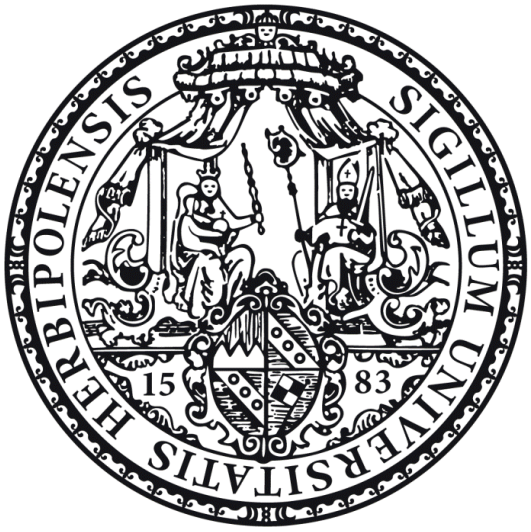
Degradation of Tumour Suppressor p53 during *Chlamydia trachomatis* Infections



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Abstract

The intracellular pathogen *Chlamydia* is the causative agent of millions of new infections per year transmitting diseases like trachoma, pelvic inflammatory disease or lymphogranuloma venereum. Undetected or recurrent infections caused by chlamydial persistence are especially likely to provoke severe pathologies. To ensure host cell survival and to facilitate long term infections *Chlamydia* induces anti-apoptotic pathways, mainly at the level of mitochondria, and restrains activity of pro-apoptotic proteins. Additionally, the pathogen seizes host energy, carbohydrates, amino acids, lipids and nucleotides to facilitate propagation of bacterial progeny and growth of the chlamydial inclusion.

At the beginning of this study, *Chlamydia*-mediated apoptosis resistance to DNA damage induced by the topoisomerase inhibitor etoposide was investigated. In the course of this, a central cellular protein crucial for etoposide-mediated apoptosis, the tumour suppressor p53, was found to be downregulated during *Chlamydia* infections. Subsequently, different chlamydial strains and serovars were examined and p53 downregulation was ascertained to be a general feature during *Chlamydia* infections of human cells. Reduction of p53 protein level was established to be mediated by the PI3K-Akt signalling pathway, activation of the E3-ubiquitin ligase HDM2 and final degradation by the proteasome. Additionally, an intriguing discrepancy between infections of human and mouse cells was detected. Both activation of the PI3K-Akt pathway as well as degradation of p53 could not be observed in *Chlamydia*-infected mouse cells. Recently, production of reactive oxygen species (ROS) and damage to host cell DNA was reported to occur during *Chlamydia* infection. Thus, degradation of p53 strongly contributes to the anti-apoptotic environment crucial for chlamydial infection.

To verify the importance of p53 degradation for chlamydial growth and development, p53 was stabilised and activated by the HDM2-inhibiting drug nutlin-3 and the DNA damage-inducing compound etoposide. Unexpectedly, chlamydial development was severely impaired and inclusion formation was defective. Completion of the chlamydial developmental cycle was prevented resulting in loss of infectivity. Intriguingly, removal of the p53 activating stimulus allowed formation of the bacterial inclusion and recovery of infectivity. A similar observation of growth recovery was made in infected cell lines deficient for p53.

As bacterial growth and inclusion formation was strongly delayed in the presence of activated p53, p53-mediated inhibitory regulation of cellular metabolism was suspected to contribute to chlamydial growth defects. To verify this, glycolytic and pentose phosphate pathways were analysed revealing the importance of a functioning PPP for chlamydial growth. In addition, increased expression of glucose-6-phosphate dehydrogenase rescued chlamydial growth inhibition induced by activated p53. The rescuing effect was even more pronounced in p53-

deficient cells treated with etoposide or nutlin-3 revealing additional p53-independent aspects of *Chlamydia* inhibition. Removal of ROS by anti-oxidant compounds was not sufficient to rescue chlamydial infectivity. Apparently, not only the anti-oxidant capacities of the PPP but also provision of precursors for nucleotide synthesis as well as contribution to DNA repair are important for successful chlamydial growth.

Modulation of host cell signalling was previously reported for a number of pathogens. As formation of ROS and DNA damage are likely to occur during infections of intracellular bacteria, several strategies to manipulate the host and to inhibit induction of apoptosis were invented. Downregulation of the tumour suppressor p53 is a crucial point during development of *Chlamydia*, ensuring both host cell survival and metabolic support conducive to chlamydial growth.

Zusammenfassung

Intrazellulär lebende Chlamydien führen jährlich zu Millionen an Neuinfektionen und lösen Krankheiten wie das Trachom, eine Entzündung des Auges, sowie entzündliche Beckenerkrankungen oder Lymphogranuloma venereum, eine venerische Lymphknotenentzündung, aus. Unentdeckte oder wiederkehrende Infektionen, ausgelöst durch chronisch persistierende Chlamydien, führen häufig zu schwerwiegenden Komplikationen. Um das Überleben der Wirtszelle und dauerhafte Infektionen zu ermöglichen, induzieren Chlamydien antiapoptotische Signalwege, hauptsächlich auf Höhe der Mitochondrien, und beeinträchtigen darüber hinaus die Aktivität proapoptotischer Proteine. Energie, Kohlenhydrate, Aminosäuren, Lipide und Nukleotide bezieht der Krankheitserreger vollständig aus der Wirtszelle. Erst dadurch wird sowohl die Vermehrung der Bakterien, als auch das Wachstum der chlamydialen Inklusion ermöglicht.

Zu Beginn dieser Arbeit wurde die Chlamydien-vermittelte Resistenz gegenüber induziertem Zelltod nach Schädigung der DNA durch den Topoisomerase-Inhibitor Etoposid untersucht. Im Zuge dessen wurde entdeckt, dass der Tumorsuppressor p53, ein zentrales zelluläres Protein entscheidend für die Etoposid-induzierte Apoptose, während Chlamydien-Infektionen herunterreguliert wird. Nachdem verschiedene chlamydiale Stämme und Serovare untersucht wurden, konnte festgestellt werden, dass es sich bei der Herunterregulierung von p53 um ein allgemeines Merkmal chlamydialer Infektionen von humanen Zellen handelt. Die Reduzierung der Proteinmenge von p53 wird dabei durch den PI3K-Akt Signalweg, Aktivierung der E3-Ubiquitin-Ligase HDM2 und abschließendem Abbau durch das Proteasom vermittelt. Zusätzlich wurde ein interessanter Unterschied zwischen Infektionen humaner und muriner Zellen entdeckt. Sowohl Aktivierung des PI3K-Akt Weges, als auch der Abbau von p53 konnten in Chlamydien-infizierten Mauszellen nicht beobachtet werden. Kürzlich wurde darüber berichtet, dass während chlamydialer Infektionen reaktive Sauerstoffspezies produziert werden und die DNA der Wirtszelle geschädigt wird. Demnach trägt der Abbau von p53 entscheidend dazu bei, ein für chlamydiale Infektionen maßgebliches, anti-apoptotisch geprägtes Umfeld zu generieren.

Um die Bedeutung des Abbaus von p53 für Wachstum und Entwicklung von Chlamydien zu ermessen, wurde p53 durch den HDM2-inhibierenden Wirkstoff Nutlin-3, sowie die DNA-Schäden induzierende Verbindung Etoposid stabilisiert bzw. aktiviert. Die Entwicklung der Chlamydien, sowie die Ausbildung der Inklusion wurden dadurch überraschenderweise stark beeinträchtigt bzw. waren fehlerhaft. Die Vollendung des chlamydialen Entwicklungszyklus wurde verhindert, was den Verlust der Infektivität nach sich zog. Interessanterweise erlaubte das Entfernen des p53-aktivierenden Stimulus die Ausbildung der bakteriellen Inklusion und

die Wiedererlangung der Infektivität. Eine ähnliche Beobachtung konnte in Zelllinien mit einer p53-Defizienz gemacht werden.

Da bakterielles Wachstum und Ausbildung der Inklusion durch aktiviertes p53 stark eingeschränkt war, wurde vermutet, dass p53-vermittelte Inhibierung des zellulären Metabolismus am fehlerhaften Wachstum der Chlamydien beteiligt ist. Analyse von Glykolyse und Pentosephosphatweg (PP-Weg) zeigten den Stellenwert eines funktionierenden PP-Wegs für das Wachstum der Chlamydien auf. Zusätzlich konnte durch Überexpression der Glucose-6-phosphat-Dehydrogenase das durch aktiviertes p53 gehemmte Wachstum der Chlamydien wiederhergestellt werden. Dieser Effekt war noch deutlicher in p53-defizienten Zellen, die mit Etoposid bzw. Nutlin-3 behandelt wurden. Demnach tragen auch p53-unabhängige Aspekte zur Einschränkung des chlamydialen Wachstums bei. Das Entfernen von reaktiven Sauerstoffspezies durch Antioxidationsmittel war jedoch nicht ausreichend zur Wiedererlangung der chlamydialen Infektivität. Demnach sind nicht nur die anti-oxidativen Eigenschaften des PP-Wegs sondern auch das Bereitstellen von Vorläufermolekülen für die Nukleotidsynthese, sowie dessen Beitrag zur DNA-Reparatur entscheidend für erfolgreiches Wachstum von Chlamydien.

Veränderung der Signaltransduktion der Wirtszelle wurde bereits bei einigen Krankheitserregern nachgewiesen. Da reaktive Sauerstoffspezies und DNA Schäden häufig bei Infektionen intrazellulärer Bakterien auftreten, entstanden unterschiedliche Strategien, den Wirt zu manipulieren und das Einleiten des Zelltodes zu verhindern. Das Herunterregulieren des Tumorsuppressors p53 ist entscheidend während der Entwicklung von Chlamydien. Sowohl das Überleben der Wirtszelle, als auch die für chlamydiales Wachstum förderliche Unterstützung durch den Stoffwechsel werden dadurch gewährleistet.

1 Introduction

1.1 *Chlamydia*

1.1.1 Chlamydial infections

Chlamydia trachomatis is a gram-negative, obligate intracellular human pathogen causing numerous diseases including trachoma and ectopic pregnancy. With more than 100 million new infections per year it is the most common sexually transmitted disease (STD) by a bacterial pathogen. Left untreated, *Chlamydia* may spread into the pelvic area, uterus, fallopian tubes and ovaries leading to pelvic inflammatory disease (PID). Long term infections over months or years can cause permanent damage, infertility, chronic pelvic pain, or lead to spontaneous abortion (Brunham *et al.*, 2005). Untreated *Chlamydia* infection in men ultimately leads to infertility. *Chlamydia* infections normally do not cause any symptoms in 75% of infected women and 50% of infected men and are therefore known as a “silent epidemic” (Bebear *et al.*, 2009). It is one of the most common sexually transmitted diseases in the U.S. and, according to health economists, causes expenses of more than $2 billion a year.

The word *Chlamydia* is of Greek origin (“chlamys”, cloak) and describes the cloak-like surrounding of the nucleus by the chlamydial inclusion. In the beginning it was claimed that *Chlamydia* are intracellular “mantled” protozoans, called *Chlamydozoa*, embedded in a matrix. The first descriptions about eye infections, today known as trachoma, are from ancient Chinese and Egyptian manuscripts. Since the early 20th century, however, isolates and descriptions of *Chlamydia* as an intracellular prokaryotic organism exist.

The order *Chlamydiales* contains four chlamydial families: the *Chlamydiaceae*, *Parachlamydiaceae*, *Simkaniaceae* and *Waddliaceae* (Everett *et al*., 1999, Rurangirwa *et al.*, 1999). Another family, the *Rhabdochlamydiaceae*, has been proposed (Kostanjsek *et al.*, 2004). The family *Chlamydiaceae* includes two genera, the *Chlamydia*, e.g. *Chlamydia trachomatis* and *Chlamydia muridarum*, and the *Chlamydophila*, e.g. *Chlamydophila pneumoniae. C. trachomatis* includes three human biovars which cause different diseases and symptoms. Serovars A, B and C are responsible for infections of the eye, called trachoma, and are the leading cause of blindness in Africa (Wright *et al.*, 2008). Serovars D to K lead to infections of the urogenital tract, causing urethritis, pelvic inflammatory disease and ectopic pregnancy (Brunham *et al.*, 2005), while serovars L1, L2 and L3 primarily infect lymphatics and lymph nodes. Infections of serovars A to K are normally restricted to the mucosal epithelium, genotypes L1 to L3 cross this barrier and lead to invasive infections called lymphogranuloma venereum (LGV) (Bebear *et al.*, 2009). Infections with *C. pneumoniae* cause pulmonary

diseases like bronchitis, pneumonia or pharyngitis (Murphy *et al.*, 2002). Chronic infections are linked to cerebrovascular diseases (Balin *et al.*, 1998) or atherosclerosis (Kuo *et al.*, 2003).

Due to genomic degradation, the chlamydial genome is only slightly larger than that of *Mycoplasma*, the organism with the smallest bacterial genome (0.8 Mbp). Within the family of *Chlamydiaceae*, the genome of *C. trachomatis* has a length of about 1.04 Mbp, coding for approximately 875 proteins, whereas that of *C. pneumoniae* is longer with 1.23 Mbp (Kalman *et al.*, 1999, Stephens *et al.*, 1998). Additionally, *C. trachomatis*, but not *C. pneumoniae*, has a cryptic plasmid of 7.5 kbp with eight open reading frames (Thomas *et al.*, 1997, Pearce *et al.*, 1991).

1.1.2 The developmental cycle of *C. trachomatis*

Chlamydia have a unique biphasic developmental life cycle consisting of two different bacterial types, elementary bodies (EBs) and reticulate bodies (RBs) (Moulder, 1991). EBs were thought to be metabolically inactive until Omsland *et al.* demonstrated chlamydial EBs to be conditionally active in cell-free culture systems (Omsland *et al.*, 2013). In addition, this specific chlamydial form has the ability to infect cells, preferentially nonprofessional phagocytes. Their surface is hydrophobic, negatively charged and resembles that of gram-negative free living bacteria, except that a network of disulfide bonds between the major outer membrane proteins (MOMPs) gives stability to the chlamydial envelope. MOMP makes up 60% of the total outer membrane protein (Caldwell *et al.*, 1981) and a large amount of the MOMPs consists of a 40 kDa form. MOMPs of molecular masses of 12, 59 and 62 kDa exist as well (Hatch *et al.*, 1984), all rich in cysteine residues to facilitate cross-linking. Recently, a new labelling technique allowed the detection of chlamydial peptidoglycan, arranged in a ring-like shape at the cellular division plane (Liechti *et al.*, 2014).

RBs on the other hand do not possess the rigid cell wall of EBs, as the MOMPs are no longer cross-linked with disulfide bonds. They are non-infectious, metabolically active and have a fibrillar nucleoid (Costerton *et al.*, 1976) instead of the highly compacted one of EBs. After reduction of disulfide bonds between MOMPs, formation of channels and porins in the chlamydial cell wall enables ATP and other metabolites to enter the bacteria (Bavoil *et al.*, 1984).

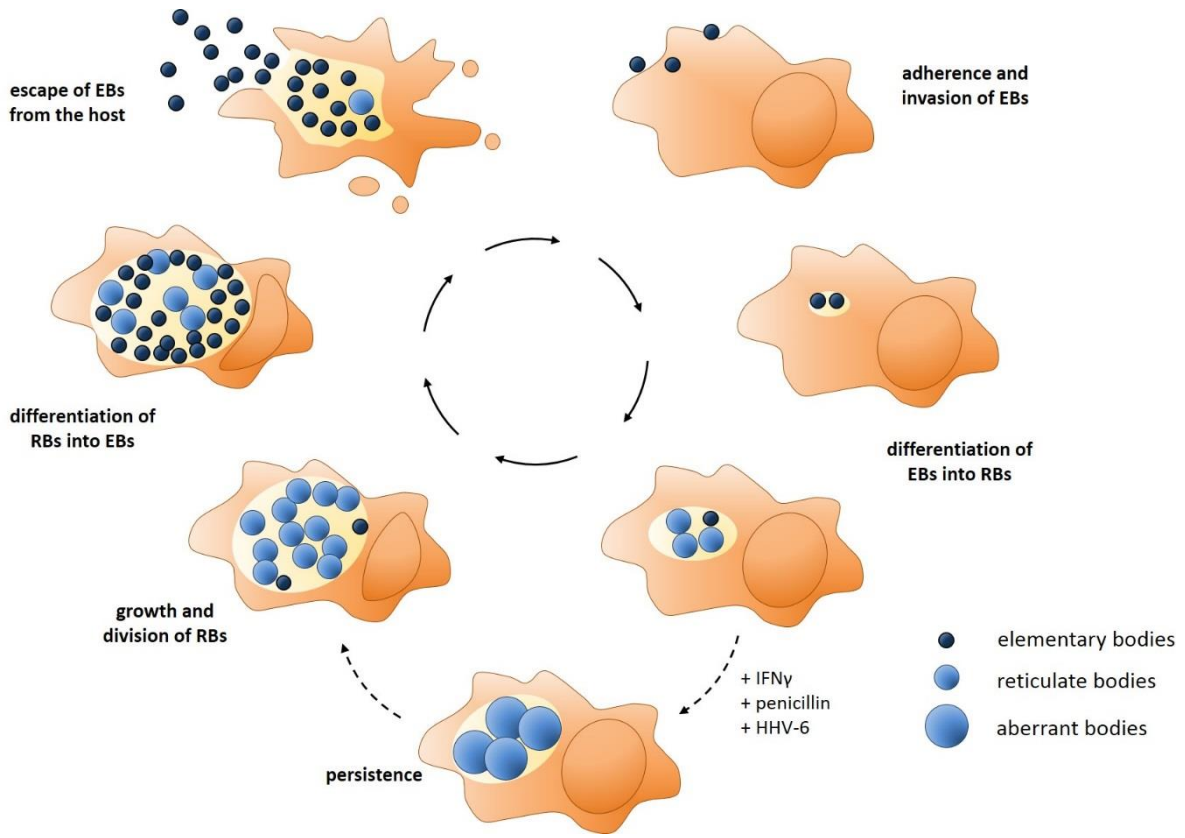


Figure 1 The chlamydial developmental cycle. Infectious EBs attach to the host cell surface and are internalised by an endocytosis-like mechanism. After conversion of EBs to RBs, *Chlamydia* multiply by binary fission. *Chlamydia* start differentiating into their infectious form after about 20 h of infection and exit their host at the end of their life cycle by either cell lysis or extrusion. Persistent, non-replicative *Chlamydia* do not differentiate into EBs and do not finish their life cycle. Persistence is initiated by different stimuli including antibiotics, cytokines like IFNγ and depletion of essential nutrients or co-infection with various human herpes viruses.

Without the provision of molecules from the host, protein synthesis of the obligate intracellular *Chlamydia* which starts less than one hour after bacterial entry into the host cell (Belland *et al.*, 2003) could not be initiated. The regulatory mechanisms conferring distinct characteristics on EBs and RBs are poorly understood, however, temporally regulated proteins (Nicholson *et al.*, 2003), cycle-specific promoters (Cheng *et al.*, 2012) and sigma factors (Shen *et al.*, 2004, Engel *et al.*, 1990) have been described and account for the conversion of EBs into RBs at the beginning of infection and of RBs to EBs at the end of the chlamydial life cycle.

After the attachment of EBs to the host cell surface, *Chlamydia* are internalised in an endocytosis-like mechanism. The exact pathway is still unclear, yet different uptake mechanisms were proposed. Clathrin-mediated (Majeed *et al.*, 1991, Hodinka *et al.*, 1988, Hybiske *et al.*, 2007a) and caveola-mediated (Norkin *et al.*, 2001, Stuart *et al.*, 2003) endocytosis were two possibilities, however, there was also evidence against these findings

(Boleti *et al.*, 1999, Gabel *et al.*, 2004). Important regulatory factors during this process are the small GTPases Rac1 and Arf6 (Balana *et al.*, 2005, Carabeo *et al.*, 2004). The large GTPase dynamin-1 (Boleti *et al.*, 1999) and the small GTPases RhoA and Cdc42 (Carabeo *et al.*, 2004) on the other hand do not play a role. Phagocytosis (Byrne *et al.*, 1978) and pinocytosis (Reynolds *et al.*, 1990) were also suggested to be possible mechanisms of chlamydial uptake. A requirement for internalization is the reorganization of the actin cytoskeleton (Carabeo *et al.*, 2002). It was proposed that *Chlamydia* translocates the chlamydial effector protein Tarp to nucleate actin filaments and promote entry of the pathogen (Clifton *et al.*, 2005, Clifton *et al.*, 2004, Jewett *et al.*, 2006). The reorganisation of EBs into RBs occurs in the first eight to twelve hours after infection. They increase in size, the structure of cell wall and nucleoid is changed and the number of ribosomes is increased to facilitate protein synthesis. *Chlamydia* multiply inside a membrane-bound vacuole in the cytoplasm termed inclusion derived from a phagosomal membrane and replaced by chlamydial proteins during infection.

Multiplication of *Chlamydia* results from binary fission and after eight to twelve doublings a total of about 100 to 1000 bacteria reside inside the inclusion. After about 20 hours of infection the first RBs start converting into EBs, however, other RBs continue to multiply. The transition from RB to EB is characterized by reduction in size, condensation and differentiation of electron-dense nucleoids. Till the end of the chlamydial life cycle a heterogeneous population of RBs, EBs and intermediate forms exists. The length of the developmental cycle ranges between 30 and 60 h but is generally stated as 48 h. By this time, the chlamydial inclusion mainly contains EBs already expressing MOMP on their surface. Cross-linking of the disulfide bonds takes place during the second half of the developmental cycle and results in their typical rigid cell wall and necessary stability. *Chlamydia* exit from the host cell occurs by either cell lysis or extrusion (Hybiske *et al.*, 2007b). Cell lysis is characterized by sequential rupture of inclusion and cellular membranes resulting in host cell death. The process called extrusion on the other hand results in the release of a portion of the chlamydial inclusion by a membranous protrusion and leaves the host cell intact. Extrusions eventually detach from host cells and release infectious EBs. It was also proposed that *Chlamydia* exit their host cell by exocytosis (Todd *et al.*, 1985) or apoptotic pathways (Perfettini *et al.*, 2003).

1.1.3 Interaction of *Chlamydia* with its host

As obligate intracellular bacteria *Chlamydia* are dependent on the host cell providing most of the required nutrients, cofactors, amino acids, and purine and pyrimidine nucleotides. During evolution loss and inactivation of non-essential genes resulted in a reduced genome. This phenomenon is also observed in other obligate intracellular pathogens, including *Mycoplasma genitalium*, the model organism for minimal genomes (Su *et al.*, 1990).

For decades *Chlamydia* were considered to be energy parasites incapable of generating their own ATP and totally depending on the host for ATP supply. This hypothesis was strongly supported by the finding of an ATP-ADP translocase allowing the exchange of host ATP for chlamydial ADP (Hatch *et al.*, 1982). However, Stephens *et al.* finally found out that *Chlamydia* encodes for several energy-producing enzymes (Stephens *et al.*, 1998) and it was further proven that *Chlamydia* contains complete and functional pathways for glycolysis and the pentose phosphate pathway, as well as parts of the tricarboxylic acid cycle (Iliffe-Lee *et al.*, 1999). Despite these discoveries, chlamydial infections stimulate ATP synthesis in the infected host, the highest levels occurring midway of the life cycle, when most of the bacteria are metabolically active RBs (Ojcius *et al.*, 1998). Glucose consumption, expression of the glucose transporter (Glut 1), lactate production and glutamate synthesis are increased in a similar way as ATP synthesis emphasising the dependency of the pathogen on the host cell, regardless of its own contribution to energy and metabolite production (Ojcius *et al.*, 1998).

Furthermore, *Chlamydia* are dependent on host amino acids as depletion of amino acids results in abnormal chlamydial growth and formation of aberrant bodies (Harper *et al.*, 2000). Sequencing of chlamydial genomes revealed that amino acid synthesis pathways are not available in either *C. trachomatis* or *C. pneumoniae* (Kalman et al., 1999). There is only one predicted chlamydial transporter for branched-chain amino acids, BrnQ, which also facilitates uptake of methionine, a feature unique among gram-negative bacteria (Braun *et al.*, 2008). Addition of excess amino acids, especially leucine, isoleucine, methionine and phenylalanine leads to competitive inhibition of the transporter, subsequent valine starvation and growth inhibition of *Chlamydia* (Braun *et al.*, 2008).

As already mentioned, *Chlamydia* are able to exchange host ATP for chlamydial ADP ensuring constant energy supply. In addition to this translocase, also called nucleoside phosphate transporter 1 of *C. trachomatis*, $Npt1_{Ct}$, *Chlamydia* possess another way of nucleotide uptake. $Npt2_{Ct}$, nucleoside phosphate transporter 2 of *C. trachomatis*, catalyses the uptake of ATP, CTP, UTP and with the highest affinity GTP. In contrast to $Npt1_{Ct}$ catalysing the exchange of nucleotides $Npt2_{Ct}$ promotes the uptake of host nucleotides energised by a proton motive force across the chlamydial cytoplasmic membrane (Tjaden *et al.*, 1999). NTPs are required for nucleic acid synthesis, cell signalling and cofactors for enzyme reactions. As *Chlamydia* are auxotrophic for ATP, GTP and UTP (Tipples *et al.*, 1993) uptake of host nucleotides is essential for chlamydial growth.

Beside glucose, amino acids and nucleotides *Chlamydia* are known to take up host-derived lipids including sphingomyelin (Hackstadt *et al.*, 1995), cholesterol (Carabeo *et al.*, 2003) and triglycerol phospholipids (Wylie *et al.*, 1997). Considering the composition of chlamydial cell membranes, the dependency on host-derived lipids becomes obvious: Chlamydial membranes

not only contain phospholipids typical for prokaryotes, like phosphatidylglycerol, cardiolipin and phosphatidylethanolamine, but also phosphatidylcholine and phosphatidylinositol normally only present in eukaryotic membranes (Hatch *et al.*, 1998, Wylie *et al.*, 1997). In addition to the normal gram-negative outer and inner membrane, *Chlamydia* are surrounded by an inclusion membrane which has to be enlarged during the replication process inside the host cell. The inclusion membrane is neither positive for markers of lysosomes, early or late endosomes, nor fluid-phase endosomes (Heinzen *et al.*, 1996, Taraska *et al.*, 1996, Scidmore *et al.*, 1996). The only cellular trafficking involving membranous vesicles takes place between the Golgi apparatus and the inclusion. Using the fluorescent ceramide analogue C6-NBD-ceramide (6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)sphingosine) converted to sphingomyelin or glucosylceramide at the Golgi apparatus (Lipsky *et al.*, 1985) vesicular trafficking between the Golgi and the chlamydial inclusion could be visualised. Subsequently incorporation of sphingomyelin into the inclusion membrane and into *Chlamydia* took place (Hackstadt *et al.*, 1995). By intercepting the exocytic pathway and incorporating typical host proteins, chlamydial inclusions obtain the character of secretory vesicles and thus avoid fusion with endosomal vesicles or lysosomes. In addition to host proteins and lipids, chlamydial proteins, e.g. Inc proteins, are inserted into the inclusion membrane.

