1, HSP60) as well as impact on host cell proteins (Mcl-1, Actin) was analyzed by immunoblot. (B) HeLa229 cells were infected with *Ctr* (MOI1) and fixed with 4 % PFA/Sucrose at the indicated time point. Indirect immunofluorescence staining with purified antibody serum against ChlaDUB1 and ChlaDUB2 (rabbit; Cy2, green) as well as monoclonal antibody detecting chlamydial HSP60 (mouse, Cy5, red) was performed and analyzed by confocal microscopy.

In immunoblot analysis (Figure 3.6 A) and immunofluorescence staining (Figure 3.6 B) of a *Ctr* infection time course experiment the time of expression as well as protein amount were evaluated for ChlaDUB1 and ChlaDUB2. The RT-PCR data from Le Negrate revealing ChlaDUB1 expression upon 16 h of infection was confirmed by immunoblot and immunofluorescence staining (Le Negrate et al., 2008). The ChlaDUB2 antibody is not suitable to detect natural amounts of ChlaDUB2 in immunoblot, albeit a strong signal for ChlaDUB2 is detected in immunofluorescence staining indicating that ChlaDUB2 is expressed 12 hpi but also in the mid phase of the developmental cycle of *Chlamydia* (Figure 3.6 B).

3.3.2 Subcellular localization of ChlaDUB1 and ChlaDUB2

Several groups set up heterologous systems to identify type 3 secreted effector proteins of *Chlamydia* (da Cunha et al., 2014b; Subtil et al., 2005). Till now it is not known how the type 3 effectors are recognized and targeted to the secretion machinery and whether N-terminal signal peptides or chaperones are involved. In 2009, Arnold and colleagues analyzed the N-terminal sequences of known T3SS effectors in detail and set up the program EffectiveT3 (http://www.chlamydiadb.org) to identify type 3 secreted proteins in *Chlamydia* (Arnold et al., 2009). Depending on standard settings, EffectiveT3 predicts ChlaDUB1 to be a type 3 secreted effector protein whereas ChlaDUB2 is not (Table 3-1). The known type 3 secreted proteins IncA and TARP serve as controls. In this analysis the selective cut-off settings were used for a high confidence prediction of T3 effectors (cut-off=0.9999), but proteins with an EffectiveT3 score of 0.95 or higher can also be type 3 secreted effectors. Taken together, ChlaDUB1 is most probably a T3 effector whereas ChlaDUB2 is a potential T3 effector protein.

Table 3-1: EffectiveT3 based type 3 secreted effector protein prediction

Protein	is T3 secreted	EffectiveT3 score
ChlaDUB1 (CTL0247)	+	1
ChlaDUB2 (CTL0246)	-	0.99883
IncA (CTL0374)	+	1
TARP (CTL0716)	+	1
GroEL (CTL0365)	-	0

Depending on this EffectiveT3 calculation, ChlaDUB1 and ChlaDUB2 are expected to be secreted into the host cell cytoplasm. Nevertheless, immunofluorescence staining of *Ctr*-infected HeLa229 or HEp-2 cells 24 hpi revealed the predominant localization of ChlaDUB1 merging with the chlamydial particles and at the chlamydial inclusion surface. It is possible that ChlaDUB1 is anchored to the inclusion membrane by its N-terminal single pass transmembrane domain after secretion (Figure 3.7 A+B). It was not possible to confirm the detection of ChlaDUB1 in the host cell cytoplasm,

however it cannot be ruled out that the amount of ChlaDUB1 present in the host cell cytoplasm is below the detection limit of the antibody. The ChlaDUB2 antibody stains the chlamydial particles, but strong unspecific background signal in uninfected cells asks for further purification approaches of the serum (Figure 3.7 C). The strong unspecific signal might overframe a ChlaDUB2 specific signal present in the host cell cytosol which cannot be detected under this condition. Furthermore, the detection limit of the ChlaDUB2 antibody might be too low as it was already postulated for the ChlaDUB1 antibody.

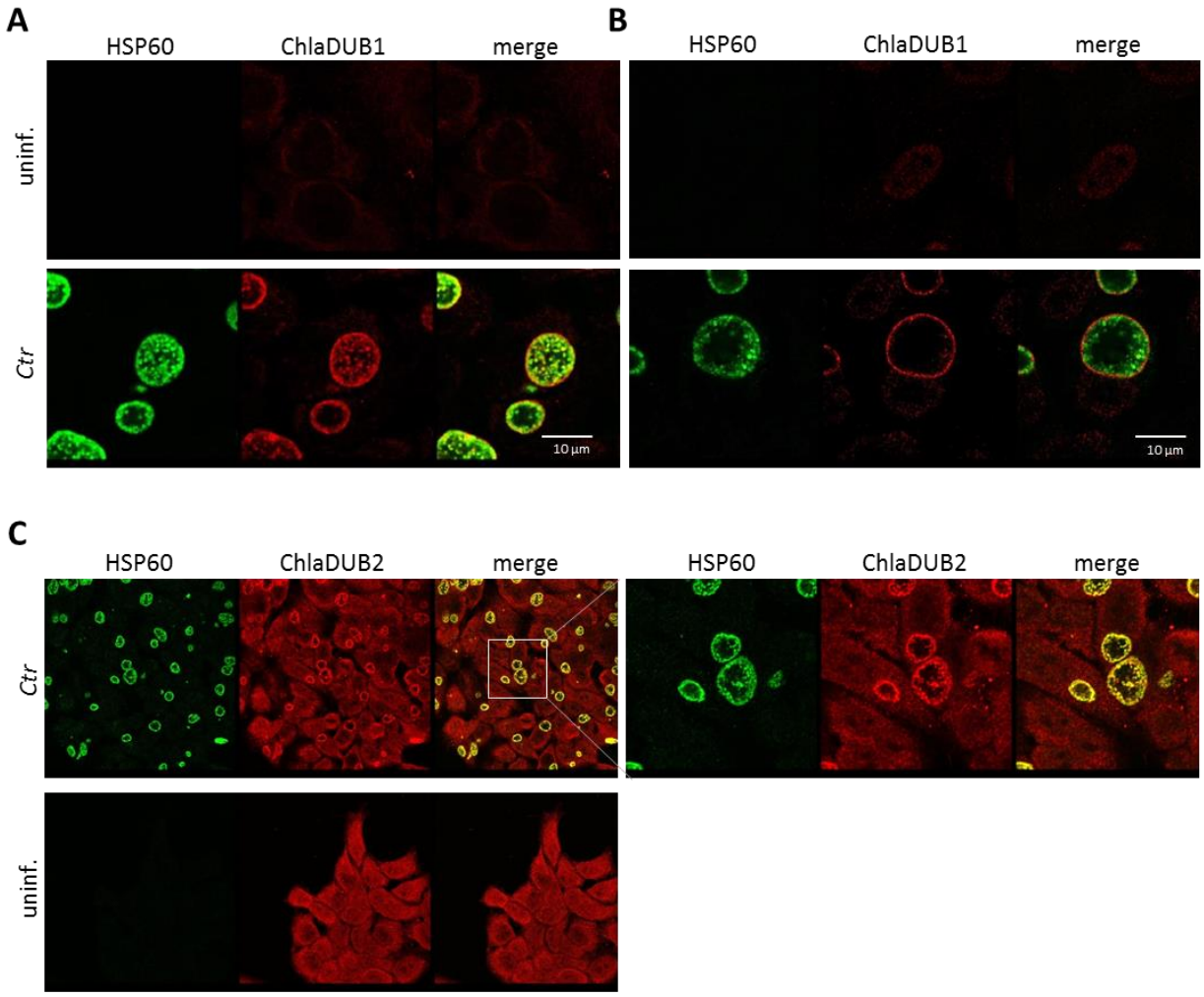

Figure 3.7: Subcellular localization of ChlaDUB1 and ChlaDUB2 during infection. (A+B) *Ctr*-infected HeLa229 (A) and HEp-2 (B) cells grown on cover slips were fixed 24 hpi. Expression and localization of ChlaDUB1 (rabbit, Cy5, red) was detected by immunofluorescence staining and confocal microscopy. Chlamydial particles were stained with anti-HSP60 antibody (mouse, Cy2; green). (C) HeLa229 cells infected with *Ctr* (MOI1) were fixed 24 hpi and stained with anti-ChlaDUB2 (rabbit, Cy5, red) and anti-HSP60 (mouse, Cy2, green) antibodies in indirect immunofluorescence staining and analyzed by confocal microscopy.

To eradicate the issue of detection limit, *Chlamydia* strains were generated suitable for the inducible overexpression of ChlaDUB1 (*Ctr* pTet/ChlaDUB1-FLAG) and a mutated ChlaDUB2 protein carrying an amino acid exchange from cysteine to alanine at position 282 (*Ctr* pTet/ChlaDUB2(C282A)-FLAG) (Auer, 2014). The *Chlamydia* overexpress the protein of interest after addition of anhydrotetracycline (AHT) and secrete it to its natural localization. Cells infected with *Ctr* induced for ChlaDUB1-FLAG overexpression were analyzed by immunofluorescence staining with ChlaDUB1 and FLAG antibodies both detecting ChlaDUB1 (Figure 3.8 A). Although high amounts of ChlaDUB1 protein due to the induced overexpression are present in the infected cell, no ChlaDUB1-signal could be detected in the host cell cytoplasm by the ChlaDUB1 antibody or the highly specific FLAG antibody. These results indicate that ChlaDUB1 is secreted by *Chlamydia* to the surface of the inclusion where it might be anchored by its single pass transmembrane domain. The overexpression experiment for ChlaDUB2 with differential antibody staining as described before reveals that ChlaDUB2 is not secreted into the host cell cytoplasm but rather is present inside the Inclusion merging with the chlamydial particles, see Figure 3.8 B.

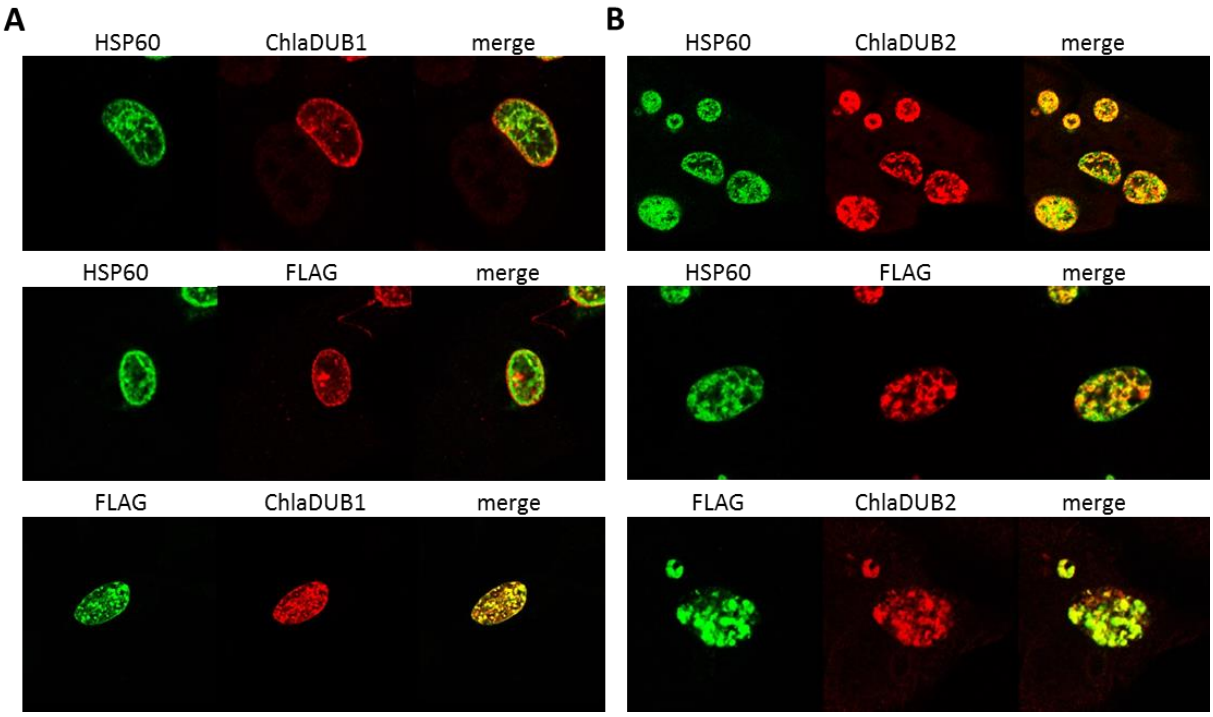

Figure 3.8: Validation of ChlaDUB1 and ChlaDUB2 antibody specificity. (A+B) HeLa229 cells were infected with *Ctr* pTet/ChlaDUB1-FLAG (A) or *Ctr* pTet/ChlaDUB2(C282A)-FLAG (B) and induced for protein overexpression with AHT over night. 24 hpi infected cells were fixed with 4 % PFA/Sucrose and analyzed by indirect immunofluorescence staining and confocal microscopy. (A) Indirect immunofluorescence staining using the following antibody combinations: anti-HSP60 (mouse, Cy2, green) and anti-ChlaDUB1 (rabbit, Cy5, red); anti-HSP60 (rabbit, Cy2, green) and anti-FLAG (mouse, Cy5, red); anti-FLAG (mouse, Cy2, green) and anti-ChlaDUB1 (rabbit, Cy5, red). (B) Immunofluorescence staining for analysis of ChlaDUB2 antibody specificity using the following antibody combinations: anti-HSP60 (mouse, Cy2, green) and anti-ChlaDUB2 (rabbit, Cy5, red); anti-HSP60 (rabbit, Cy2, green) and anti-FLAG (mouse, Cy5, red); anti-FLAG (mouse, Cy2, green) and anti-ChlaDUB2 (rabbit, Cy5, red).

3.4 Mcl-1 as ChlaDUB1 substrate

3.4.1 Subcellular localization of Mcl-1 during infection

The infection timeframe experiments analyzed by immunoblot and immunofluorescence staining indicate that Mcl-1 stabilization upon 16 hpi is on par with time of ChlaDUB1 but not with ChlaDUB2 expression (see Figure 3.6 B). Furthermore, the detailed study of subcellular localization of the two chlamydial deubiquitinases indicates that only ChlaDUB1 has direct access to the host cell cytoplasm (Figure 3.7 and Figure 3.8). Mcl-1 is known to be present in the cytoplasm where it can be associated with intracellular membranes like the mitochondrial outer membrane or the Endoplasmic reticulum (ER) (Leuenroth et al., 2000; Yang et al., 1995). This subcellular localization was confirmed by immunofluorescence staining of uninfected HeLa229 and HEp-2 cells, see Figure 3.9, upper panel. In *Ctr*-infected cells Mcl-1 is dramatically enriched in close proximity to the chlamydial inclusion (Figure 3.9, lower panel) allowing the assumption of an interaction of Mcl-1 with ChlaDUB1 which is also located at the surface of the inclusion (Figure 3.7 A+B). Since the available antibodies targeting ChlaDUB1 and Mcl-1 are both raised in rabbit a co-staining for ChlaDUB1 and Mcl-1 was not possible.

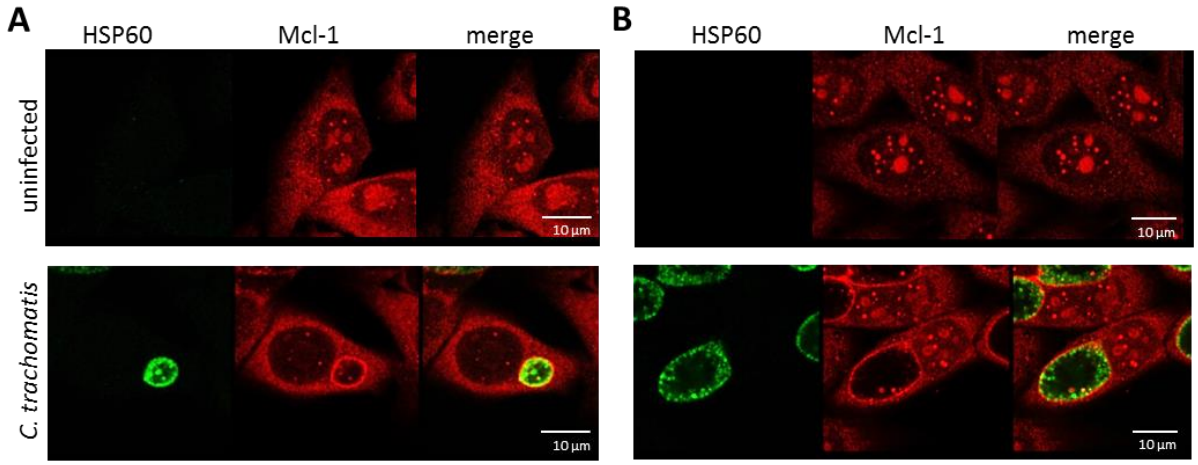

Figure 3.9: Subcellular localization of Mcl-1 during *Ctr* infection. (A+B) HeLa229 (A) or HEp-2 (B) cells were seeded on cover slips and infected with *Ctr* (MOI1) for 24 h. Subcellular localization of Mcl-1 in uninfected and *Ctr*-infected cells was detected by immunofluorescence staining using anti-HSP60 (mouse, Cy2, green) and anti-Mcl-1 (rabbit, Cy5, red) antibodies. Samples were analyzed by confocal microscopy.

3.4.2 Generation and characterization of the *Ctr* CTL0247-FLAG strain

The *Chlamydia* strain overexpressing ChlaDUB1-FLAG is a versatile tool to reveal subcellular localization of the secreted protein. However, permanent overexpression of enzymes can cause adverse effects on fitness and pathogenicity of the bacteria. Therefore, a strategy to replace the natural CTL0247 gene by a FLAG-tagged ChlaDUB1 variant was set up. The strategy was based on homologues recombination of plasmid DNA into the chlamydial genome dependent on the natural competence of *Chlamydia* to integrate DNA into their genome. After successful recombination the

transformed *Chlamydia* strain (*Ctr* CTL0247-FLAG) expresses a FLAG-tagged ChlaDUB1 enzyme under control of its natural promoter assuring natural protein expression regarding amount and time of expression. Considering these aspects, the *Ctr* CTL0247-FLAG strain can be used like the wild type strain and the ChlaDUB1-FLAG protein can be detected with the help of the highly specific FLAG-antibody. As template for homologues recombination the plasmid pAH1 was constructed. The pAH1 plasmid (see Figure 3.10) is a chlamydial suicidal plasmid which can replicate in *E. coli* but not in *Chlamydia*.

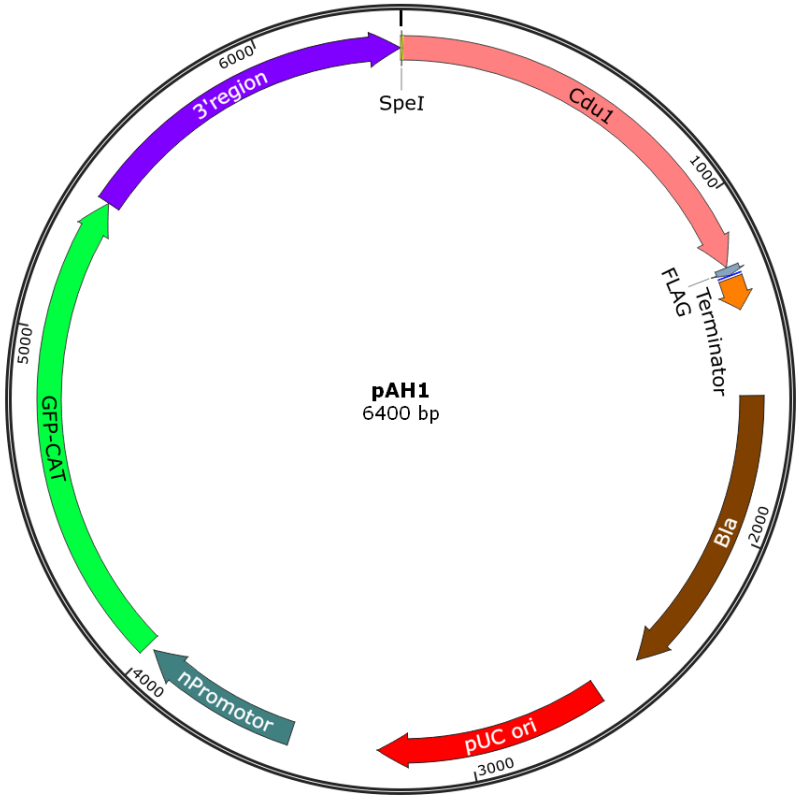

Figure 3.10: Vector map of the pAH1 plasmid. The plasmid pAH1 is designed for targeted homologues recombination into the chlamydial genome at the CTL0247 locus. The plasmid can replicate in *E. coli* but is a suicidal plasmid in *C. trachomatis* forcing a homologues recombination into the chlamydial genome to keep the β-lactamase gene. As recombination matrices serve the CTL0247 gene as well as the 1 kb downstream region. By recombination the CTL0247 gene is replaced by a modified form encoding for a FLAG-tagged ChlaDUB1 enzyme under its native promoter followed by a selection cassette consistent of a β-lactamase and a GFP fusion protein.