1.1.4 Apoptosis inhibition during chlamydial infections

Effective infection of the host is dependent on completion of the chlamydial life cycle. *Chlamydia* prevents lysis of the host cell by inhibition of apoptosis. Since its first mentioning in 1997 several anti-apoptotic mechanisms inhibiting chemically induced apoptosis during mid and late stages of the chlamydial cycle have been demonstrated. Two cellular pathways lead to apoptosis induction: the extrinsic, death receptor-mediated pathway and the intrinsic, mitochondria-mediated pathway.

The extrinsic pathway is activated by ligands, which bind to death receptors like Fas, the TNF (tumour necrosis factor) receptor and TRAIL (TNF-related apoptosis-inducing ligand) receptors. Subsequently a large multi-protein complex known as DISC (Death Initiation Signalling Complex) is assembled and serves as an activation platform for initiator caspases. The death receptor Fas, for example, recruits the adaptor molecule FADD (Fas-associated protein with a DD) via a death domain (DD), which in turn binds to caspase-8 with its death effector domain (DED), activating further caspase 8 molecules and ultimately effector caspase 3 (Lavrik *et al.*, 2012, Peter *et al.*, 2003). The intrinsic pathway can be activated as a result of stimuli like DNA damage, oxidative stress and many others. The signals converge at the mitochondria and lead to mitochondrial outer membrane permeabilisation, resulting in the release of cytochrome c, AIF (apoptosis-inducing factor), endonuclease G and DIABLO (direct

inhibitor of apoptosis protein-binding protein with low PI, also known as SMAC (second mitochondrial-derived activator of caspase)). Once released, cytochrome c directly binds to Apaf-1 (Apoptotic protease activating factor 1), inducing the formation of a large complex, the apoptosome. Caspase 9 is recruited and leads to the activation of down-stream effectors like caspase 3 (Green *et al.*, 2004). Mitochondrial outer membrane permeabilisation is induced in two different ways: Either the permeability transition pore complex (PTPC) in the inner mitochondrial membrane is opened or pro-apoptotic members of the Bcl-2 family disrupt the integrity of the outer mitochondrial membrane (Green *et al.*, 2004). Characteristic for the Bcl-2 family are the Bcl-2 homology domains (BH), present in different numbers in all anti- or pro-apoptotic members. The anti-apoptotic members (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1) all contain three or four BH domains. The pro-apoptotic Bcl-2 family members either possess two or three BH domains (Bax, Bak, Bcl-x_S, Bok/Mtd and Bcl-G_L) or only the BH3 domain (Bad, Bik, Blk, Bid, Bim, Bmf, Noxa, Puma) (Adams *et al.*, 1998, Gross *et al.*, 1999, Martinou *et al.*, 2011). These BH3-only proteins either interact with anti-apoptotic proteins to inhibit their function or activate multidomain proteins like Bax or Bak. The latter are translocated from the cytosol to mitochondria, where they insert with the help of tBid into the outer mitochondrial membrane, oligomerise and subsequently form a pore (Lovell *et al.*, 2008, Desagher *et al.*, 1999, Eskes *et al.*, 2000). Beside pore formation, pro-apoptotic proteins induce mitochondrial outer membrane permeabilisation through binding to mitochondrial channel proteins such as VDAC (voltage dependent anion channel) or ANT (adenine nucleotide transporter) (Favaloro *et al.*, 2012). The cIAPs (cellular inhibitor of apoptosis) are a family of endogenous apoptosis inhibitors. Characteristic for these proteins is the presence of one to three BIR-domains (Baculovirus IAP Repeat). cIAPs exert their anti-apoptotic function by binding to caspases, XIAP (X-linked IAP) for instance binds to caspase 3, 7 and 9, thereby inhibiting their activation (Deveraux *et al.*, 1999). XIAP activity on the other hand is blocked by binding of DIABLO (Gao *et al.*, 2007), which is released by mitochondria during the course of apoptosis.

Chlamydia infection of cells prevents apoptosis induced by different components, like granzyme B/perforin, TNFα, staurosporine, etoposide, antibodies against the Fas receptor (Fan *et al.*, 1998, Rajalingam *et al.*, 2008) or UV-light (Fischer *et al.*, 2004). Further investigations into the mechanism of apoptosis prevention revealed different sites of action affected by *Chlamydia* (Sharma *et al.*, 2009). As cytochrome c release as well as downstream activation of caspase 9 and 3 could no longer be observed, a block at the mitochondrial level became apparent (Fan *et al.*, 1998). Moreover, Fischer *et al*. found out that there is an inhibition of apoptosis induction downstream of mitochondria as well (Fischer *et al.*, 2001). Some groups observed a degradation of BH3-only proteins (Dong *et al.*, 2005, Fischer *et al.*, 2004, Ying *et al.*, 2005) mediated by the chlamydial protease-like activity factor (CPAF), but others could not confirm these findings (Rajalingam *et al.*, 2008, Chen *et al.*, 2012). Rajalingam *et al*. observed

an upregulation of cIAP-2 during *Chlamydia* infection and could show that silencing of cIAP-2 sensitised infected cells for TNFα-induced apoptosis (Rajalingam *et al.*, 2006). Although only cIAP-2 is upregulated, the presence of cIAP-1 and XIAP is required for preventing cell death. cIAP-1, cIAP-2 and XIAP form a heteromeric complex, also called IAPosome, stabilise each other and prevent the activation of effector caspases (Rajalingam *et al.*, 2006, Salvesen *et al.*, 2002, Vaux *et al.*, 2005). Beside cIAP-2, the anti-apoptotic protein Mcl-1 was also shown to be upregulated during *Chlamydia* infection. Su *et al*., as well as Rajalingam *et al.*, demonstrated that *C. trachomatis* leads to activation of the PI3K-Akt and the Raf-MEK-ERK pathway (Su *et al.*, 2004, Rajalingam *et al.*, 2008, Verbeke *et al.*, 2006) and that both Mcl-1 and cIAP-2 upregulation were dependent on the activation of these pathways (Rajalingam *et al.*, 2008). Upon upregulation, Mcl-1 strongly binds to the BH3-only protein Bim and forms a complex with Bak on the outer mitochondrial membrane, thus preventing the formation of pores and the release of DIABLO and cytochrome c. PI3K-Akt pathway activation was also deemed responsible for the phosphorylation of the BH3-only protein Bad which is subsequently sequestered to the surface of the chlamydial inclusion through its interaction with the host-cell adapter protein 14-3-3β and the chlamydial inclusion protein IncG (Verbeke *et al.*, 2006, Scidmore *et al.*, 2001).

1.2 Tumour suppressor p53

1.2.1 Functions of p53

The tumour suppressor p53 was first identified in 1979 by DeLeo *et al.* and cloned in 1984 (DeLeo *et al.*, 1979, Matlashewski *et al.*, 1984). Interestingly, p53 was initially characterised as an oncogene as isolation of tumour cell mRNA resulted in the discovery of a mutated version of the *tp53* gene. Its true nature as a tumour suppressor was revealed in 1989 by Baker *et al.* (Baker *et al.*, 1989). To this day, p53 turned out to be one of the most important proteins in the cell, as a transcription factor, an activator or repressor of target genes or an activator of growth arrest, cellular senescence or apoptotic pathways. For its role in genomic stability control and DNA damage response p53 was called “guardian of the genome” (Lane, 1992). In recent years p53 was assigned an emerging role in development and metabolism. Around 50% of human cancers carry missense mutations in the *tp53* gene (Petitjean *et al.*, 2007). These mutations result in general protein loss, loss of function or gain of function. Especially a gain of protein function often turns out to actively contribute to tumour development and to increased resistance to anticancer treatments (Oren *et al.*, 2010).

Among the first discoveries about p53 was its activation after UV-irradiation of cells and its important role in DNA damage response and regulation of the cell cycle (Maltzman *et al.*, 1984,

Kastan *et al.*, 1993, Canman *et al.*, 1994, Nelson *et al.*, 1994). After detection of DNA damage p53 is rapidly stabilised and activated. The DNA repair pathway is very sensitive: A small number of double strand breaks (DSB) or single strand gaps are enough to set the activation of p53 in motion (Huang *et al.*, 1996). DSBs are detected by the MRN (MRE11–RAD50–NBS1) complex, which mediates the activation of the protein kinases ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) (Dupre *et al.*, 2006). ATM, ATR and DNA-dependent protein kinase (DNA-PK) are members of the phosphatidylinositol-3 kinase-like kinase family and initiate the phosphorylation of a range of proteins, e.g. the DSB marker H2Ax, p53 and its negative regulators MDM2 (murine double minute 2) and MDM4, but also 53BP1 (p53-binding protein 1), Chk1 and Chk2 (checkpoint kinase 1 and 2), thus inducing the complex signalling pathways of the DNA damage response (DDR) (Meulmeester *et al.*, 2005, Stommel *et al.*, 2004, Schultz *et al.*, 2000). Nuclear foci with phosphorylated H2Ax (γH2Ax) attract DNA repair proteins and thus promote DSB repair and genome stability (Srivastava *et al.*, 2009). How stress-activated p53 differentiates between induction of temporary cell growth arrest, permanent senescence or apoptosis is still not clear. However, early tumorigenic events are often accompanied by DNA damage and p53-controlled checkpoints in cell cycle and DNA damage response are important steps in preventing tumour development (Meek, 2009).

Cell growth arrest is initiated through p53-induced expression of the *waf1/cip1* gene encoding for p21 which subsequently binds to and inhibits the cyclin-CDK (cyclin dependent kinase) complexes, G1-S/CDK and S/CDK, important for the G1/S transition in the cell cycle (el-Deiry *et al.*, 1993). After successful DNA repair p53 releases the block on G1/S transition and allows cell cycle progression. In addition to the G1/S checkpoint, p53 has also been shown to regulate the G2/M checkpoint. This checkpoint is activated upon detection of DNA damage after completion of the S-phase or unbalanced nucleotide pools as a result of blocked purine or pyrimidine synthesis. p53 represses gene transcription of Cdc2, the cyclin necessary to enter mitosis, and the gene *cyclin B1*. Cyclin B1 has to bind to Cdc2 for its activation. Inhibition of Cdc2 is further achieved by three transcriptional targets of p53, Gadd45, p21, and 14-3-3σ, which additionally repress activation of the Cdc2/Cyclin B1 complex (Taylor *et al.*, 2001). Aside from apoptosis, cellular senescence is an important way of silencing damaged cells. Irreversible cell cycle arrest is triggered through p53-mediated activation of genes involved in senescence, like p21 and PAI-1 (plasminogen activator inhibitor-1) (Kortlever *et al.*, 2006, Deng *et al.*, 2008). Characteristic for senescence is often a persistently activated DDR arresting cells in a non-proliferative state. Thus a crucial barrier against tumour development is created (Reinhardt *et al.*, 2012). Prevention of tumour development is profoundly dependent on p53-induced apoptosis. Both mitochondrial and death-receptor induced apoptotic pathways are targeted. p53 induces gene expression of the pro-apoptotic proteins PUMA (Jeffers *et al.*, 2003, Villunger *et al.*, 2003, Nakano *et al.*, 2001), Bax (Miyashita *et al.*, 1995) and Noxa (Oda

et al., 2000). Cytochrome c release from mitochondria and formation of the apoptosome complex are triggered. Moreover, p53 directly activates Bax (Chipuk *et al.*, 2004) or Bak (Leu *et al.*, 2004) in the cytoplasm and blocks the anti-apoptotic protein Bcl-xL (Chipuk *et al.*, 2004). As a consequence pro-apoptotic proteins sequestered by Bcl-xL are released.

1.2.2 p53 and metabolism

Metabolic transformation is a common characteristic observed in cancerous cells. Changes in metabolism during tumour development, referred to as the Warburg effect, include high uptake of glucose for ATP synthesis, aerobic glycolysis instead of the more effective oxidative phosphorylation and uncontrolled cell cycle progression (Warburg, 1956). The tumour suppressor p53 is involved in the regulation of many pathways in cellular metabolism, including glycolysis, the pentose phosphate pathway (PPP), oxidative phosphorylation, glutaminolysis, nucleotide biosynthesis and fatty acid metabolism, autophagy and mTOR (mammalian target of rapamycin) signalling (Gottlieb, 2011, Gottlieb *et al.*, 2010, Puzio-Kuter, 2011, Hu *et al.*, 2010, Maddocks *et al.*, 2011). p53 supports efficient energy yield of glucose through slowdown of glycolysis, inhibition of the PPP and promotion of oxidative phosphorylation (Bensaad *et al.*, 2007). To limit glycolytic flux, p53 represses expression of Glut 1 and 4 (glucose transporter 1 and 4) (Schwartzenberg-Bar-Yoseph *et al.*, 2004) and the insulin receptor (Webster *et al.*, 1996) and negatively regulates protein stability of PGM (phosphoglycerate mutase) (Kondoh *et al.*, 2005). TIGAR (TP53-induced glycolysis and apoptosis regulator), a negative regulator of glycolysis, is transcriptionally induced by p53 and dephosphorylates either fructose-1,6-bisphosphate or fructose-2,6-bisphosphate, counteracting the enzyme reaction of the phosphofructokinase (PFK). As p53 increases expression of TIGAR, it indirectly promotes glucose flux through the PPP, advantageous during situations of oxidative stress but also for cancer development (Bensaad *et al.*, 2006). However, recent findings showed that in many types of cancer TIGAR is under the control of other proteins and can be expressed independently of the transcriptional activity of p53 (Wanka *et al.*, 2012, Won *et al.*, 2012, Lui *et al.*, 2011, Yin *et al.*, 2012). Oxidative phosphorylation has been shown to be promoted by p53 through induction of SCO2 (synthesis of cytochrome c oxidase 2) expression (Matoba *et al.*, 2006). While glycolysis is very important for energy production, the PPP has a biosynthetic role, generating the reducing equivalent NADPH (nicotinamide adenine dinucleotide phosphate), ribose-5-phosphate, the precursor for nucleotide biosynthesis, and erythrose-4-phosphate, used for the synthesis of aromatic amino acids. Moreover, the PPP has a protecting role against reactive oxygen species: NADPH is utilised to prevent oxidative stress in the cell. Glutathione reductase uses NADPH to reduce glutathione, subsequently applied for the conversion of H_2O_2 to H_2O by glutathione peroxidase (GPx). Activation of the PPP is achieved

by allosterical stimulation of the first enzyme of the pathway, the glucose-6-phosphate dehydrogenase (G6PD), by $NADP^+$ resulting in NADPH production. Moreover, Jiang *et al.* demonstrated that p53 blocks the activity of the enzyme through direct protein-protein interactions and blocking of active dimer formation of G6PD. Inhibition of G6PD in an *in vitro* assay continued after removal of p53 suggesting a catalytic process (Jiang *et al.*, 2011). In addition, p53 is involved in controlling oxidative stress and production of ROS. While low levels of ROS induce proliferation or transcription of host defence genes, high levels are harmful for DNA, RNA and proteins. p53 initiates an antioxidant response by promoting gene expression of GPx1, MnSOD (Manganese superoxide dismutase) and ALDH4 (aldehyde dehydrogenase 4 family, member A1) (Hussain *et al.*, 2004, Yoon *et al.*, 2004). Oxidative stress-induced DNA damage is cleared by p53 (Ueno *et al.*, 1999). After detection of severe cellular stress, p53 deliberately promotes ROS production to induce apoptosis (Johnson *et al.*, 1996, Bensaad *et al.*, 2005).

1.2.3 Regulation of p53 activation

The transcription factor p53 is composed of several conserved domains: the N-terminal domain contains a region required for transcriptional transactivation (TAD, also known as activation domain 1, AD1) and a proline-rich region important for apoptotic activity, a central sequence-specific DNA binding core domain (DBD) and a C-terminal domain composed of a tetramerisation domain (homo-oligomerisation domain, OD), a strongly basic regulatory domain, a nuclear localization signal sequence and three nuclear export signals (Bode *et al.*, 2004, Vousden *et al.*, 2002). The protein is encoded by the *tp53* gene, located on the short arm of chromosome 17 (Isobe *et al.*, 1986) and contains 11 exons and 10 introns (Lamb *et al.*, 1986).

In healthy cells p53 is kept at very low concentrations due to constant degradation by the proteasome. Only certain stimuli like DNA damage by UV-light or chemicals, oxidative stress, osmotic shock, ribonucleotide depletion or deregulated oncogene expression, prolong its very short half-life and lead to the stabilisation of p53 protein levels and its accumulation inside the cell (Siliciano *et al.*, 1997). Posttranslational modifications of p53 are normally accompanied by abolished interaction of HDM2 (the human form of murine double minute 2, MDM2) with p53 or degradation of HDM2. Activation of p53 can be achieved by phosphorylation (Meek, 1999), acetylation (Gu *et al.*, 1997), ADP-ribosylation, methylation (Chuikov *et al.*, 2004), ubiquitination, sumoylation (Gostissa *et al.*, 1999) or neddylation (Bode *et al.*, 2004, Vousden *et al.*, 2002, Vogelstein *et al.*, 2000). Transcriptional activity is thereby mainly increased by phosphorylation of the N-terminal domain which contains a large number of possible phosphorylation sites and is therefore the primary target of protein kinases transducing stress

signals. Phosphorylation of this domain leads to conformational changes, subsequent tetramerisation and transcriptional activity in the nucleus (Appella *et al.*, 2001, Xu, 2003, Brooks *et al.*, 2003). Kinases responsible for phosphorylation of p53 can be divided into two classes: the MAPK-family (JNK1-3, ERK1-2, p38 MAPK) (Lin *et al.*, 2008, Huang *et al.*, 1999, Buschmann *et al.*, 2001, Fuchs *et al.*, 1998), which transmits stress signals from membrane damage, oxidative stress, osmotic shock and heat shock, and kinases involved in genome integrity and DDR (ATR, ATM, Chk1 and Chk2, DNA-PK, TP53RK) (Shieh *et al.*, 1997, Kruse *et al.*, 2009a, Appella *et al.*, 2001).

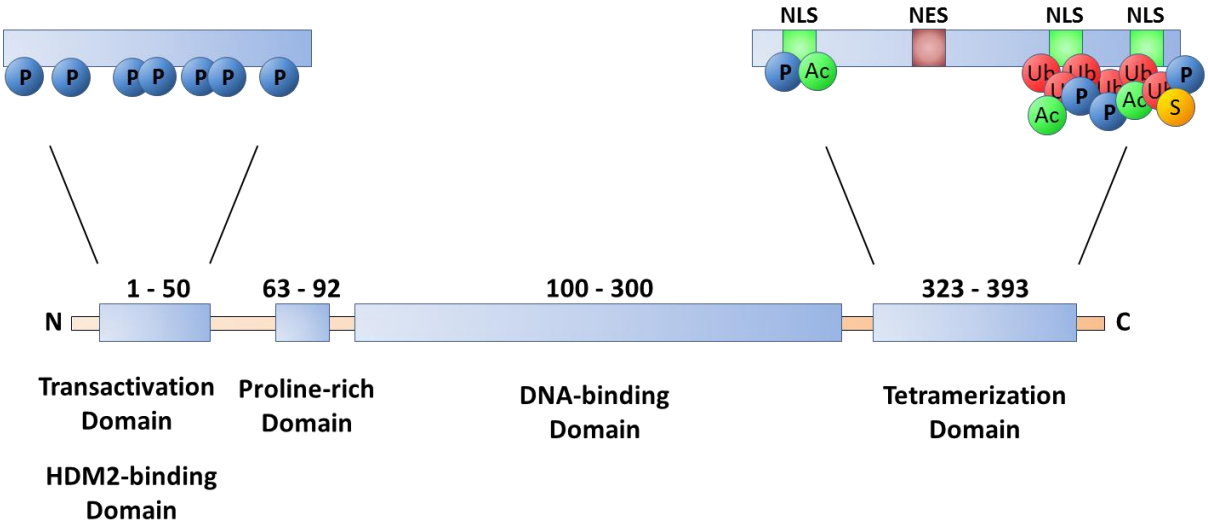


Figure 2 Conserved domains and modification sites of tumour suppressor p53. P: phosphorylation site; Ac: acetylation site; Ub: ubiquitination site; S: sumoylation site; NLS: nuclear localisation signal; NES: nuclear export signal.

However, it was also demonstrated that phosphorylation is not the main mechanism of p53 activation and unnecessary for activating the tumour suppressor in response to a number of stress signals (Ashcroft *et al.*, 1999, Ashcroft *et al.*, 2000, de Stanchina *et al.*, 1998). A phosphorylation-independent way of p53 activation involves the tumour suppressor ARF (alternate reading frame) of the INK4a/ARF gene locus (CDKN2A). ARF is involved in cell cycle progression and activated after sustained mitogenic stimulation e.g. aberrant growth signalling from oncogenes like Myc and Ras (Abida *et al.*, 2008). It sequesters HDM2 in the nucleolus thus releasing p53 from its negative regulator (Zhang *et al.*, 1998).

Next to phosphorylation, acetylation is another posttranslational modification leading to p53 activation. Acetylations are catalysed by histone acetyltransferases, such as p300/CBP (CREB (cAMP-response element-binding protein) binding protein) and pCAF (p300/CBP-associated factor), on lysine residues of the carboxyl terminus of p53 (Ito *et al.*, 2001). Acetylation of the C-terminus inhibits the p53-HDM2 interaction and the ubiquitination dependent proteolysis. HDM2 itself gets acetylated by CBP and to a lesser extent also by p300 which inhibits its

enzyme activity (Wang *et al.*, 2004). HDM2 is competing with acetyltransferases for the lysine residues as the acetylated lysines are no longer ubiquitinated (Li *et al.*, 2002). However, Chan *et al.* demonstrated that in addition to the six key lysine residues in the C-terminal domain of p53, other specific lysine residues, located in the DNA binding domain, are ubiquitinated by HDM2 *in vitro* (Chan *et al.*, 2006).

1.2.4 Regulation of p53 stability

Despite posttranslational modifications for activation of p53 after stress stimuli, the tumour suppressor is an inherently active protein and therefore needs to be tightly regulated. The low basal level of p53 in normal, unstressed cells is maintained by its key regulator HDM2 which represses p53 protein levels through constant ubiquitination and degradation. HDM2, transcriptionally induced by p53 itself (Haupt *et al.*, 1997), continuously shuttles p53 from the nucleus to the cytoplasm and marks it for proteasomal degradation. However, ubiquitination of p53 is reversible and is subjected to further restrictions. The ubiquitin-specific proteases USP7 (also called HAUSP (herpes virus-associated ubiquitin-specific protease)), USP10 and USP42 catalyse deubiquitination of p53. Additionally, USP7 is able to deubiquitinate HDM2, thus inhibiting its function. HDMX, also known as HDM4 and structurally related to HDM2, is equally important for regulating p53 (Marine *et al.*, 2004, Marine *et al.*, 2005). Despite having a RING-domain and an E3 ligase activity like HDM2, HDMX does not ubiquitinate p53 but rather inhibits its transcriptional activity by direct interaction with the transcription factor at the promoter site. Together with HDM2, HDMX forms a complex with p53 and represses gene transcription (Tang *et al.*, 2008). HDM2 and HDMX are subjected to phosphorylations and ubiquitinations by other regulatory proteins, further fine-tuning the regulation process around p53 (Pereg *et al.*, 2005).

Enhanced degradation of p53 is initiated after activation of the PI3K-Akt pathway and phosphorylation of HDM2. Induction of the PI3K pathway can be mediated by growth factors, cytokines or certain oncogenes, which promote inhibition of apoptosis. Activated PI3K catalyses the formation of phosphoinositide-3,4,5-trisphosphate (PIP3), which in turn recruits both the serine/threonine kinase Akt (also known as protein kinase B, PKB) and the 3'-phosphoinositide-dependent kinase PDK1. PDK1 activates Akt by phosphorylation, which then phosphorylates HDM2 at Ser166 and Ser186 (Ogawara *et al.*, 2002). HDM2 binds to p53 in the nucleus, mediates its export to the cytoplasm and catalyses formation of ubiquitin chains on p53 via its E3 ligase activity. Mono-ubiquitination leads to nuclear export and allows p53 to exert its functions in the cytoplasm e.g. induction of apoptosis. Poly-ubiquitination on the other hand marks p53 for proteasomal degradation (Lee *et al.*, 2010).

Other ways of p53 degradation involve the E3 ubiquitin ligases ARF-BP1/Mule (ARF binding protein1/Mcl-1 ubiquitin ligase E3) (Chen *et al.*, 2005), COP1 (constitutively photomorphogenic

1) (Dornan *et al.*, 2004), Pirh2 (p53-induced RING H2) (Leng *et al.*, 2003) and MSL2 (male-specific lethal 2) (Kruse *et al.*, 2009b). Sumoylation and neddylation represent further modifications of p53 and are important for its regulation (Stehmeier *et al.*, 2009, Abida *et al.*, 2007). In addition to the ubiquitin-mediated 26S proteasome-dependent degradation pathways, cytoplasmic calpain-dependent degradation plays a role in regulation of p53 protein amount (Kubbutat *et al.*, 1997), as well as a nucleolar pathway for p53 protein turnover, dependent on Def (digestive organ expansion factor) and the nucleolar cysteine protease Calpain 3 (Tao *et al.*, 2013). In response to stress signals, p53 both induces cleavage of Akt through caspases and activates expression of the tumour suppressor PTEN, which dephosphorylates PI3K. Subsequently, Akt and HDM2 activity is inhibited leading to stabilisation of p53 (Mayo *et al.*, 2002, Gottlieb *et al.*, 2002).

1.2.5 p53 and cancer

During carcinogenesis normal cells are transformed into cancer cells. Several changes at the cellular or genetic level ultimately result in uncontrolled cell division and the growth of a malignant tumour. If the balance between proliferation and cell death is disturbed, the development of benign tumours can occur. In contrast to benign tumours, malignant tumours spread to and invade other organs or tissues (metastasis). For the transformation of a healthy cell to a cancer cell, several mutations in certain genes, e.g. proto-oncogenes or genes involved in cell cycle control or apoptosis are necessary (Fearon *et al.*, 1990). One of these is the tumour suppressor p53, which is mutated in a large percentage of human cancers. Mutations in the *tp53* gene occur through single base substitutions, loss of alleles or inactivation by cellular or viral proteins, and are classified into somatic mutations, germ line mutations and polymorphisms. Mutations of the *tp53* gene rarely result in simple protein loss, they rather lead to disruption of wild-type p53 function and accumulation of high levels in the cell.

Somatic mutations of p53 exist in 30 – 50% of ovarian, oesophageal, colorectal and lung cancers, at lower incidences in primary leukaemia, sarcoma, testicular cancer, malignant melanoma and cervical cancer (Hainaut *et al.*, 1997, Hollstein *et al.*, 1994). Especially the DNA binding domain is affected by mutations (86%), which are mostly missense mutations, resulting in impaired DNA binding affinity or irregular protein folding (Pavletich *et al.*, 1993, Petitjean *et al.*, 2007). Mutations mainly occur at CpG sites: about 4% of cytosines in the human genome are methylated by DNA methyltransferases, a process restricted to CpG dinucleotides. These methylcytosines are prone to transformation into thymines due to spontaneous deamination (Jones *et al.*, 1992, Schmutte *et al.*, 1996). As expected, the three most frequently mutated codons of p53 (amino acid 175, 248 and 273), accounting for 60% of mutations, are all located

at CpG sites (Chen *et al.*, 1993, Olivier *et al.*, 2010). Other regions of p53 mostly contain nonsense or frame shift mutations resulting in inactive proteins.

The Li-Fraumeni syndrome (LFS) is a very rare autosomal dominant disorder, characterized by the inheritance of a *tp53* mutation in the germ line. One allele of the *tp53* gene is either mutated or partly deleted, predisposing the patients for early-onset cancers, like breast cancer and soft tissue and bone sarcoma (Li *et al.*, 1988, Malkin *et al.*, 1990). Moreover, only one additional mutation in the second p53 allele severely impairs prevention of tumour development, leading to a 25-fold increased risk of developing cancer by the age of 50. So far, about 500 families were diagnosed positive for the syndrome (Olivier *et al.*, 2003).

Numerous p53 missense mutations are deleterious for the cell as mutated proteins may exert dominant-negative effects over wild-type p53 or possess gain-of-function activities. Prominent among those are interference with p53-family proteins p63 and p73, the DNA repair pathway or other transcription factors, activation of normally repressed genes or resistance to drugs and chemotherapy (Song *et al.*, 2007, Irwin, 2004, Sigal *et al.*, 2000, Kim *et al.*, 2004).

1.3 Modulation of p53 during bacterial infections

Modulation of p53 during viral infections e.g. degradation mediated by Epstein-Barr virus (EBV) or Human Papillomavirus (HPV) and suppression of transcriptional activity by Simian virus 40 (SV40) was extensively studied (Sato *et al.*, 2009, Dobbelstein *et al.*, 1998, Werness *et al.*, 1990). Inactivation or degradation of p53 by binding of viral proteins are often critical for replication of DNA tumour viruses. Thus, cell cycle transition of quiescent cells into S-phase is achieved, necessary for efficient viral replication. Cell cycle modulation also occurs during bacterial infections and is thought to aid during attachment, survival, growth and dissemination, as well as proliferative-dependent mechanisms of the immune system. Host DNA is thereby often damaged and cellular DDR signalling is inhibited by bacterial strategies leading to severe consequences for the host. In recent years infections are increasingly recognised as major contributors to cancer development (de Martel *et al.*, 2012). Inactivation of the tumour suppressor p53 was also demonstrated during bacterial infections as a means of apoptosis prevention after induction of DNA damage.

The gram-negative, microaerophilic bacterium *Helicobacter pylori* colonises the upper gastrointestinal tract of more than 50% of the human population. Although it is associated with gastric cancer, ulcers and gastric mucosa-associated lymphoid tissue (MALT) lymphoma, around 80% of infected people are asymptomatic. During *H. pylori* infection an increase of p53 levels was observed, followed by rapid degradation of the tumour suppressor (Wei *et al.*, 2010). CagA, an effector protein which is injected into the cell via a type IV secretion system, induced

activation of the PI3K-Akt pathway, leading to HDM2 activation and p53 degradation. Additionally, Buti *et al*. demonstrated that CagA binds to the tumour suppressor apoptosis-stimulating protein of p53-2 (ASPP2), which normally binds to and activates p53 after DNA damage or oncogenic stimulation. However, binding of ASPP2 to p53 now results in its rapid degradation by the proteasome (Buti *et al.*, 2011). During *H. pylori* infection cells are harmed by DNA damage: by inducing degradation of p53 the pathogen is avoiding an apoptotic response.

Neisseria gonorrhoeae infection causes DNA damage as well. Vielfort *et al.* demonstrated the degradation or downregulation of the tumour suppressor p53 during *N. gonorrhoeae* infection by an as yet unknown mechanism (Vielfort *et al.*, 2013). Moreover, in the absence of p53, *N. gonorrhoeae* promotes upregulation of the cdk inhibitors p21 and p27 leading to cell cycle arrest in the G1 phase. As a consequence, infected cells are able to repair single and double strand breaks, are not subjected to apoptosis and subsequently primed for persistent gonococcal infections (Vielfort *et al.*, 2013, Jones *et al.*, 2007).

Another way of evading the consequences of genotoxic stress is pursued by the pathogen *Shigella*. p53 response and cell death are avoided by cleaving p53 molecules through calpain protease activation. *Shigella* achieves activation of the protease by depleting cells of the endogenous calpain inhibitor calpastatin. Responsible is VirA, a type III secreted virulence effector protein (Bergounioux *et al.*, 2012). However, p53 degradation is only delaying cellular death, as activation of calpains ultimately results in necrosis induction. Yet, prolonged survival of the host is apparently enough for the pathogen to successfully multiply.

Opposed to *Helicobacter*, *Neisseria* and *Shigella*, *Salmonella typhimurium* induces activation of p53 pathways for its own benefit. *Salmonella* injects bacterial effectors including AvrA into the cytosol of intestinal epithelial cells in a TTSS dependent way which induce acetylation of p53. Subsequently, p53 target proteins like the cyclin-dependent kinase inhibitor p21 are activated leading to cell cycle arrest, apoptosis inhibition and cellular survival (Wu *et al.*, 2010).

1.4 Aim of this work

Apoptosis modulation is one of the most well studied fields in *Chlamydia* research. Nonetheless, many aspects remain controversial or unknown. Upregulation of the anti-apoptotic proteins Mcl-1 and cIAP-2, as well as downregulation of pro-apoptotic BH3-only proteins were demonstrated to contribute to prevention of host cell death. All these findings however were challenged by others. During the course of this study, Chumduri *et al.* demonstrated ROS production and subsequent damage to host DNA to occur during *Chlamydia* infections. Moreover, interference with recruitment of pATM and 53BP1 of the host DDR to sites of DSBs was reported (Chumduri *et al.*, 2013). Bacterial modulation of host cell signalling to prevent onset of apoptosis due to DNA damage was revealed for pathogens like *Helicobacter*, *Neisseria*, *Shigella* and *Listeria*. Still, little is known about the exact mechanisms, manipulation strategies and how it affects the host during long-term infections, in particular about *Chlamydia* infections.

The present study aimed to elucidate the strategy *Chlamydia* devised to avoid apoptosis induction in response to genotoxic stress. As cellular DDR signalling converges at the central regulator p53, a special focus was set on the tumour suppressor. In addition, crucial steps of cellular signalling mediating host survival were disclosed. The question about species-specific and host cell-specific differences during infection were also addressed. In order to reveal the benefits of p53 modulation for the pathogen, impact of pharmacological stabilisation and activation of the tumour suppressor on chlamydial growth were investigated. As a result, an antibacterial role of p53 was revealed.

As earlier studies exposed dependency of the intracellular *Chlamydia* on host cell energy, amino acids, lipids and nucleotides and as the pathogen was shown to intercept cellular pathways and trafficking to ensure supply of metabolites, influence of activated p53 on host cell metabolism and consequently on *Chlamydia* was evaluated. Recent publications stress on the crucial role of p53 in controlling cellular metabolism and energy production as part of its anti-tumour activity. Concluding, implications of *Chlamydia* infections and modulation of the tumour suppressor on host cell integrity and risk of cancer development were discussed.

2 Material

2.1 Antibodies

2.1.1 Primary antibodies

Table 1: Primary antibodies for immunoblotting (IB) and immunofluorescence (IF)

Antibody	Host	Dilution	Company
anti-Akt	polyclonal rabbit	IB 1:1000	Cell Signaling
anti-pAkt (phospho S473)	polyclonal rabbit	IB 1:1000	Cell Signaling
anti-yH2Ax (ab11174) (phospho S139)	polyclonal rabbit	IB 1:1000	Abcam
anti-G6PD (H-160)	polyclonal rabbit IgG	IB 1:500	Santa Cruz
anti-GAPDH (FL-335)	polyclonal rabbit IgG	IB 1:500	Santa Cruz
anti-Glut1 (5B12.3)	monoclonal mouse IgG1κ	IB 1:1000	Millipore
anti-Glut4 (IF8)	monoclonal mouse IgG_1	IB 1:500	Santa Cruz
anti-Hsp60 (chlamydial, A57-B9)	monoclonal mouse IgG_1	IB/IF 1:500	Santa Cruz
anti-IncA	polyclonal rabbit	IF 1:50	*
anti-MDM2 (ab16895)	monoclonal mouse IgG2a	IB 1:500	Abcam
anti-pMDM2 (3521)	polyclonal rabbit	IB 1:1000	Cell Signaling
anti-OmpA	polyclonal rabbit	IB 1:500	*
anti-p21 (2947)	monoclonal rabbit	IB 1:1000	Cell Signaling
anti-p53 (DO-1)	monoclonal mouse IgG_{2a}	IB 1:500	Santa Cruz
anti-p53 (FL-393)	polyclonal rabbit IgG	IB 1:500	Santa Cruz
anti-Puma (12450)	monoclonal rabbit	IB 1:1000	Cell Signaling

anti-TIGAR	polyclonal rabbit IgG	IB 1:250	Abcam

* Antibody against IncA was designed, purified and tested by Prema Subbarayal (AG Rudel, Microbiology, Würzburg), OmpA by Annette Huber (AG Rudel, Microbiology, Würzburg).

2.1.2 Secondary antibodies

Table 2: Secondary antibodies

Antibody	Source	Dilution	Company
Anti-rabbit IgG Cy2-linked	Goat	IF 1:150	Dianova
Anti-rabbit IgG Cy3-linked	Goat	IF 1:150	Dianova
Anti-mouse IgG Cy2-linked	Goat	IF 1:150	Dianova
Anti-mouse IgG Cy3-linked	Goat	IF 1:150	Dianova
ECL™ anti-mouse IgG HRP-linked	Goat	IB 1:3000	Santa Cruz
ECL™ anti-rabbit IgG HRP-linked	Goat	IB 1:3000	Santa Cruz

2.2 Reaction kits

Table 3: Reaction kits

Reaction kit	Company
GeneJet™ Gel Extraction Kit	Fermentas
PureYield™ Plasmid Midiprep System	Promega
AxyPrep™ Plasmid Miniprep Kit	Axygen
Glucose 6 Phosphate Dehydrogenase Assay Kit (Colorimetric)	Abcam
Illustra Microspin G-25 purification column	GE Healthcare

2.3 Plasmids

Table 4: Plasmids

Plasmid	Comment	Source
pLVTHM	shRNA expression vector; Addgene plasmid 12247	(Wiznerowicz et al., 2003)
psPAX	viral packaging vector; Addgene plasmid 12260	(Wiznerowicz et al., 2003)
pCMV-VSV-G	viral envelope vector; Addgene plasmid 8454	(Stewart et al., 2003)
pcDNA3-HA-p53	a gift from Prof. Dr. Stefan Gaubatz (Physiological Chemistry, Würzburg)	(Marin et al., 2000)
pCMV6-G6PD	Myc-DDK-tagged vector, origene RC201807	Origene

2.4 Protein and DNA ladders

DNA and protein markers used in this work were GeneRulerTM 1kb DNA ladder (Fermentas) and PageRuler™ Prestained Protein Ladder (Fermentas).

2.5 Oligonucleotides

Table 5: shRNA oligonucleotides

oligonucleotide	sequence
HDM2-1	5’-AGACAAAGAAGAGAGTGTGGAATCTAGTT-3’
HDM2-2	5’-AATGCCATTGAACCTTGTGTGATTTGTCA-3’
HDM2-3	5’-TTGTTTGGCGTGCCAAGCTTCTCTGTGAA-3’
HDM2-4	5’-TGAGGAGCAGGCAAATGTGCAATACCAAC-3’
p53-1	5’-AGTAGATTACCACTGGAGTCTT-3’
p53-2	5’-GTGCAGCTGTGGGTTGATT-3’
p53-3	5’-GAAATTTGCGTGTGGAGTA-3’

Table 6: siRNA oligonucleotides

oligonucleotide	sequence
HDM2	ON-TARGETplus SMARTpool siRNA (Dharmacon) J-003279-11 GCCAGUAUAUUAUGACUAA J-003279-12 GAACAAGAGACCCUGGUUA J-003279-13 GAAUUUAGACAACCUGAAA J-003279-14 GAUGAGAAGCAACAACAUA

Table 7: oligonucleotides for EMSA

oligonucleotide	sequence
p53	5'-TACAGAACATGTCTAAGCATGCTGGGGACT-3' 5'-AGTCCCCAGCATGCTTAGACATGTTCTGTA-3'

2.6 Eukaryotic cells

Huvecs — adherent primary Human Umbilical Vein Endothelial Cells (HUVEC), isolated from the vein of the umbilical cord; cultivated in Medium 200 supplemented with LSGS (low serum growth supplement, Gibco BRL) Invitrogen

Hela229 — adherent *Homo sapiens* cervix adenocarcinoma; cultivated in RPMI 1640 (10% FCS)

293T — adherent HEK293T cells, human embryonic kidney cells, expressing large T antigen from SV40 virus; cultivated in DMEM (10% FCS)

H1299 — adherent human non-small cell lung carcinoma cell line derived from the lymph node; homozygous partial deletion of the *tp53* gene; cultivated in RPMI 1640 (10% FCS)

H1299 R175H — adherent human non-small cell lung carcinoma cell line derived from the lymph node; homozygous partial deletion of the *tp53* gene; expressing mutant p53 R175H; cultivated in RPMI 1640 (10% FCS)

H1299 R273H — adherent human non-small cell lung carcinoma cell line derived from the lymph node; homozygous partial deletion of the *tp53* gene; expressing mutant p53 R273H; cultivated in RPMI 1640 (10% FCS)

MEF — adherent mouse embryonic fibroblasts; cultivated in RPMI 1640 (10% FCS)

MEF $p53^{-/-}$ — adherent mouse embryonic fibroblasts; deficient for the *tp53* gene; cultivated in RPMI 1640 (10% FCS)

HCT116 wt — adherent isogenic human colorectal carcinoma cells; cultivated in RPMI 1640 (10% FCS)

HCT116 $p53^{-/-}$ — adherent isogenic human colorectal carcinoma cells deficient for the *tp53* gene; cultivated in RPMI 1640 (10% FCS)

H1299, H1299 R175H, H1299 R273H, MEF, MEF $p53^{-/-}$, HCT116 wt and HCT116 $p53^{-/-}$ cells were a kind gift of Prof. Martin Eilers, Chair of Biochemistry and Molecular Biology, Biocentre, Würzburg.

2.7 Bacterial strains

Table 8: Bacterial strains

Bacterial strain	Source
E. coli DH5α	Hanahan, 1983
C. trachomatis L2	ATCC
C. trachomatis D	ATCC
C. pneumoniae VR1310	a gift of Gunna Christiansen
C. muridarum	a gift of Thomas Miethke

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

2.8 Enzymes

All restriction endonucleases were purchased from Fermentas. T4 DNA ligase (Fermentas) and FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific).

2.9 Chemicals

Acrylamid Rotiphorese Gel 30 (37,5:1)	Roth
Albumin Fraktion V (BSA)	Roth
Agarose	Serva
6-Aminonicotinamide (6-AN)	Sigma
Ammonium persulfate (APS)	Merck
Ampicillin	Sigma
Bromophenol blue	Roth
Coumaric acid	Sigma
2-Deoxy-glucose	Sigma
Dimethyl sulfoxide (DMSO)	Roth
Diphenyleneiodonium (DPI)	Sigma
Disodium hydrogen phosphate (Na_2HPO_4)	Merck
Dithiothreitol (DTT)	Sigma
Doxycycline	Sigma
EDTA	Serva
Ethidium bromide	Roth
Etoposide	Sigma
Glutamic acid	Sigma
Glutaraldehyde	Merck
Glycine	Sigma
HEPES	Serva
Lipofectamine™ 2000	Invitrogen
Luminol	Fluka
LY294002	Cell Signaling
Magnesium chloride ($MgCl_2$)	Merck
2-Mercaptoethanol	Roth
MG-132	Sigma
Mowiol	Roth
N-Acetyl-L-cysteine (NAC)	Sigma
Non-fat dry milk powder, blotting grade	AppliChem

Nutlin-3	Sigma
Opti-MEM®	Gibco
Paraformaldehyde	Roth
Penicillin (5000 U/ml)/Streptomycin (5 mg/ml)	Sigma
Potassium chloride (KCl)	Merck
Potassium dihydrogen phosphate (KH_2PO_4)	Merck
Sodium cacodylate	Sigma
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck
Sodium dodecyl sulfate (SDS)	Roth
Sucrose	Roth
Superoxide dismutase (SOD)	Sigma
Tetramethylethylenediamine (TEMED)	Fluka
Tiron	Sigma
Tris(hydroxymethyl)aminomethane (Tris)	Sigma
Triton X-100	AppliChem
Tryptone	BD
Tween 20	Sigma
Yeast extract	BD
740-YP	Tocris

Chemicals not listed separately were purchased from Sigma, Roth, Serva or Merck.

2.10 Buffers, solutions and culture media

2.10.1 Buffers for SDS-PAGE, Western blot and electrophoresis

12% Separating gel (20 ml)

H_2O	6.6 ml
30% Acrylamide sol.	8.0 ml
1.5 M Tris-HCl (pH 8.8)	5.0 ml
10% SDS	0.2 ml
10% APS	0.2 ml
TEMED	0.008 ml

Stacking gel (4 ml)

H_2O	2.7 ml
30% Acrylamide sol.	0.67 ml

1.0 M Tris-HCl (pH 6.8)	0.5 ml
10% SDS	0.04 ml
10% APS	0.04 ml
TEMED	0.004 ml

SDS-PAGE buffer (10x)

Tris	30.25 g
Glycine	144 g
SDS	10 g
H_2O	ad 1 l

Western Transferpuffer (10x)

Tris	24 g
Glycine	113 g
SDS	2 g
H_2O	ad 1 l

Preparation of 1x Transferbuffer

10x Transferbuffer	100 ml
Methanol	200 ml
H_2O	700 ml

TBS-T (10x)

Tris	48.44 g
NaCl	175.32 g
Tween 20	10 ml
H_2O	ad 2 l
adjust pH to 7.5	

Stripping Buffer

Glycine	7.5 g
SDS	0.5 g
DTT	1.54 g
Tween 20	5 ml
H_2O	ad 500 ml
adjust pH to 2.2	

Laemmli buffer (2x)

Tris/HCl pH 6.8	100 mM
SDS	4%
Glycerol	20%
β-mercaptoethanol	1.5%
bromophenol blue	0.2%

ECL-solutions

ECL solution 1	100 mM	Tris HCl pH 8.5
	2.5 mM	Luminol
	0.4 mM	p-coumaric acid
ECL solution 2	100 mM	Tris HCl pH 8.5
	0.02 %	H_2O_2

50x TAE

Tris	242 g
acetic acid, glacial	57.1 ml
EDTA (0.5 M, pH 8.0)	100 ml
H_2O	ad 1 l
adjust pH to 8.3	

2.10.2 Bacterial culture media

LB-Medium

Tryptone	10 g
Yeast	5 g
NaCl	10 g
(Agar)	15 g
H_2O	ad 1 l

SPG-buffer (sucrose-phosphate-glutamic acid buffer)

Sucrose	75 g
KH_2PO_4	0.52 g
Na_2HPO_4	1.22 g
L-glutamic acid	0.72 g
H_2O	ad 1 l
adjust pH to 7.4	

sterile filter and store at 4°C

2.10.3 Buffers for TEM, immunostaining and SA β-gal staining

Buffers for transmission electron microscopy (TEM):

2.5% glutaraldehyde solution

glutaraldehyde	2.5%
Na cacodylate pH 7.2	50 mM
KCl	50 mM
$MgCl_2$	2.5 mM

Buffers for immunostaining:

Paraformaldehyde (4% solution)

PFA	20 g
PBS	480 ml
heat to 60°C	
adjust pH to 7.2	
PBS	ad 500 ml

Permeabilization buffer

PBS	
Triton X-100	0.2%

Blocking buffer

PBS	
FCS	10%

Antibody dilution buffer

PBS	
FCS	2%

Mowiol 4-88 (mounting medium)

Glycerol	6 g
Mowiol	2.4 g
stir 1 h at RT	

H_2O	6 ml
stir 1 h at RT	
Tris-HCl (0.2 M, pH 8.5)	12 ml
incubate 2 h at 50°C, stir every 20 min	
aliquot and freeze at -20°C	

Buffers for senescence-associated β-galactosidase staining:

Fixation solution

Formaldehyde	2%
Glutaraldehyde	0.2%
in PBS	

Staining solution

citric acid/Na phosphate	40 mM
$K_4[Fe(CN)_6]3H_2O$	5 mM
$K_3[Fe(CN)_6]$	5 mM
NaCl	150 mM
$MgCl_2$	2 mM
X-gal	1 mg/ml
in H_2O dest.	

2.10.4 Buffers for cell culture and flow cytometry

10x PBS

NaCl	1.37 M
KCl	27 mM
Na_2HPO_4	100 mM
KH_2PO_4	18 mM
H_2O	ad 1 l

2x HBS

Hepes pH 7.00	50 mM
NaCl	280 mM
Na_2HPO_4	1.5 mM

Freezing medium

FCS	90%
DMSO	10%

Annexin V binding buffer

HEPES/NaOH (pH 7.4)	10 mM
NaCl	150 mM
KCl	5 mM
$MgCl_2$	5 mM
$CaCl_2$	1.8 mM

Annealing buffer for shRNA oligonucleotides

K-acetate	100 mM
Hepes-KOH (pH 7.4)	30 mM
Mg-acetate	2 mM

2.10.5 Cell culture media and chemicals

Table 9: Cell culture media and supplements

Medium/Chemical	Company
RPMI 1640	GibcoBRL
DMEM	Sigma Aldrich
Opti-MEM®	GibcoBRL
DPBS	GibcoBRL/Sigma Aldrich
TrypLE™ Express	GibcoBRL
Fetal calf serum (FCS)	PAA
Penicillin/Streptomycin	Sigma Aldrich
Pyruvate	GibcoBRL

2.11 Consumables

Consumables like culture plates, disposable pipettes, pipette tips, cryotubes and reaction tubes were purchased from Sarstedt (Nümbrecht). Culture dishes, flasks, 15 ml and 50 ml reaction tubes, well plates, medium bottles and filters for sterile filtration were purchased from Corning Incorporated (Corning, USA). PVDF membrane for immunoblotting was purchased from GE Healthcare (Buckinghamshire, Great Britain), blotting paper from Hartenstein (Würzburg). All other consumables were purchased from Sigma (St. Louis, USA), VWR (Radnor, USA) and Hartenstein (Würzburg).

2.12 Laboratory equipment

1000/500 Constant Voltage Power Supply	Bio-Rad
Accu-jet® pro	Brandt
Avanti™ J-25 I centrifuge	Beckman Coulter
Avanti™ J-25 XP centrifuge	Beckman Coulter
BD FACSAria™ III Cell Sorter	BD Biosciences
Captair™ ductless fume hood	Erlab
Chemiluminescence imager	Intas
Darkhood DH-40/50	biostep GmbH
DM IL LED microscope	Leica
DMIRB Inverted Fluorescence Microscope	Leica
Electronic balance ABS 80-4	Kern
Electronic balance EW 1500-2M	Kern
Heidolph™ Reax Top Vortex Mixer	Thermo Fisher Scientific
Heracell™ 240i incubator	Thermo Fisher Scientific
Heraeus Megafuge 1.0R	Thermo Fisher Scientific
HERAsafe® 2020 safety hood	Thermo Fisher Scientific
HERAsafe® sterile hood	Thermo Fisher Scientific
Infinite® M200 Multimode microplate reader	Tecan
inoLab® pH 720 Benchtop Meter	WTW GmbH
Labinco L-81 Hotplate Stirrer	Wolf Laboratories
MR-1 Mini Rocker Shaker	Molecular Technology GmbH
NanoDrop 1000 spectrophotometer	Peqlab Biotechnology
Optima™ L-80 XP ultracentrifuge	Beckman Coulter
Owl™ Separation HEP-1 Semi Dry Electroblotting System	Thermo Fisher Scientific

PerfectBlue™ Vertical Double Gel Systems	Peqlab Biotechnology
Power Pack P25 T	Biometra
Refrigerated Microcentrifuge 5417R	Eppendorf
Rotanta 460 centrifuge	Hettich Instruments
Scanjet G4010	Hewlett Packard
Spectrophotometer Ultrospec 3100 pro	Amersham Bioscience
Synergy® Ultrapure Water Systems	Merck Millipore
Systec VX-150 autoclave	Systec GmbH
TCS SPE Confocal Microscope	Leica
Thermal cycler GS1	G-Storm
ThermoCell Mixing Block	Bioer Technology
Thermomixer® comfort	Eppendorf
Typhoon 9200 Imager	GE Healthcare
Vacupack Plus F380 70	Krups
Vacusafe comfort	Integra Biosciences
Water bath WNB 7	Memmert

3 Methods

3.1 Cell biological methods

3.1.1 Cultivation of eukaryotic cell lines

All cell lines were cultivated under water-saturated atmosphere at 5% CO_2 and 37°C. Cells infected with *C. trachomatis* were cultivated at 5% CO_2 at 35°C. HeLa229, H1299, H1299 R175H, H1299 R273H, HCT116 wt and HCT116 $p53^{-/-}$, MEF wt and MEF $p53^{-/-}$ cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco BRL). 293T were cultured in DMEM containing 10% FCS. Huvecs were cultured in Medium 200 supplemented with LSGS (low serum growth supplement, Gibco BRL).

With the exception of primary Huvec cells, cell lines were cultivated through continuous passaging. Adherent cells were washed with sterile 1x PBS, followed by incubation with Trypsin/EDTA. Detaching of cells was supported by placing cell culture flasks into the incubator (37°C) for 1-5 min depending on the cell line. Medium containing 10% FCS was added to the cells, efficiently stopping enzyme activity of Trypsin. After pipetting of cell suspension to ensure removal of cell clumps, cells were seeded at appropriate dilution ratios. Huvecs, in general, were freshly thawed and cultivated in a T75 cell culture flask. The next day, cells were detached and spread to three culture flasks, cultivated until cells almost reached confluence and finally seeded in cell culture plates for experiments.

3.1.2 Freezing and thawing of eukaryotic cell lines

For long term storage of cells in a liquid nitrogen tank, cells were detached from the cell culture flasks as described above and centrifuged at 800 g for 5 min at 4°C. Medium or PBS was discarded and cell pellet was resuspended in 1 ml freezing medium (90% FCS, 10% DMSO). Cells were pipetted into cryotubes and placed into a freezing container (Thermo Scientific), ensuring a slow freezing process. The isopropyl alcohol filled device allows a slow and gentle freezing procedure, cooling down cells with -1°C/min. After 24 h, the cryotubes were transferred to the liquid nitrogen tank for long term storage. To recultivate cell lines, frozen cryotubes were thawed at RT, diluted with pre-warmed culture medium containing 10% FCS and centrifuged at 800 g, 5 min, 4°C. Cells were resuspended in culture media and seeded in T75 cell culture flasks.