The plasmid consists of two homology regions allowing the site directed homologues recombination at the ChlaDUB1 gene CTL0247. By recombination a FLAG-tagged version of the ChlaDUB1 gene as well as a selection cassette is integrated into the chlamydial genome. The pAH1 plasmid was generated by overlap PCR fusing three fragments together. Fragment 1 consists of the ChlaDUB1-gene followed by a bacterial lambda t_0 transcriptional terminator and was amplified from the plasmid pQE30/ChlaDUB1. Fragment 2 contains the origin of replication for *E. coli* and a selection

cassette encoding for β-lactamase and a GFP-CAT fusion protein and was amplified from the plasmid pGFP::SW2. The third fragment consists of the 1 kb downstream region of CTL0247 and was transcribed from chlamydial genomic DNA. At the ends of fragment 1 and fragment 3 a restriction site for SpeI endonuclease was attached by which the plasmid can be circularized. The newly formed plasmid was amplified to integrate a FLAG-tag in frame of the ChlaDUB1 gene and a MluI restriction site for circularization. The pAH1 plasmid isolated out of *E. coli* JM110 was used to transform *C. trachomatis* to obtain the *Ctr* CTL0247-FLAG strain.

Prior to routine use of the *Ctr* CTL0247-FLAG strain the correct site specific integration as well as expression of the FLAG-tagged version of ChlaDUB1 was confirmed. Furthermore, any side effects altering normal growth and development of the *Chlamydia* evoked by the integration of the selection cassette into the chlamydial genome were ruled out. First evidence for the successful transformation and recombination of the selection cassette into the chlamydial genome was the resistance towards penicillin as well as GFP-expression detected by flow cytometry of infected HeLa229 cells (Figure 3.11).

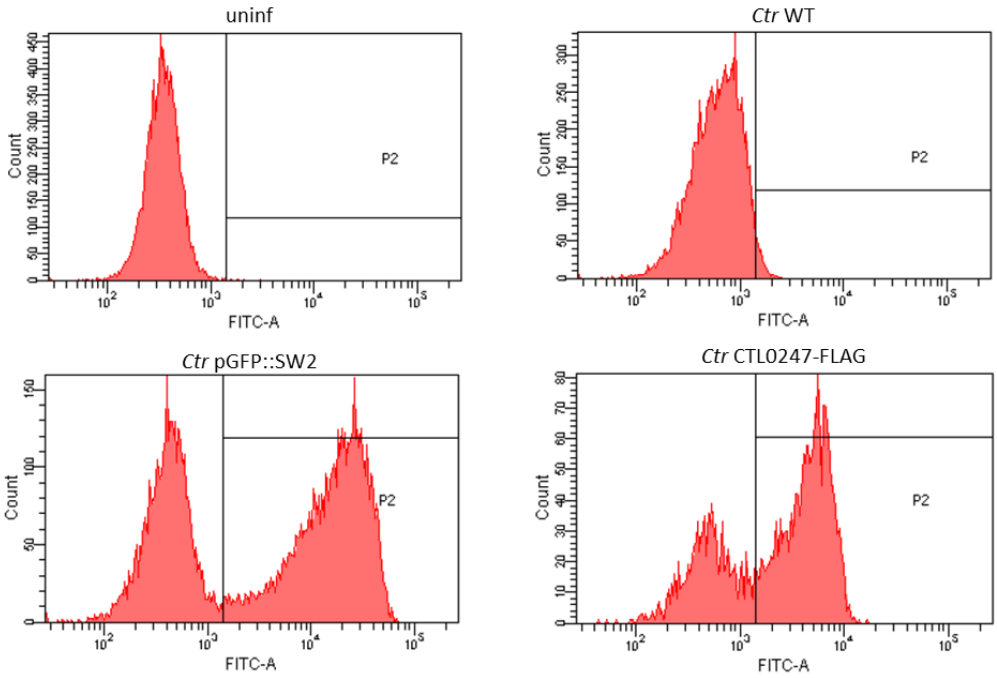

Figure 3.11: Flow cytometry analysis of infected HeLa229 cells for GFP-positive *Ctr* CTL0247-FLAG. HeLa229 cells were infected with the *Chlamydia* strains *Ctr* WT, *Ctr* pGFP::SW2 or *Ctr* CTL0247-FLAG as indicated. Infected cells were trypsinized 24 hpi and GFP-positive *Chlamydia* were detected by flow cytometry.

The site-specific integration of the ChlaDUB1-FLAG gene followed by the selection cassette was demonstrated by PCR with primer pairs annealing to the upstream genomic region of CTL0247 and to the newly integrated selection cassette (Figure 3.12 A). Only the transformed *Ctr* CTL0247-FLAG strain gave a positive PCR result with the selective primer pair 1 whereas the control reaction with primer pair 2 resulted in amplification in all samples (Figure 3.12 B). However, the selective PCR with primer pairs proofing the recombination at the 3' region of CTL0247 did not work (data not shown).

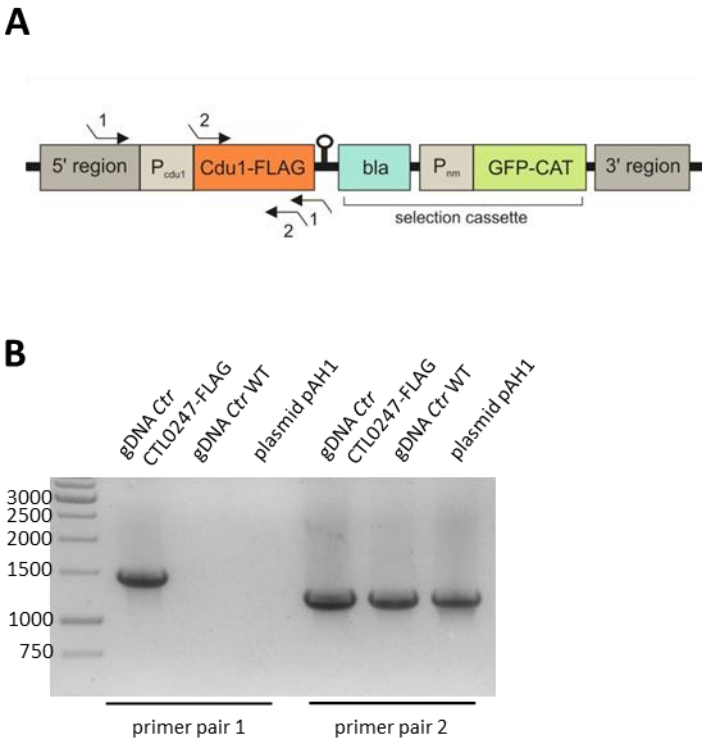

Figure 3.12: Confirmation of recombination by selective PCR. (A) Genomic locus of the transformed CTL0247-FLAG *Chlamydia* with annealing positions of the primer pairs used to prove correct integration of the CTL0247-FLAG gene as well as selection cassette. (B) Agarose gel of the PCR products amplified with the primer pairs 1 and 2 from genomic DNA isolated from *Ctr* WT, *Ctr* CTL0247-FLAG or from the pAH1 plasmid used for recombination.

An additional proof for successful recombination provided southern hybridization experiments with probes targeting the β-lactamase gene or the 5' region of CTL0247 which were performed in collaboration with Dr. B. Prusty. The cutting scheme for *Ctr* WT and *Ctr* CTL0247-FLAG genomic DNA with EcoRI endonuclease is depicted in Figure 3.13 A. Probe 1 targeting the β-lactamase gene gives only a strong signal in the transformed *Chlamydia* strain and not in the WT whilst the control probe binding to the 5' region give signals in both *Chlamydia* strains at the expected sizes (Figure 3.13 B).

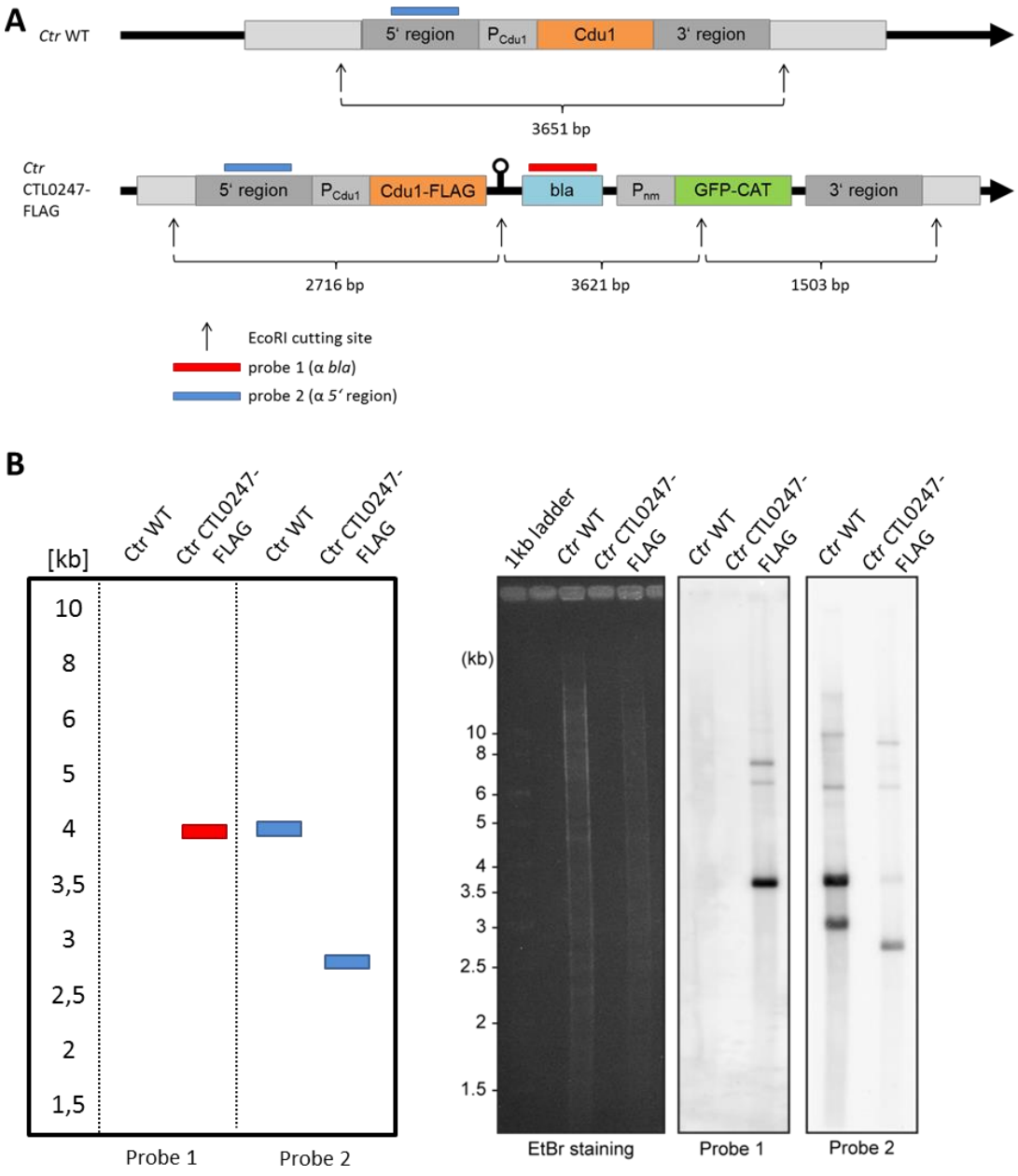

Figure 3.13: Southern hybridization to confirm recombination of pAH1 into the chlamydial genome. (A) Cutting scheme of the CTL0247 locus with EcoRI endonuclease digestion and probe annealing positions in WT and CTL0247-FLAG *Chlamydia*. (B) Expected (left) and actual (right) results of the southern hybridization of EcoRI digested genomic DNA of WT and CTL0247-FLAG *Chlamydia*. Southern hybridization experiments were performed in collaboration with Dr. B. Prusty.

After the site-specific homologues recombination into the chlamydial genome was confirmed by PCR and southern hybridization experiments, the expression of ChlaDUB1-FLAG protein instead of wild type ChlaDUB1 was analyzed and approved by immunoblot (Figure 3.14). No double band for ChlaDUB1 in the transformed strain is detected and the size of ChlaDUB1-FLAG is approximately 1 kDa higher than the wild type protein according to the additional FLAG-tag which can be detected by immunoblot, too (Figure 3.14 A).

Furthermore, time frame experiments analyzed by immunofluorescence staining revealed the contemporary expression of ChlaDUB1 in wild type *Chlamydia* and the *Ctr* CTL0247-FLAG strain upon 16 h of infection proofing the expression of ChlaDUB1-FLAG under control of its native promoter (Figure 3.14 B+C).

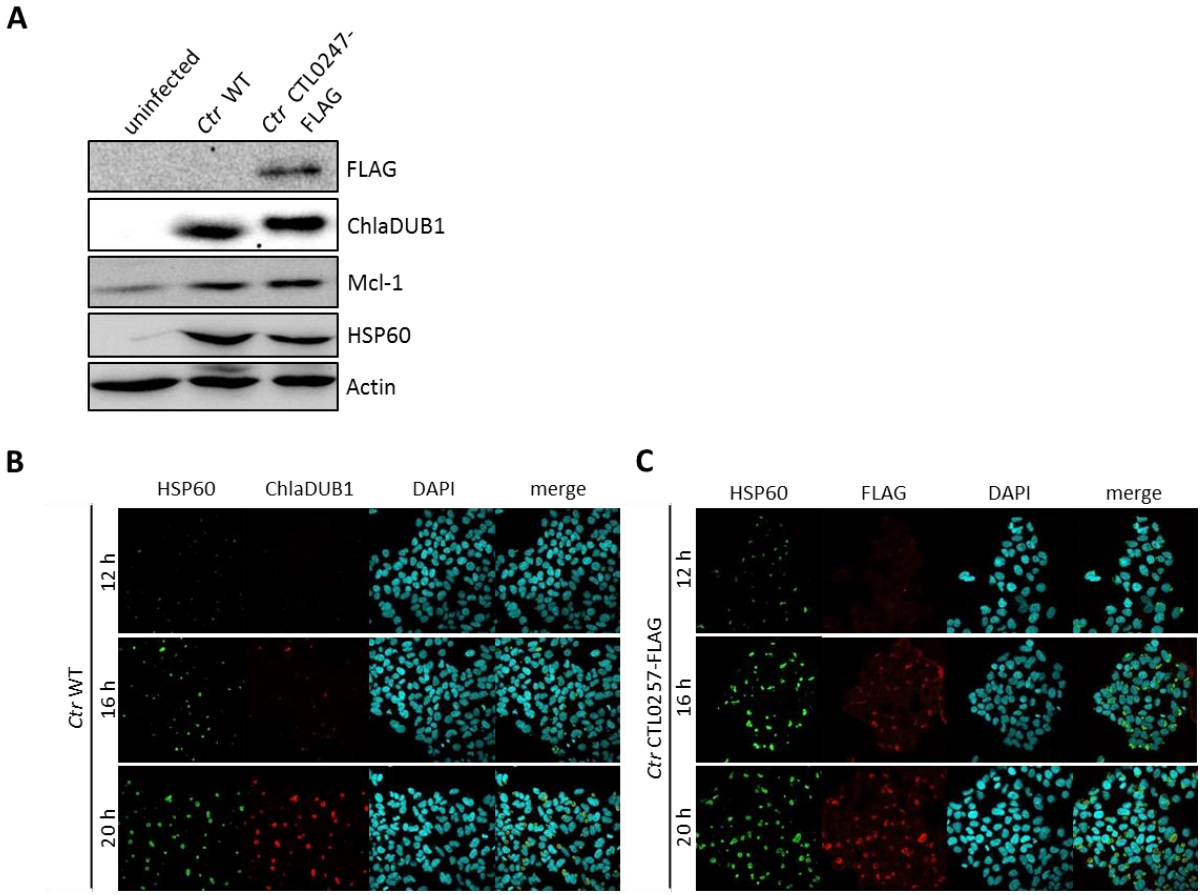

Figure 3.14: Expression of ChlaDUB1-FLAG by *Ctr* CTL0247-FLAG. (A) HeLa229 cells were infected with *Ctr* WT or *Ctr* CTL0247-FLAG and lysed 24 hpi for immunoblot analysis. Proteins were separated by SDS-PAGE and expression of the FLAG-tagged ChlaDUB1 protein was verified using anti-FLAG and anti-ChlaDUB1 antibody in immunoblot analysis. *Chlamydia*-induced Mcl-1 stabilization was shown using anti-Mcl-1 antibody in immunoblot. Signals for HSP60 and Actin served as controls. HeLa229 cells were grown on cover slips and infected with *Ctr* WT (B) or *Ctr* CTL0247-FLAG (C) and fixed after the indicated time post infection. *Chlamydia* were stained by HSP60-antibody (mouse, Cy3, green) and DNA was marked with DAPI. (B) ChlaDUB1 expression was detected using an anti-ChlaDUB1 (rabbit, Cy5, red) antibody. (C) ChlaDUB1-FLAG protein was detected using an anti-FLAG antibody (mouse, Cy5, red).

Prior to routine use of the *Ctr* CTL0247-FLAG strain the bacteria were tested for proper secretion of C-terminal FLAG-tagged ChlaDUB1 enzyme and if the additional tag interfered with chlamydial fitness. Detailed immunofluorescence analysis of infected cells 24 hpi depicted in Figure 3.15 showed that the additional C-terminal FLAG-tag did not affect the proper secretion of the enzyme to the surface of the inclusion.

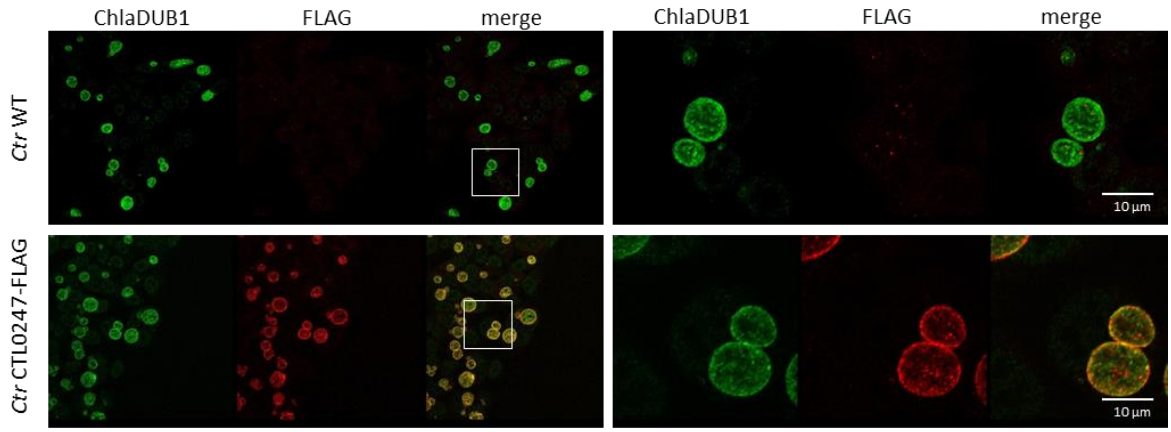

Figure 3.15: Secretion of ChlaDUB1-FLAG by *Ctr* CTL0247-FLAG. HeLa229 cells infected with *Ctr* WT or *Ctr* CTL0247-FLAG were fixed 24 hpi and subcellular localization of ChlaDUB1 was visualized by anti-FLAG (mouse, Cy5, red) and anti-ChlaDUB1 (rabbit, Cy3, green) staining. All samples were analyzed by confocal microscopy.

In addition, infectivity and duration of the developmental cycle as well as anti-apoptotic features of *Ctr* CTL0247-FLAG were investigated. The insertion of the selection cassette and replacement of the CTL0247 gene by the modified form did not affect duration of the developmental cycle and infectivity (Figure 3.16 A) nor was the ability to stabilize Mcl-1 and block host cell apoptosis induced by TNFα/CHX affected (Figure 3.16 B+C). Since no major adverse effects caused by the homologues recombination at the CTL0247 locus were observed, the *Ctr* CTL0247-FLAG strain was used to study ChlaDUB1 and Mcl-1 interaction.