3.1.3 Inhibitor treatment of eukaryotic cell lines

Analysis of signalling pathways during *Chlamydia* infection was performed using pathway specific inhibitors. PI3K signalling and subsequent Akt phosphorylation was blocked using the inhibitor LY294002, diluted in DMSO and applied at a concentration of 10 µM. The HDM-2 inhibitor nutlin-3, highly specific and commonly used in anti-cancer studies, binds to HDM2 and inhibits its interaction with p53. Nutlin-3 is very effective and stabilises the protein amount of p53 in minutes (van Leeuwen *et al.*, 2011) and was applied at a concentration of 10 µM. The proteasome inhibitor MG-132 was used at 3 µM.

The topoisomerase II inhibitor etoposide forms a ternary complex with DNA and the topoisomerase II enzyme. It prevents re-ligation of the DNA strands and induces DNA double strand breaks. As a consequence, DNA damage response is initiated in the cell, resulting in activation of the tumour suppressor p53. Etoposide was used at a concentration of 50 µM for 6 h, efficiently inducing DNA damage and p53. To induce immediate cell death, etoposide was applied at 500 µM for 2 h. Cells were normally treated with the inhibitors 1 h before infection. Etoposide was added to the cells for 6 h to ensure sufficient p53 stabilisation. Cells were washed with fresh medium and subsequently infected with *C. trachomatis*.

3.1.4 Isolation of human and mouse fimbriae

Isolation of human and mouse fimbriae was performed by the group of Prof. Dr. Jörg Wischhusen (University Clinic Würzburg) and by Dr. Karthika Karunakaran (Microbiology, University Würzburg). Human fimbriae of uterine tubes were collected from patients. The tissues were collected in Hank‘s balanced salt solution. After washing, the tissue was cut into 3 mm size and incubated with 10% liberase at 37°C for 1 h. The cells were separated by pipetting up and down. After centrifuging at 1000 rpm for 5 min, the cell pellet was collected and grown on collagen coated plates with RPMI and 10% FCS. Confluent monolayers were formed after 13-20 days and subcultured one to three times. The monolayers mostly had a cobblestone appearance, fusiform or polygonal cells were also observed. The cells were subjected to sorting with a BD FACS Aria III cell sorter to separate EpCAM positive cells for propagation.

3.2 Molecular biological methods

3.2.1 Gel electrophoresis of nucleic acids

Nucleic acid electrophoresis is an analytical technique used to separate DNA and RNA by size. Voltage application to an agarose gel, loaded with DNA or RNA samples, induces migration of the nucleic acids to the anode, due to the net negative charge of their sugar-phosphate backbone. After electrophoresis, DNA fragments are visualized using an intercalating, fluorescent dye, such as ethidium bromide. Fragment size can be determined comparing bands with DNA markers containing linear DNA fragments of known length. 1% agarose gels were cast after dissolving appropriate amount of agarose in 1x TAE buffer by boiling several times in the microwave. A comb was immediately inserted into the agarose. After polymerization, the gel was placed into an electrophoresis chamber, filled with 1x TAE buffer. DNA samples were supplemented with 6x DNA loading dye and loaded into the wells together with a DNA ladder. Electrophoresis was performed at 100 – 120 V. Subsequently, the gel was placed into an ethidium bromide bath for 15 min. Ethidium bromide intercalates in between the bases of DNA, visualizing it under UV light (λ = 312 nm).

3.2.2 Digestion and ligation of DNA

Restriction enzymes as well as T4 DNA ligase were obtained from Fermentas and reactions were performed according to manufacturer's protocol. pLVTHM vector was digested using restriction endonucleases ClaI and MluI. Subsequently, the digested vector was loaded onto an agarose gel and isolated using a purification kit. The digested vector and the annealed shRNA oligonucleotides were ligated using the T4 DNA ligase.

3.2.3 Transformation of ligated vectors into DH5α

Before transformation, aliquots of DH5α cells were thawed on ice. *E. coli* were directly frozen in 100 µl aliquots in liquid nitrogen after generation of competent bacteria. 5 µl of the respective ligation products were added to the competent bacteria and gently mixed. After incubation on ice for 30 min, the bacteria were subjected to heat-shock transformation in a water bath set to 42°C for 60 s. The increase in temperature creates pores in the plasma membrane of the bacteria and allows uptake of the vectors into the bacterial cell. After the heat-shock, DH5α were rapidly transferred to ice and incubated for 2 min. Subsequently 350 µl of LB media was added and bacteria were incubated on a shaker for 1 h at 37°C and 900 rpm. Cultures were plated on LB agar plates containing the appropriate antibiotic for selecting successfully

transformed bacteria. The bacteria were incubated over night at 37°C. The next day, colonies were counted and used for plasmid purification and determination of correct insertion.

3.2.4 Plasmid purification using a Plasmid Mini Kit

Small scale purification of plasmids was conducted using a Plasmid Mini Kit. Bacterial colonies grown on LB agar plates were picked and transferred to culture tubes containing 5 ml of LB medium supplemented with ampicillin (100 µg/ml) or kanamycin (50 µg/ml). Bacteria were incubated on a shaker overnight at 37°C and 100 rpm. 2 ml of these cultures were utilised for small scale purification of the plasmid DNA. Cells were pelleted through centrifugation at 11000 g for 1 min. Lysis of the bacteria and plasmid isolation was achieved using the AxyPrepTM Plasmid Miniprep Kit (Axygen) according to the manufacturer´s instructions. The DNA was eluted in 50 µl EB buffer (provided with the kit). The correct insertion of the inserts was analysed using appropriate restriction enzymes and gel electrophoresis.

3.2.5 Plasmid purification using a Plasmid Midi Kit

For large scale purification of plasmids, 1 ml of an overnight culture used for plasmid purification using a Mini Kit was added to 50 ml LB-medium (containing 100 µg/ml ampicillin or 50 µg/ml kanamycin). Cells were incubated in the incubator shaker over night at 37°C and 100 rpm. The next day, bacteria were transferred to a 50 ml centrifuge tube and pelleted through centrifugation at 5100 rpm for 12 min. Lysis of the cells and plasmid purification was performed using a PureYield^{TM} Plasmid Midiprep System (Promega) according to the instructions of the manufacturer. The purified DNA was eluted in 600 µl H_2O provided with the kit.

3.2.6 Annealing of shRNA oligonucleotides

Lyophilized shRNA oligonucleotides were resuspended with H_2O to a concentration of 100 µM. 1 µl of the forward and the reverse oligonucleotide, 1 µl of 10x annealing buffer and 7 µl H_2O were mixed. The sample was first incubated at 96°C for 5 min and then left at RT to facilitate annealing of the oligonucleotides. The fragment which contained restriction sites for the enzymes ClaI and MluI was ligated into the pLVTHM vector. Chemo-competent *E. coli* DH5 were transformed with the ligated construct and positive clones were isolated by the PureYield™ Plasmid Midiprep System (Promega).

3.2.7 Lentiviral shRNA-mediated gene silencing

Huvecs with short hairpin RNA (shRNA)-mediated knockdown for HDM2 and HeLa229 with knockdown for p53 were generated by lentivirus-mediated transduction of expression constructs containing shRNA oligonucleotides. shRNA constructs with restriction endonuclease cutting sites for MluI and ClaI were ligated into the lentiviral vector pLVTHM. The marker gene GFP, encoded by the vector, indicates expression efficiency. For generation of lentiviral particles, two 15 cm^2 cell culture plates of 293T cells, seeded the previous day, were transfected using the calcium phosphate method. For this, 20 µg of the pLVTHM-shp53 vector, 10 µg of VSVG (envelope plasmid) and 15 µg of psPAX2 (packaging plasmid) were mixed in a 5 ml polypropylene microfuge tube. Subsequently, 400 µl of 2.5 M $CaCl_2$ and 1.6 ml H_2O were added and mixed by tapping gently. In the last step, 2 ml of 2x HBS were added drop wise to the mixture while bubbling with a pipette. The transfection mixture was added drop wise to the cells and spread evenly by gently swirling the plate. After 5-6 h incubation, media was removed, cells were washed twice with 1x PBS and fresh media was added to the plates. 36-48 h after transfection, supernatant containing lentiviral particles was removed, collected in a 50 ml centrifuge tube and centrifuged at 2000 rpm for 7 min at 4°C. Subsequently, the supernatant was passed through a 0.45 µm filter. Target cells were seeded in 6-well cell culture plates, supernatant containing lentiviral particles was added in different concentrations to the cells. To increase transduction efficiency, the cationic polymere polybrene (hexadimethrine bromide) was added to the mixture. 2-3 days after transduction, cells were inspected for GFP expression and subjected to single cell sorting using a BD FACSAria III cell sorter. Cells were cultivated in RPMI-1640 (Gibco-BRL) supplemented with 10% fetal calf serum (FCS) and checked for gene silencing by Western blot analysis.

3.2.8 Transfections

Transfection of plasmid DNA was performed using Lipofectamine® 2000 transfection reagent. HeLa229 or H1299 cells were seeded to reach 70-90% confluence at the day of transfection. Lipofectamine® 2000 reagent (3 µl/µg DNA), as well as DNA was separately prediluted in Eppendorf reaction tubes using Opti-MEM® medium. Diluted DNA was subsequently added to diluted Lipofectamin® 2000 reagent at a 1:1 ratio, followed by incubation for 10 min at RT. After this, the DNA-lipid complex was added drop wise to the cells; plates were gently swirled to ensure even distribution. 6 h after transfection, medium was removed, cells were washed using 1x PBS and fresh medium was added to the cells. 24 h after transfection, cells were infected with *Chlamydia* or treated with inhibitors. The transfection efficiencies were analysed by western blotting. For overexpression of G6PD, the construct pCMV6-Myc-DDK-G6PD

(OriGene) was transfected, overexpression of p53 was achieved by transfecting pCDNA3-HA-p53 (a kind gift from Stefan Gaubatz).

3.2.9 Small interfering RNA transfection

For siRNA transfections, HeLa229 cells were seeded in 12-well cell culture plates. After 24 h incubation, growth medium was replaced. HiPerFect® transfection reagent (Qiagen) was chosen for transient siRNA transfections according to the manufacturer's instructions. siRNA against HDM2 (ON-TARGETplus smartpool, Dharmacon, Thermo Fisher Scientific), as well as control siRNA were prediluted in Opti-MEM®. HiPerFect® transfection reagent was added, mixed by vortexing and incubated for 10 min at RT to allow formation of transfection complexes. HeLa229 cells were transfected with a final concentration of 160 nM siRNA. After the incubation period, the mixture was added drop wise to the plate and cells were incubated for 48 h at 37°C and 5% CO2. Subsequently, cells were infected or treated with inhibitors as mentioned in the figure legends. The transfection efficiencies were analysed by western blotting.

3.2.10 SDS-PAGE

The separation of proteins according to their mass was performed using the denaturing SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). Because of the anionic part of the detergent SDS, which binds to side chains of proteins, electrical charges of the proteins are masked and proteins gain a net negative charge. Proteins can now be separated according to their size irrespective of their charge. Moreover, SDS interrupts hydrogen bonds and in combination with β-mercaptoethanol denatures polypeptides. SDS gels were cast according to the above mentioned gel formula. The separating gel was cast and overlaid with 2-propanol. After polymerisation of the gel, the isopropyl alcohol was discarded and the stacking gel was cast. An appropriate comb was immediately inserted. After polymerisation of the stacking gel, the comb was carefully removed, the pockets were washed and the gel chamber filled with 1x SDS PAGE buffer. The experiments were all performed using 12% separating gels. After loading the samples, electrophoresis was conducted at 40-50 mA for 2-3 h. To facilitate detection of the size of proteins, the PageRuler™ Prestained Protein Ladder was loaded to the gel.

3.2.11 Semi-dry western blotting

After separation of proteins according to their size via SDS-polyacrylamide gel electrophoresis, they were transferred to a PVDF (polyvinylidene fluoride) membrane via semi-dry western blotting, to make the proteins accessible to antibody detection. The membrane and 4 layers of whatman paper were cut to the required gel size. The PVDF-membrane was activated through immersion in 100% methanol for 1 min. Afterwards the membrane was washed in 1x transfer buffer. Meanwhile, the gel was extracted from the glass plates and washed in H_2O. The whatman papers were equilibrated in 1x transfer buffer and the assembly of the Western blot was performed as follows (from the bottom (anode) to the top (cathode)): 2 whatman paper soaked in transfer buffer, the PVDF-membrane, the gel and 2 whatman paper. Avoiding bubble formation and removal of excessive buffer is essential for successful protein transfer. The blotting chamber was gently closed and the transfer was performed at 150 mA for 120 min. After protein transfer, membranes were blocked using 5% milk/TBS-T for 1 h. Membranes were briefly rinsed in 1x TBS-T and subsequently incubated over night with indicated primary antibodies at 4°C on a shaker. The next day, membranes were washed thrice for 10 minutes each in 1x TBS-T, incubated with the respective horseradish peroxidase (HRP) conjugated secondary antibody for 1-2 h at room temperature, followed again by three washing steps. Detection of the protein of interest was finalised by using an enhanced chemiluminescence kit (ECL, Amersham) according to the manufacturer's instructions. For detecting the chemiluminescence signal a quantitative western blot imager from Intas Science Imaging was used. Signal was detected in the linear response range. To verify equal loading an appropriate loading control was analysed. Before washing, blocking and reprobing of the blots to detect the unphosphorylated protein loading controls, the membranes were incubated in stripping buffer (at 70°C for 20 min) to remove primary and secondary antibodies. Analysis of western blots was performed using ImageJ software.

3.2.12 Immunofluorescence

Immunofluorescence was performed with Hela229 and Huvecs cells during infectivity assays and determination of chlamydial localization in the cytoplasm. HeLa229 or Huvecs were seeded in 24-well plates on glass coverslips. The next day, cells were infected with *C. trachomatis* and/or treated with etoposide (50 µM) for 6 h or nutlin-3 (10 µM) for 1 h. Cells were washed twice with 1x PBS and fixed with 4% paraformaldehyde for 15 min at RT. After three washing steps with 1x PBS, cells were permeabilised using 0.2% Triton-X100/PBS for 15 min followed again by three washing steps. Blocking of unspecific binding sites was achieved by incubating cells in blocking buffer (10% FCS/PBS) for 45 min at RT. Subsequently, cover slips

were incubated with primary antibodies, diluted in 2% FCS/PBS. After three washing steps, cells were incubated with secondary antibodies, again diluted in 2% FCS/PBS. Cell nuclei were either stained with the intercalating agents DAPI or Draq5, depending on the microscope intended for analysis. Incubation with DAPI or Draq5 was carried out together with secondary antibody staining. Finally, cells were again washed in 1x PBS, rinsed once with dH_2O and carefully mounted on glass slides using Mowiol (Carl Roth, Germany). Stained samples were analysed on either a Leica DMR epifluorescence or Leica SPE confocal microscope.

3.2.13 Electrophoretic mobility shift assay (EMSA)

Transcriptional activity of p53 during a *Chlamydia* infection was analysed by electrophoretic mobility shift assay (EMSA). This technique is one of the most sensitive methods for studying the DNA-binding properties of a protein and also useful for studying higher order complexes with several proteins, called supershift assay. In an EMSA a 32P-labeled specific DNA fragment is incubated with a DNA-binding protein. By electrophoresis through a non-denaturing polyacrylamide gel the DNA-protein complexes are separated from unbound DNA, as the protein slows down mobility of the bound DNA fragments. Thus, free DNA migrates faster through the gel than DNA-protein complexes.

Chlamydia infected HeLa protein extracts for EMSA were prepared as described in Prusty *et al*. (Prusty *et al.*, 2005). DNA oligos (5'-TACAGAACATGTCTAA GCATGCTGGGGACT-3' and 5'-AGTCCCCAGCATGCTTAGACATGTTCTGTA-3') having p53 binding sites were annealed and were end-labelled using T4 polynucleotide kinase. Unincorporated nucleotide was removed using an Illustra Microspin G-25 purification column (GE Healthcare Life Sciences). The binding reaction was performed in a 25 µl reaction volume containing 50% glycerol, 60 mM HEPES (pH 7.9), 20 mM Tris- HCl (pH7.9), 300 mM KCl, 5 mM EDTA, 5 mM DTT, 100 µg of BSA/ml, 2.5 µg of poly (dI-dC) and 10 µg of protein extract. After 5 min, 10.000 cpm of the end labelled double-stranded oligonucleotide probe was added and the incubation was continued for additional 25 min at RT. The DNA-protein complexes were resolved on 4.5% non-denaturing polyacrylamide gel (cross-linking ratio, 29:1), fixed, dried, exposed to a phosphor screen overnight and analysed by phosphor autoradiography.

3.2.14 Transmission electron microscopy

Analysis of impaired chlamydial inclusion formation was analysed by transmission electron microscopy. For this, cells were grown on glass coverslips, treated with etoposide (50 µM) for 6 h and infected with *C. trachomatis* for 24 h. Subsequently, cells were washed twice with 1x PBS, fixed for 45 min with 2.5% glutaraldehyde solution at 4°C and washed twice with 50 mM

cacodylate buffer. All further steps were carried out by the working group of Prof. Georg Krohne. Coverslips were incubated for 2 h at 4°C with 2% OsO4 buffered with 50 mM sodium cacodylate (pH 7.2), washed with dH_2O and incubated overnight at 4°C with 0.5% uranyl acetate (in dH_2O). Cells were dehydrated, embedded in the epoxy embedding medium Epon^{TM}812 and ultrathin sectioned at 50 nm. Sections were stained with 2% uranyl acetate in ethanol, followed by staining with lead citrate. Analysis of sections was carried out on a Zeiss EM10 transmission electron microscope (Zeiss, Oberkochen, Germany).

3.2.15 Glucose-6-Phosphate-Dehydrogenase activity measurement

G6PD activity during a *Chlamydia* infection and in the presence of the DNA damaging agent etoposide was measured using a Glucose-6-Phosphate Dehydrogenase Activity Colorimetric Assay Kit (Abcam) according to the manufacturer's instructions. In this assay, glucose-6-phosphate is oxidized to 6-phosphoglucono-δ-lactone with the generation of NADH. The amount of NADH is detected colorimetrically with a Tecan Microplate Reader at an absorbance of 450 nm. Detection limit is 0.04 mU G6PD per well. G6PD activity was measured in H1299, H1299 transfected with HA-tagged p53, H1299 R175H and R273H cells. Cells were either left uninfected as a control or infected with *C. trachomatis* for 24 h. To measure negative influence of p53 on G6PD, cells were treated with etoposide (50 µM) for 6 h. Cells were lysed and directly transferred to a 96-well plate. A Reaction Mix containing G6PD Assay Buffer, G6PD Substrate and G6PD Developer was added to the wells and directly subjected to colorimetric analysis (T1). OD was taken at 450 nm. Subsequently, the plate was incubated at 37°C for 30 min followed by a second measurement (T2). B is the NADH amount that was generated between T1 and T2 (in nmol).

G6PD activity = B/(T1 - T2) * V * sample dilution
= nmol/min/ml
= mU/ml

T1: time of first reading (in min)
T2: time of second reading (in min)
V: pre-treated sample volume added into the reaction well (in ml)

One unit defines as the amount of enzyme that catalyses the conversion of 1 µmol of glucose 6 phosphate into 6-phosphoglucono-δ-lactone and generates 1 µmol of NAD^+ to NADH per minute at 37°C.

3.2.16 Flow cytometry

Cell death during *C. trachomatis* infection was analysed using Annexin-V-FLUOS/Propidium Iodide staining, followed by flow cytometry. Annexin-V-FLUOS, a cellular protein of the annexin family conjugated to a fluorescent marker, binds with high affinity to phosphatidylserine (PS) in a Ca^{2+}-dependent process. In early apoptotic cells, PS is expressed on the cell surface. To distinguish between early apoptotic and late apoptotic/necrotic cells, which also stain positive for Annexin-V-FLUOS due to a damaged cellular membrane, the fluorescent dye propidium iodide (PI) is utilized. PI is excluded from viable cells and only able to enter cells and intercalate with nucleic acids when cell membranes are damaged.

To analyse cytotoxicity of *Chlamydia* infections, Huvecs, mouse fimb cells, MEF wt and MEF $p53^{-/-}$ cells were infected for 24 or 48 h with *C. trachomatis*, detached using 5% EDTA/PBS and stained with Annexin-V-FLUOS and propidium iodide according to the manufacturer's instructions. Cells were incubated with the fluorescent dyes, diluted in 1x Annexin V binding buffer, for 10 min on ice. Prior to flow cytometric analysis, cells in staining solution were diluted 1:5 with 1x binding buffer. Analysis of cell death was carried out using a BD FACSAria III Cell Sorter and FACSDiva Software. Based on forward and side scatter characteristics (FSC/SSC), cell debris was excluded, however intact and dying cells were gated for further analysis. Fluorescence signals were detected in FITC and PE channel and depicted in dot plots with quadrant gates. Healthy cells are shown in quadrant Q3, early apoptotic cells in Q4 and late apoptotic/necrotic cells in Q2.

3.2.17 Senescence-associated β-galactosidase staining

Senescence-associated β-galactosidase activity (SA-β gal) is used as a marker for detection of senescent cells. An increase in the abundance of the lysosomal enzyme β-galactosidase can be observed in these cells. Cytochemical or histochemical detection of SA-β gal activity requires incubation of samples with the chromogenic β gal substrate X-gal at pH 6.0. Subsequently, a blue colour develops in some cells within 2 h, but staining is maximal after 12–16 h. In the experiments, SA-β gal activity was measured using a chromogenic assay described by Debacq-Chainiaux *et al*. (Debacq-Chainiaux *et al.*, 2009). Huvecs were transfected with pCMV6-Myc-DDK-G6PD or empty vector control, media was changed after 6 h. After 24 h cells were treated with etoposide (50 µM) or nutlin-3 (10 µM) and infected with *C. trachomatis* for 24 h. Cells were washed twice with PBS, fixed for 8 min, washed again twice with PBS and stained (for buffers see Material section 2.9.3). Incubation with staining solution was carried out for 10 – 12 h at 37°C. Samples were analysed by bright-field microscopy.

3.2.18 Statistical analysis

For statistical analysis Microsoft Office Excel was used. Statistical significance was calculated using the Students t-test, p-value < 0.05 (*), p-value < 0.01 (**).

3.3 *Chlamydia*

3.3.1 Propagation of *Chlamydia*

Infections were performed using the following *Chlamydia* strains and serovars: *C. trachomatis* LGV serovar L2, *C. trachomatis* serovar D (ATCC), *C. pneumoniae* strain VR1310 (a gift of Gunna Christiansen) and *C. muridarum* (a gift of Thomas Miethke).

For propagation of *Chlamydia*, a T75 cell culture flask with HeLa229 was infected with *C. trachomatis* at MOI 1. In parallel, 10 T150 cell culture flasks were seeded with HeLa229 in such a way that cells reach 80% confluence two days after plating. After 48 h of *Chlamydia* infection, cells were scraped off with a rubber policeman and collected in a 50 ml centrifuge tube containing around 7 ml of autoclaved glass beads. Cells were lysed by vortexing the tubes for 3 min. After changing media of the 10 T150 cell culture flasks, each flask was inoculated with 150 µl of the EB containing cell lysate and incubated for another 48 h. Cells were checked for successful growth of *Chlamydia* and again lysed by scraping off cells and vigorous vortexing together with glass beads. To remove cell debris, the supernatant was carefully transferred to a new 50 ml centrifuge tube and spun down at 2000 rpm for 10 min at 4°C in a Hermle centrifuge. The supernatant containing chlamydial EBs was subsequently transferred to an autoclaved SS34-centrifuge tube and pelleted for 30 min at 25000 g and 4°C in a Sorvall centrifuge. After completely removing the supernatant, *Chlamydia* were washed with 10 ml of SPG-buffer, resuspending the pellet in the process. The tubes were again centrifuged for 30 min at 25000 g and 4°C. The supernatant was again carefully removed and the pellet according to its size resuspended in about 5 ml of SPG buffer and transferred to a 50 ml centrifuge tube. To remove residual clumps, the solution was passed three times through a G20 Sterican needle and once through a G18 needle. In the last step, *Chlamydia* were pipetted into Eppendorf reaction tubes in 25 µl aliquots and immediately transferred to a -80°C freezer for long term storage. All experiments were conducted with fresh *Chlamydia* aliquots to ensure comparable infection rates. Propagation of *C. muridarum* was performed in MEF cells, infection was conducted for 72 h, all further steps of the protocol remained the same.

Calculation of infection units (IFU)/ml was performed as follows: HeLa229 cells were seeded in a 12-well plate and infected with different dilutions of *Chlamydia* (1:1000, 1:5000, 1:10000,

1:20000) for 24 h. Subsequently, inclusions of 10 visible fields at 40x magnification were counted. IFU/ml were calculated with the following formula:
IFU/ml = Ø of counted inclusions per visible field x 2975.2 x dilution factor x 4
2975.2: number of visible fields per 40x objective

3.3.2 Infection of eukaryotic cells with *Chlamydia*

Cells were seeded in 12-well or 24-well cell culture plates and incubated at 37°C and 5% CO_2. The following day, media was aspirated and replaced by fresh media containing 5% FCS. Infection with *C. trachomatis* in HeLa229 cells, MEFs and H1299 cells was performed at MOI 1, Huvecs were infected at MOI 0.5. After incubation at 35°C for 2.5 h, medium was replaced by the respective medium containing 10% FCS and infection was continued at 35°C for the time points mentioned in the figure legends. Infection with *C. pneumoniae* was conducted at MOI 5 and improved by centrifugation of the cell culture plates directly after infection for 1 h at 800 g and 35°C with appropriate safety measurements. Infection of MEFs and HeLa229 with *C. muridarum* was performed at MOI 5.

3.3.3 Chlamydial Infectivity Assay

Quantification of chlamydial progeny formation and infectivity was monitored using infectivity assays. Primary infection of cells with *C. trachomatis* for 48 h is followed by a secondary infection of new cells after lysing cells with glass beads and releasing infectious EBs. Incubation with inhibitors or usage of specific knock-down or knock-out cell lines provide information about signalling pathways activated during *Chlamydia* infection or proteins necessary for chlamydial development. Only *Chlamydia* which successfully finished their life cycle in the primary infection are capable of forming new inclusions in the secondary infection. Quantification of chlamydial growth was monitored by western blotting for both primary and secondary infection. Additionally, size and number of inclusions in the secondary infection can be assessed by staining chlamydial inclusions with antibodies against chlamydial Hsp60, followed by evaluation with fluorescence microscopy.

A first set of cells, Huvecs, HeLa229, MEFs or H1299 p53 mutant cells, was seeded in 12-well cell culture plates and infected with *C. trachomatis* at MOI 1 the next day, left uninfected as control or treated with the respective inhibitors followed by *Chlamydia* infection. A second set of cells (HeLa229), plated in 12-well culture plates for western blot analysis and 24-well culture plates containing coverslips for immunostaining, was prepared. After 48 h, cells were washed with preheated 1x PBS. Subsequently, 100 µl of 1x PBS and glass beads were added to the wells. Lysis of cells and release of EBs was achieved by vigorous shaking of the culture plates.