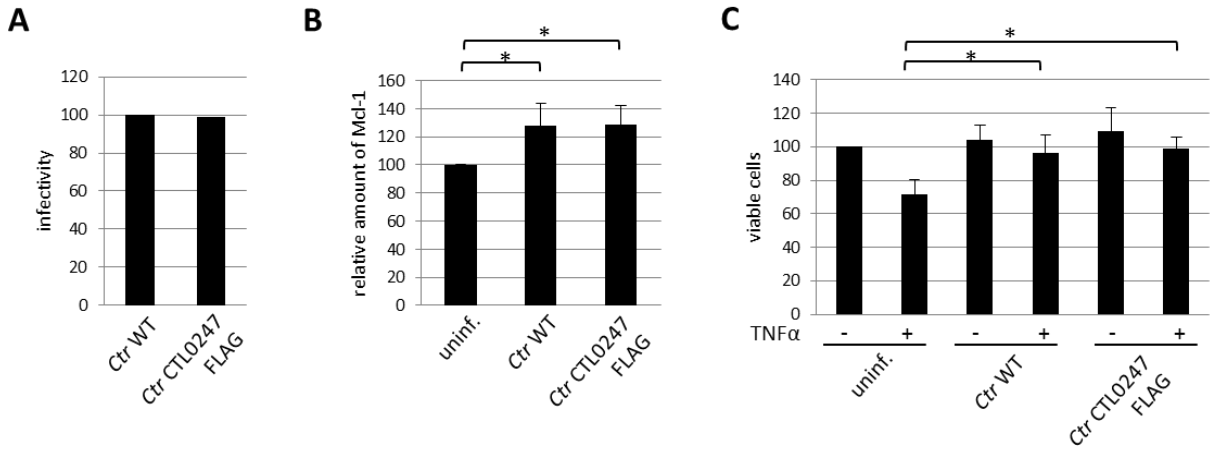

Figure 3.16: Fitness and anti-apoptotic features of the *Ctr* CTL0247-FLAG strain. (A) Infectivity assay. HeLa229 cells were infected with either wild type *Chlamydia* or the *Ctr* CTL0247-FLAG strain. 24 hpi lysates of primary infection control were collected. The second set of infected cells was lysed 48 hpi and used for infection of fresh cells. Progeny infection was harvested 24 hpi and chlamydial growth was measured by HSP60 signal in immunoblot with Actin as loading control. Infectivity was calculated from three individual experiments. Shown are mean values ±SD. (B) Mcl-1 stabilization. HeLa229 cells were infected with *Ctr* WT or *Ctr* CTL0247-FLAG for 24 h. Cells were harvested and Mcl-1 stabilization was analyzed by immunoblot using anti-Mcl-1 antibody with Actin as loading control. Relative amount of Mcl-1 was calculated out of three individual experiments. Depicted are mean values ±SD. (C) Apoptosis assay. HeLa229 cells infected with *Ctr* WT or *Ctr* CTL0247-FLAG for 24 h were induced for apoptosis by TNFα/CHX. Lysates were prepared and proteins were separated by SDS-PAGE. Amount of apoptotic cells was measured by PARP-cleavage in immunoblot with anti-HSP60 and anti-Actin as controls. Relative values for viable cells were calculated out of three individual experiments. Shown are mean values ±SD. * = $p<0.05$.

3.4.3 Interaction study of ChlaDUB1 and Mcl-1

Initial immunofluorescence studies with Mcl-1 or ChlaDUB1 staining indicated the same subcellular localization of both proteins (see Figure 3.7 and Figure 3.9). With the help of the *Ctr* CTL0247-FLAG strain a co-staining for both potential interaction partners was possible. The ChlaDUB1 enzyme was detected by its FLAG-tag and is present at the surface of the chlamydial inclusion as already predicted. Mcl-1 is enriched at the same subcellular compartment and the signals for ChlaDUB1 and Mcl-1 merge at this position (Figure 3.17 A).

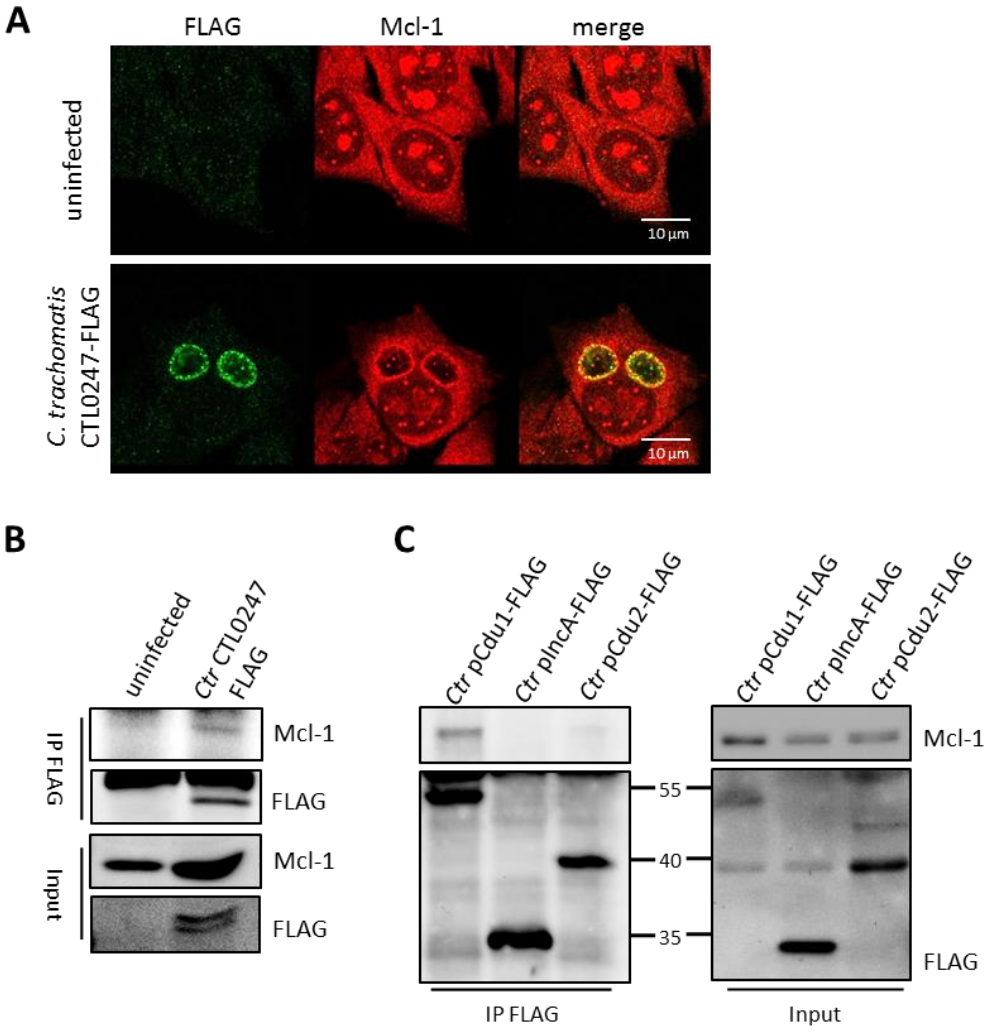

Figure 3.17: Interaction study for ChlaDUB1 and Mcl-1 during infection. (A) HeLa229 cells were infected with *Ctr* CTL0247-FLAG for 24 h and fixed with 4 % PFA/Sucrose. Immunofluorescence staining for FLAG-tagged ChlaDUB1 (anti-FLAG, mouse, Cy3, green) and Mcl-1 (anti-Mcl-1, rabbit, Cy5, red) was performed and analyzed by confocal microscopy. (B) HeLa229 cells were infected with *Ctr* CTL0247-FLAG (MOI1) for 24 h and lysed in Co-IP lysis buffer. Samples were used for immunoprecipitation of the FLAG-tagged ChlaDUB1 using an anti-FLAG antibody. ChlaDUB1-FLAG and co-precipitated endogenous Mcl-1 was detected by immunoblot. (C) HeLa229 cells were infected with the indicated *Ctr* strain overexpressing either ChlaDUB1-FLAG, ChlaDUB2(C282A)-FLAG or IncA-FLAG for 24 h. Cells were lysed in co-IP lysis buffer and FLAG-tagged proteins were precipitated using anti-FLAG antibody. Samples were separated by SDS-PAGE and precipitated FLAG-tagged proteins and co-precipitated Mcl-1 were detected by immunoblot using anti-FLAG and anti-Mcl-1 antibodies.

To further prove the interaction of both proteins co-immunoprecipitation experiments were performed. HeLa229 cells were infected with *Ctr* CTL0247-FLAG and ChlaDUB1 was precipitated using an anti-FLAG antibody and co-precipitated endogenous Mcl-1 was visualized by immunoblot (Figure 3.17 B). To rule out any unspecific binding of Mcl-1 to the FLAG-tag, HeLa229 cells were infected with *Chlamydia* overexpressing ChlaDUB1-FLAG, IncA-FLAG or ChlaDUB2(C282A)-FLAG which were precipitated by their FLAG-tag. However, endogenous Mcl-1 was solely co-precipitated with ChlaDUB1-FLAG, but not with the control proteins (Figure 3.17 C). In addition, the specific interaction between ChlaDUB1 and Mcl-1 was shown by co-precipitation experiments with cells overexpressing ChlaDUB1-FLAG (Figure 3.18 A) or ChlaDUB1-FLAG and Mcl-1 together after transfection (Figure 3.18 B) confirming the previous results.

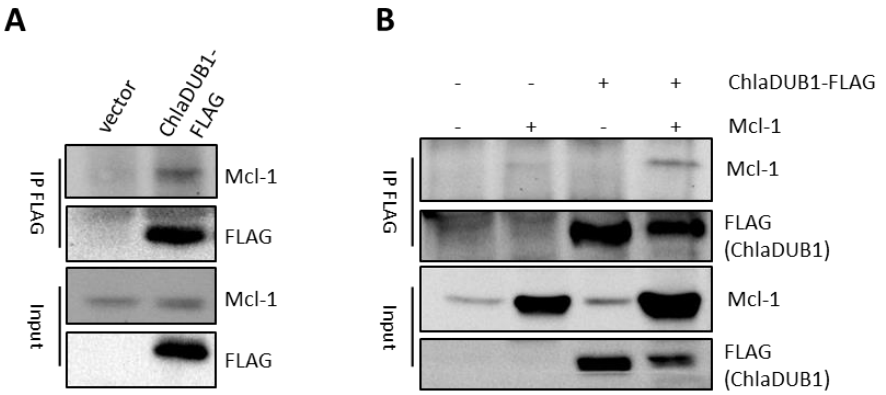

Figure 3.18: Co-Immunoprecipitation of ChlaDUB1 and Mcl-1 overexpressed in mammalian cells. (A) 293T cells were transfected with the empty vector control or pCDNA3/ChlaDUB1-FLAG plasmid for 24 h and lysed in co-IP lysis buffer. Immunoprecipitation using anti-FLAG antibody was performed and precipitated ChlaDUB1-FLAG as well as co-precipitated endogenous Mcl-1 was detected by immunoblot. (B) 293T cells were transfected with pcDNA3/ChlaDUB1-FLAG, pcDNA3/ Mcl-1 or empty vector control in the indicated combination for 24 h. Cells were lysed in co-IP lysis buffer and ChlaDUB1-FLAG was precipitated with an anti-FLAG antibody. Precipitated ChlaDUB1-FLAG and co-precipitated Mcl-1 were detected in immunoblot using anti-FLAG and anti-Mcl-1 antibodies.

Using the method of co-immunoprecipitation an interaction of proteins is shown, but if this interaction is direct or if any other factors are involved in this interaction cannot be clarified. To solve this issue an *in vitro* binding assay with purified proteins was performed. Besides His-tagged ChlaDUB1, the control proteins His-ChlaDUB2 and a truncated His-GroEL were used and specific binding to GST-Mcl-1 was determined in an *in vitro* binding assay and analyzed by immunoblot (Figure 3.19). Solely ChlaDUB1 binds to Mcl-1 whereas the control proteins show no interaction with Mcl-1. Furthermore, the *in vitro* binding assay demonstrates that ChlaDUB1 binds directly to Mcl-1 and no additional factors or modifications are needed for this interaction.

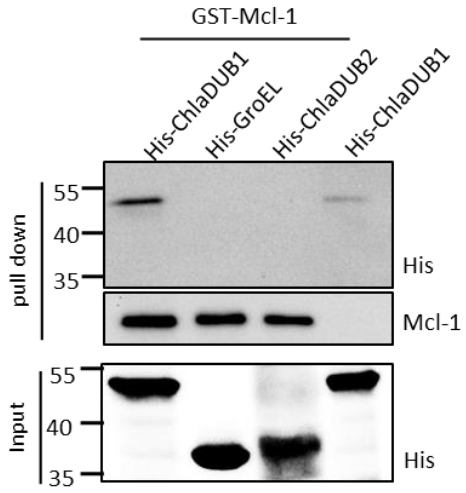

Figure 3.19: *in vitro* binding assay of ChlaDUB1 and Mcl-1. Purified recombinant His-ChlaDUB1 or control proteins His-GroEL and His-ChlaDUB2 were used in an *in vitro* binding assay with recombinant GST-Mcl-1. GST-Mcl-1 was bound to glutathione sepharose beads and then incubated with equal amounts of His-ChlaDUB1, His-GroEL or His-ChlaDUB2, respectively. After washing, proteins were separated by SDS-PAGE and binding of His-tagged proteins to GST-Mcl-1 was investigated by immunoblot analysis using anti-His and anti-Mcl-1 antibodies.

3.4.4 ChlaDUB1 mediates Mcl-1 stabilization by deubiquitination

The previous results indicate that ChlaDUB1 and Mcl-1 interact with each other during infection. However, the evidence that Mcl-1 is a substrate of ChlaDUB1 and that the reduced ubiquitination of Mcl-1 is a result of ChlaDUB1 activity has to be confirmed. Overexpression of ChlaDUB1 either with a C-terminal FLAG-tag (Figure 3.20 A+B) or as a GFP-fusion protein (Figure 3.20 C) in human embryonic kidney 293T cells resulted in a massive increase of Mcl-1 quantity in transfected cells comparable to the phenotype in *C. trachomatis* infection. The overexpression of a catalytically inactive mutant with a cysteine to alanine replacement at amino acid position 345 (ChlaDUB1(C345A)-FLAG) showed reduced ability to enrich Mcl-1 in the cell (Figure 3.20 A+B). Moreover, the complete deletion of the catalytic domain (GFP-ChlaDUB1ΔCD) resulted in complete loss of Mcl-1 stabilization (Figure 3.20 C). The ability of overexpressed ChlaDUB1 to stabilize Mcl-1 was also confirmed in HeLa229 cells routinely used for *Ctr* infection experiments (Figure 3.20 D).

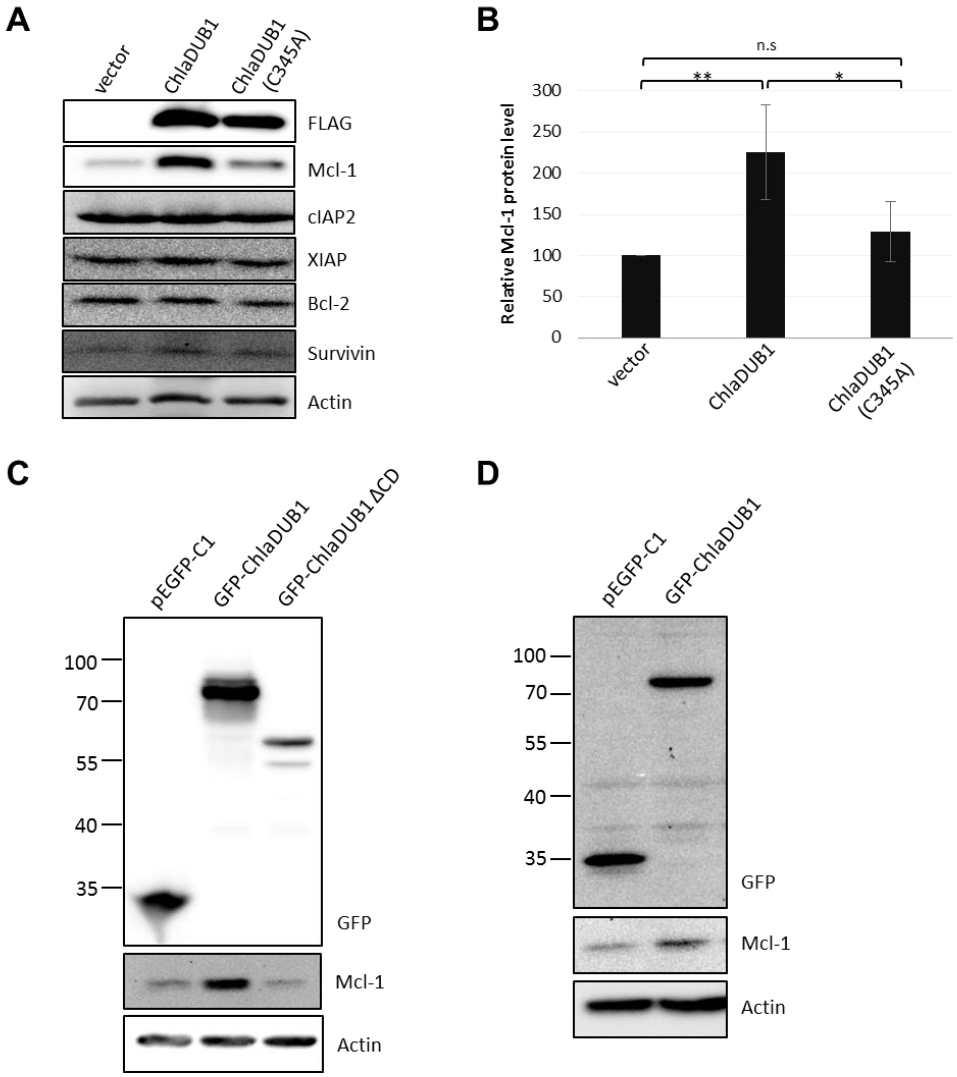

Figure 3.20: ChlaDUB1 overexpression results in Mcl-1 stabilization. (A) 293T cells were transfected with pcDNA3/ChlaDUB1-FLAG, pcDNA3/ChlaDUB1(C345A)-FLAG and empty vector control for 24 h before cells were lysed and proteins separated by SDS-PAGE. Expression of FLAG-tagged proteins and impact on Mcl-1 stabilization was monitored by immunoblot with anti-FLAG and anti-Mcl-1 antibodies. Protein level of anti-apoptotic proteins was tested using anti-cIAP2, XIAP, Bcl-2 and Survivin antibodies. Actin immunoblot served as loading control. (B) Relative amount of Mcl-1 in transfected cells from (A) calculated from three individual experiments. Depicted are mean values ±SD. * = $p<0.05$; ** = $p<0.01$. (C) Transfection of 293T cells with pEGFP-C1 empty vector control or plasmids encoding for the GFP-ChlaDUB1 and GFP-ChlaDUB1ΔCD fusion proteins for 24 h. Cells were lysed and samples were analyzed by immunoblot for protein expression with anti-GFP antibody and Mcl-1 stabilization with anti-Mcl-1 antibody. Actin serves as loading control. (D) GFP-ChlaDUB1 fusion protein was overexpressed in HeLa229 cells by transfection with pEGFP-ChlaDUB1 vector or empty vector control. Lysates were prepared 24 h post transfection and ChlaDUB1 expression and resulting Mcl-1 stabilization were visualized by immunoblot using anti-GFP and anti-Mcl-1 antibodies.

With the following experiments the enzymatic activity of ChlaDUB1 towards Mcl-1 was investigated. 293T cells were transfected with plasmids promoting the overexpression of HA-Ubiquitin, myc-tagged Mcl-1 and either empty vector control, ChlaDUB1-FLAG or ChlaDUB1(C345A)-FLAG. Mcl-1 was precipitated out of these cells by its myc-tag under denaturing conditions and stained for ubiquitin in immunoblot. The results of the *in vivo* DUB assay are depicted in Figure 3.21 A and reveal the deubiquitination of Mcl-1 in presence of ChlaDUB1. The deubiquitination of Mcl-1 is related to the

enzymatic activity of ChlaDUB1 since the catalytic inactive mutant shows more poly-ubiquitinated Mcl-1 comparable to the empty vector control. However, the *in vivo* DUB assay is unsuitable to confirm Mcl-1 as a direct substrate of ChlaDUB1 because the reduced ubiquitination of Mcl-1 might also be a secondary consequence of ChlaDUB1 activity. To approve Mcl-1 as a direct substrate of ChlaDUB1 an *in vitro* DUB assay was performed. Recombinant Mcl-1 was first ubiquitinated and subsequently used as substrate for ChlaDUB1 or control enzymes. Mcl-1 is deubiquitinated in presence of ChlaDUB1 whereas the enzymatic inactive point mutant ChlaDUB1(C345A) or a chemically inactivated ChlaDUB1 (ChlaDUB1 NEM) is not able to deubiquitinate Mcl-1. This is also the case for the unrelated control DUB UCH-L3 (Figure 3.21 B). With the help of *in vitro* DUB assays Mcl-1 was confirmed as a direct substrate of ChlaDUB1.

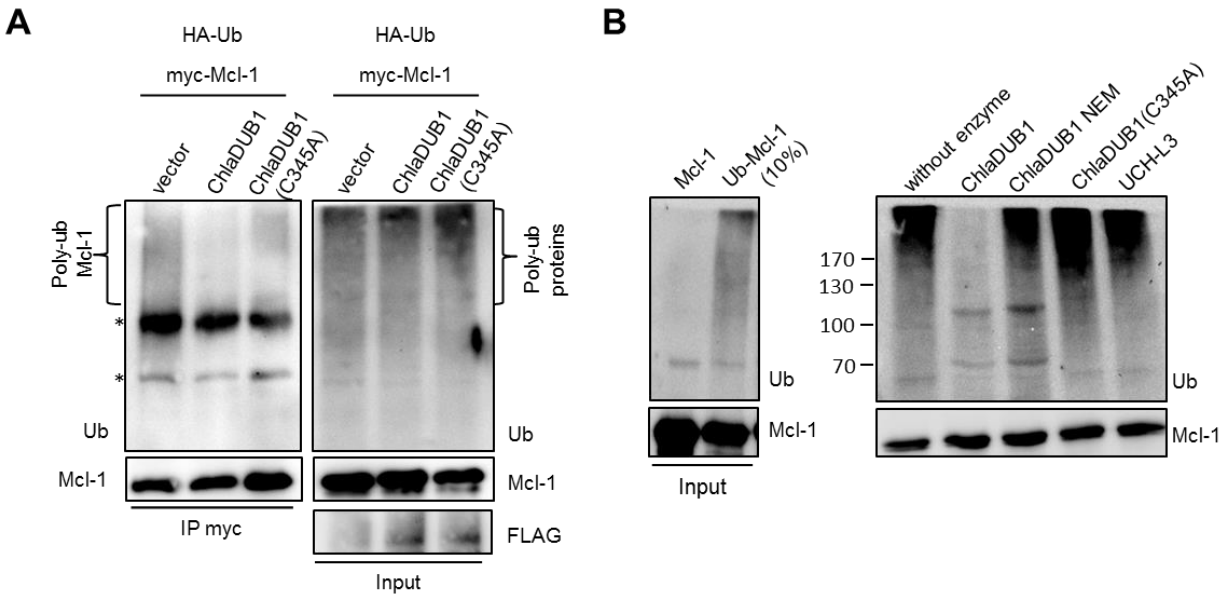

Figure 3.21: ChlaDUB1 as deubiquitinase of Mcl-1 *in vivo* and *in vitro*. (A) *In vivo* DUB assay. 293T cells were transfected with plasmids promoting the overexpression of HA-Ubiquitin (pcDNA3.1/HA-Ubiquitin), myc-tagged Mcl-1 (pcDNA3/myc-Mcl-1) and ChlaDUB1-FLAG (pcDNA3/ChlaDUB1-FLAG), ChlaDUB1(C345A)-FLAG (pcDNA3/ChlaDUB1(C345A)-FLAG) or empty vector control (pcDNA3). Cells were cultivated in presence of the proteasome inhibitor MG132 to block degradation of ubiquitinated proteins. 24 h post transfection cells were lysed under denaturing conditions and Mcl-1 was immunoprecipitated under stringent conditions using anti-myc-tag antibody. Ubiquitination pattern of precipitated Mcl-1 was analyzed by immunoblot using anti-ubiquitin antibody. Mcl-1 was detected by anti-Mcl-1 antibodies and expression of FLAG-tagged proteins was monitored by immunoblot with an anti-FLAG antibody. (B) *In vitro* DUB assay. Recombinant GST-Mcl-1 was incubated with E1, E2 and ΔN-HECT-H9 E3 ligase, ubiquitin and ATP-regenerating buffer to obtain poly-ubiquitinated Mcl-1 (left panel). Poly-ubiquitinated Mcl-1 was incubated with 20 nM recombinant His-ChlaDUB1, His-ChlaDUB1(C345A) or by NEM chemically inactivated His-ChlaDUB1. As negative control served the unrelated DUB UCH-L3 in a final concentration of 0.4 nM. Enzyme activity was stopped by addition of 2x Laemmli and deubiquitination of Mcl-1 was visualized by immunoblot with an anti-ubiquitin antibody (right panel). Presence of equal amounts of Mcl-1 was tested with anti-Mcl-1 immunoblot.

3.5 Role of ChlaDUB1 during infection and apoptosis induction

3.5.1 ChlaDUB1 knock-out and dominant-negative *Ctr* strains

With the previous *in vitro* experiments and transgene overexpression of the chlamydial effector ChlaDUB1 in eukaryotic cells it was possible to demonstrate that ChlaDUB1 is responsible for Mcl-1 stabilization by deubiquitination. The stabilization of Mcl-1 by its deubiquitination was also detected during chlamydial infection (see Figure 3.2 A). To verify the participation of ChlaDUB1 in this process during infection a strategy to create ChlaDUB1 knock-out mutants in *Chlamydia* was designed.

3.5.1.1 ChlaDUB1 knock-out strategy

To obtain ChlaDUB1-negative mutants of *Chlamydia* the pAH3 plasmid (Figure 3.22) was designed which contains homology regions targeting the integration of the already described selection cassette (Figure 3.13 A) inside the CTL0247 gene causing its disruption.

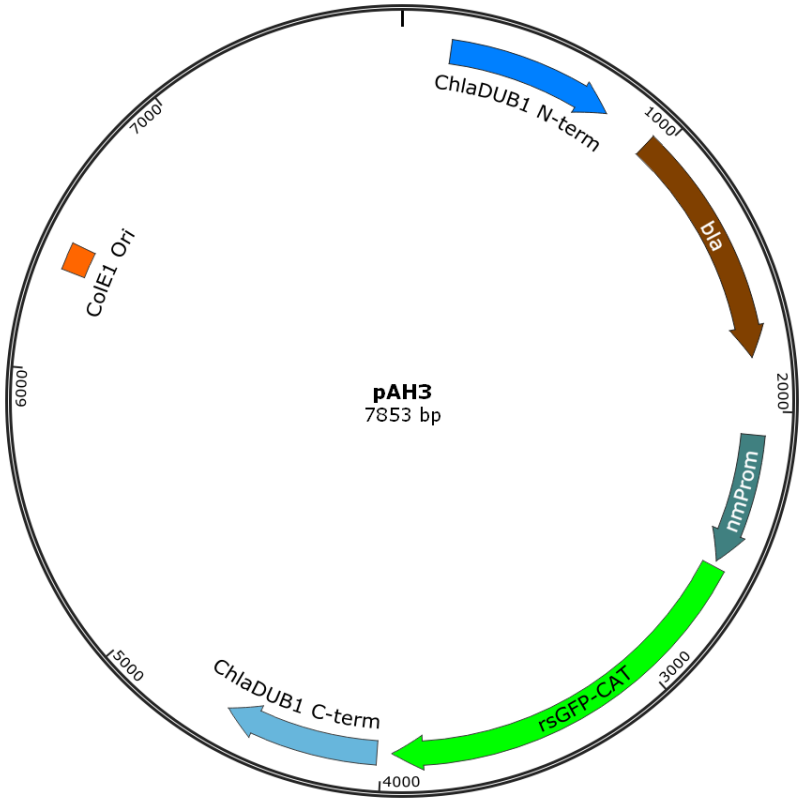

Figure 3.22: Vector map of pAH3 plasmid. As backbone of the pAH3 plasmid serves the pQE30/Cdu1 construct which was opened by PCR in the middle of the ChlaDUB1 gene. A modified selection cassette originating from the pGFP::SW2 plasmid lacking the pUC origin of replication was generated by overlap PCR and cloned into the opened pQE30/Cdu1 plasmid by DiSecTriSec method. The pAH3 plasmid can replicate in *E. coli* but not in *C. trachomatis* by which the integration of the selection cassette into the chlamydial genome inside the CTL0247 gene is favored in presence of the selection antibiotic penicillin G.

The backbone of the pAH3 plasmid originates from the pQE30/Cdu1 plasmid. The pQE30 plasmid was opened by PCR amplification and a modified selection cassette lacking the pUC origin of replication was inserted. As homology domains mediating the homologues recombination into the CTL0247 gene serve the 500 bp sequences of the N-terminal and C-terminal part of the ChlaDUB1 gene. The pAH3 plasmid isolated out of *E. coli* JM110 was used to transform *C. trachomatis*. *Ctr* were transformed with the pAH3 plasmid but no viable transformants could be obtained. Since the homologues recombination into the chlamydial genome at the CTL0247 locus to create the *Ctr* CTL0247-FLAG strain was already performed once it can be assumed that CTL0247 deficient *Chlamydia* are not viable and ChlaDUB1 represents an essential gene.

3.5.1.2 ChlaDUB1 dominant-negative strategy

Since ChlaDUB1 appears to be crucial for chlamydial replication, a dominant-negative approach was set up which can be used to weaken but not to completely diminish the effect of an enzyme. The chlamydial expression plasmid pCdu1(C345A)-FLAG::SW2 encodes for the point mutant of ChlaDUB1 under the control of the constantly active neisserial promoter. *Chlamydia* harboring this plasmid will overexpress the FLAG-tagged mutant enzyme contemporary with the endogenous wild type enzyme. *Chlamydia* were transformed with the pCdu1(C345A)-FLAG::SW2 plasmid and selected to obtain the *Chlamydia* stain *Ctr* pCdu1(C345A)-FLAG::SW2.

To test if transgene encoded ChlaDUB1(C345A)-FLAG is expressed an immunoblot analysis of infected cells was performed (Figure 3.23 A). The immunoblot reveals that the FLAG-tagged mutant enzyme is massively overexpressed in the *Ctr* pCdu1(C345A)-FLAG::SW2 strain compared to the wild type *Chlamydia*. The proper secretion to the surface of the inclusion was visualized by immunofluorescence staining. The results depicted in Figure 3.23 B show that the transgene expressed enzyme distinguishable from the wild type ChlaDUB1 by the additional FLAG-tag is secreted to the surface of the inclusion but high amounts of the massively overexpressed protein are also enriched inside the chlamydial particles.

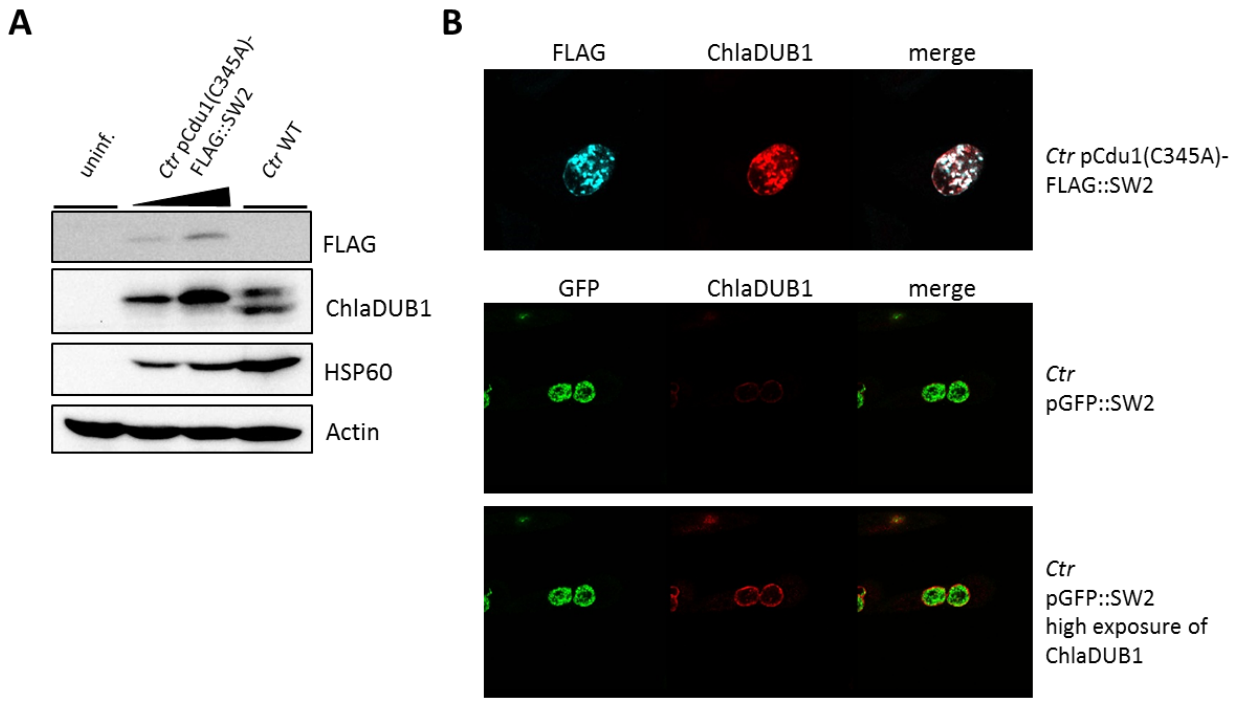

Figure 3.23: Characterization of the ChlaDUB1 dominant-negative strain *Ctr* pCdu1(C345A)-FLAG::SW2. (A) HeLa229 cells were infected with *Ctr* WT (MOI1) or *Ctr* pCdu1(C345A)-FLAG::SW2 with two different MOIs for 24 h. Lysates were prepared and expression of ChlaDUB1(C345A)-FLAG was detected with anti-FLAG and anti-ChlaDUB1 antibodies in immunoblot analysis. Anti-HSP60 immunoblot indicates degree of infection and Actin immunoblot serves as loading control. (B) HeLa229 cells were infected with *Ctr* pCdu1(C345A)-FLAG::SW2 or *Ctr* pEGFP::SW2 for 24 h. Cells were fixed and stained for the FLAG-tag (anti-FLAG, mouse, Cy3, cyan) and ChlaDUB1 (anti-ChlaDUB1, rabbit, Cy5, red). Samples were analyzed by confocal microscopy.

Additional immunofluorescence staining against the FLAG-tag and HSP60 to mark *Chlamydia* uncovered that the stringent selected stock (50 U PenG/ml) with penicillin-resistant *Chlamydia* consists of a *Chlamydia* population expressing the FLAG-tagged mutant ChlaDUB1 enzyme and a population of *Chlamydia* that are resistant to penicillin indicating the presence of the pCdu1(C345A)-FLAG::SW2 plasmid but with no expression of ChlaDUB1(C345A)-FLAG (Figure 3.24 A). By single cell sorting of *Ctr* pCdu1(C345A)-FLAG::SW2-infected HeLa229 cells *Chlamydia* populations derived from one single bacterium were obtained. Each clone was tested for ChlaDUB1(C345A)-FLAG expression, thereby clone B9 of the *Ctr* pCdu1(C345A)-FLAG::SW2 strain showed a medium overexpression of the FLAG-tagged mutant enzyme whereas clone G7 showed very weak expression (Figure 3.24 B). However, immunoblot analysis of *Ctr* WT and *Ctr* pCdu1(C345A)-FLAG::SW2 clone B9-infected cells revealed that the overexpression of the mutant enzyme from the beginning of infection on had no effect on Mcl-1 stabilization (Figure 3.24 C).

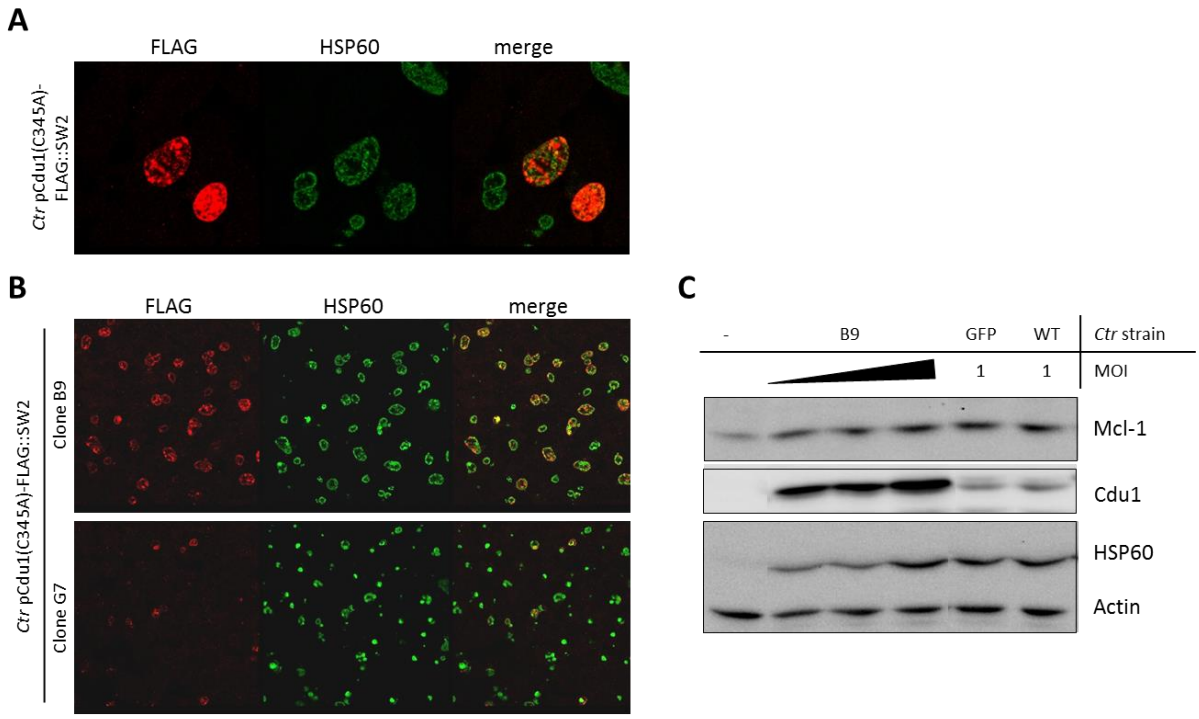

Figure 3.24: Single clone selection of *Ctr* pCdu1(C345A)-FLAG::SW2. (A) HeLa229 cells were infected with the *Ctr* pCdu1(C345A)-FLAG::SW2 strain which was selected with 50 units penicillin for multiple passages. Infected cells were fixed 24 hpi and stained with anti-FLAG (mouse, Cy5, red) and anti-HSP60 (rabbit, Cy2, green) antibodies. Immunofluorescence pictures were prepared by confocal microscopy. (B) HeLa229 cells were infected with *Ctr* pCdu1(C345A)-FLAG::SW2 single clones B9 and G7 for 24 h. Cells were fixed and ChlaDUB1(C345A)-FLAG expression was visualized by anti-FLAG (mouse, Cy5, red) immunofluorescence staining. Chlamydial particles were detected using an anti-HSP60 antibody (rabbit, Cy2, green). Staining was analyzed by confocal microscopy. (C) HeLa229 cells infected with *Ctr* WT, *Ctr* pGFP::SW2 or *Ctr* pCdu1(C345A)-FLAG::SW2 clone B9 were lysed 24 hpi and analyzed by immunoblot. Expression of ChlaDUB1 wild type enzyme or ChlaDUB1(C345A)-FLAG was detected with anti-ChlaDUB1 antibody and impact on Mcl-1 quantity was investigated using anti-Mcl-1 antibody. Immunoblot against HSP60 indicated degree of infection and Actin represents loading control.

It is possible that the dominant-negative effect of ChlaDUB1(C345A)-FLAG is compensated by the amount of mutant enzyme present in the infected cell. Furthermore, the overexpression of the point mutant in eukaryotic cells showed a slight positive effect on Mcl-1 stability whereas the mutant lacking the catalytic domain did not (see Figure 3.20). Therefore, a second plasmid encoding for the overexpression of ChlaDUB1ΔCD-FLAG was constructed and transformed into *Ctr*. However, no viable transformants containing the pCdu1ΔCD-FLAG::SW2 plasmid expressing ChlaDUB1ΔCD-FLAG could be obtained.

The overexpression of the ChlaDUB1(C345A)-FLAG point mutant showed no dominant-negative effect and *Chlamydia* overexpressing the ChlaDUB1 enzyme lacking the whole catalytic domain were not viable. By these results it became obvious that if this well balanced system of protease expression is influenced too much, the bacteria do not survive or are disadvantaged. Therefore, an inducible expression system for controlled overexpression with respect to time and amount of expression has to be introduced.