For the secondary infection, one hundredth of the *Chlamydia* containing cell lysate was transferred to the prepared plates and incubated at 35°C for 24 h. 2x Laemmli buffer was added to the cells of the primary infection and samples were boiled at 96°C for 6 min. Cells of the secondary infection were also lysed with 2x Laemmli buffer for western blot analysis, and fixed with 4% PFA for immunostaining. After fixation, cells were permeabilised with PBS/0.2% Triton X-100 for 15 min at RT, followed by three washing steps with 1x PBS. Blocking of unspecific binding sites was achieved by incubating coverslips with 10% FCS/PBS for 45 min at RT. The primary antibody against chlamydial Hsp60 was prediluted 1:500 in 2% FCS/PBS and placed drop wise on parafilm. Coverslips were carefully taken out of the wells with forceps and placed upside down on the antibody drops. After incubation at RT for 1 h, coverslips were placed back into the wells and washed three times with 1x PBS. Samples were subsequently incubated with the respective Cy2- or Cy3-labelled secondary antibodies, again prediluted 1:100 in 2% FCS/PBS for 1 h in the dark. Cell nuclei were stained with DAPI (1:500) together with the secondary antibody. Cover slips were washed twice in PBS, once in distilled water to remove PBS and were mounted with Mowiol (Carl Roth) on glass slides. The number of inclusions was determined by counting ten random fields using an epifluorescence microscope (Leica) at 40x magnification. For confocal laser scanning microscopy, nuclei were stained with Draq5 and analysed using a Leica TCS SPE. Overlay images of the single channels were obtained using ImageJ. Infectivity was also determined by quantifying chlamydial Hsp60 (cHsp60) by immunoblotting using antibodies against *Chlamydia*-specific Hsp60. The values were normalised to GAPDH after signal quantification using densitometric analysis and ImageJ software.

4 Results

4.1 *C. trachomatis* infection induces degradation of tumour suppressor p53

4.1.1 *Chlamydia* and DNA damage induced cell death

The intracellular pathogen *Chlamydia* is increasingly successful in its dissemination, leading to millions of new infections per year. Often undetected for months due to relatively symptom free infections the pathogen survives and multiplies inside its host. During their developmental cycle *Chlamydia* are strongly dependent on the provision of energy and metabolites by the host. To avoid recognition by the immune system or induction of apoptosis by the host cell, *Chlamydia* invented a series of manipulative strategies to ensure survival of the cell. As previously mentioned *Chlamydia* infected cells are resistant to a series of apoptotic stimuli like granzyme B/perforin, TNFα, staurosporine, etoposide or UV-light (Fan *et al.*, 1998, Rajalingam *et al.*, 2008, Fischer *et al.*, 2004).

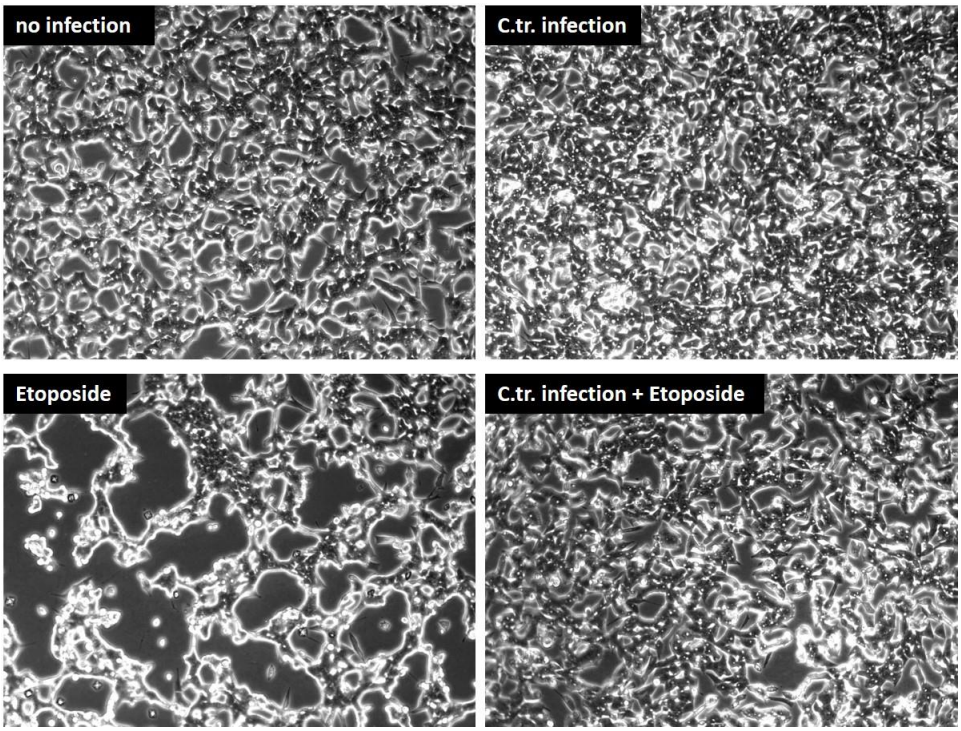
Chlamydia infected cells are resistant to etoposide induced cell death

Similar to other pathogens like *Neisseria* and *Shigella*, *Chlamydia* was recently shown to induce DNA damage during its course of infection. Chumduri *et al.* demonstrated that markers for DNA double strand breaks (DSBs) and senescence-associated heterochromatin foci (SAHF) were upregulated in infected cells (Chumduri *et al.*, 2013). However, *Chlamydia* apparently interferes with the cellular DNA repair machinery in order to avoid host cell death. Recruitment of the DDR proteins pATM and 53BP1 to the sites of DSBs was prevented in an as yet unknown way (Chumduri *et al.*, 2013). Furthermore, *Chlamydia* was shown to alter pathways involving cell aging and survival (Padberg *et al.*, 2013).

The topoisomerase II inhibitor etoposide is often used to study DNA damage induced apoptosis (Mizumoto *et al.*, 1994). Topoisomerase II induces the controlled induction of DNA DSBs to allow the passage of another DNA double strand through the break, thus promoting chromosome disentanglement. After this process the DSB gets re-ligated. Activity of the enzyme is particularly important during DNA replication and transcription. Etoposide stabilises the complexes formed by topoisomerase II and the cleaved DNA strands, thus preventing the re-ligation of the DSBs (Burden *et al.*, 1998). As a consequence, the cellular DNA damage response gets activated. DNA-PK recognizes the DSBs and induces phosphorylation of the tumour suppressor p53. Activation of p53 results in upregulation of the pro-apoptotic protein

Bax which translocates to the mitochondria and induces the release of cytochrome c. Induction of the mitochondrial permeability transition (MPT) ultimately leads to etoposide-induced apoptosis (Karpinich *et al.*, 2002).

A



B

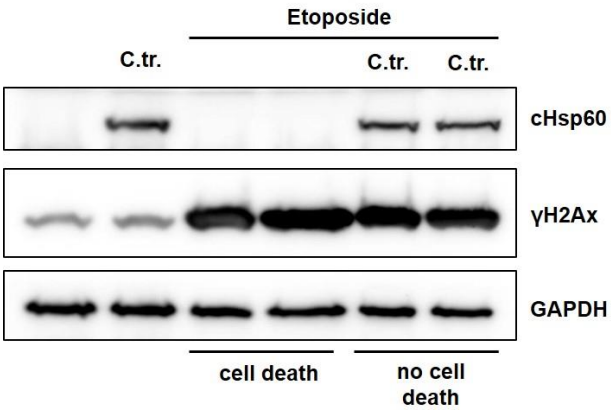


Figure 3 (A) *C. trachomatis* infection prevents DSB-induced cell death. Hela229 were infected with *C. trachomatis* (MOI 1, 24 h). Control cells were left uninfected. Subsequently, cells were treated with etoposide (200 µM, 2 h). Samples were analysed by light microscopy. (B) Phosphorylation of DSB-marker H2Ax. HeLa229 were infected with *C. trachomatis* (MOI 1, 24 h) and treated with etoposide (200 µM, 2 h). Samples were analysed by SDS-PAGE and western blotting. Chlamydial growth was detected using an antibody against chlamydial Hsp60 (cHsp60). (B) Phosphorylation status of H2Ax was assessed using an antibody against γH2Ax. GAPDH was used as loading control. C.tr.: *C. trachomatis*. Parts of this figure were first published in Siegl *et al.*, 2014.

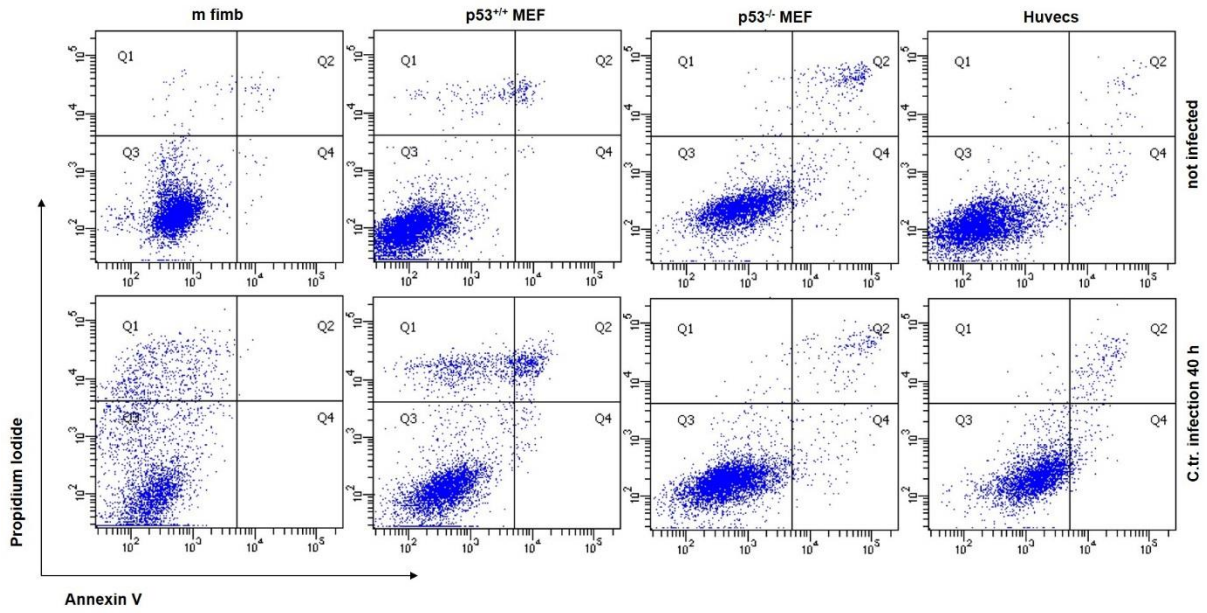
To investigate the anti-apoptotic effect of *Chlamydia* after induction of DNA damage, HeLa229 cells were infected with *C. trachomatis* for 24 h. To strongly induce apoptosis, infected and uninfected control cells were incubated with high concentration of etoposide (200 µM) for 2 h. Apoptosis induction was monitored by light microscopy. After 2 h of incubation, strong cell death and detachment of cells in the non-infected control group treated with etoposide could be observed (Figure 3A). HeLa229 infected with *C. trachomatis* prior to etoposide treatment did not display cell death. Cellular morphology was comparable to infected cells not stimulated with etoposide (Figure 3A).

A consequence of etoposide treatment is the formation of DNA DSBs and the activation of the cellular DDR. A marker for DSBs is the histone H2Ax, a member of the H2A histone family. H2Ax becomes phosphorylated on serine 139 (γH2Ax) by the kinases of the PI3-family (ATM, ATR, DNA-PK) in response to damaged DNA. To investigate the phosphorylation status of this histone HeLa229 cells were again infected with *C. trachomatis* for 24 h and treated with 200 µM etoposide to induce apoptosis. As described in the methods section protein lysates of the cells were analysed by SDS-PAGE and western blotting. H2Ax was strongly phosphorylated in etoposide treated samples (Figure 3B). *C. trachomatis* infected cells showed baseline phosphorylation status of H2Ax comparable to uninfected cells, clearly demonstrating that *Chlamydia* do not induce DSBs after 24 h of infection. Like uninfected cells, *C. trachomatis* infected cells treated with etoposide showed strong H2Ax activation. Taken together, inhibition of apoptosis induction by *Chlamydia* infection is mediated downstream of histone H2Ax activation.

Chlamydia induces strong cytotoxicity in mouse but not in human cells

In order to study species- and cell type specific cell death induction after *Chlamydia* infection, viability of Huvecs, mouse fimb (mfimb) and mouse embryonic fibroblasts (MEFs) was investigated. As Chumduri *et al*. reported formation of DSBs during chlamydial infections and as p53 is a central regulator of apoptosis induction after DNA damage, cytotoxicity in p53 deficient MEFs ($p53^{-/-}$ MEF) was analysed as well (Chumduri *et al.*, 2013). Cells were infected with *C. trachomatis* for 40 h and subsequently subjected to Annexin-V-FLUOS/PI staining to quantify cytotoxicity using flow cytometric analysis as described in the methods section. Healthy cells are displayed in quadrant Q3, early apoptotic cells in Q4 and late apoptotic/necrotic cells in Q2. *Chlamydia* infection of human and mouse cells resulted in differing amounts of early and late apoptotic, as well as necrotic cells.

A



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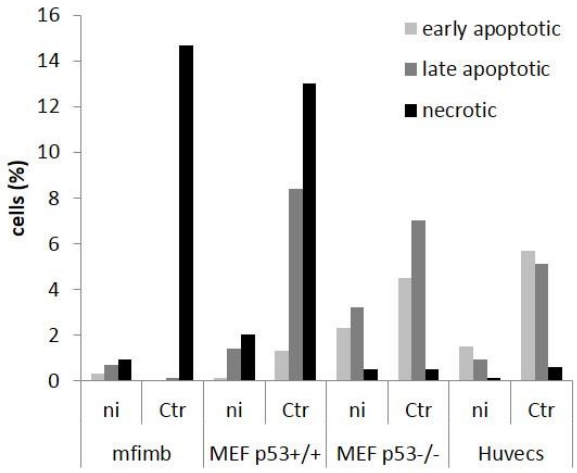


Figure 4 (A) Infection of mouse fimb cells and wild type MEFs with *C. trachomatis* induces cytotoxicity. Mouse fimb cells, $p53^{+/+}$ and $p53^{-/-}$ MEFs and Huvecs were infected with *C. trachomatis* for 40 h, followed by Annexin-V-FLUOS/Propidium Iodide staining. Samples were analysed by flow cytometry. Q1: necrotic cells; Q2: late apoptotic cells; Q3: healthy cells; Q4: early apoptotic cells. (B) Quantification of flow cytometry analysis of cytotoxicity during *C. trachomatis* infection. Mouse fimb cells, $p53^{+/+}$ and $p53^{-/-}$ MEFs and Huvecs were infected with *C. trachomatis* for 40 h. Annexin-V-FLUOS/Propidium Iodide staining was performed, followed by flow cytometric analysis. Early apoptotic cells after 40 h *Chlamydia* infection are displayed in Q4, late apoptotic cells in Q2 and necrotic cells in Q1. ni: not infected; C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

A small population of infected Huvecs was detected as late-apoptotic (5.1%, Figure 4A, B), the same observation was made for $p53^{-/-}$ MEFs (7.0%). Both cell types showed low levels of necrotic cells, 0.6% and 0.5% respectively. In contrast to this, mouse fimb and wild type MEFs

exhibited strongly elevated levels of necrotic cell death (14.7 and 13%, Figure 4A, B). Surprisingly, populations of early or late apoptotic cells could not be detected in mouse fimb cells, in contrast to wild type MEFs (8.4% late apoptotic cells, Figure 4A, B). The exclusive induction of necrotic cell death in wild type p53 mouse cells but not in human or $p53^{-/-}$ mouse cells indicates a significant contribution of p53 to *Chlamydia* induced cytotoxicity in mouse cells. Additionally, the results suggest species-specific regulation of host cell pathways after *C. trachomatis* infection.

To further investigate the observation of increased cytotoxicity during *Chlamydia* infection of mouse cells and its impact on bacterial load mouse fimb, $p53^{-/-}$ MEFs, $p53^{-/-}$ MEFs transfected with HA-tagged p53, as well as Huvecs were infected with *C. trachomatis* for 48 h, followed by an infectivity assay. For this, samples were lysed using glass beads in order to release EBs to the supernatant. A hundredth of the lysate was subsequently transferred to a second set of HeLa229 cells and incubated for 24 h. Protein lysates were analysed by SDS-PAGE and western blotting. An antibody against the chlamydial heat shock protein Hsp60 was used to determine the amount of the pathogen. Interestingly, quantification of chlamydial progeny formation revealed that bacterial load was increased in $p53^{-/-}$ MEFs compared to mouse fimb and $p53^{-/-}$ MEFs transfected with HA-p53 (Figure 5). Restoration of p53 resulted in similar growth inhibition as in mouse fimb cells. Primary human cells on the other hand allowed normal chlamydial growth as observed in $p53^{-/-}$ MEFs. Taken together, the data show that p53 plays a critical part in increased cytotoxicity in mouse cells and causes a reduction of chlamydial load.

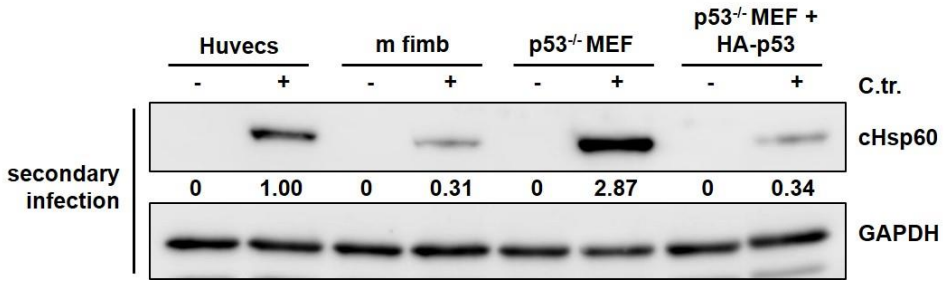


Figure 5 Cytotoxicity in MEFs reduces chlamydial infectivity. Mouse fimb cells, $p53^{-/-}$ MEFs, $p53^{-/-}$ MEFs transfected with HA-tagged p53 and Huvecs were infected with *C. trachomatis* (MOI 1, 48 h), followed by an infectivity assay. Samples were analysed by SDS-PAGE and western blotting. Bacterial progeny was quantified in the secondary infection using an antibody against chlamydial Hsp60 (cHsp60). GAPDH was used as loading control. C.tr.: *C. trachomatis*.

4.1.2 *Chlamydia* induces downregulation of tumour suppressor p53 in human cells

In recent years, several publications reported about regulation of p53 during bacterial infections. Degradation of the tumour suppressor was first described after infection with *H. pylori* (Wei *et al.*, 2010). The bacterial effector protein CagA induced activation of the PI3K-Akt pathway, leading to HDM2 activation and p53 degradation. In a similar way *Neisseria* infection was demonstrated to induce p53 downregulation in specific cell types, together with an upregulation of regulators of cell cycle (Vielfort *et al.*, 2013). *Shigella* utilizes cellular calpain proteases to facilitate cleavage of p53 (Bergounioux *et al.*, 2012). The common feature of these pathogens is the induction of DNA damage after infection. Consequently, in order to avoid apoptosis bacteria mediate degradation of p53 which otherwise becomes activated after detection of genotoxic stress.

Several apoptosis-inhibiting regulations at the site of mitochondria and downstream of mitochondria were detected during *Chlamydia* infection. Recently, induction of DNA damage was also demonstrated during *Chlamydia* infection. Cellular DDR was shown to be abrogated as DDR proteins pATM and 53BP1 were not recruited to damaged sites (Chumduri *et al.*, 2013). Because of these data and together with our own observations of *Chlamydia*-mediated protection of cells against p53-induced apoptosis and strongly reduced cytotoxicity in $p53^{-/-}$ mouse cells, we focused more closely on the tumour suppressor and its regulation during infection.

Protein levels of p53 are downregulated during *C. trachomatis* infection

The *tp53* gene coding for the tumour suppressor is often mutated in human cancers and cell lines used in cell culture. Primary Huvecs with wild type p53 were therefore used for experiments. Firstly, protein levels of the tumour suppressor were assessed at several time points during infection. Protein lysates were analysed by SDS-PAGE and western blotting.

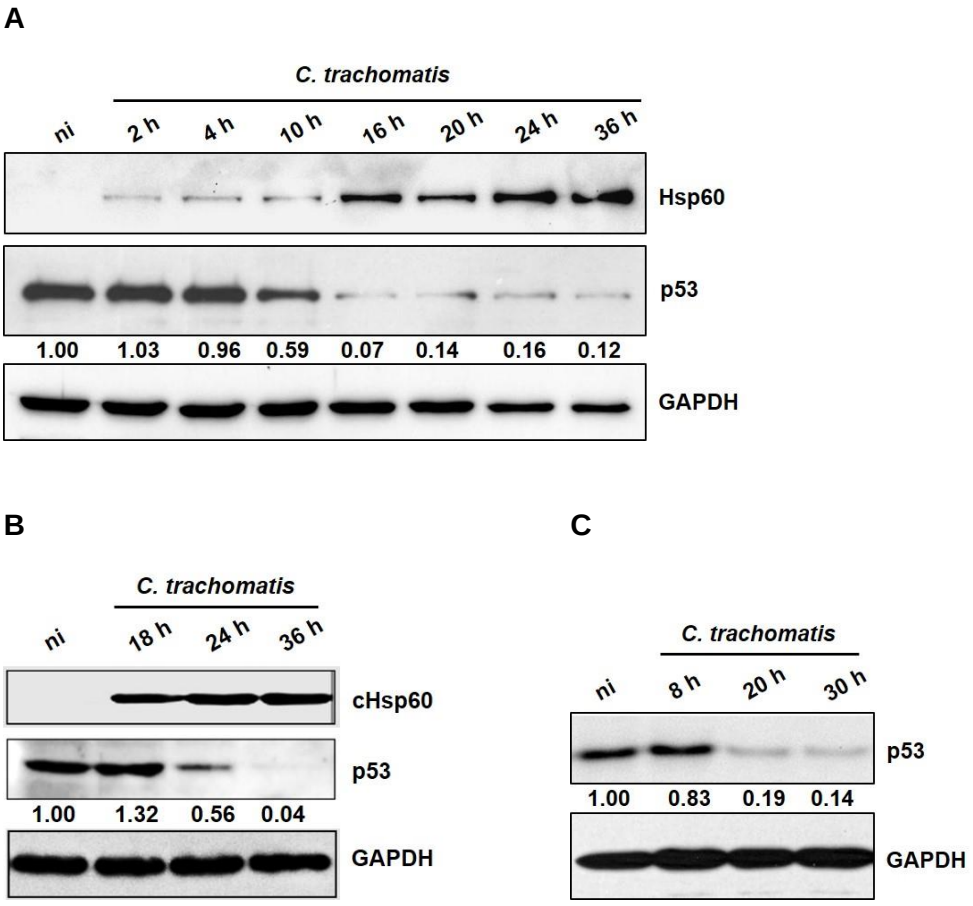


Figure 6 Downregulation of the tumour suppressor p53 in *Chlamydia* infected human cells. (A) Huvecs were infected with *C. trachomatis* for different time points, followed by analysis by SDS-PAGE and western blotting. Blots were probed with antibodies against p53 and chlamydial Hsp60. GAPDH was used as loading control. (B) Epithelial cells of human fallopian tube fimbriae were infected with *C. trachomatis* for different time points and processed as mentioned above. (C) HeLa229 were infected with *C. trachomatis* for different time points and processed as mentioned above. ni: not infected. This figure was first published in Siegl *et al*., 2014.

Intriguingly, a time-dependent downregulation of p53 during the course of *Chlamydia* infection could be observed (Figure 6A). At 10 h post infection (hpi) a decrease to 59% of the normal protein level was apparent which further dropped to about 15%. The amount of p53 remained at low level until the end of the chlamydial life cycle without disappearing completely.

During an undiagnosed infection of *C. trachomatis* the pathogen spreads from the cervix to the uterus and finally the fallopian tubes potentially causing pelvic inflammatory disease (PID). To monitor regulation of p53 in a cell type natural for *Chlamydia,* fimbriae of uterine tubes were collected from patients and prepared as described in the methods section (performed by the group of Prof. Dr. Jörg Wischhusen (University Clinic Würzburg) and by Dr. Karthika Karunakaran at the chair of microbiology (University Würzburg). Western blot analysis

confirmed the results obtained by infected Huvecs and showed a strong downregulation of p53 24 h after infection (Figure 6B).

Despite its wild type status, function of p53 in HeLa229 cells was characterized to be absent or diminished due to the presence of the HPV18-specific protein E6 leading to inactivation of p53. Elsewhere the protein was reported to be DNA-binding-competent and transcriptionally active (Hoppeseyler *et al.*, 1993). We investigated protein level of p53 in HeLa229 cells infected with *C. trachomatis* (MOI 1) for different time points. Here again, the protein level of p53 was downregulated in the cancer cell line comparable to primary cells (Figure 6C).

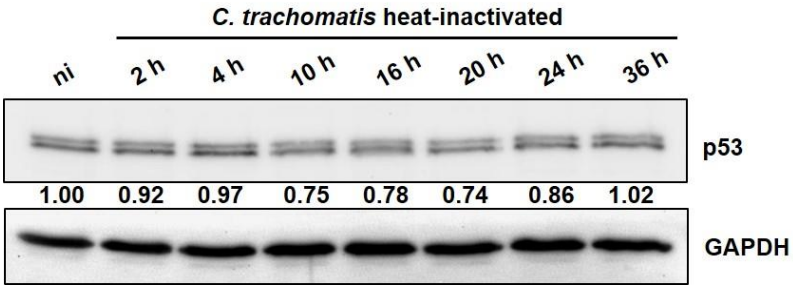


Figure 7 Downregulation of p53 requires live bacteria. *C. trachomatis* was heat-inactivated by incubation at 96°C for 10 min. Huvecs were subsequently infected for the indicated time points and subjected to SDS-PAGE and western blotting. ni: not infected. This figure was first published in Siegl *et al*., 2014.

To demonstrate that live bacteria are necessary for p53 downregulation and not sensing of pathogen associated molecular patterns (PAMPs) such as bacterial LPS, *Chlamydia* were heat-inactivated prior to infection by incubation at 96°C for 10 min. Infection with heat-inactivated *Chlamydia* failed to induce downregulation of p53, protein levels remained unaltered during the course of infection (Figure 7).

Downregulation of p53 by different chlamydial strains

C. trachomatis serovar L2 is a member of the chlamydial biovar (L1 - L3) causative for the disease lymphogranuloma venereum (LGV). Serovars D to K are responsible for STDs like urethritis, cervicitis, salpingitis and epididymitis. Also belonging to the genus *Chlamydia* is *C. muridarum*, naturally infecting mice and hamsters. Another member of the family *Chlamydiaceae* is the bacterium *C. pneumoniae*, which causes diseases like pharyngitis, bronchitis and atypical pneumonia.