3.5.2 *In vitro* ChlaDUB1 inhibitor screen

It was shown that *Ctr*-infected cells depleted in Mcl-1 are sensitized for apoptosis. Consequently, the identification of the chlamydial effector protein ChlaDUB1 mediating Mcl-1 stabilization during infection represents a promising target for anti-chlamydial therapy. Moreover, several experiments indicated ChlaDUB1 to be an essential factor for *Chlamydia*. Blocking ChlaDUB1 activity would on the one hand result in loss of Mcl-1 thereby sensitizing the infected cell for apoptosis and facilitating its removal by the immune system; on the other hand, inactivation of ChlaDUB1 will limit chlamydial growth and thereby course of disease.

DUB activity can be monitored *in vitro* by hydrolysis of the ubiquitin derivate Ub-AMC (7-amino-4-methylcoumarin). Ub-AMC is non-fluorescent until the ubiquitin moiety is cleaved and AMC fluorescence can be detected. Initially, the ability of ChlaDUB1 and the unrelated DUB UCH-L3 to hydrolyze Ub-AMC was monitored in the Ub-AMC assay. As controls the broad cysteine protease inhibitor NEM and different solvents were used. Figure 3.25 A shows DUB activity measured by number of RFUs (relative fluorescence units) which can be blocked completely by inhibitor addition. A library consistent of 25 potential cysteine protease inhibitors was provided by the group of Prof. Dr. Tanja Schirmeister, University of Mainz. These compounds were tested in the *in vitro* Ub-AMC assay for their ability to block ChlaDUB1 activity. Recombinant ChlaDUB1 and UCH-L3 were pre-incubated with the inhibitors and DUB activity was measured over time by quantity of fluorescence signal resulting from Ub-AMC cleavage (Figure 3.25 B). The Ub-AMC assay identified compound 8 as a strong ChlaDUB1 inhibitor which showed only weak impact on UCH-L3 control enzyme. Compound 8 is an endoperoxide called CYN312, isolated out of the marine sponge *Plakortis halichondrioides* and was later identified as Plakortide E (Sun et al., 2011).

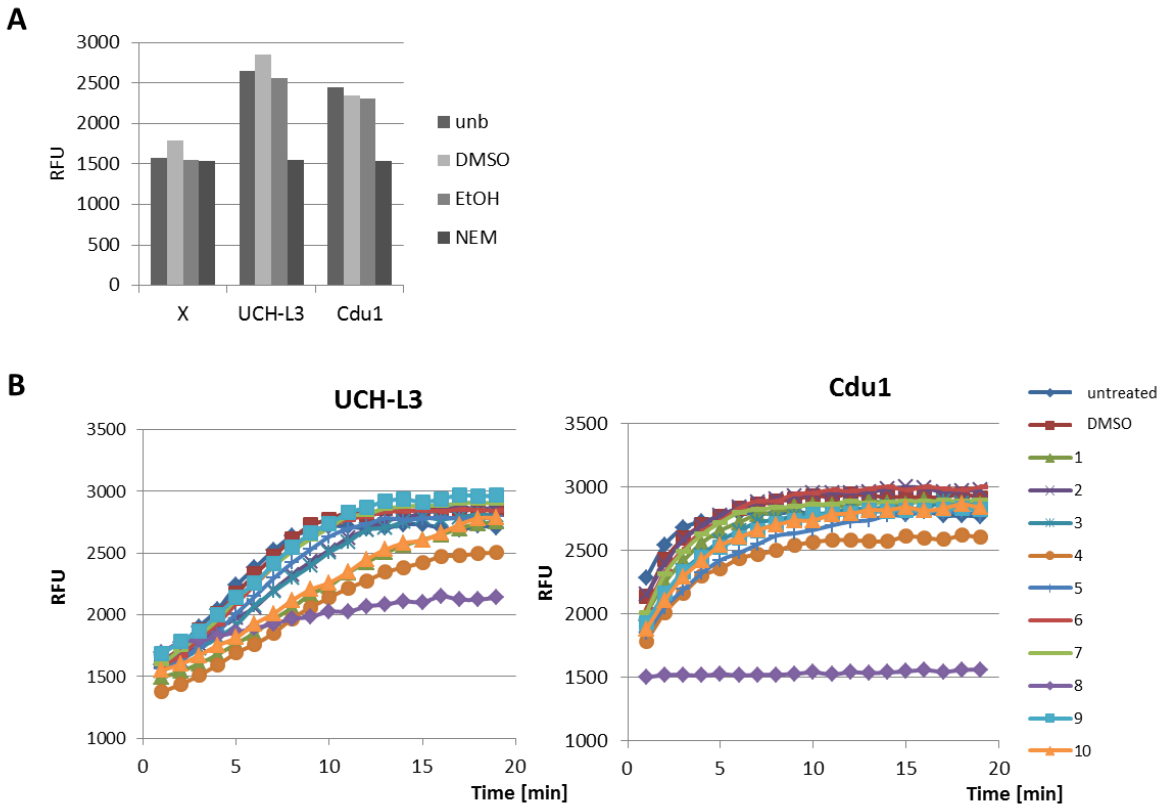

Figure 3.25: *in vitro* ChlaDUB1 inhibitor screen with Ub-AMC assay. (A) Ub-AMC assay with 15 nM ChlaDUB1 and 0.4 nM UCH-L3. Enzymes were pre-incubated with common cysteine protease inhibitor NEM (10 mM) or solvents (DMSO, ethanol) before fluorogenic substrate Ub-AMC was added. DUB activity was measured by fluorescence signal (RFU) in a TECAN multiplate reader at Ex_{380}/Em_{460nm}. (B) ChlaDUB1 inhibitor screen with compounds one to ten of potential cysteine protease inhibitor library (T. Schirmeister, University of Mainz) in Ub-AMC assay. Enzymes were treated with potential DUB inhibitors (100 µM) as described in (A) and activity was measured by Ub-AMC hydrolysis and fluorescence signal (RFU) over time in a TECAN multiplate reader (Ex_{380}/Em_{460nm}).

The inhibitory properties of CYN312 towards ChlaDUB1 were further investigated by inhibitor titration experiments. In the Ub-AMC assay the half maximal inhibitory concentration (IC_{50}) of CYN312 for ChlaDUB1 was defined to 11.5 µM (Figure 3.26 A+B). Furthermore, the inhibitory mode of action of CYN312 for ChlaDUB1 was investigated by Lineweaver-Burk calculation of the Michaelis constant K_m for ChlaDUB1 or ChlaDUB1 blocked by CYN312. The K_m values for both samples were calculated to 825 nM or 865 nM, respectively. Not the K_m values, but the maximum speed of enzymatic reaction after CYN312 treatment was degreased from 1.64 nM/s to 0.46 nM/s defining CYN312 as a non-competitive inhibitor of ChlaDUB1 (Figure 3.26 C).

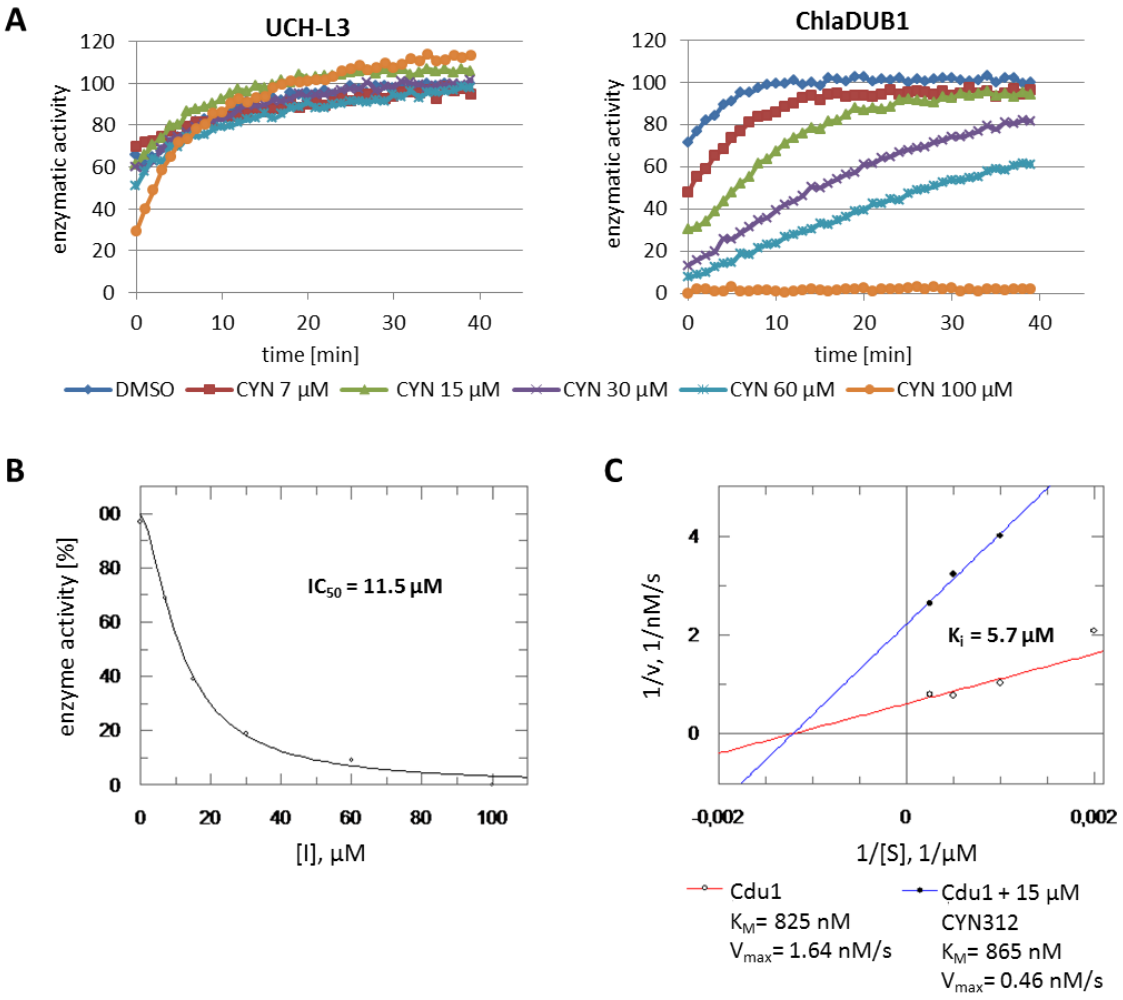

Figure 3.26: Determination of inhibitory properties of CYN312 towards ChlaDUB1. (A) Ub-AMC assay with 15 nM ChlaDUB1 and 0.4 nM UCH-L3. Activity of enzymes pretreated with increasing concentrations of CYN312 (7-100 µM) as described before was analyzed in Ub-AMC assay over time. Relative enzyme activities of UCH-L3 control enzyme and ChlaDUB1 were calculated and are depicted. (B) Determination of the IC_{50} concentration of CYN312 for ChlaDUB1. Data from A (right panel) was analyzed with GraFit (Version 5.0.13, Erithacus Software Ltd.) program and IC_{50} value was calculated. (C) Determination of inhibitory mode of action of CYN312 towards ChlaDUB1. ChlaDUB1 (5 nM) was pre-incubated with DMSO control or 15 µM CYN312 and Ub-AMC assay with increasing substrate concentrations was performed. Michaelis-Menten value K_M and v_{max} were calculated with GraFit software.

3.5.3 CYN312 in *Ctr* infection and apoptosis induction

With the help of the strong ChlaDUB1 inhibitor CYN312 it was now possible to block ChlaDUB1 activity at a given time point during infection and investigate the function of ChlaDUB1 regarding Mcl-1 stabilization and apoptosis resistance. Initially, the cytotoxicity of CYN312 towards host cells was tested by AnnexinV-APC and 7AAD staining analyzed in flow cytometry. To approve the effects of CYN312 as specific, several compounds of the inhibitor library were used as controls and the results for compound 7 serve as representative example. Figure 3.27 shows that CYN312 as well as control compound 7 are non-cytotoxic towards uninfected as well as *Ctr*-infected HeLa229 cells.

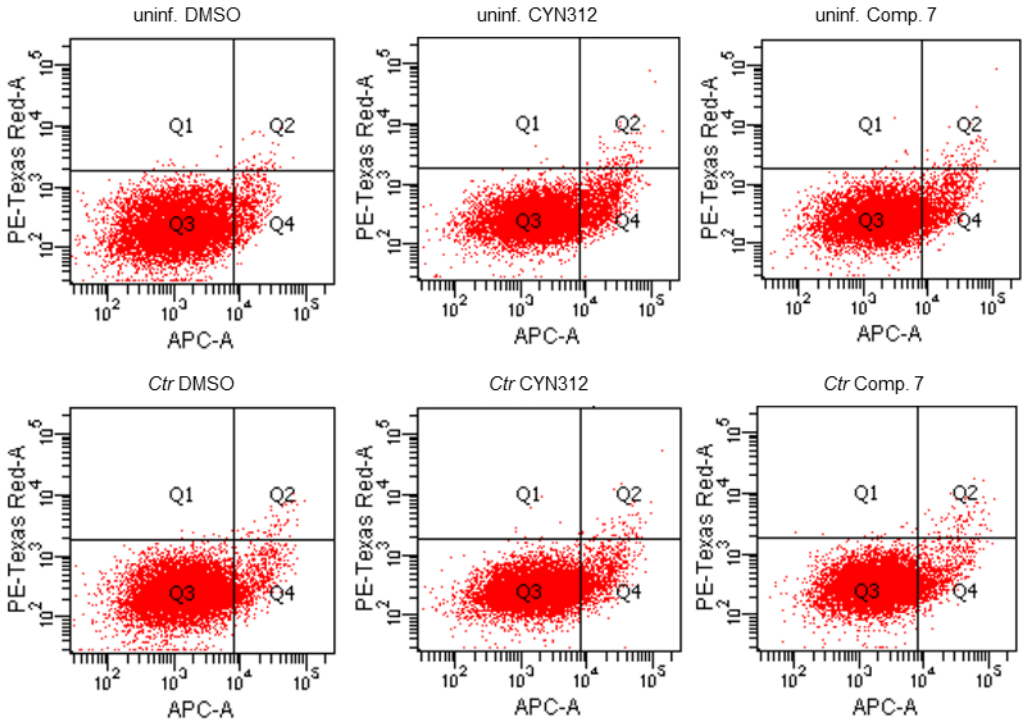

Figure 3.27: Cytotoxic effect of CYN312 on HeLa229 cells. HeLa229 cells were seeded in 12 Well plates and infected with *Ctr*. 8 hpi 20 µM CYN312, compound 7 and DMSO were added over night. Cells were trypsinized, pelleted and resuspended in Annexin binding buffer containing AnnexinV-APC and 7AAD. Cells were stained at RT in the dark for 15 min and subsequently analyzed by flow cytometry. Apoptotic cells are simply Annexin-APC positive (Q4) whereas necrotic or dead cells are Annexin-APC and 7AAD positive (Q2). Viable cell population is double negative and located in the Q3 area.

Subsequently, the impact of CYN312 on chlamydial development was tested in infection experiments. Cells infected with GFP-expressing *Ctr* were treated with CYN312 and controls upon 8 h of infection and chlamydial growth was investigated by bright field microscopy and GFP-signal 24 hpi (Figure 3.28 A). Afterwards, cells were lysed and quantity of Mcl-1 was analyzed by immunoblot (see Figure 3.28 B-E). According to the FACS analysis no cytotoxicity of CYN312 towards the host cell could be observed in bright field (BF) analysis of infected cells treated with indicated compounds (Figure 3.28 A). But, the chlamydial growth is tremendous affected at CYN312 concentrations of 20 µM indicated by the reduction of inclusion size and GFP-signal correlating with amount of chlamydial particles (Figure 3.28 A). The HSP60 immunoblot results confirmed these observations (Figure 3.28 B). Additionally, besides reduced chlamydial growth the capability to stabilize Mcl-1 is lost (Figure 3.28 B+C). No effect on chlamydial growth or Mcl-1 stabilization was observed in cells treated with compound 7 indicating that the anti-chlamydial property of CYN312 is not only a side effect (Figure 3.28 A, D+E).

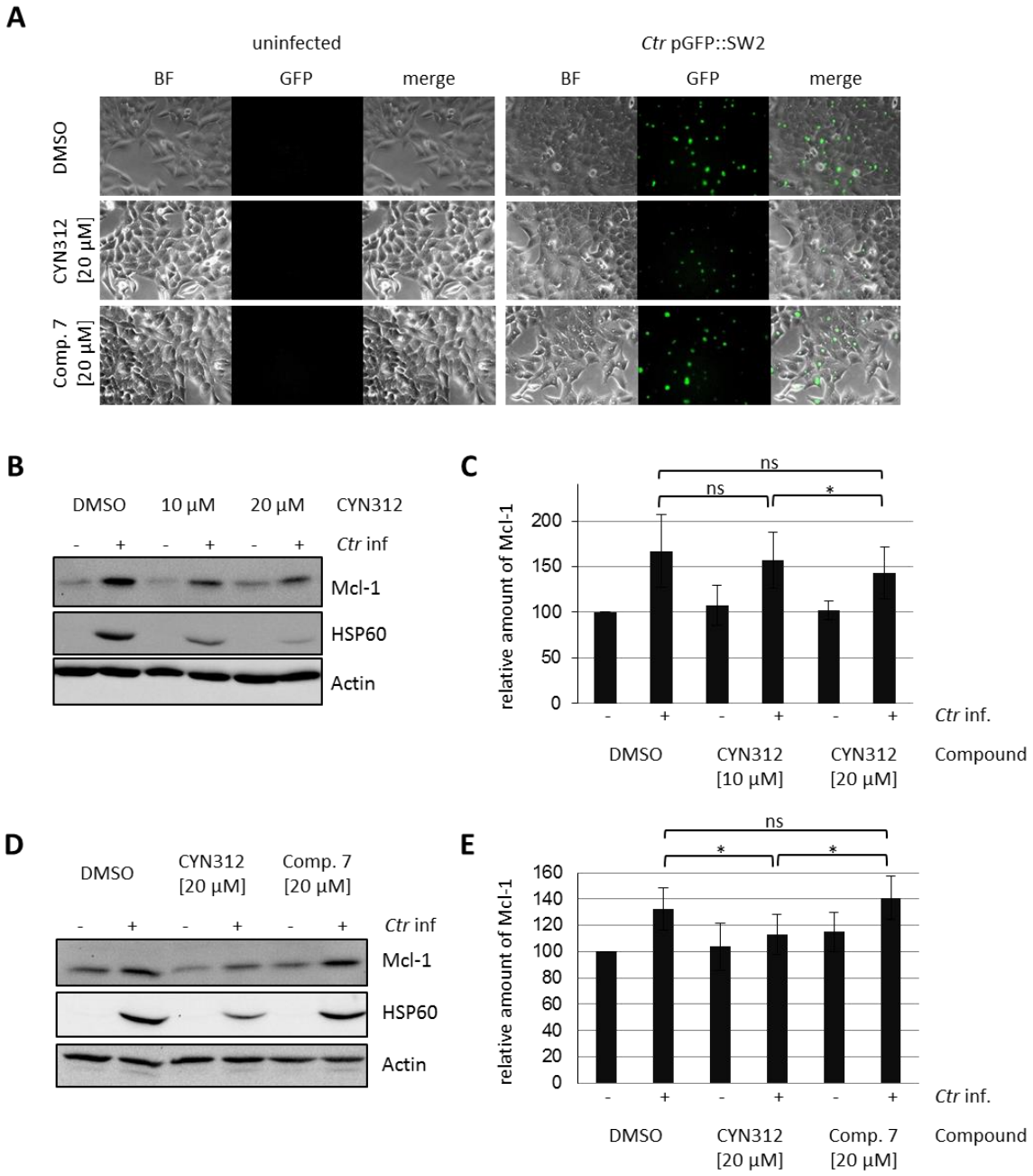

Figure 3.28: Application of the ChlaDUB1 inhibitor CYN312 in cell culture during *Ctr* infection. (A-D) HeLa229 cells were infected with *Ctr* pGFP::SW2 (MOI1) and CYN312 or control compound were added 8 hpi in the indicated concentrations. (A) Degree of *Ctr* infection (24 hpi) after inhibitor treatment was monitored by GFP signal in fluorescence microscopy and bright field microscopy. (B) Cell lysates of *Ctr*-infected HeLa229 cells 24 hpi treated with increasing concentrations of CYN312 upon 8 h of infection were prepared and analyzed by immunoblot. Protein amount of Mcl-1 was checked by immunoblot using anti-Mcl-1 antibody and degree of infection was visualized by HSP60 signal. Actin was used as loading control. (C) Relative amount of Mcl-1 calculated from three individual experiments described in (B) are depicted ±SD. (D) Infected HeLa229 cells were treated with 20 µM CYN312, compound 7 or DMSO as controls upon 8 h of infection. Lysates were prepared 24 hpi and proteins were separated by SDS-PAGE. Anti-HSP60 and anti-Actin immunoblot represent degree of infection and loading control. Mcl-1 stabilization was monitored with anti-Mcl-1 immunoblot. (E) Relative values for Mcl-1 protein level were calculated from three individual experiments described in (D). Depicted are mean values ±SD; * = p<0.05.