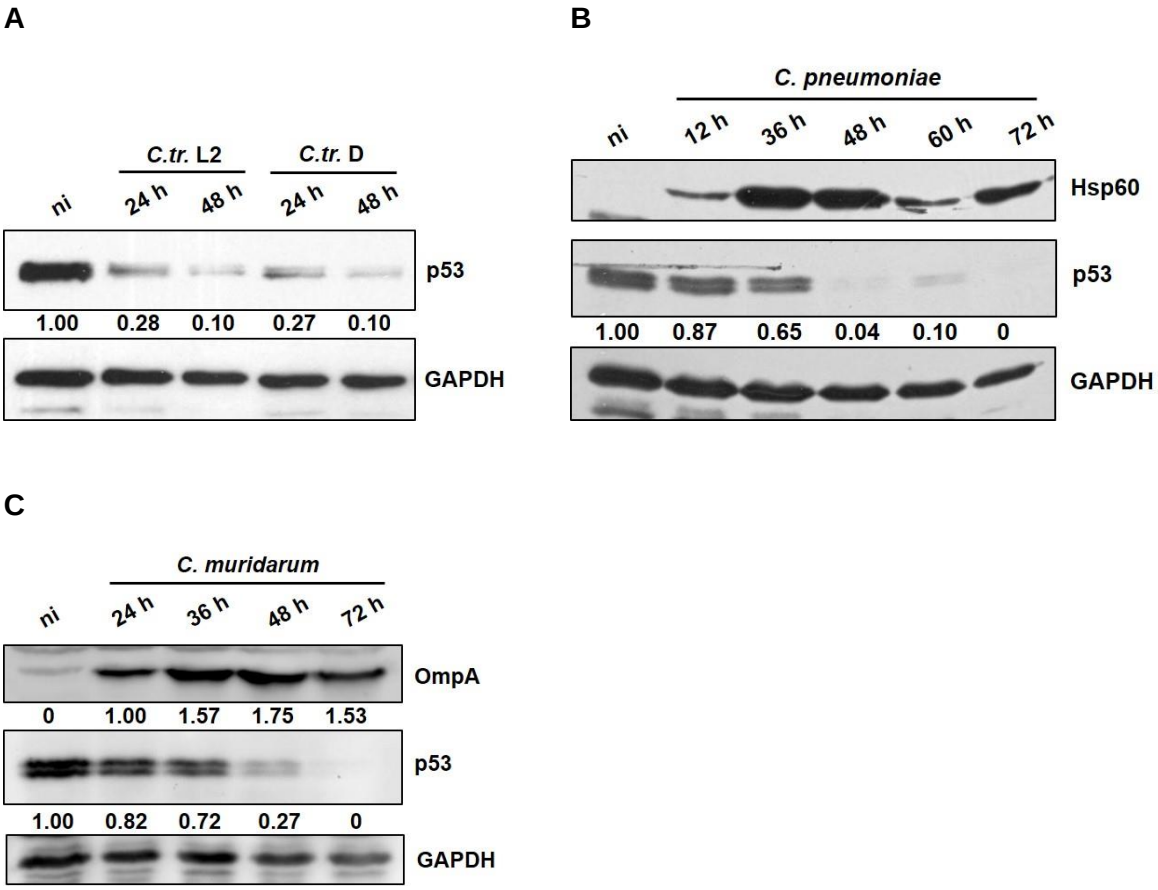


Figure 8 (A) Huvecs were infected with *C. trachomatis* serotype L2 and serotype D for the indicated time points. Samples were analysed by SDS-PAGE and western blotting. Blots were probed with p53, chlamydial Hsp60. GAPDH was used as loading control. (B) Huvecs were infected with *C. pneumoniae* for the indicated time points and processed as mentioned above. (C) *C. muridarum* infection of human cells induces downregulation of the tumour suppressor p53. Huvecs were infected with *C. muridarum* for the indicated time points and samples were analysed by SDS-PAGE and western blotting. OmpA antibody was used to quantify *C. muridarum* infection. GAPDH was used as loading control. ni: not infected; C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

To address the question if downregulation of p53 is serovar-specific or a general feature of chlamydial infections, p53 protein level was assessed during infections with *C. trachomatis* D, *C. pneumoniae* and *C. muridarum*. Growth of *C. muridarum* was quantified using an antibody against the outer membrane protein OmpA. Western blot analysis revealed that all chlamydial serovars and strains had the same effect on the protein level of the tumour suppressor. A strong downregulation of p53 during *C. trachomatis* D infection could be observed at 24 and 48 hpi (Figure 8A). *C. pneumoniae* and *C. muridarum* infections showed downregulation of p53 at later time points compared to *C. trachomatis* infections. *C. pneumoniae* infected cells displayed a decrease in p53 protein level of 35% at 36 hpi (Figure 8B), *C. muridarum* infected cells 28% at 36 hpi (Figure 8C). At later time points protein levels of p53 were strongly diminished. Longer chlamydial developmental cycles of *C. pneumoniae* and *C. muridarum*

explain the delay in p53 downregulation. Lysis of host cells is initiated at about 72 hpi. Taken together, infections with various chlamydial strains demonstrated that p53 downregulation can be regarded as a general observation among *Chlamydiaceae*.

p53 is not downregulated after *Chlamydia* infection in mouse cells

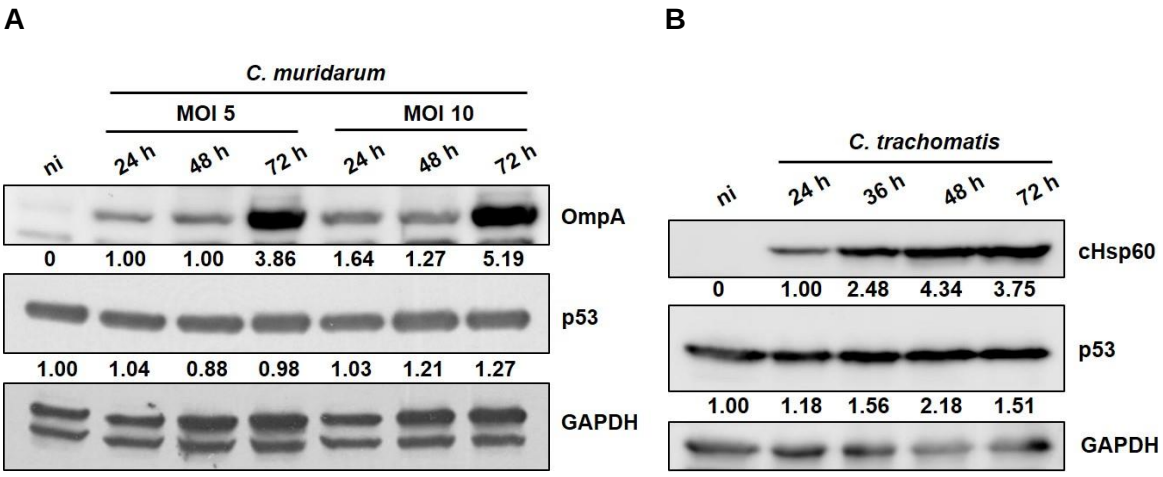


Figure 9 p53 is not downregulated in mouse cells. (A) MEFs were infected with *C. muridarum* (MOI 5 and MOI 10) for different time points and analysed by SDS-PAGE and western blotting. Blots were probed with antibodies against OmpA and p53. GAPDH was used as loading control. (B) MEFs were infected with *C. trachomatis* for different time points. Samples were processed as mentioned above. ni: not infected. This figure was first published in Siegl *et al*., 2014.

Comparing *Chlamydia* infections of mouse and human cells differences regarding induction of cytotoxicity were detected. Reduced cell viability of mouse cells resulted in decreased bacterial load. In $p53^{-/-}$ mouse cells on the other hand cell survival and chlamydial infectivity was restored. So far, strong evidence for a general downregulation of p53 in human cells was found. To clarify regulation of the tumour suppressor in mouse cells $p53^{+/+}$ MEFs were infected with both *C. muridarum* and *C. trachomatis* for different time points. Chlamydial growth and level of p53 was detected by immunoblotting. Intriguingly, *C. muridarum* infection of mouse cells did not initiate downregulation of p53. Despite strong infection at 72 h protein level of p53 did not decrease (Figure 9A). A similar observation was made after infecting mouse cells with the human pathogen *C. trachomatis* (Figure 9B). Consequently, downregulation of the tumour suppressor is thought to be cell type specific. These data confirm the critical role of p53 established after analysis of cell viability of infected mouse and human cells.

Transcriptional activity of p53 is decreased during *C. trachomatis* infections

The transcription factor and tumour suppressor p53 plays a central role in the complex signalling network of cells. Various stimuli and signals like replication stress, DNA damage, oncogene activation or hypoxia lead to stabilisation of p53 and induction of transcriptional activity. Depending on the stress signal, posttranslational modifications and target promoter genes, various transcriptional outputs are possible. Under basal conditions a certain amount of p53 is bound to selected DNA-sites. In addtion to its core DBD p53 possesses a highly basic, lysine rich C-terminal domain which recognizes DNA unspecifically (McKinney *et al.*, 2004, Liu *et al.*, 2006).

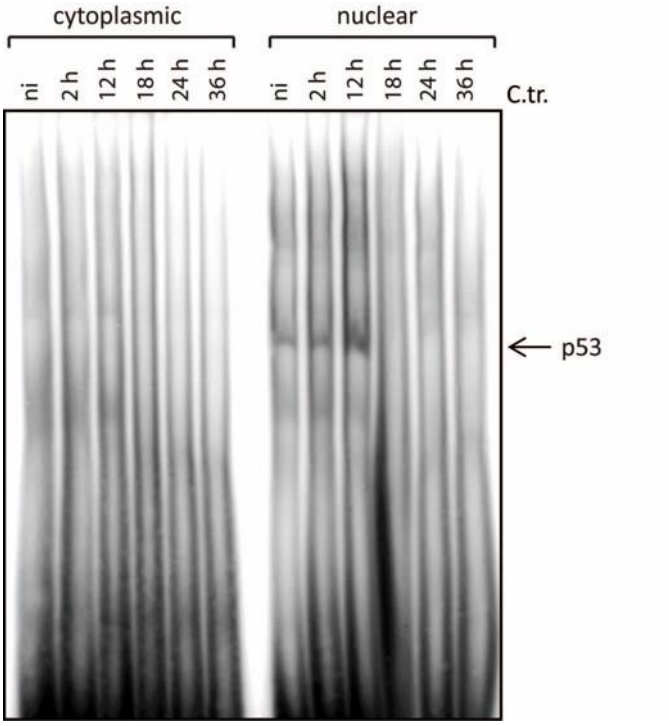


Figure 10 DNA binding activity of the transcription factor p53 during *C. trachomatis* infection was analyzed by electrophoretic mobility shift assay (EMSA). ni: not infected; C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

After detecting downregulation of p53 during chlamydial infections in human cells, transcriptional binding activity of p53 in the nucleus was measured to verify these findings. In collaboration with Dr. Bhupesh Prusty (microbiology, University Würzburg) an electrophoretic mobility shift assay (EMSA) was performed. Cytoplasmic and nuclear fractions of protein extracts were prepared as described in Prusty *et al*. (Prusty *et al.*, 2005), the EMSA was performed as described in the methods section. DNA-protein complexes were analysed by phosphor autoradiography. Consistent with our previous findings, DNA-binding activity of the

tumour suppressor decreased at later time points of *Chlamydia* infection (Figure 10). In the non-infected sample a basal level of DNA-binding of the transcription factor could be observed confirming the non-specific binding of p53 to DNA. At 12 and 24 h after infection a slight increase of transcriptional binding occurred followed by the rapid disappearance of the DNA-protein complex. The decline of transcriptional activity further strengthens the previously detected decrease of p53 protein level.

4.1.3 p53 degradation is mediated by the PI3K-Akt-signalling pathway

In healthy, unstressed cells p53 is continuously ubiquitinated by its main regulator HDM2 and proteasomally degraded. HDM2 shuttles between the nucleus and the cytoplasm and mediates the export of the tumour suppressor. To further diminish p53 certain stimuli like growth factors, cytokines or oncogenes are necessary to activate the PI3K-Akt-signalling pathway. Activation of this pathway reinforces degradation of p53 by the proteasome. To clarify the mechanism *Chlamydia* employs to induce p53 downregulation crucial steps of the PI3K-pathway like phosphorylation state of Akt and HDM2 were analysed.

Activation of the PI3K-Akt-pathway by *C. trachomatis*

As already published by Verbeke *et al*. and Rajalingam *et al.*, chlamydial infections of cells result in the activation of PI3K and Akt (Verbeke *et al.*, 2006, Rajalingam *et al.*, 2008). Until now, this activation was only linked to the inhibition of apoptosis induction through the sequestration of Bad to the chlamydial inclusion and to the expression and stabilisation of Mcl-1 during *Chlamydia* infections.

Phosphorylation state of Akt (Ser473) and HDM2 (Ser166) was assessed after infecting Huvecs with *C. trachomatis* at different MOIs and for different time points. Strong activation of Akt was detected after 24 h of infection at MOI 0.5 (Figure 11A). Infection of cells with *Chlamydia* at MOI 1.0 resulted in a 5.48 fold induction of Akt compared to uninfected control cells. Analysing several time points of infection, activation of Akt could be confirmed (Figure 11B). Phosphorylation and activation of HDM2 follows activation of Akt in the signalling cascade leading to enhanced degradation of p53. HDM2 activation requires phosphorylation at serine residues 166 and 188 by Akt, leading to inhibition of self-ubiquitination and subsequent stabilisation of the E3-ligase. Phosphorylation of HDM2 was analysed in a MOI and time dependent manner.

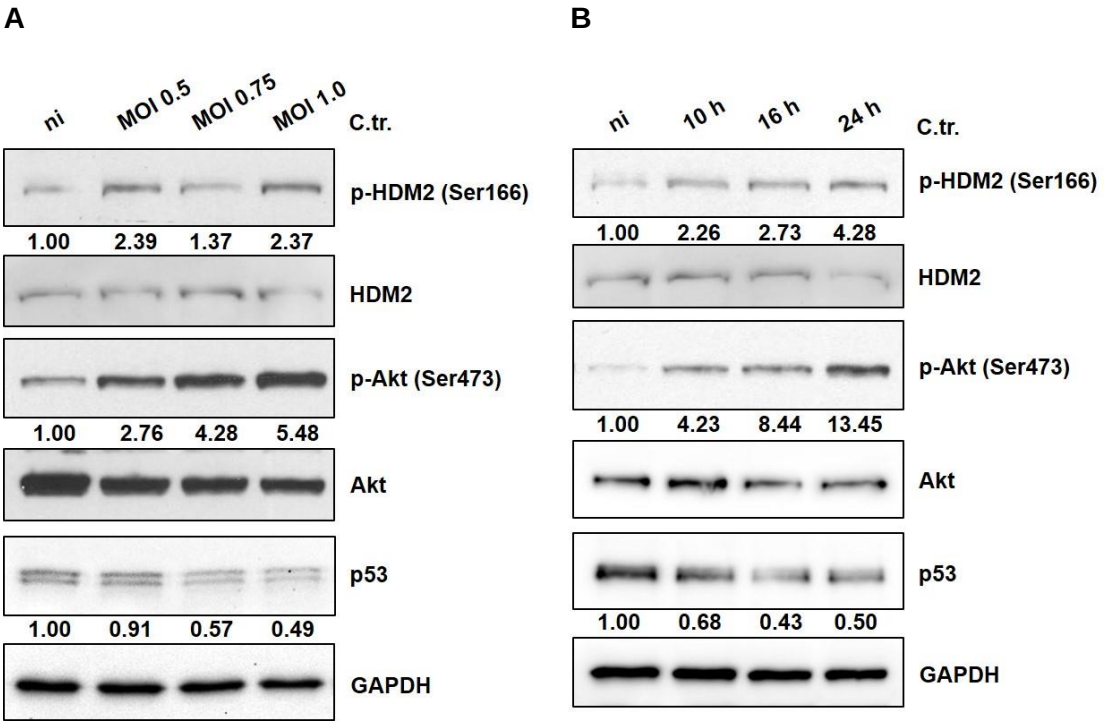


Figure 11 *C. trachomatis* infection induces phosphorylation of Akt and HDM2. Huvecs were infected with *C. trachomatis* at different MOI (A) or for different time points (B). Samples were analysed by SDS-PAGE and western blotting. Membranes were probed with antibodies against p-HDM2 (Ser166), HDM2, p-Akt (Ser473), Akt, cHsp60 and p53. GAPDH was used as loading control. ni: not infected; C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

As expected, phosphorylation of HDM2 with a 2.55 fold-induction at MOI 1.0 could be detected (Figure 11A). Analysis of several time points of infection affirmed activation of the PI3K-pathway: After 24 h a 3.17 fold-induction of phosphorylated HDM2 (Figure 11B) was detected. PI3Ks are potently inhibited by the chemical compound LY294002 which acts on the ATP binding site of the enzyme. Contrary to wortmannin, another well-known PI3K-inhibitor, LY294002 is a reversible inhibitor of PI3K. Stabilisation of p53 after application of this inhibitor was verified by infecting Huvecs with *C. trachomatis* for 12 and 16 h. The inhibitor was added to the cell culture 1 hpi at a concentration of 10 µM. Cell lysates were analysed by SDS-PAGE and western blotting. As already demonstrated *Chlamydia* infection of control cells resulted in p53 downregulation. Inhibitor treatment effectively blocked degradation of the tumour suppressor during *Chlamydia* infection as shown in Figure 12. To sum up, the data provide evidence for activation of both the PI3K-Akt-signalling pathway and the main regulator of p53, HDM2, during *Chlamydia* infections.

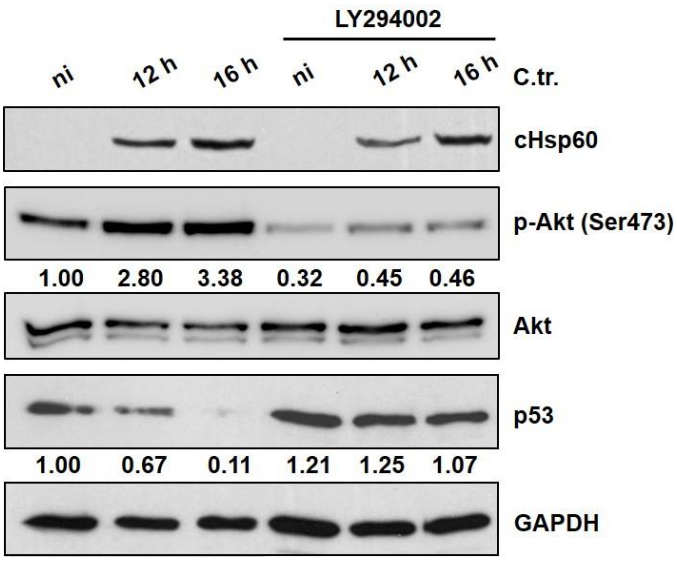


Figure 12 Inhibition of PI3K blocks p53 depletion. Huvecs were treated with 10 µM PI3K inhibitor LY294002 1 hpi with *C. trachomatis*. Samples were analysed by SDS-PAGE and immunoblotting. Membranes were probed with antibodies against chlamydial Hsp60, p53, pAkt and Akt, GAPDH was used as loading control. ni: not infected; C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

Blocking of HDM2 activity rescues p53 protein level

The degradation signal of p53 is transferred by the E3-ubiquitin ligase HDM2. To verify activation of HDM2 during *Chlamydia* infections activity of this enzyme was blocked. A potent and selective small-molecule inhibitor of the interaction between HDM2 and p53 is the cis-imidazoline analogue nutlin-3. Nutlins are designed to fit into the p53 binding pocket of the E3 ligase HDM2. Nutlin-3 not only induces the stabilisation of p53 but also activates p53-dependent signalling pathways causing cell cycle arrest in G1 and G2 phases (Vassilev *et al.*, 2004). At higher concentrations it is a potent inducer of apoptosis.

Huvecs were infected with *C. trachomatis* for 24 h and incubated 6 h before lysis of the samples with 5 and 10 µM nutlin-3. The inhibitor strongly stabilised p53 during *Chlamydia* infection (Figure 13A). At a concentration of 5 µM a slight decrease in p53 protein level could be detected, while a concentration of 10 µM completely stabilised the tumour suppressor.

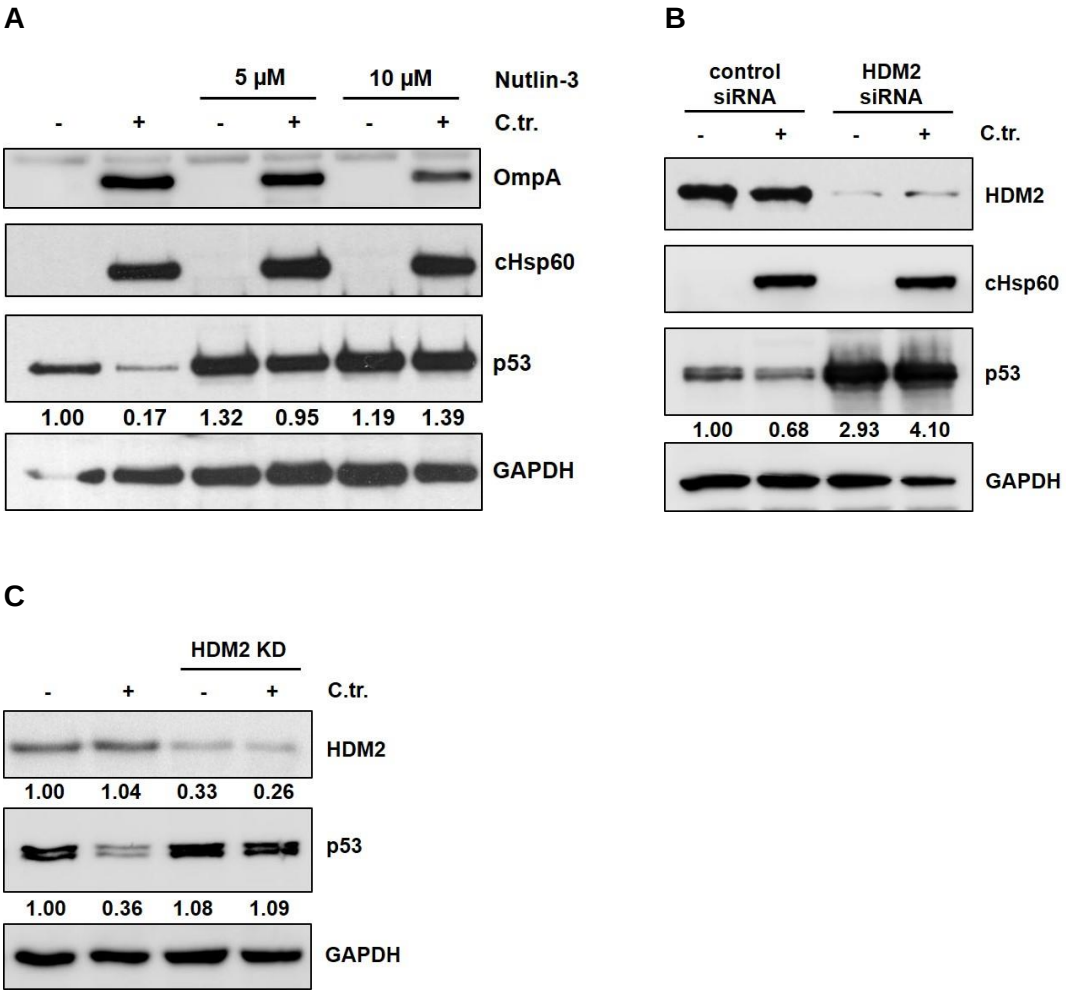


Figure 13 (A) Inhibition of HDM2 results in p53 stabilisation. Huvecs were treated with 5 and 10 µM HDM2 inhibitor nutlin-3 and infected with *C. trachomatis* (MOI 1, 24 h). p53, chlamydial Hsp60 and chlamydial OmpA were detected by immunoblotting and GAPDH was used as loading control. (B) Silencing of HDM2 resulted in stabilisation of p53 in HeLa229 cells. siRNA-based gene silencing of HDM2 was performed, followed by infection of cells with *C. trachomatis* for 24 h. (C) Silencing of HDM2 resulted in stabilisation of p53 in Huvecs. HDM2 was silenced by lentivirus-mediated transduction of shRNA-constructs in Huvecs, the knock down efficiency was checked with an antibody against HDM2. C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

To confirm the results obtained by inhibition of HDM2 siRNA-based gene silencing of the E3-ubiquitin ligase was performed in HeLa229 cells. Successful knockdown was controlled by immunoblotting. 24 h after transfection of cells with siRNA against HDM2 cells were infected with *C. trachomatis*. As observed after nutlin-3 treatment silencing of HDM2 resulted in strong stabilisation of p53 during *Chlamydia* infection (Figure 13B). The same experiment was repeated in Huvecs with HDM2 silenced by lentivirus-mediated transduction of shRNA-constructs. Again the tumour suppressor was strongly enriched (Figure 13C). Silencing of HDM2 and inhibition of its interaction with p53 efficiently rescued protein level of p53 during *Chlamydia* infection.

Degradation of p53 is proteasome dependent

The 26S proteasome is a protein complex primarily located in the nucleus, the cytoplasm and at the membrane of the endoplasmic reticulum (ER). Most of the cellular proteins, marked by poly-ubiquitin chains, are degraded by the ubiquitin-proteasome pathway, others are cleaved in lysosomes or by calpains (Lecker *et al.*, 2006). Mono- and poly-ubiquitinations are involved in many cellular processes. Mono-ubiquitination was found to play a role during endocytosis or transcriptional regulation, whereas poly-ubiquitination targets proteins for degradation (Hicke *et al.*, 2003).

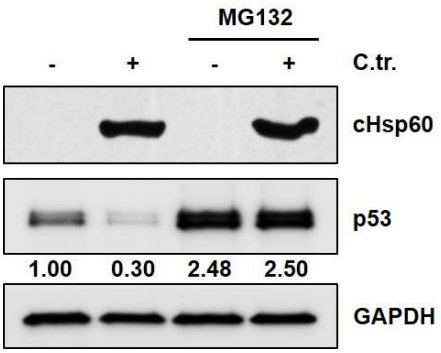


Figure 14 Inhibition of the proteasome prevents p53 degradation. Huvecs were infected with *C. trachomatis* (MOI 1, 24 h) and treated with 3 µM proteasome inhibitor MG-132. Samples were analysed by immunoblotting. Membranes were probed with antibodies against cHsp60 and p53. GAPDH was used as loading control. C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

HDM2 catalyses both mono- and poly-ubiquitination on p53 depending on the protein level of HDM2 inside the cell. In unstressed cells, low levels of HDM2 mono-ubiquitinate p53 and only mediate its nuclear export, high levels lead to poly-ubiquitination and proteasomal degradation suppressing p53 function e.g. in response to cellular stress (Li *et al.*, 2003).