To prove a specific effect of CYN312 towards ChlaDUB1 and to no other chlamydial factor, the infection experiments were repeated and CYN312 and control compounds were removed from the cells by repeated washing steps at defined time points of infection. Initially, CYN312 was added 2 hpi and removed 12 hpi prior to hypothesized time of ChlaDUB1 expression. Immunoblots shown in Figure 3.29 A reveal that early removal of CYN312 completely recovers Mcl-1 stabilization and only slightly affects bacterial replication. Furthermore, removal of CYN312 upon 24 hpi with analysis 36 hpi exhibits the same tendency. The ability to stabilize Mcl-1 is fully recovered and block of chlamydial replication is reversed (Figure 3.29 B).

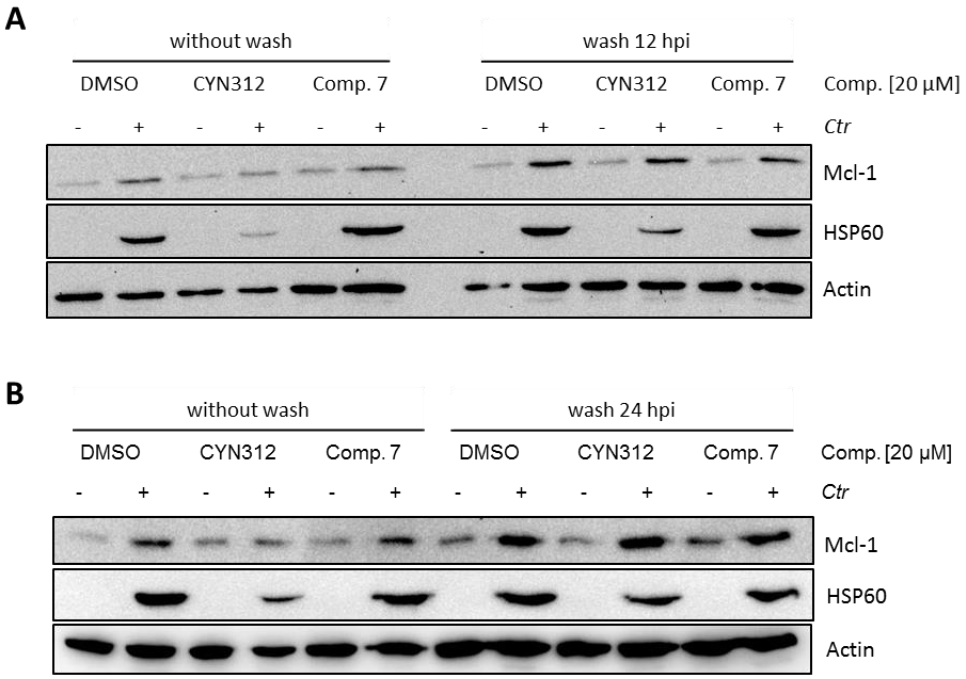

Figure 3.29: CYN312 treatment of *Ctr*-infected cells at defined time points of infection. (A) HeLa229 cells were infected with *Ctr* (MOI1) and 2 hpi CYN312 or controls (compound 7, DMSO) were added to the cells in the final concentration of 20 µM. Upon 12 h of infection cells were washed repeatedly and lysed for immunoblot analysis 24 hpi. Mcl-1 protein level was checked by anti-Mcl-1 immunoblot and degree of infection and equal loading was controlled by anti-HSP60 and anti-Actin immunoblot. (B) *Ctr*-infected HeLa229 cells were treated with 20 µM CYN312, compound 7 or DMSO upon 8 hpi and 24 hpi chemicals were removed by repeated washing steps. Cell lysates for immunoblot analysis were prepared 36 hpi and chlamydial growth (anti-HSP60) and ability to stabilize Mcl-1 (anti-Mcl-1) was analyzed. Anti-Actin immunoblot represents loading control.

The infection experiments repeated with *C. trachomatis* D (*Ctr* D) expressing an enzyme homologue to ChlaDUB1 of *C. trachomatis* L2 and with *C. pneumoniae* (*Cpn*) which do not express deubiquitinating enzymes during infection brought further proof for a specific effect of CYN312 towards ChlaDUB1. Whilst CYN312 blocked *Ctr* D replication and ability to stabilize Mcl-1 (Figure 3.30 A), prolonged treatment of *Cpn*-infected cells with CYN312 showed no effect on bacterial growth nor on Mcl-1 quantity in the cell (Figure 3.30 B).

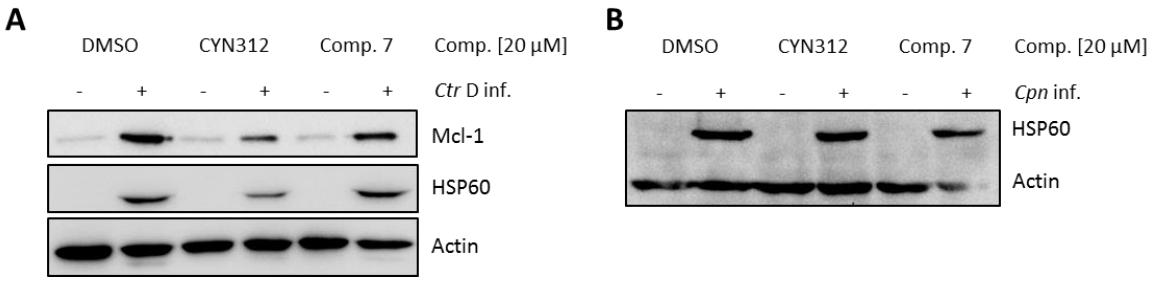

Figure 3.30: CYN312 treatment in *Ctr* D and *Cpn* infection. (A) HeLa229 cells were infected with *Ctr* D and 8 hpi CYN312 and controls comp. 7 and DMSO were added in the final concentration of 20 µM to the cells. 24 hpi cells were lysed and chlamydial growth (anti-HSP60) as well as amount of Mcl-1 (anti-Mcl-1) were determined by immunoblot. Actin represents loading control. (B) HeLa229 cells infected with *Cpn* were treated with 20 µM CYN312, comp. 7 or DMSO upon 8 h of infection. 72 hpi cells were lysed and degree of infection was analyzed by immunoblot using an anti-HSP60 antibody.

The treatment of *Ctr*-infected cells with CYN312 resulted in a massive growth defect of the bacteria when applied early in infection. This phenotype strengthens the hypothesis that ChlaDUB1 is essential for chlamydial replication or survival in the cell. The second function of ChlaDUB1 during infection is the stabilization of Mcl-1 important for apoptosis resistance of infected cells. The impact of CYN312 on *Chlamydia*-mediated apoptosis resistance was tested in an apoptosis assay. Infected cells were treated with CYN312 or control compounds upon 14 hpi. Apoptosis was induced 24 hpi by TNFα/CHX and lysates were prepared and analyzed by immunoblot. Apoptosis induction was detected by PARP-cleavage in immunoblot using anti-PARP antibodies. Relative values of viable cells were calculated and are depicted in Figure 3.31. The control compound 7 as well as DMSO control had no effect on apoptosis resistance whilst CYN312 significantly sensitized *Ctr*-infected cells for apoptosis.

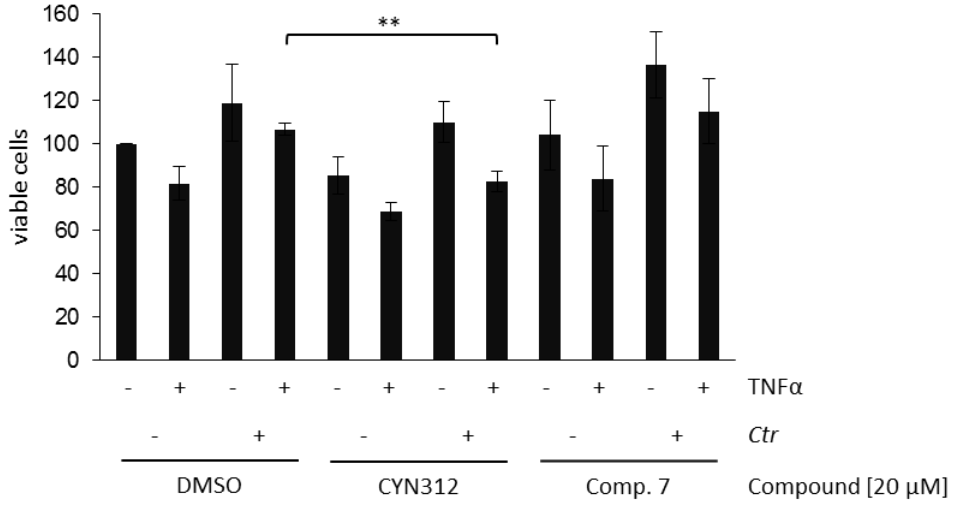

Figure 3.31: Apoptosis assay with CYN312 during *Ctr*-infection. CYN312 in apoptosis assay. HeLa229 cells were infected with *Ctr* (MOI1) and CYN312 or compound 7 inhibitors or DMSO were added to the infection 14 hpi. 24 hpi apoptosis was induced by addition of TNFα/CHX and lysates were prepared for immunoblot. Proteins were separated by SDS-PAGE and apoptosis induction was detected by PARP-cleavage using anti-PARP antibody. Relative values of viable cells were calculated using ImageJ depending on PARP cleavage and Actin loading control. Depicted are mean values calculated from three individual experiments ±SD. ** = $p<0.01$.

4 Discussion

Chlamydia trachomatis are obligate intracellular pathogens which completely depend on their host cell with respect to replication niche and nutrition acquisition. Upon infection, *Chlamydia* take control over the host cell by manipulation of multiple cellular processes such as signaling pathways, cell cycle and metabolism and by alteration of the proteome of the cell (Bavoil et al., 2000; Olive et al., 2014; Valdivia, 2008). *Chlamydia* have an incomplete metabolism and many metabolites have to be obtained from the host cell, for example lipids or nucleotides (Iliffe-Lee and McClarty, 1999; Ojcius et al., 1998). Furthermore, as soon as infectious EBs enter the cell they differentiate into the non-infectious, replicative RBs meaning that a premature host cell death would cause the complete loss of one generation of *Chlamydia*. To ensure the completion of the developmental cycle and the production of infectious progeny RBs mediate strong resistance to host cell apoptosis (Bohme et al., 2010; Fan et al., 1998; Rajalingam et al., 2008; Rajalingam et al., 2006; Sharma et al., 2011; Xiao et al., 2004).

With a RNAi screen the host cell proteins Survivin, HIF1α and Mcl-1, all massively stabilized at defined time points during infection, as well as components of the MAPK signaling pathways have been identified to be important for apoptosis resistance of *Ctr*-infected cells (Sharma et al., 2011). Especially Mcl-1 represents the key player in *Ctr*-mediated apoptosis resistance since HIF1α regulates Mcl-1 expression on transcriptional level and activation of the MAPK pathway prolongs Mcl-1 half-life (Rajalingam et al., 2008; Sharma et al., 2011). The activation of the MAPK pathway during mid and late phase of infection is associated with apoptosis resistance whereas MAPK signaling in early phase of infection is essential for the establishment of chlamydial infection (Sharma et al., 2011). However, the detailed molecular mechanisms how the anti-apoptotic proteins are stabilized and how the activation of the survival signaling pathways is triggered to mediate apoptosis resistance is so far unknown. Infection experiments with heat inactivated *Chlamydia* or chloramphenicol treatment to block bacterial protein synthesis proved an involvement of secreted bacterial factors (Fan et al., 1998; Rajalingam et al., 2001). Multiple host cell proteins important for *Ctr*-mediated apoptosis resistance are known. On the other hand, the associated bacterial effectors involved in this process and the detailed molecular mechanisms are still unknown. With this work the molecular mechanism leading to the massive stabilization of Mcl-1 in the *Ctr*-infected cell crucial for apoptosis resistance should be revealed.

4.1 Infection-induced deubiquitination of Mcl-1

Mcl-1 as the key player of *Ctr*-mediated apoptosis resistance is regulated in multiple ways. During early phase of infection (16-20 hpi) HIF1α and the activation of the MEK/ERK pathway cause increased transcription of *MCL1* and the PI3K/AKT as well as MEK/ERK signaling pathway trigger Mcl-1 stabilization on protein level (Piret et al., 2005; Rajalingam et al., 2008; Sharma et al., 2011; Yamaguchi et al., 2012). However, inhibition of the two signaling pathways during mid and late phase of infection does not result in complete loss of Mcl-1 stabilization in infected cells indicating that additional mechanisms are involved in the regulation of Mcl-1 protein level (Figure 3.1 B). Mcl-1 has a very short half-life of about 30 min (Nijhawan et al., 2003) and is permanently ubiquitinated and degraded by the 26S proteasome. Detailed analysis of ubiquitination level of Mcl-1 in uninfected and *Ctr*-infected cells revealed reduced ubiquitination of Mcl-1 in infected cells regardless of apoptotic stimuli (Figure 3.2 A). This reduced ubiquitination seems to be independent of the host UPS machinery since no infection induced degradation of cellular UPS proteins regulating Mcl-1 turnover could be observed nor were the known modifications such as neddylation regulating enzyme activity altered (Figure 3.3). Furthermore, complete depletion of the host deubiquitinase USP9X did not result in significant loss of *Ctr*-mediated Mcl-1 stabilization (Figure 3.4).

Nevertheless, the substrates of many host UPS factors are unknown and therefore so far unrelated E3 ubiquitin ligases targeting cellular UPS components or Mcl-1 specific deubiquitinases might be involved in *Ctr*-mediated Mcl-1 stabilization (Deshaies and Joazeiro, 2009; Li et al., 2008). Especially the SCF ubiquitin ligase family represents an interesting weak point. The cullin protein family consists only of a small number of members (Sarikas et al., 2011), but these cullins are present in a variety of SCF E3 ubiquitin ligases initiating the degradation of all kinds of proteins (Zhao and Sun, 2013). By deneddylation of one type of cullin multiple SCF complexes can be inactivated or with the loss of one subunit the assembly of the whole complex is blocked. The aspect of enzyme inactivation by deneddylation is even more interesting due to the fact that *C. trachomatis* express two effectors with deneddylating activity shown *in vitro* (Misaghi et al., 2006). A common pathogenicity mechanism of bacteria is the secretion of effector proteins exhibiting direct E3 ligase activity or effectors hijacking cellular E3 ligase complexes (Collier-Hyams et al., 2005; Perrett et al., 2011). The substrate specificity of SCF E3 ligase complexes is mediated by the F-box subunit (Lee and Diehl, 2014). Known F-box proteins specific for Mcl-1 are Fbw7 and β-TrCP (Ding et al., 2007; Inuzuka et al., 2011), but no infection induced degradation of these factors could be observed (Figure 3.3). Some bacteria, for example *Legionella* secrete multiple F-box proteins into the host cell to capture cellular SCF E3 ligases and cause specific protein ubiquitination and degradation (Price and Kwaik, 2010). However, for *C. trachomatis* no F-box proteins have been identified so far which could cause targeted protein degradation of host cell or bacterial effector proteins.

Substrate recognition by the E3 ligase frequently requires priming modifications like phosphorylation. Phosphorylation of Mcl-1 at amino acid residue Ser 121 and Thr 92 by ERK signaling stabilizes Mcl-1 while GSK3 catalyzed Mcl-1 phosphorylation at Ser 155 and Ser 159 induces ubiquitination and proteasomal degradation (Thomas et al., 2010; Yamaguchi et al., 2012). Immunoblot analysis of Mcl-1 in uninfected and *Ctr*-infected cells revealed a variation in mobility of Mcl-1, seen in Figure 3.1 and Figure 3.2. A reduced mobility in SDS-PAGE is most often associated with post-translational modification like phosphorylation (Shirai et al., 2008). Corresponding to this, the immunoblot results indicate that Mcl-1 is less modified, most likely phosphorylated in infected cells which can be linked to reduced accessibility of Mcl-1 to its E3 ligase and thereby reduced ubiquitination and degradation. Deubiquitination being the reason for the observed size shift is doubtful because one ubiquitin molecule has a molecular mass of 9 kDa causing a stronger size shift. Detailed information about the post-translational modification of Mcl-1 in *Ctr*-infected versus uninfected cells would be obtained by mass spectrometry analysis of purified Mcl-1. How *Chlamydia* manipulate the post-translational modification of defined target proteins is unknown. Nevertheless, for multiple open reading frames of *Chlamydia* the expression and function of the encoded proteins are still unknown but might be associated with targeted protein modification, destruction or stabilization.

For a long time the bacterial protease CPAF was thought to mediate apoptosis resistance during mid and late phase of infection by cleaving the pro-apoptotic BH3-only proteins Puma, Bim or Bik (Ying et al., 2005). However, all known CPAF substrates were disproved as post lysis artefacts by application of CPAF inhibitors (Chen et al., 2012) or the use of a CPAF mutant *Ctr* strain (Snavely et al., 2014). Under survival conditions, Mcl-1 and other anti-apoptotic Bcl-2 protein family members entrap the direct activator BH3-only proteins (e.g. Bim and Puma). Upon apoptosis induction sensitizer BH3-only proteins (like Bad, Noxa or Bmf) replace the direct activator molecules from the complex with anti-apoptotic proteins which can then activate Bax and Bak to cause MOMP (Kim et al., 2006; Letai et al., 2002). The interaction of Mcl-1 with the pro-apoptotic BH3-only protein Bim is important for the anti-apoptotic mode of action of Mcl-1 (Chen et al., 2005; Labi et al., 2008). By co-immunoprecipitation experiments of Mcl-1 and Bim (see Figure 3.2 B) it was shown that this interaction is not lost upon infection indicating that Mcl-1 still functions as anti-apoptotic protein in the infected cells by complex formation with Bim.

4.2 Chlamydial deubiquitinating enzymes

Mcl-1 is massively enriched in *Ctr*-infected cells due to reduced ubiquitination. The reduced ubiquitination seems to be independent from host cell proteins but might be linked to the activity of bacterial effector proteins. *Chlamydia trachomatis* express the two deubiquitinating and deneddylating effectors ChlaDUB1 and ChlaDUB2 (Misaghi et al., 2006). To characterize the bacterial DUBs antibodies against the two proteins were raised. ChlaDUB1 protein could be detected in time course experiments upon 16 hpi simultaneously with Mcl-1 stabilization while ChlaDUB2 was already detected upon 12 h of infection, albeit earlier time points have not been tested (Figure 3.6). Effective T3 analysis hypothesized ChlaDUB1 to be a type 3 secreted effector and ChlaDUB2 as a potential type 3 secreted effector (Table 3-1). Dependent on this analysis, it was assumed to detect ChlaDUB1 and ChlaDUB2 secreted into the host cell cytoplasm as it was reported twice (Claessen et al., 2013; Misaghi et al., 2006). However, detailed analysis of immunofluorescence staining 24 hpi revealed a predominant localization of ChlaDUB1 secreted to the surface of the chlamydial inclusion membrane whereas ChlaDUB2 was simply present within the chlamydial particles (Figure 3.7). This subcellular localization of the two chlamydial effectors was confirmed by immunofluorescence staining with *Chlamydia* strains overexpressing FLAG-tagged proteins at 24 hpi. Even with the highly sensitive and specific anti-FLAG antibody no signal for the two overexpressed DUBs in the host cell cytoplasm was observed (Figure 3.8). It is possible that ChlaDUB2 is secreted at another time point of infection and that the chaperons necessary for proper secretion are not expressed in this period of infection. Thus, immunofluorescence staining with the ChlaDUB2-FLAG overexpressing *Ctr* strain should be repeated at different time points of infection.

ChlaDUB1 might be anchored by its N-terminal transmembrane domain to the inclusion membrane after secretion where it is able to interact with cytoplasmic proteins or fulfill functions regarding structural stability and maintenance of the inclusion. Since ChlaDUB2 is exclusively detected within the chlamydial particles it is possible that ChlaDUB2 plays a role as a bacterial protease. Substrates for ChlaDUB2 could either be bacterial proteins or host cell proteins actively transported into the inclusion lumen (Pollack et al., 2008); this can be adapted to ChlaDUB1, too.

Another role of the two chlamydial DUBs could be linked to early infection events and inhibition of autophagy. Since ChlaDUB1 and ChlaDUB2 are expressed till the end of the developmental cycle, the proteins can be accumulated inside the chlamydial EBs and secreted directly upon infection to block ubiquitination and subsequent autophagy of the bacteria containing vacuole (Gomes and Dikic, 2014). The secretion of effectors by intracellular pathogens to block autophagy has been observed for *Salmonella* and *Legionella*. *Salmonella typhimurium* secretes the DUB SseL which catalyzes the deubiquitination of ALIS. ALIS is ubiquitinated upon infection and associates with the autophagy marker proteins p62 and LC3 directing the autophagy machinery to the *Salmonella*

containing vacuole. SseL reverses ALIS ubiquitination and thereby formation of the autophagosome is blocked (Thomas et al., 2012). On the contrary, the *Legionella pneumophila* effector RavZ is a cysteine protease that cleaves LC3 conjugates blocking the formation of the autophagosome (Ashida et al., 2014). In 2013, the group of A. Subtil identified the type 3 secreted effector protein ChlaOTU of *Chlamydia caviae* which exhibit deubiquitinating activity with homology to the OTU protein superfamily (Furtado et al., 2013). Upon infection with *Chlamydia caviae* a local transient accumulation of ubiquitinated proteins at the bacterial entry site was observed which was cleared within 20 min after infection. ChlaOTU shows affinity for ubiquitin and NDP52 and is supposed to be responsible for clearance of ubiquitinated proteins after bacterial invasion. Upon invasion and escape into the cytosol bacteria are directly tagged with ubiquitin. NDP52 represents a linker protein between ubiquitinated bacteria and the autophagic system to facilitate clearance of the infection (Ivanov and Roy, 2009). *C. trachomatis* do not express DUBs belonging to the OUT protein superfamily. However, regarding these examples *C. trachomatis* might interfere with autophagy and the lysosomal pathway by secretion of ChlaDUB1 and ChlaDUB2 to ensure establishment of the inclusion as replication niche soon after infection

4.3 Mcl-1 is a ChlaDUB1 substrate

The obvious correlation between ChlaDUB1 expression and time of Mcl-1 stabilization (see Figure 3.6) asked for further investigation of this circumstance. ChlaDUB1 is secreted to the surface of the chlamydial inclusion with potential access to the host cell cytoplasm (Figure 3.7) whereas Mcl-1 is known to be a cytoplasmic protein partially associated with internal membranes like the ER or mitochondria (Thomas et al., 2010). However, immunofluorescence staining for Mcl-1 in *Ctr*-infected cells revealed a massive enrichment of Mcl-1 in close proximity to the inclusion, thus to ChlaDUB1 (Figure 3.9). The co-localization of ChlaDUB1 and Mcl-1 was confirmed by immunostaining of ChlaDUB1-FLAG and Mcl-1 in cells infected with the CTL0247-FLAG strain (see Figure 3.17 A).

As a first step to approve Mcl-1 as a ChlaDUB1 substrate the interaction of both proteins on molecular level had to be confirmed by co-immunoprecipitation experiments with ChlaDUB1-FLAG and Mcl-1 derived either from HeLa229 cells infected with the CTL0247-FLAG *Ctr* strain (Figure 3.17 B+C) or 293T cells overexpressing recombinant ChlaDUB1-FLAG (Figure 3.18). Under both conditions a co-precipitation of ChlaDUB1-FLAG and Mcl-1 was detected. Especially the missing interaction between ChlaDUB2 and Mcl-1 implies substrate specificity of the chlamydial DUBs.

It is not clear if Mcl-1 is actively recruited to the inclusion or if the accumulation is the result of permanent deubiquitination of Mcl-1 and thereby stabilization in this area. Nevertheless, the anti-apoptotic feature of Mcl-1 is dependent on its interaction with pro-apoptotic BH3-only proteins like Bim or Puma (Labi et al., 2008; Willis et al., 2005) which are cytoplasmatic proteins or associated with mitochondria (Huang and Strasser, 2000; O'Connor et al., 1998). This implies a dissociation of deubiquitinated Mcl-1 from the inclusion back to the cytoplasm where it can interact with BH3-only proteins to ensure host cell survival. Only small amounts of Mcl-1 were co-precipitated together with ChlaDUB1 compared to co-precipitation experiments of Mcl-1 and Bim which form a stable complex (Figure 3.2) (Chen et al., 2005; Labi et al., 2008). This circumstance supports the hypothesis that ChlaDUB1 and Mcl-1 show only a weak and transient interaction facilitating the dissociation between enzyme and substrate after the deubiquitination reaction. Many DUBs show only weak affinity towards ubiquitin or the ubiquitinated protein, respectively, but exhibit high catalytic capability (Ventii and Wilkinson, 2008). In contrast to other proteases, DUBs are not produced as inactive zymogenes but are activated by conformational rearrangements of the active center induced by protein binding. The bound proteins are either the substrate itself or regulatory binding partners which control enzyme activity or mediate specific interaction with the substrate (Reyes-Turcu et al., 2009; Wolberger, 2014). One example for direct substrate recognition and binding by the DUB is USP7 (HAUSP) which catalyzes p53 deubiquitination (Sheng et al., 2006). How the so far identified bacterial DUBs recognize their substrate and if additional scaffolding proteins are needed is in many cases still unclear (Edelmann and Kessler, 2008; Rytkonen and Holden, 2007). This question can be addressed to ChlaDUB1 substrate recognition, too. It seems that recognition and binding of Mcl-1 to ChlaDUB1 is independent of its ubiquitination status and do not need additional factors demonstrated by *in vitro* binding assays with purified recombinant proteins (Figure 3.19). Furthermore, ectopically expressed ChlaDUB1 mediates accumulation of Mcl-1 inside the cell comparable to the phenotype observed in *Ctr*-infected cells, but no bacterial factor other than ChlaDUB1 is present in these cells. The stabilization of Mcl-1 is related to the enzymatic activity of ChlaDUB1 because expression of mutant ChlaDUB1 (ChlaDUB1(C345A) or ChlaDUB1ΔCD) is not able to stabilize Mcl-1 (Figure 3.20). Nevertheless, unknown factors might be involved in the recruitment of Mcl-1 to the ChlaDUB1-containing compartment increasing the chance of an interaction between ChlaDUB1 and Mcl-1. The impact of ChlaDUB1 expression on other anti-apoptotic proteins was tested, too. None of the other Bcl-2 protein family members or cIAPs were altered in their protein level upon ChlaDUB1 expression strengthening the earlier statement about specificity of ChlaDUB1 towards Mcl-1.

To test whether Mcl-1 accumulation in cells overexpressing ChlaDUB1 is a result of reduced ubiquitination, Mcl-1 was immunoprecipitated under denaturing conditions out of these cells. Solely Mcl-1 derived from cells overexpressing ChlaDUB1 is massively deubiquitinated whereas control cells or cells overexpressing the mutated enzyme (ChlaDUB1(C345A)-FLAG) contain more poly-ubiquitinated Mcl-1 (see Figure 3.21 A). To reinforce the previous hypothesis that ChlaDUB1 alone despite of any other factors is able to bind Mcl-1 and catalyze its deubiquitination, an *in vitro* DUB assay with purified proteins was performed. Just ChlaDUB1 but not the control DUB UCH-L3 nor the inactivated ChlaDUB1 were able to deubiquitinate Mcl-1 approving it as a ChlaDUB1 substrate (see Figure 3.21 B).

4.4 Chlamydial genetics

With multiple biochemical approaches it was confirmed that Mcl-1 is deubiquitinated by ChlaDUB1 mediating its stabilization (see Figure 3.20 and Figure 3.21). However, the physiological role of ChlaDUB1 during *Ctr* infection and the real impact on Mcl-1 stabilization and inhibition of host cell apoptosis still had to be determined. In other fields of infection research the way to characterize bacterial effector proteins and their physiological role during infection is the creation of knock-out mutants (Thomas et al., 2012; Wu et al., 2012; Zurawski et al., 2009). Unfortunately, within the last decades this approach was not feasible for *Chlamydia*. Only a very time consuming and indefinite way by creating EMS mutant libraries gave a first idea about the role of particular chlamydial genes (Nguyen and Valdivia, 2012). But, with the method described by Wang et al. in 2011 how to transform *Ctr* with artificial DNA, the basis for targeted genetic manipulation of *Chlamydia* was formed (Wang et al., 2011). Within a tremendous short time this method was adapted for multiple *Chlamydia* strains as well as different purposes. By exchanging the SW2-backbone of the shuttle vector with the strain specific equivalent, transformation and replication of the shuttle vector in *C. muridarum* was established (Song et al., 2013b). Surprisingly, even *C. pneumoniae* which naturally do not have a cryptic plasmid was able to replicate a plasmid with the SW2 backbone of *C. trachomatis* after dendrimer-enabled DNA delivery for multiple passages (Gerard et al., 2013). On the contrary, by exchanging the open reading frame of GFP to mCherry or CFP multi-color *Chlamydia* strains were established (Agaisse and Derre, 2013) and additional selection markers like chloramphenicol (Xu et al., 2013) or blasticidine (Ding et al., 2013) resistance were introduced.

The tool of genetic manipulation by introduction of artificial DNA into *Chlamydia* was used to characterize the physiological role of ChlaDUB1. *Chlamydia* are known to be able to recombine foreign DNA into their genome by homologous recombination, shown during natural infection (Joseph et al., 2012; Putman et al., 2013) and *in vitro* (Jeffrey et al., 2013; Suchland et al., 2009). A strategy based on

the natural ability to integrate DNA into the chlamydial genome was designed to obtain ChlaDUB1 knock-out mutants. Unfortunately, even after multiple approaches of *Ctr* transformation with the pAH3 suicidal plasmid (Figure 3.22) no viable progeny could be obtained. To eliminate any doubt about technical problems or steric hindrance *Ctr* were transformed with the pAH1 suicidal plasmid. The plasmid pAH1 (Figure 3.10) contains homology arms directing the integration of a modified CTL0247 gene and a selection cassette to the CTL0247 locus (Figure 3.13). After transformation viable *Chlamydia* were obtained which were resistant to PenG, GFP-positive (Figure 3.11) and expressed a FLAG-tagged ChlaDUB1 protein instead of the wild type protein (Figure 3.14) proving the successful targeted integration of artificial DNA into the chlamydial genome. The newly formed *Chlamydia* strain *Ctr* CTL0247-FLAG exhibited multiple advantages for the biochemical analysis of ChlaDUB1 function. On the one hand a commercial highly affine and specific FLAG-antibody could be used to detect ChlaDUB1-FLAG (Figure 3.15 and Figure 3.17) and on the other hand the time of ChlaDUB1-FLAG expression as well as the amount was not altered because the CTL0247-FLAG gene was still under the control of its natural promoter (Figure 3.14 A+B). Nevertheless, after the insertion of DNA material into the chlamydial genome the correct integration (see Figure 3.13 and Figure 3.14), fitness as well as anti-apoptotic features of the new *Chlamydia* strain was tested (Figure 3.16). The CTL0247 gene is not located in an operon (Albrecht et al., 2009), but if this approach will be used for other genes present in an operon either a second copy of the promoter has to be introduced downstream the recombination position or special analysis of the expression pattern of the other genes present in that operon has to be performed.

An easier way to proof intracellular localization by immunofluorescence staining or to identify interaction partners by co-immunoprecipitation would be the overexpression of the tagged protein from a plasmid as it was shown for GFP (Agaisse and Derre, 2013; Wang et al., 2011). However, as it became obvious from the permanent and strong overexpression of the ChlaDUB1(C345A)-FLAG protein an interference into the well balanced interplay of multiple bacterial effector proteins can cause adverse effects which are either toxic for the bacterium or are not related to the real function of the protein at all (Figure 3.24). The dominant-negative strain *Ctr* ChlaDUB1(C345A)-FLAG did not show reduced Mcl-1 amounts in the cell. It is very likely that the point mutant ChlaDUB1(C345A)-FLAG is still able to bind Mcl-1 and that the binding itself can protect Mcl-1 from proteasomal degradation to a certain degree, especially if the dominant-negative *Ctr* strain expresses extremely more ChlaDUB1 protein than the wild type strain (Figure 3.23 and Figure 3.24). Furthermore, a *Chlamydia* strain permanently overexpressing the ChlaDUB1 mutant lacking the whole catalytic domain (ChlaDUB1ΔCD-FLAG) could not be obtained. The fact that strong interference in ChlaDUB1 expression and activity results in lethality for the *Chlamydia* underlines the importance of this virulence factor for the bacteria once more.

Since manipulation of *Chlamydia* to interfere with ChlaDUB1 activity by dominant-negative approaches on a permanent basis was not possible, the use of inducible promoters represented an alternative. ChlaDUB1 and its mutant variants can be cloned under control of an inducible promoter and protein expression can be induced at defined time points of infection in parallel to natural ChlaDUB1 expression to investigate dominant-negative effects. By characterization of a *Chlamydia* strain expressing GFP under a tetracycline-inducible promoter we showed that the promoter reacts upon 1-2 hours after induction and even very low concentrations of AHT (10 ng/ml) result in a strong protein expression (Auer, 2014) precluding the possibility to regulate amount of expressed protein. Nevertheless, the preparation of the inducible ChlaDUB1ΔCD dominant-negative strain would provide new insights in the physiological role of ChlaDUB1.

A common mechanism of gene regulation involves small RNAs (sRNAs) which control either gene transcription or translation by multiple mechanisms, reviewed by Water and Storz (Waters and Storz, 2009). By deep sequencing approaches of *Ctr*-infected HeLa229 cells so far unknown sRNAs in *Ctr* were identified (Albrecht et al., 2009). Trans-acting sRNAs need the RNA chaperon Hfq for stability and function, however a homologue in *Chlamydia* was not found so far. For the first time, Mishra and colleagues used dedrimers to deliver asRNAs into *Ctr*-infected cells and were able to induce gene knock-down of chlamydial HSP60 (Mishra et al., 2012). With this work they showed that sRNA-mediated protein knock-down in *Chlamydia* does not depend on Hfq. Nevertheless, the detailed mechanism is unclear and so far no additional examples were presented nor reported any other group of asRNA-mediated gene silencing in *Chlamydia*. A new system for targeted protein knock-down is introduced by the CRISPR interference (CRISPRi) system derived from *Streptococcus pyogenes* (Larson et al., 2013). The CRISPRi system consists of the deactivated Cas9 endonuclease (dCas9) and a customized single guide RNA (sgRNA). The sgRNA binds to the Cas9 protein and directs the complex to the specific target DNA sequence on the genome. Since the dCas9 endonuclease is enzymatic inactive, the protein simply blocks transcription initiation or elongation dependent on the position of the target sequence. One big advantage of this system is that all components needed for the knock-down of any gene of interest can be delivered by one plasmid and no cellular components have to be present as it is the case for siRNA induced knock-down depending on the cellular proteins DICER and Drosha. A further benefit is that the system is inducible and multiple sgRNAs can be introduced in parallel (Gilbert et al., 2013). The CRISPRi system can be incorporated into *Chlamydia* by one single plasmid fused with a SW2-backbone needed for plasmid maintenance. The plasmid containing *Chlamydia* can be selected by the common strategy and gene silencing can be induced by anhydrotetracycline triggering the dCas9 expression responsible for gene knock-down after a stable and pure *Chlamydia* population was selected. Due to the extremely small number of genes in *Chlamydia* and their altered expression with respect to the developmental cycle it is supposed that nearly all genes are essential at

defined stages during chlamydial replication. Hence, an inducible as well as reversible knock-down system is needed which cannot be rendered by common methods like transposon mutagenesis but is enabled by the CRISPRi system.

4.5 ChlaDUB1 inhibitor CYN312 sensitizes *Ctr*-infected cells for apoptosis

So far, the common genetic approaches such as knock-out mutants or dominant-negative strains did not succeed to reveal the natural role of ChlaDUB1 during infection and apoptosis inhibition. Especially the absence of viable ChlaDUB1 knock-out mutants indicates that ChlaDUB1 is an essential gene important for chlamydial growth and replication. The clearest way to uncover ChlaDUB1 substrates and its role during infection would be the inducible knock-down by asRNA or CRISPRi. Unfortunately, these techniques are not yet fully adapted for the use in *Chlamydia* but will be available in future. Till then, other strategies like the use of enzyme specific inhibitors have to be pursued. The advantage of use of inhibitors compared to permanent knock-down is the possibility to define time of inhibitor treatment, concentration and removal of inhibitor to recover the initial state.

Deduced from the amino acid sequence ChlaDUB1 was assigned to be a member of the family of cysteine proteases with an active center located at the C-terminus of the protein and a N-terminal transmembrane domain (Le Negrate et al., 2008; Misaghi et al., 2006). However, no crystal structure or further domains important for substrate recognition or protein-protein interaction have been identified. Enzymatic activity of deubiquitinating enzymes can be monitored *in vitro* with the fluorogenic substrate Ub-AMC (Dang et al., 1998) and incubation of ChlaDUB1 or control enzyme UCH-L3 with the universal cysteine protease inhibitor NEM did completely block enzymatic activity (Figure 3.25 A). A library with 25 potential, non-commercially available cysteine protease inhibitors was tested in an Ub-AMC assay for their ability to specifically block ChlaDUB1 activity (Figure 3.25 B). The compound CYN312 is an endoperoxide isolated from the marine sponge *Plakortis halichondrioides* (Oli et al., 2014) and blocks ChlaDUB1 activity *in vitro* in a concentration dependent way. Further enzymatic characterization revealed that CYN312 acts as a non-competitive inhibitor on ChlaDUB1 meaning that CYN312 does not bind to the active center of the enzyme but to any other allosteric center of the protein (Figure 3.26).

With the application of CYN312 in cell culture during *Ctr* infection the natural role of ChlaDUB1 in bacterial replication and apoptosis inhibition was investigated. By flow cytometry analysis no cytotoxicity of CYN312 or control compounds towards the host cell was observed (Figure 3.27). On the contrary, CYN312 treatment of *Ctr*-infected cells dramatically affected chlamydial growth in a concentration dependent manner (Figure 3.28). To restrict the observed effects of CYN312 towards its activity against ChlaDUB1, the inhibitor was removed prior to ChlaDUB1 expression 16 hpi, see

Figure 3.29 A. *Ctr* were able to recover after removal of ChlaDUB1, although bacterial growth was still affected. CYN312 was removed by multiple washing steps upon 12 hpi. However, slight amounts of the compound could still have been present in the cell. Furthermore, time of ChlaDUB1 expression is supposed to start around 16 hpi, but detailed analysis for the exact time was not done and expression of slight amounts of the protein beyond the detection limit earlier during infection is possible. Application of CYN312 early during infection and removal upon 24 hpi resulted in almost complete recovery of *Ctr* replication (Figure 3.29 B). An additional evidence for the specificity of CYN312 towards ChlaDUB1 and to no other factor was delivered by infection experiments with *C. pneumoniae* which do not express deubiquitinating enzymes belonging to the family of cysteine proteases (Misaghi et al., 2006). Intracellular growth of *Cpn* is not affected at all by CYN312 treatment whereas *Ctr* serovar D which expresses a close homolog of ChlaDUB1 is blocked comparable to *Ctr* L2 (Figure 3.30).

The substrate of ChlaDUB1, among others, is supposed to be Mcl-1. Therefore, inhibition of ChlaDUB1 by CYN312 should result in loss of Mcl-1 stabilization during infection which is indeed the case. Upon CYN312 treatment the amount of Mcl-1 in the infected cell dramatically drops but can be recovered completely by removal of CYN312 (see Figure 3.28 and Figure 3.29). The fact that CYN312 treatment of *Ctr*-infected cells results in growth inhibition of the bacteria strengthens the hypothesis of ChlaDUB1 being an essential gene once more. *Chlamydia* do not depend on Mcl-1 or IκBα as known substrates of ChlaDUB1 for their replication and intracellular survival (Rajalingam et al., 2008) hence ChlaDUB1 must have additional substrates on host cell or bacterial side important for chlamydial intracellular survival and growth. However, Mcl-1 is the most important factor for *Chlamydia*-mediated apoptosis inhibition of the host cell and depletion of Mcl-1 dramatically sensitizes infected cells for TNFα-induced apoptosis (Figure 3.31). Multiple mechanisms utilized by *Chlamydia* to enrich Mcl-1 in the cell have been uncovered such as activation of survival signaling pathways or the enrichment of the transcription regulator HIF1α to increase transcription of the *MCL1* gene. Nevertheless, the manipulation of *MCL1* gene transcription takes place only at early and mid-phase of the infection cycle and inactivation of the signaling pathways does not completely prevent Mcl-1 stabilization in infected cells (Figure 3.1) (Rajalingam et al., 2008; Sharma et al., 2011). With this work it was shown that the chlamydial deubiquitinase ChlaDUB1 catalyzes Mcl-1 deubiquitination and stabilization upon mid and late phase of infection. Inactivation of ChlaDUB1 by the newly identified specific inhibitor CYN312 interferes with Mcl-1 stabilization and significantly sensitizes *Ctr*-infected cells for apoptosis induction (Figure 3.31).