Proteasome-mediated degradation of p53 was assessed by inhibitor treatment. *C. trachomatis*-infected Huvecs were supplemented with proteasome inhibitor MG-132 12 h before sample lysis. Western blot analysis confirmed degradation of p53 by the proteasome. The tumour suppressor was stabilised in infected and uninfected cells (Figure 14). Taken together, our findings clarified the signalling pathway initiated by *C. trachomatis* infection to mediate degradation of the tumour suppressor p53.

Regulation of transcriptional targets of p53

Over 125 protein-coding genes and non-coding RNAs were discovered to be transcriptionally activated by p53. Moreover, the transcription factor directly targets several hundred and indirectly thousands of genes. Direct targets are characterized by the presence of p53 response elements (Beckerman *et al.*, 2010).

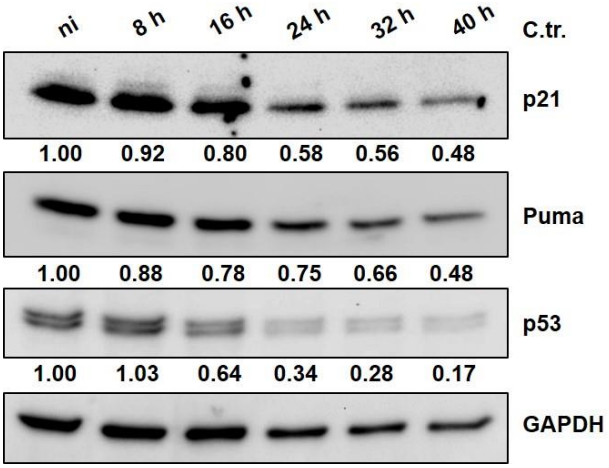


Figure 15 Direct transcriptional targets of p53 are affected by the degradation of the tumour suppressor. Huvecs were infected with *C. trachomatis* for different time points. p21, Puma and p53 were detected by immunoblotting and GAPDH was used as loading control. ni: not infected; C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

Two well-known targets of p53 are the cyclin-dependent kinase inhibitor p21 and the pro-apoptotic protein Puma. Puma gets activated upon p53-induced apoptosis. In addition, recent publications demonstrated that cells with mutated p53 have lower levels of Puma (Kabacik *et al.*, 2011). To verify impact of p53 degradation on transcriptional targets, protein levels of p21 and Puma were analysed after *Chlamydia* infection. Both p21 and Puma were reduced by 50% after 40 h of infection. As a consequence of p53 degradation direct targets of the transcription factor display reduced protein expression (Figure 15).

4.1.4 The PI3K-Akt-signalling pathway is not activated in mouse cells during *Chlamydia* infection

The absence of p53 degradation in mouse cells led to strong cytotoxicity and reduced chlamydial infectivity and progeny formation. To clarify this inconsistence between *Chlamydia* infections of human and mouse cells activation of the PI3K-pathway was analysed in mouse cells.

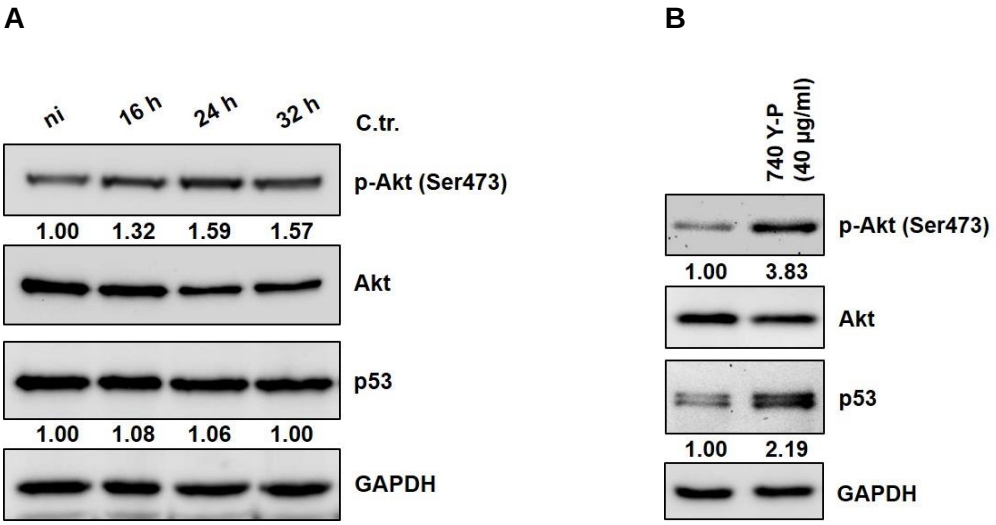


Figure 16 (A) *C. trachomatis* infection does not activate the PI3K-Akt-pathway in endothelial cells of mouse fimb cells. p-Akt (Ser473), Akt and p53 were detected by immunoblotting and GAPDH was used as loading control. Fold change values were derived by normalization to GAPDH and are mentioned below each lane. (B) Artificial activation of PI3K signalling does not induce degradation of p53. Huvecs were treated with PI3K activator 740 Y-P (40 µg/ml) for 6 h. p-Akt (Ser473), Akt and p53 were detected by immunoblotting and GAPDH was used as loading control. ni: not infected; C.tr.: *C. trachomatis*. Parts of this figure were first published in Siegl *et al*., 2014.

Mouse fimb cells were infected with *C. trachomatis* for several time points and analysed by immunoblotting. Phosphorylation of Akt was not initiated upon *Chlamydia* infection as observed in human cells (Figure 16A). Even at late time points Akt was not activated explaining the stable level of p53. The question why PI3K-Akt-signalling was not initiated in mouse cells after *Chlamydia* infection remains to be investigated. To exclude defective signalling, a PI3K activator, 740 Y-P, was administered to Huvecs. By detecting phosphorylated levels of Akt, PI3K activation could be demonstrated. However, activated signalling resulted in stabilisation of p53, not degradation (Figure 16B). This effect is mentioned in publications of Lee *et al*. and Astle et al. (Lee *et al.*, 2007, Astle *et al.*, 2012), showing that activating mutations of PI3K and hyper activation of Akt lead to stabilisation of p53. Thus, the activator could not be applied for activation of PI3K-Akt signalling in mouse cells to demonstrate functional degradation of p53.

4.1.5 Silencing of p53 does not interfere with chlamydial growth

Degradation of the tumour suppressor in *Chlamydia*-infected cells is initiated after 10 to 16 h at the beginning of enhanced metabolic activity after conversion of EBs to RBs. The strong downregulation of p53 until the end of the life cycle suggests to be beneficial for chlamydial development.

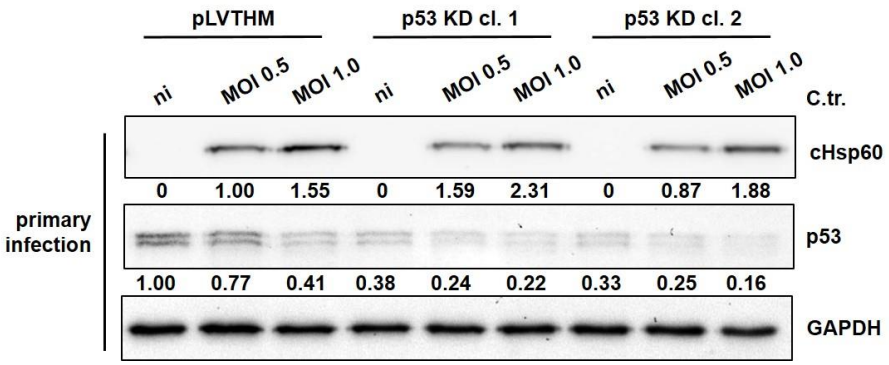
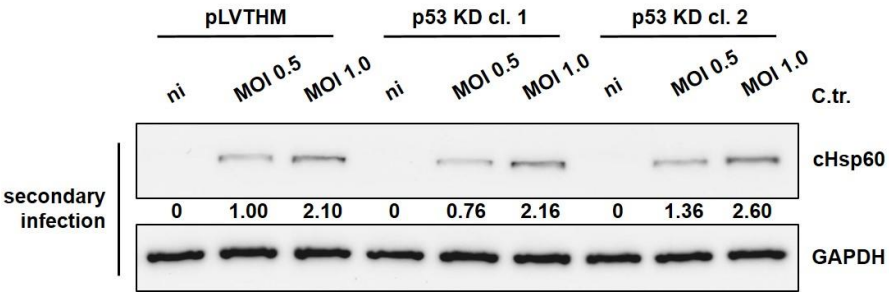


Figure 17 Low cellular levels of p53 do not interfere with chlamydial growth. Expression of p53 was silenced using specific short hairpin RNAs expressed by lentiviral gene transduction in HeLa229 cells. Two single-cell clones were tested for chlamydial growth and infectivity. p53 was detected by immunoblotting and fold change values were derived by normalization to GAPDH. ni: not infected; C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

To address the question if an environment depleted of p53 negatively affects chlamydial growth protein expression of p53 was silenced using lentivirus-mediated shRNA-based gene silencing as described in the methods section. After generation of single cell clones of the knock down cells p53 protein expression was verified by western blot analysis. Two clones, exhibiting knock down of p53 level to 38% and 33%, were selected for further experiments. Chlamydial growth was observed by performing an infectivity assay. Empty vector control cells as well as the two knockdown clones were infected with *C. trachomatis* for 48 h at MOI 0.5 and 1.0.

A



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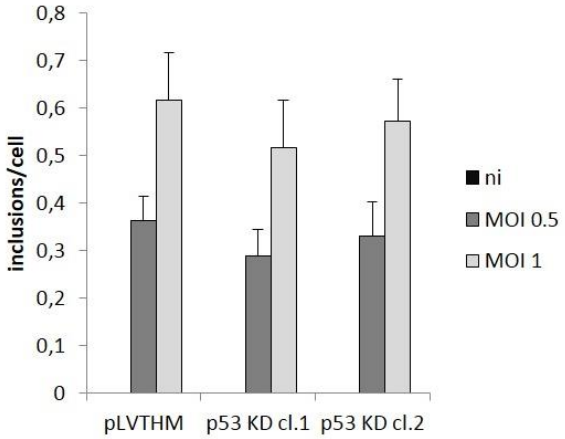


Figure 18 (A) *C. trachomatis* growth and infectivity in p53 knock-down cells was analysed using an infectivity assay. Control cells and two single cell knock-down clones of p53 were infected with *C. trachomatis* (MOI 0.5 and 1.0, 48 h). Cells were lysed with glass beads and one hundredth of the cell lysate containing infectious EB was used to infect a second set of HeLa229 cells. (B) Number of chlamydial inclusions is not significantly changed in p53 knock-down cells. Inclusion numbers were determined by microscopical examination and counting of ten random fields at 40x magnification. ni: not infected; C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

As described previously, cells were manually lysed and EB containing lysate was transferred to new cells. Primary and secondary infection were analysed by immunoblotting. Additionally, inclusions of the secondary infection were quantified by immunostaining and microscopic examination. Degradation of p53 was initiated after *Chlamydia* infection in a MOI dependent way in both control and p53 KD cell lines (Figure 17). Decreased amounts of the tumour suppressor did not interfere with chlamydial growth. Infectivity and amount of chlamydial progeny in p53 knock down cells was comparable to bacterial load of control cells (Figure 18A). Quantification of chlamydial inclusions revealed no differences between control and knock down cells (Figure 18B).

4.1.6 Degradation of p53 is also mediated by calpains

Aside from ubiquitin-dependent proteasomal degradation representing the major route of p53 turnover, cytoplasmic calcium-dependent proteases termed calpains directly target p53 and induce its breakdown. Calpains can be divided into ubiquitous and tissue-specific calpains. Ubiquitous calpains belong to either milli- or microcalpains (m- and µ-calpains) according to the concentration of calcium necessary for enzymatic activity (mM and µM range). Instead of being specific for peptide motifs like other proteases calpains recognize structural determinants. Inhibition of calpains is mediated by their highly specific inhibitor calpastatin

(Croall *et al.*, 1991, Saido *et al.*, 1994). To understand the contribution of calpain-mediated cleavage of p53 during *Chlamydia* infection, infected Huvecs were treated with a calpain specific inhibitor (calpain inhibitor I). The inhibitor was applied at a concentration of 10 µM 0 and 5 h prior to *Chlamydia* infection. In uninfected Huvecs inhibition of calpain lead to a 1.22 fold increase of p53 protein levels (Figure 19) demonstrating the contribution of calpains to general turnover of the tumour suppressor. Calpastatin, the endogenous inhibitor of the calpain system is regulated by negative feedback and cleaved by calpain (Murachi *et al.*, 1980). Thus, inhibition of calpains results in stabilisation of calpastatin (Figure 19). As previously shown *Chlamydia*-infected Huvecs displayed p53 degradation.

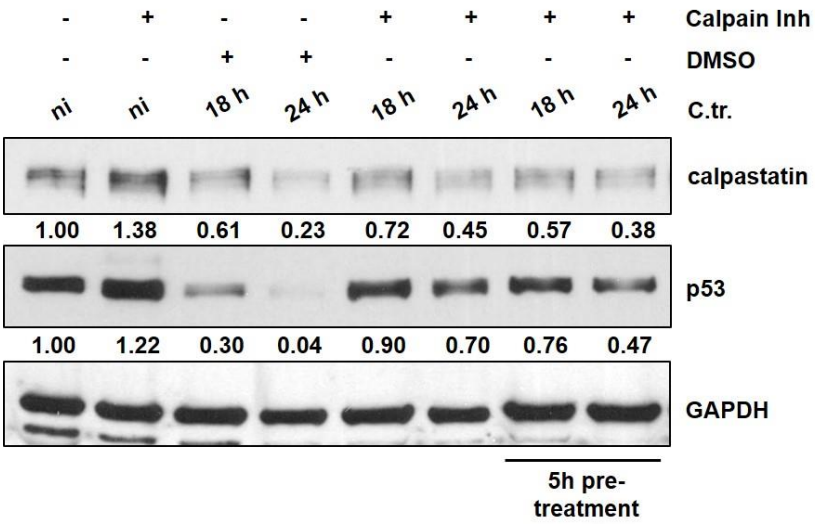


Figure 19 Cellular calpains target p53 during *C. trachomatis* infections. Huvecs were treated 5 h and 0 h before infection with 10 µM calpain inhibitor I and infected with *C. trachomatis* (MOI 1) for the indicated time points. Samples were analysed by SDS-PAGE and western blotting. Membranes were probed with an antibody against the endogenous inhibitor of calpain, calpastatin, as well as p53 and GAPDH. ni: not infected; C.tr.: *C. trachomatis*.

Treatment with the calpain inhibitor I stabilised p53 levels substantially: 24 h and 48 h after infection p53 levels were reduced to 90 and 70%, respectively. Inhibitor treatment 5 h before infection with *Chlamydia* was less efficient possibly because of a low stability of calpain inhibitor I. Interestingly, calpastatin levels were decreased in *Chlamydia* infected cells. In inhibitor treated and infected cells calpastatin remained diminished suggesting a *Chlamydia*-mediated degradation of calpastatin. Downregulation of calpastatin releases enzyme activity of calpains and thus facilitates p53 cleavage. However, further experiments have to be conducted to strengthen the hypothesis of an additional route of degradation for the tumour suppressor during *Chlamydia* infections.

4.2 Activation of p53 inhibits chlamydial growth and infectivity

The majority of human cancers is characterised by the loss of p53 activity making it an attractive target for tumour therapy. In 1996, the first retroviral vector based p53 gene therapy was attempted to cure non-small cell lung carcinoma (NSCLC) resulting in an increased p53 production in infected cells (Roth *et al.*, 1996). Today, p53 gene therapy is already approved in China (Shi *et al.*, 2009) and tested in clinical trials in the U.S. Other approaches like adenovirus based therapy, antisense and siRNA based therapy, as well as p53 vaccines are being developed (Lane *et al.*, 2010). Additionally, a number of small molecule inhibitors are validated in clinical trials with inhibitors of the interaction between p53 and HDM2, the nutlins, being among the most promising p53 activators (Vassilev *et al.*, 2004). One of the most widely used chemotherapy agents is the topoisomerase II inhibitor etoposide (Hande, 1998), a synthetic derivative of podophyllotoxin. In 1983, approval was granted for etoposide by the FDA (Food and Drug Administration). Due to its antineoplastic activity etoposide is applied in cancer therapy including lung cancer, lymphomas and genital tumours.

4.2.1 Inhibition of chlamydial inclusion formation and loss of infectivity after activation of p53

Stabilisation of p53 with the chemical inhibitors etoposide and nutlin-3

To induce activation of the tumour suppressor p53 in human primary cells, two of the most well-known p53 activators, nutlin-3 and etoposide, were applied. Incubation of cells with etoposide was performed for 6 h during which time the activity of the topoisomerase II is blocked. DSBs regularly occurring during replication and transcription are not repaired leading to permanent DNA DSB formation. Efficiency of etoposide was verified monitoring upregulation of p53 protein level. Depending on the concentration a constant increase of protein amount could be observed. As activation of p53 reached maximum levels at application of 50 µM experiments were conducted with this concentration (Figure 20). Concentrations higher than 200 µM resulted in onset of apoptosis within several hours.

Off-target effects on *Chlamydia* were investigated by incubating pure EBs with the inhibitors before infection of cells (Bowie, 1990, Park *et al.*, 2009). *Chlamyd*
ia were incubated for 30 min with etoposide (50 µM), nutlin-3 (10 µM) or DMSO on ice. Subsequently, to monitor chlamydial growth Huvecs were infected with pre-treated EBs. As demonstrated by an infectivity assay inhibitor treatment did not interfere with chlamydial inclusion formation and development (Figure 21).

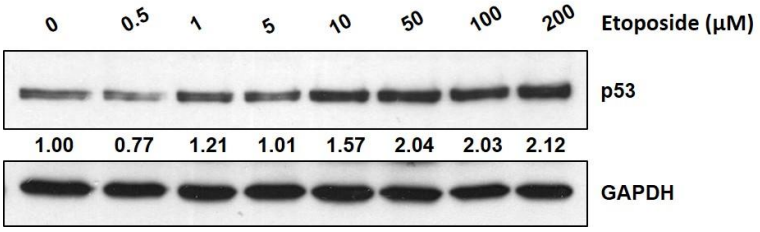


Figure 20 Induction of p53 expression in etoposide-treated cells. Huvecs were treated with different concentrations (0.5 – 200 µM) of etoposide for 6 h to stabilise p53 expression. 50 µM was the lowest concentration that induced maximal p53 stabilisation. This figure was first published in Siegl *et al*., 2014.

To further avoid off-target effects media of etoposide-incubated cells was replaced by fresh media before infection with *Chlamydia*. Due to the permanent formation of double strand breaks activation of p53 persisted after removal of etoposide. After incubation of cells with nutlin-3 media could not be replaced as its inhibitory effect is reversible. Removal of nutlin-3 restores levels of p53 noticeably after 15 min. Normal endogenous values of p53 are reached in less than 1 h (van Leeuwen *et al.*, 2011).

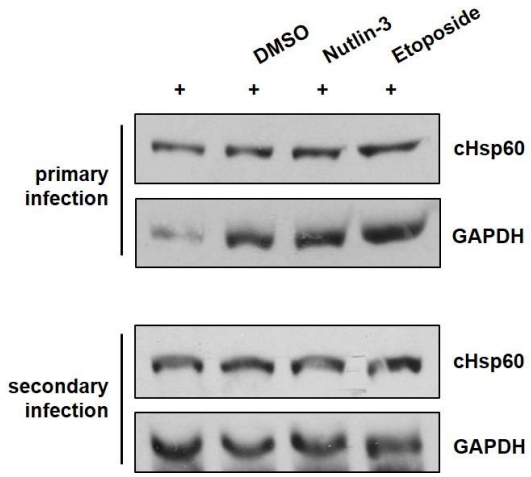


Figure 21 Nutlin-3 or etoposide have no direct adverse effect on chlamydial development. EB were pre-treated with the solvent DMSO, 10 µM nutlin-3 or 50 µM etoposide for 30 min on ice and used for infectivity assays. Bacterial load monitored by Hsp60 immunoblotting revealed no change in chlamydial development. This figure was first published in Siegl *et al*., 2014.

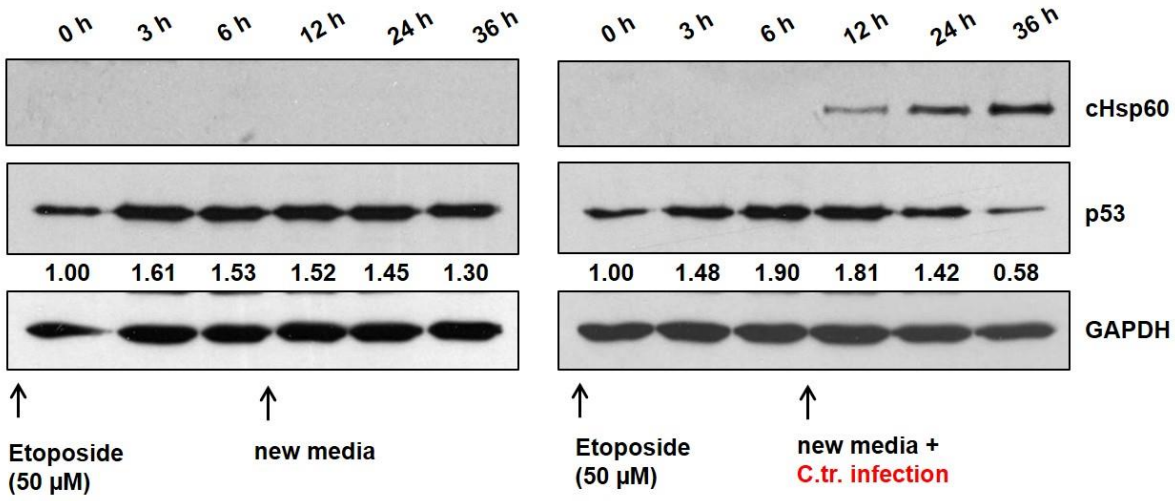
Loss of chlamydial infectivity after activation of p53 by etoposide

So far, this study demonstrated the induction of p53 degradation during *Chlamydia* infections. Silencing of the tumour suppressor further strengthened the observation that p53 is dispensable for chlamydial development. To address the question about the necessity of p53 degradation for *Chlamydia*, stabilisation and activation of the tumour suppressor was chemically induced. Furthermore, experiments should clarify if *Chlamydia* mediates downregulation of activated p53. Etoposide induces transcriptional upregulation of p53 and phosphorylation of key serine residues Ser6, Ser15 and Ser20. Phosphorylation of Ser15 and Ser20 strongly weakens the interaction of p53 with HDM2 (Unger *et al.*, 1999, Shieh *et al.*, 1997).

As shown in Figure 22, Huvecs were treated with 50 µM etoposide for 6 h, media was replaced and cells were infected with *C. trachomatis* for different time points. Control cells remained uninfected. Samples were analysed by immunoblotting and chlamydial growth was again verified using a *Chlamydia*-specific Hsp60 antibody. Etoposide-treatment strongly increased p53 protein amounts after 6 h. In control cells, removal of the topoisomerase inhibitor resulted in minor reduction of p53 in the next 30 h. Surprisingly, *Chlamydia* infection facilitated rapid removal of the tumour suppressor despite its activation state. After 30 h of *C. trachomatis* infection p53 was reduced to 58% of the normal protein level found in unstressed cells. Further experiments are needed to investigate if degradation is still mediated by HDM2 or if calpain-mediated cleavage of p53 is responsible for the reduction of protein level.

Before samples were processed for western blot analysis, *C. trachomatis* infection was monitored by light microscopy. Intriguingly, chlamydial inclusion formation could not be observed even at late time points of infection. Presence of *Chlamydia* and bacterial growth could still be verified by increasing amounts of chlamydial Hsp60 by western blot analysis. Chlamydial development occurs inside an inclusion and as infectivity is only regained after conversion of RBs to EBs, bacterial progeny formation was tested in an infectivity assay. Due to the absence of inclusions chlamydial development was considered to be defective. As expected from our observations, activation of p53 resulted in a complete loss of chlamydial infectivity (Figure 22B).

A



B

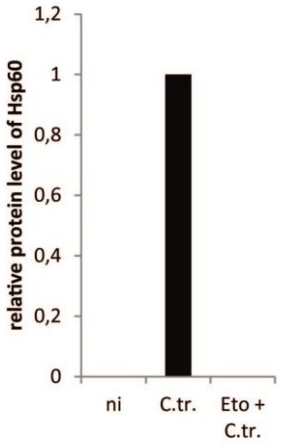


Figure 22 (A) *Chlamydia* induce downregulation of activated p53. Huvecs were treated with etoposide (50 µM) for 6 h to induce p53 stabilisation. Cells were washed with fresh medium to remove the inhibitor and subsequently infected with *C. trachomatis* (MOI 1, 30 h) or left uninfected. Cells were lysed at indicated time points and analysed by SDS-PAGE and western blotting. Membranes were probed with antibodies against p53 and chlamydial Hsp60. GAPDH was used as loading control. (B) High levels of p53 result in loss of chlamydial infectivity. HeLa229 cells were infected and treated as mentioned above. An infectivity assay was performed; for quantification chlamydial Hsp60 was normalised to GAPDH. ni: not infected; C.tr.: *C. trachomatis*; Eto: etoposide. This figure was first published in Siegl *et al*., 2014.

Etoposide and nutlin-3 treatment changes chlamydial inclusion formation

Taken together, the data so far revealed that after activation of p53 normal chlamydial inclusion formation was prevented, chlamydial growth, however, could still be detected by western blotting. To further investigate the changes during chlamydial development, immunostaining of infected cells was performed. For this, cells were incubated with the p53-activating drugs and at the same time infected with *C. trachomatis* for 24 h. Cells were fixed and stained as described in the methods section and analysed by confocal microscopy. Normal *Chlamydia* infection resulted in inclusion formation as shown in Figure 23. Chlamydial Hsp60 was visualized by Cy3-conjugated secondary antibody, nuclei were stained using Draq5. Etoposide-treatment did not interfere with inclusion formation, however, the size of inclusions was clearly reduced (Figure 23).