Inactivation of ChlaDUB1 by CYN312 represents a promising strategy to treat *Chlamydia* infections. The increasing number of bacteria resistant to antibiotics highlights the urgency to find new drugs interfering with bacterial infection. The most prominent examples are the MRSA strains of *Staphylococcus aureus* (Antibiotic resistance surveillance, Robert Koch Institut, 2008), albeit resistance

of other pathogenic bacteria like *Neisseria gonorrhoeae* to the generally used antibiotics is increasing (Ison, 2012). The common treatment of *Chlamydia* infections is based on doxycycline or azithromycin. Within the last years more and more reports about resistant *Chlamydia* were published and a tetracyclin-resistant *Chlamydia suis* strain was isolated from pigs (Horner, 2012; Sandoz and Rockey, 2010). However, it should be mentioned that the reported antibiotic resistance of *Ctr* in patients is very often linked to treatment failures and reliable studies are difficult since the possibility of re-infection is hard to control (Horner, 2012). A pathogen-specific therapy either targets host cell factors involved in pathogenicity or directly the bacterial effector proteins. Both strategies exhibit advantages as well as disadvantages. The intervention in cellular processes can cause severe side effects but the risk of resistance development on bacterial side is less. On the contrary, specific therapy against a bacterial virulence factor shows reduced adverse effects but the possibility to acquire resistance due to mutagenesis events is high. ChlaDUB1 represents a suitable target for drug design against *Chlamydia trachomatis*. ChlaDUB1 is essential for chlamydial growth and inactivation of ChlaDUB1 by CYN312 would block spread of infection and transforms *Ctr*-infected cells back into an apoptosis-susceptible state facilitating the removal of infected cells by the immune system. ChlaDUB1 plays an important role for chlamydial growth and apoptosis resistance in cultured cell lines. If inactivation of ChlaDUB1 by CYN312 in infection models with primary cells from the fallopian tubes or in mouse experiments shows the same phenotype remains to be confirmed.

With this work we revealed the molecular mechanisms of Mcl-1 stabilization during *Ctr* infection, depicted in Figure 4.1. High amounts of Mcl-1 in the infected cells ensure inhibition of apoptosis induction accompanied with mitochondrial outer membrane permeabilization and cytochrome c release responsible for the activation of the caspase cascade. Under normal conditions Mcl-1 is constantly ubiquitinated by multiple E3 ligases and subsequently degraded by the 26S proteasome. The half-life of Mcl-1 can be altered by differential phosphorylation regulating its affinity towards its E3 ligases and deubiquitinase USP9X. However, we showed that all these basic processes only form a part of the molecular mechanism regulating Mcl-1 stabilization during *Ctr* infection. Another important mechanism of Mcl-1 stabilization during infection is related to the secreted chlamydial deubiquitinase ChlaDUB1 which catalyzes the deubiquitination of Mcl-1 at the surface of the inclusion and subsequently releases the deubiquitinated Mcl-1 to the cytoplasm where is can act as anti-apoptotic protein. The compound CYN312 was shown to be a ChlaDUB1-inhibitor which interferes with Mcl-1 stabilization and apoptosis resistance during *Ctr* infection. Furthermore, CYN312 blocks chlamydial growth supporting the hypothesis of ChlaDUB1 being an essential gene for *C. trachomatis* growth and replication.

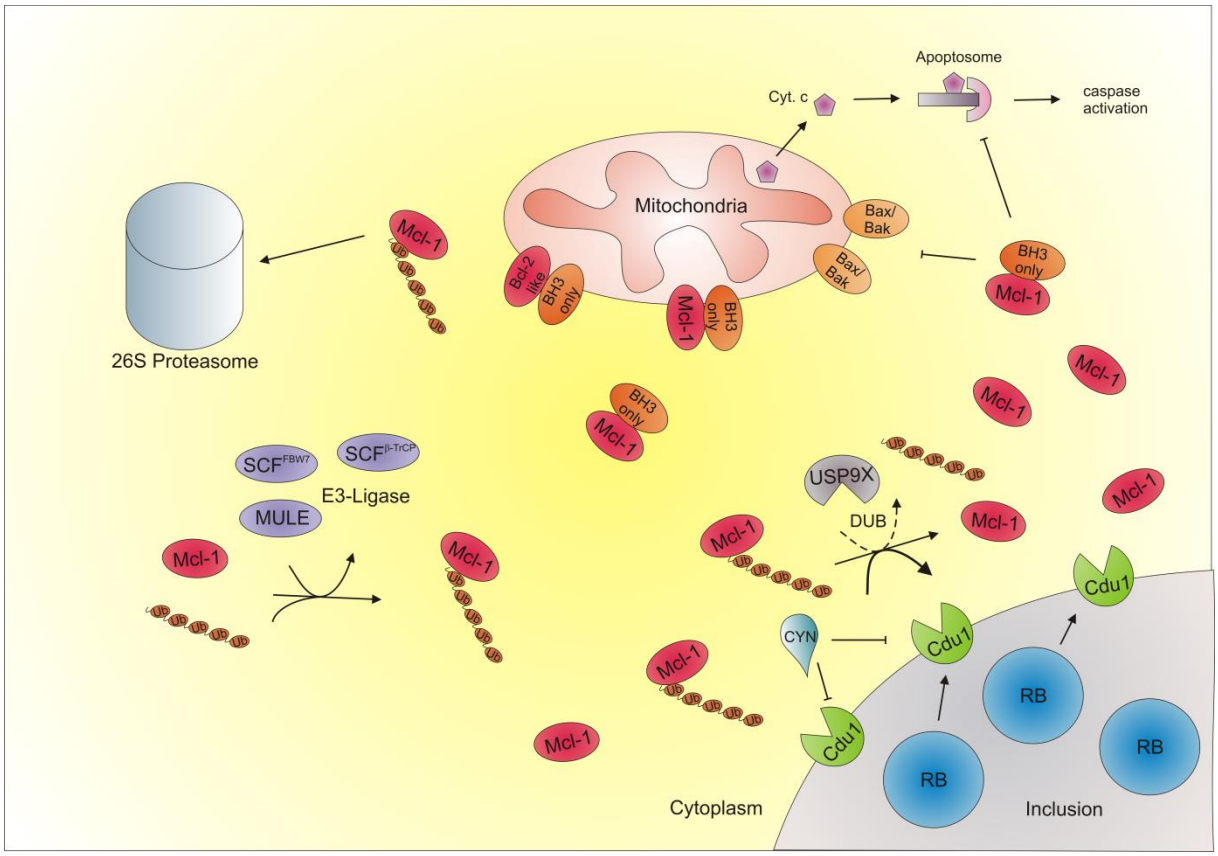

Figure 4.1: *Chlamydia*-mediated Mcl-1 stabilization. Mcl-1 turnover is catalyzed by the host ubiquitin proteasome system. The E3 ubiquitin ligases MULE, SCF^{Fbw7} and $SCF^{\beta TrCP}$ catalyze Mcl-1 ubiquitination whereas USP9X is the Mcl-1 specific deubiquitinase. Ubiquitinated Mcl-1 is recruited to the 26S proteasome and degraded. The anti-apoptotic function of Mcl-1 relies on its interaction with pro-apoptotic BH3-only proteins, e.g. Bim or Puma to keep them in an inactive state. Upon apoptosis induction the BH3-only proteins are replaced by sensitizer proteins form the complex with Mcl-1 and are able to trigger Bax and Bak oligomerization to cause mitochondrial outer membrane permeabilization and cytochrome c release which results in the activation of the caspase cascade. *Chlamydia trachomatis* interferes in Mcl-1 degradation by secretion of the deubiquitinase ChlaDUB1 which permanently deubiquitinates and thereby stabilizes Mcl-1 in the infected cell. Hence, high amounts of Mcl-1 protect the cells form apoptosis induction.

4.6 Outlook and perspectives

With this work the fundamental role of ChlaDUB1 for Mcl-1 stabilization which is linked to apoptosis resistance of *Ctr*-infected cells was demonstrated. However, various unresolved issues remain to be answered. It is still unclear how ChlaDUB1 recognizes its substrates, especially since the two identified substrates IκBα and Mcl-1 share no homology or similar domains. By *in vitro* binding assays with ChlaDUB1 and Mcl-1 variants lacking defined domains the region of protein-protein interaction can be localized. The crystallization of ChlaDUB1 alone or in complex with Mcl-1 would then resolve the enzyme-substrate interaction in more detail. Also the allosteric regulation of ChlaDUB1 by CYN312 can be clarified by co-crystallization. CYN312 is a natural compound which is extremely difficult to synthesize synthetically (Sun et al., 2011). The co-crystallization of ChlaDUB1 together with CYN312 would be of great advantage to identify the position and structure of the allosteric center and how CYN312 binds to this.

Two classical approaches are used for inhibitor design of cysteine proteases. If the peptide sequence important for substrate cleavage is known, small peptides coupled with electrophilic groups reacting with the nucleophile of the active center can be designed (Rizzi et al., 2011). The 3C protease of the foot-and-mouth-disease virus (FMDV) belongs to the family of chymotrypsin-like cysteine proteases. The cleavage site of the substrate as well as the peptide sequence important for selectivity is known. Dependent on this information a peptide consistent of four amino acids coupled to a Michaelis acceptor was designed which represents a suitable 3Cpro inhibitor (Roque Rosell et al., 2014). *Clostridium difficile* possess the two virulence factors TcdA and TcdB which both contain a cysteine protease domain. It is known that TcdB is allosterically regulated by Inositol hexakisphosphate. Di- or tri-peptides similar to the protease cleavage site coupled with an electrophilic group targeting the active group of the catalytic center were designed and tested in a SAR (structure activity relationship) study to obtain highly efficient inhibitors (Puri et al., 2010). However, all DUBs cleave the linkage between glycine 76 of ubiquitin and the internal lysine at the same position precluding the peptide-based approach for inhibitor design.

On the contrary, many natural cysteine protease inhibitors named cystatins are known. Depending on the structure and interacting domains of effective cystatins new compounds can be designed and tested (Rizzi et al., 2011). By high throughput screening (HTS) two compounds active against the SARS-CoV (severe acute respiratory syndrome corona virus) protease PLpro were identified. PLpro is a papain-like protease and exhibits proteolytic, deubiquitinating and deISGylating activity. The compound hits were co-crystallized together with PLpro and modified to obtain highly specific and active inhibitors (Akaji et al., 2011; Baez-Santos et al., 2014). The cysteine protease Falciparin-2 of *Plasmodium falciparum* can be inactivated by suramin and analogues by a so far unknown allosteric mechanism. Co-crystallization of Falciparin-2 and suramin was performed and newly designed suramin analogues were tested for increased specificity (Marques et al., 2013). Depending on the information derived from co-crystallization of ChlaDUB1 and CYN312, analogues of CYN312 which possess a higher specificity and inhibitory properties can be designed, synthesized in large scale and tested. With the identification of the substrate binding domain of ChlaDUB1 peptides can be generated which interfere with substrate binding as it was already shown for Falciparin-2 and its hemoglobin binding domain (Pandey et al., 2005) or the SMAC-mimetics blocking XIAP anti-apoptotic activity (Cossu et al., 2009; Reingewertz et al., 2011; Zobel et al., 2006). If co-crystallization succeeds and suitable CYN312 analogues or compounds masking the substrate binding site can be designed and synthesized, their effect on ChlaDUB1 activity can be tested *in vitro*, in cell culture infection experiments and finally in mouse experiments.

Chlamydia trachomatis expresses the two effector proteins ChlaDUB1 and ChlaDUB2 with deubiquitinating and deneddylating activity. With the analysis of the ubiquitome or the neddylation

pattern of infected cells in comparison to uninfected control cells proteins targeted by *Chlamydia* and thereby important for pathogenicity can be identified. All ubiquitinated proteins of a cell can be isolated easily with TUBE (tandem ubiquitin binding entity) based methods and abundance of each protein, respectively, can be defined by SILAC (stable isotope labeling with amino acids in cell culture) and mass spectrometry. Which alteration in ubiquitination can be linked to ChlaDUB1 or ChlaDUB2 activity can be investigated with CRISPRi mediated gene knock-down and SILAC analysis. Furthermore, whole proteome analysis of *Ctr*-infected cells lacking either one or both of the chlamydial DUBs would reveal complete pathways manipulated by *Chlamydia* and their DUBs. The identification of the other ChlaDUB substrates, especially the ones important for chlamydial replication would be of great advantage in understanding how *Chlamydia* survive inside the host cell. The investigation of the neddylation pattern will render additional understanding of the molecular processes during infection. Only limited number of cellular proteins are neddylated and the most prominent proteins belong to the cullin family regulating RING E3 ligase activity (Duda et al., 2008; Sarikas et al., 2011). The neddylation pattern can either be analyzed by 2D gel electrophoreses and anti-Nedd8 immunoblot or by precipitation of neddylated proteins in a SILAC experiment followed by mass spectrometry. Of course, the experiments can also be performed under ChlaDUB-lacking conditions to formulate their participation.

The CRISPRi system can be applied for multiple approaches. A library of *Chlamydia* clones containing the CRISPRi system targeting one of the approximately 1000 genes encoded by the chlamydial genome, respectively, can be prepared. Gene knock-down can be induced at defined time points of infection and survival or resulting phenotype of the *Chlamydia* can be analyzed to map the function of the respective genes. Especially the identification of essential genes, genes important for apoptosis resistance, genes involved in invasion, EB to RB or RB to EB transition as well as genes important for persistence state of *Chlamydia* would be of great importance. With this global analysis the understanding of pathogenicity of the obligate intracellular human bacterium *Chlamydia trachomatis* would take a huge step forward.

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6 Appendix

6.1 Abbreviations

AB	aberrant body
AHT	Anhydrotetracycline
AIF	apoptosis inducing factor
AMC	7-amino-4-methylcoumarin
APF-1	ATP-dependent proteolysis factor
APS	ammonium persulfate
Asp	aspartic acid
Atg	autophagy-related protein
ATP	adenosine triphosphate
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated x protein
Bcl-2	B cell lymphoma 2
BH	Bcl-2 homology
Bid	BH3 interacting-domain death agonist
BIR	baculovirus IAP repeat
BSA	bovine serum albumin
C. trachomatis	*Chlamydia trachomatis*
CAD	caspase activated DNase
CD	catalytic domain
CHX	cycloheximide
cIAP	cellular inhibitor of apoptosis protein
CnBr	Cyanogen bromide
CP	catalytic component
CPAF	chlamydial protease-like activity factor
Cpn	*Chlamydophila pneumoniae*
C-terminal	carboxyl-terminal
Ctr	*Chlamydia trachomatis*
Cys	cysteine
DD	death domain
DED	death effector domain
dH_2O	distilled water
DISC	death induced signaling complex
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	desoxynucleosid triphosphate
Dox	doxycycline
DTT	dithiothreitol
DUB	deubiquitinase
E. coli	*Escherichia coli*
EB	elementary body
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetetraacetic acid
EMS	ethyl methyl sulfonate
EndoG	endonuclease G
ERK	extracellular-signal-regulated kinase
ESCRT	Endosomal sorting complexes required for transport
FADD	Fas associated protein with death domain
Fbw7	F-box and WD repeat domain-containing 7
FCS	fetale calf serum
Fig.	figure
gDNA	genomic DNA
GFP	green fluorescent protein

GS	Glutathione sepharose
GSK3	glycogen synthase kinase 3
gt	goat
GTP	guanosine-5'-triphosphate
h	hour(s)
HECT	homologous to the E6-AP carboxyl terminus
HIF1α	hypoxia-induced factor 1 α
His	histidine
hpi	hours post infection
HRP	horseradish peroxidase
HSP60	heat shock protein 60
IB	Immunoblotting
IF	immunofluorescence
IFU	inclusion forming units
Ig	Immunoglobulin
INF-γ	interferon γ
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactopyranosid
ISG	interferon-stimulated gene
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
K	lysine
kb	kilobases
kDa	Kilodalton
K_M	Michaelis constant
l	liter
LB	lysogeny broth
M	Molar
MAPK	mitogen-activated protein kinase
Mb	megabases
Mcl-1	myeloid cell leukemia 1
Mdm2	Mouse double minute 2 homolog
MHC	major histocompatibility complex
min	minutes
MOI	multiplicity of infection
MOMP	mitochondrial outer membrane permeabilization
mRNA	messenger-RNA
ms	mouse
MULE	Mcl-1 ubiquitin ligase E3
Nedd8	neural precursor cell expressed, developmentally downregulated 8
NEM	N-methylmaleimide
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
N-terminal	amino-terminal
OD600	optical density measured at a wavelength of 600nm
OmpA	outer membrane protein A
ORF	open reading frame
OTU	ovarian tumor protease
PAGE	polyacrylamide gel electrophoresis
pAKT	phosphorylated AKT
PBS	phosphate buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction
PEI	Polyethylenimine
pERK	phosphorylated ERK
PFA	Paraformaldehyde
PI3K	phosphoinositide 3-kinase
RB	reticulate body
rb	rabbit

RFU	relative fluorescence unit
RING	really interesting new gene
RNA	ribonucleic acid
RNAi	RNA interference
RP	regulatory component
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
s	Seconds
SAR	structure activity relationship
SCF-complex	Skp, cullin, F-box containing complex
SD	standard deviation
SDS	sodium dodecyl sulphate
SENP	sentrin-specific protease
Ser	serine
SILAC	stable isotope labeling with amino acids in cell culture
SIM	SUMO-interaction motif
siRNA	small interfering RNA
Smac	second mitochondria-derived activator of caspases
SNARE	SNAP receptor
SOC	super optimal broth with catabolite repression
SUMO	small ubiquitin-related modifier
T3SS	type three secretion system
TACE	TNFα converting enzyme
TARP	translocated actin recruiting phosphoprotein
tBid	truncated Bid
TBS	tris buffered saline
TEMED	Tetramethylethylenediamine
Thr	threonine
TM	transmembrane
TNFR	tumor necrosis factor receptor
TNFα	tumor necrosis factor α
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
TRAIL	Tumor necrosis factor-related apoptosis binding ligand
Tris	tris(hydroxymethyl)aminomethane
TUBE	tandem ubiquitin binding entity
U	enzyme unit
Ub	ubiquitin
UBA	ubiquitin-associated domain
UBL	ubiquitin-like protein
UbVME	ubiquitin-vinylmethylester
UCH	ubiquitin C-terminal hydrolases
UIM	ubiquitin-interacting motif
UPS	ubiquitin proteasome system
USP	ubiquitin-specific protease
UV	ultra violet
v/v	volume per volume
vamp	vesicle-associated membrane protein
w/v	weight per volume
WHO	World Health Organization
WT	wild type
XIAP	x-linked inhibitor of apoptosis protein
µ	micro

6.2 Publications and presentations

Publications

Huber A., Oli S., Prusty B., Subbarayal P., Schirmeister T., Rudel T., *Chlamydia trachomatis* secreted deubiquitinase stabilises Mcl-1 to mediate apoptosis resistance. (under revision)

Talks and poster presentations

Huber A., Rudel T.; Identification and characterization of a chlamydial factor mediating Mcl-1 stabilization during infection. 6th Biennial meeting of the Chlamydia basic research society (CBRS) 2013, San Antonio, Texas, USA. (Poster)

Huber A., Rudel T.; *Chlamydia* trachomatis regulate Mcl-1 stabilization by secretion of the deubiquitinating enzyme ChlaDUB1. 11. Deutscher Chlamydienworkshop 2013, Würzburg. (Talk)

Huber A., Schirmeister T., Rudel T.; Plakortide E blocks ChlaDUB1 deubiquitinating activity and sensitizes *C. trachomatis* infected cells for apoptosis. International Symposium SFB630 2013 on “Novel agents against infectious diseases – an interdisciplinary approach”, Würzburg. (Poster)

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6.4 Selbstständigkeitserklärung

Ich erkläre ehrenwörtlich, dass die vorliegende Arbeit von mir selbstständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt wurde.

Diese Dissertation hat weder in der gleichen noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen.

Ich habe früher, außer den mit dem Zulassungsgesuch urkundlich vorgelegten Graden, keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Würzburg, den 15.12.2014