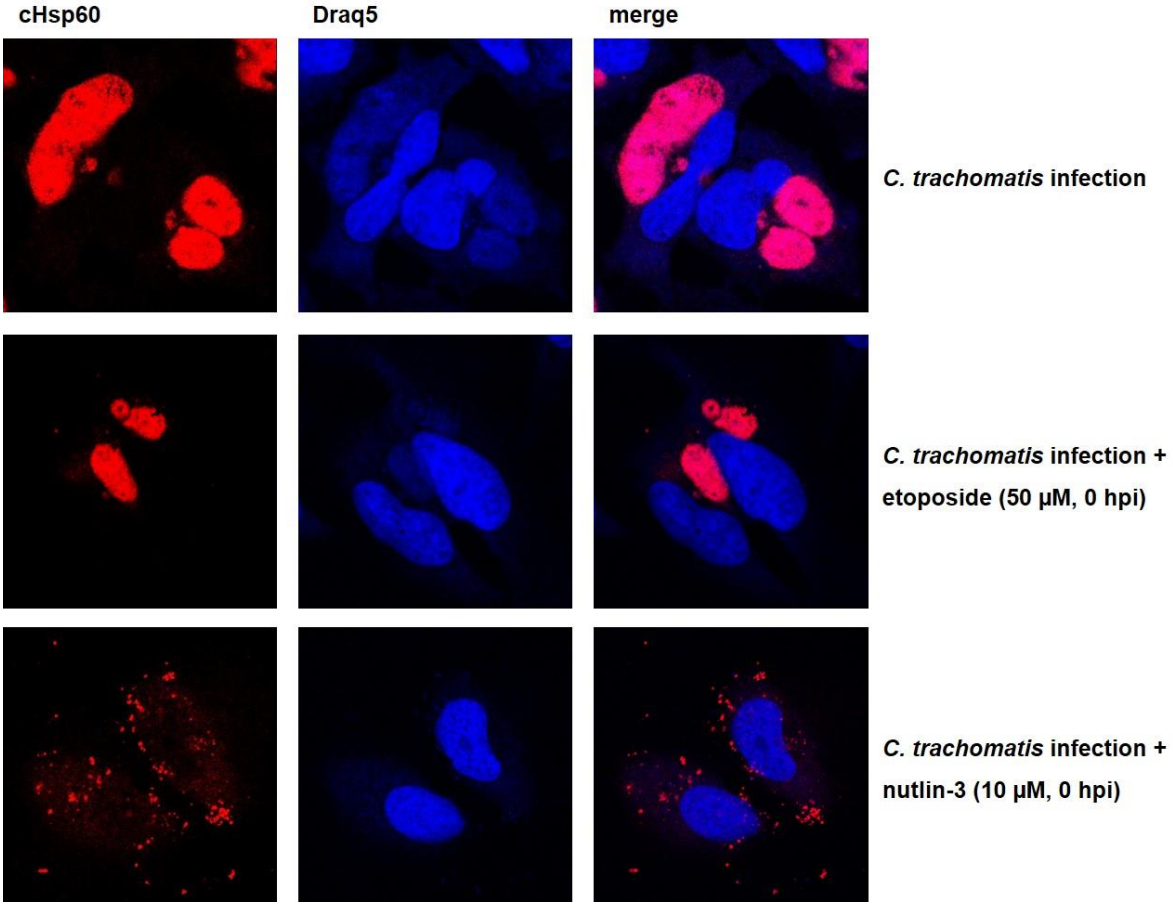


Figure 23 Upregulation of p53 results in impaired chlamydial inclusion formation. HeLa229 cells were treated with etoposide (50 µM, 0 hpi) and nutlin-3 (10 µM, 0 hpi) and infected with *C. trachomatis* (MOI 1, 24 h). Cells were fixed and stained with an antibody against chlamydial Hsp60 (cHsp60) and Draq5 to visualise nuclei and analysed by immunofluorescence.

The HDM2 inhibitor nutlin-3 resulted in disperse distribution of chlamydial particles after 24 h of infection. Its inhibitory action takes effect immediately and is detectable after 15 min (van Leeuwen *et al.*, 2011). Trafficking of *Chlamydia* to the perinuclear region and inclusion building in close proximity to the Golgi apparatus could not be detected. The dispersion of bacteria throughout the cytoplasm explains the lack of a visible inclusion.

As previously shown activation of p53 by etoposide requires several hours. To verify if growth phenotype of nutlin-3 can be reproduced by etoposide-activated p53 cells were pre-incubated with the DNA-damaging agent. Similar to nutlin-3 treatment activation of p53 by etoposide resulted in evenly distributed bacteria in the host cell after 12 h of infection (Figure 24). After 24 and 36 h of infection, several *Chlamydia* succeeded to aggregate and fuse to form small inclusions. Despite removal of the inhibitor, continued activation of p53 prevented a release of chlamydial growth inhibition even at late time points of infection.

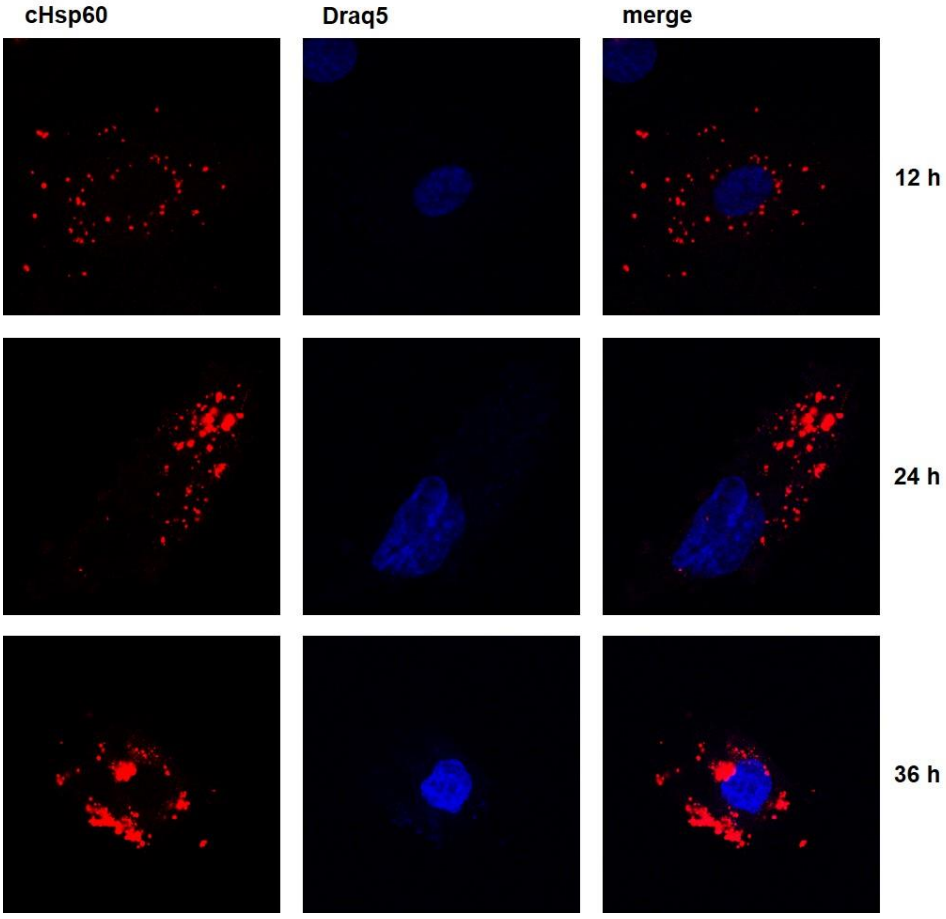


Figure 24 Upregulation of p53 results in impaired chlamydial inclusion formation. HeLa229 cells were pre-treated with etoposide (50 µM, 6 hpi) and subsequently infected with *C. trachomatis* (MOI 1) for the indicated time points. Cells were fixed and stained with an antibody against chlamydial Hsp60 (cHsp60) and Draq5 to visualise nuclei and analysed by immunofluorescence.

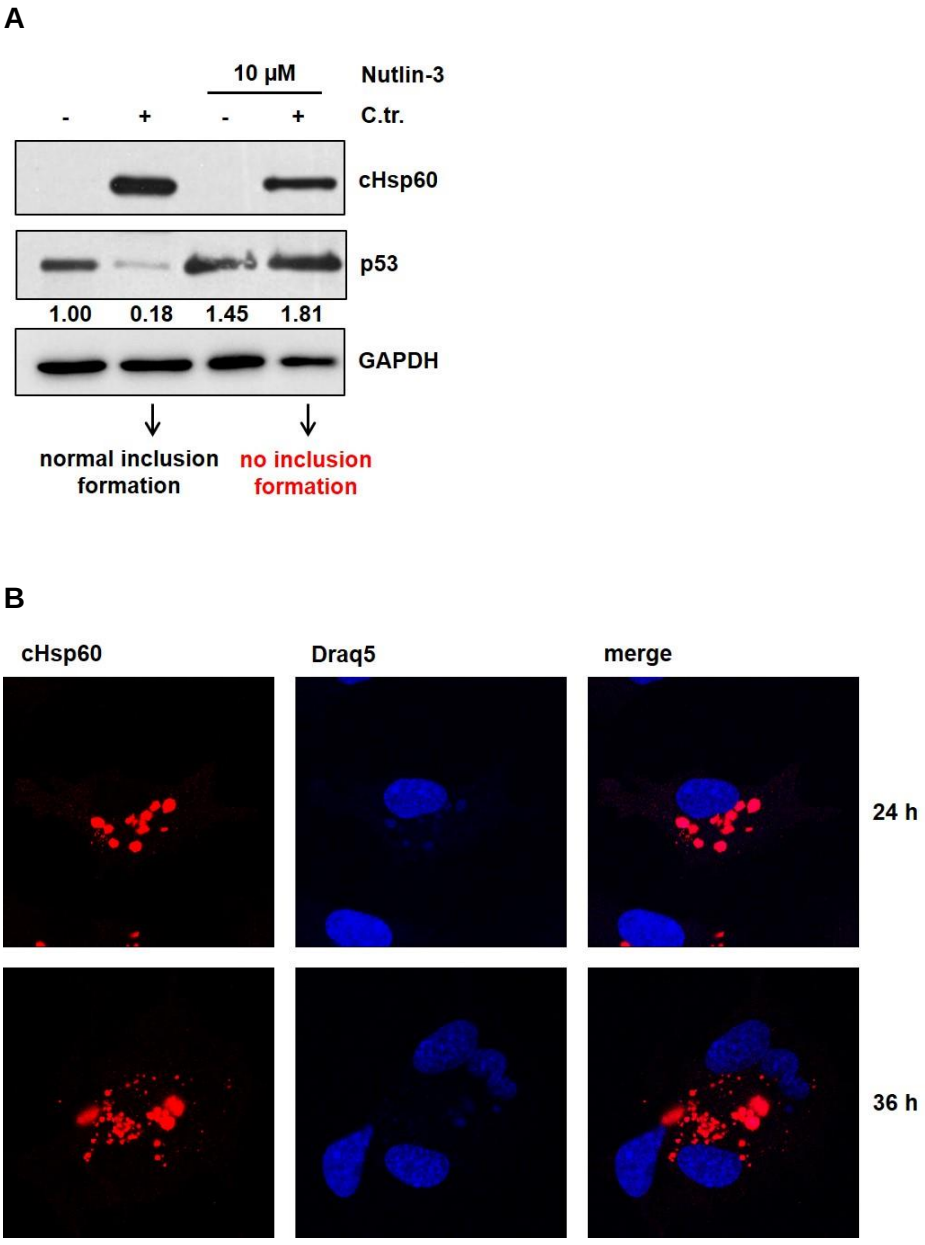


Figure 25 (A) Huvecs were treated with 10 µM nutlin-3 and infected with *C. trachomatis* (MOI 1, 24 h). Samples were analysed by light microscopy and subsequently subjected to SDS-PAGE and western blotting. Membranes were probed with antibodies against p53, chlamydial Hsp60 and GAPDH. Light microscopy revealed the absence of inclusion formation in nutlin-3 treated cells. (B) To show impaired inclusion formation after nutlin-3 treatment, cells infected with *C. trachomatis* (MOI 1) for the indicated time points were fixed and stained with an antibody against chlamydial Hsp60 (cHsp60) and Draq5 to visualise nuclei and analysed by immunofluorescence. C.tr.: *C. trachomatis*. Parts of this figure were first published in Siegl *et al*., 2014.

Chlamydial growth inhibition was further investigated in the presence of nutlin-3. The HDM2 inhibitor strongly stabilises protein amounts of p53 in cells as already shown during pathway analysis of p53 degradation (Figure 13). Huvecs were treated with 10 µM nutlin-3 or left untreated. After infecting cells with *C. trachomatis* for 24 h, a similar phenotype as after

etoposide-treatment became apparent. While untreated cells displayed chlamydial inclusion formation nutlin-3 incubation resulted in inhibition of normal chlamydial growth. Western blot analysis revealed reduced amount of bacterial growth compared to untreated cells (Figure 25A). Immunostaining was again performed to clarify developmental defects of *Chlamydia*. At 24 and 36 h after infection, *Chlamydia* were unable to form an inclusion (Figure 25B). As demonstrated after etoposide-treatment several small inclusions were built.

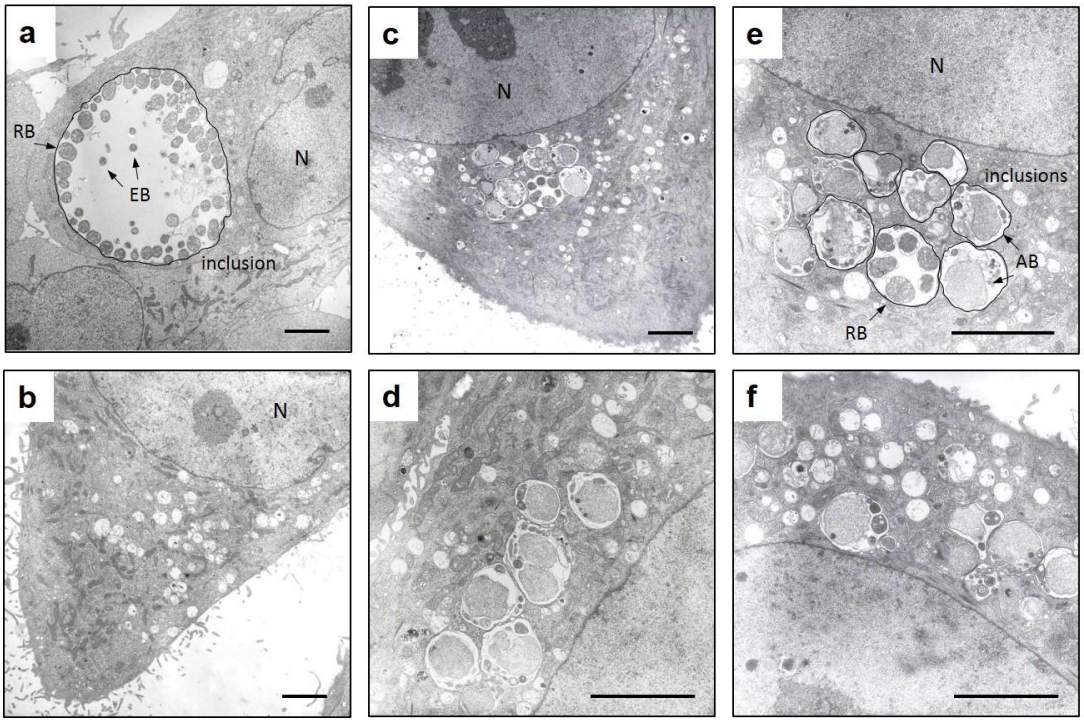


Figure 26 Transmission electron microscopy (TEM) was performed to investigate the ultrastructure of single dispersed inclusions. Huvecs were infected with *C. trachomatis* (MOI 1, 24 h) (a), treated with etoposide (50 µM, 24 h) (b) or treated with etoposide for 6 h followed by *C. trachomatis* infection (c, d, e, f) for 24 h. Panel (e) is a magnification of panel (c). Bars represent 2 µm at 3000x (a-c) and 7000x (d-f) magnification. RB: reticulate body; EB: elementary body; AB: aberrant body. Samples were fixed with a 2.5% glutaraldehyde solution and further processed as described in the methods section. Parts of this figure were first published in Siegl *et al*., 2014.

In order to specify the ultrastructure of dispersed bacteria and small inclusions during infection, transmission electron microscopy (TEM) was performed. Huvecs were treated with etoposide for 6 h and infected with *C. trachomatis* for 24 h. Samples were processed for TEM as described in the methods section. Figure 26 (a) shows a typical chlamydial inclusion containing mostly RBs, (b) shows cells treated with etoposide. Activation of p53 resulted in formation of several small inclusions, some containing only a single bacterium (Figure 26 (c), magnified in (e)). The morphology of several particles is similar to aberrant bodies, characteristic during chlamydial persistence (Figure 26 (d) and (f)). It has to be noted that *Chlamydia* are surrounded

by membranes and in close vicinity to each other suggesting inhibition of membrane fusion. Taken together our data demonstrate severe inhibition of chlamydial growth and inclusion formation after activation of the tumour suppressor p53.

4.2.2 Recovery of chlamydial growth after decrease of p53 levels

To further validate the role of p53 during chlamydial development reversal of growth inhibition was investigated. For this, elevated p53 amounts had to be decreased to normal levels. The formation of permanent double strand breaks after etoposide treatment prevents p53 downregulation due to a persisting cellular DDR. Opposed to this, nutlin-3 removal leads to an immediate return of p53 to normal levels. p53-mediated transcriptional activation of HDM2 caused by nutlin-3 facilitates rapid degradation of p53 after removal of the HDM2 inhibitor (van Leeuwen *et al.*, 2011).

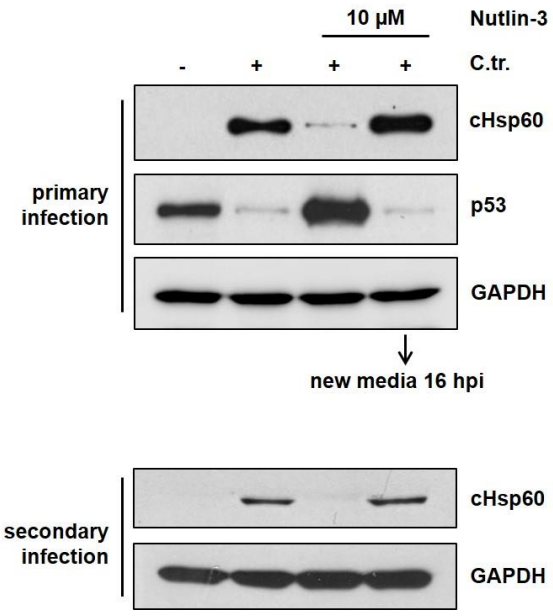


Figure 27 Chlamydial growth can be restored after removal of nutlin-3. Huvecs were treated with 10 µM nutlin-3 and infected with *C. trachomatis* (MOI 1, 48 h). After 16 h of infection, media was changed in one sample to remove nutlin-3 (lane 4). After 48 h of infection an infectivity assay was performed. Cells of the primary infection were lysed and supernatants were used to infect fresh cells. Quantification of chlamydial growth recovery was determined by SDS-PAGE and western blotting. This figure was first published in Siegl *et al*., 2014.

Huvecs were again treated with 10 µM nutlin-3 and infected with *C. trachomatis*. Control cells were not infected or only infected with *C. trachomatis*. To investigate the possibility of growth recovery, media of one sample both infected and nutlin-3-treated was replaced after 16 h. Cells were incubated for a time frame of 48 h followed by an infectivity assay. Lysates were

subsequently analysed by western blotting. While degradation of p53 could again be observed in *Chlamydia* infected control cells nutlin-3 led to a strong accumulation of p53 and reduced bacterial load in the primary infection (Figure 27). Removal of the p53-stabilising agent after 16 h led to recovery of *Chlamydia* and initiation of p53 degradation. Apparently, chlamydial development continued and resulted in successful infection of new cells. In summary, restoration of chlamydial growth and infectivity is possible after decreasing p53 to its normal level.

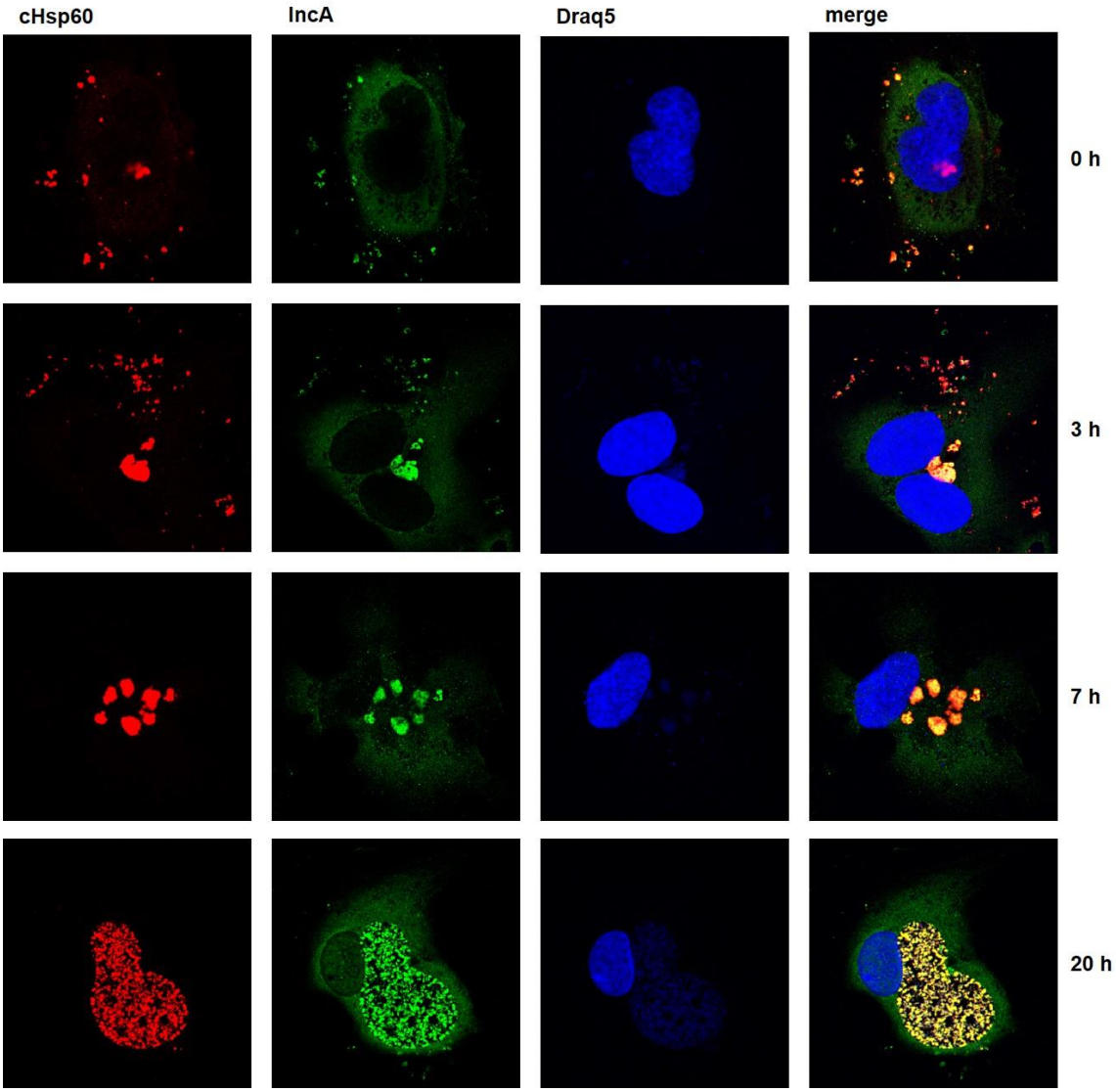


Figure 28 Immunofluorescence analysis of chlamydial inclusion recovery after removal of nutlin-3. Huvecs were treated with 10 µM nutlin-3 and infected with *C. trachomatis* (MOI 1, 16 h). After 16 h of infection, medium was replaced to remove nutlin-3. Cells were fixed at the indicated time points and subjected to immunostaining. *Chlamydia* were stained using antibodies against chlamydial Hsp60 and the inclusion protein IncA. Nuclei and chlamydial DNA were stained by Draq5. Parts of this figure were first published in Siegl *et al*., 2014.

To monitor restoration of chlamydial growth, we fixed samples at different time points after removing the inhibitor. Immunostaining was performed and *Chlamydia* were visualized with an antibody against chlamydial Hsp60 (secondary antibody Cy3-conjugated) and the chlamydial inclusion protein IncA (secondary antibody Cy2-conjugated). Nuclei were again stained with Draq5. As shown in Figure 28 incubation of cells with nutlin-3 prevented inclusion formation. Surprisingly, 3 h after fresh media was added to the cells *Chlamydia* appeared to aggregate next to the nucleus. 7 h after removing the inhibitor, the previously dispersed bacteria were contained within several small inclusions. In the end, restoration of a normal chlamydial inclusion was completed and apparently enabled finishing of the developmental cycle. This, in turn, led to a recovery of chlamydial infectivity (Figure 27).

4.2.3 Rescue of chlamydial growth in p53-deficient cells

So far, an inhibitory effect of stabilised p53 on bacterial growth could be established. By using two different p53-activating agents and demonstrating reversibility of the blocking effect, a decisive role of the tumour suppressor during chlamydial infections could be proven. To investigate if p53-activating chemicals cause any change in bacterial growth in cells deficient for p53, isogenic $p53^{+/+}$ and $p53^{-/-}$ HCT116 cell lines were used. Cells were treated with etoposide for 6 h, media was changed and cells were infected with *C. trachomatis* for 48 h.

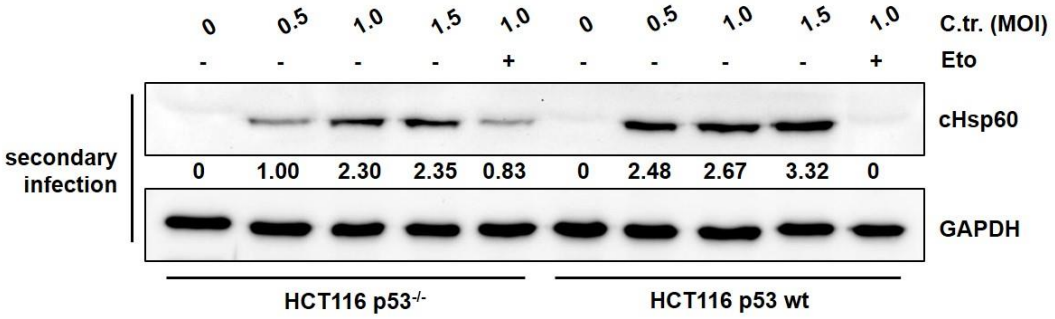


Figure 29 Infectivity of *Chlamydia* is partially restored in etoposide-treated HCT116 p53-/- cells. HCT116 p53 wt and HCT116 p53-/- cells were treated with etoposide (50 µM) for 6 h, followed by *C. trachomatis* (MOI 1, 48 h) infection. Chlamydial infectivity was quantified in an infectivity assay. C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

Subsequently, an infectivity assay gave indication about chlamydial progeny formation. As shown in Figure 29, chlamydial growth was strongly inhibited in $p53^{+/+}$ HCT116 cells after p53 activation. In $p53^{-/-}$ HCT116 cells chlamydial growth could be detected in the secondary infection. However, comparison of samples infected with MOI 1 revealed that after etoposide treatment only 36% of *Chlamydia* could be recovered compared to control cells (Figure 29).

Thus, on the one hand the experiment confirmed the inhibitory role of p53, on the other hand, a p53-independent aspect was revealed.

4.3 Regulation of cellular metabolism by p53 limits chlamydial growth

p53 is a transcription factor that restricts uncontrolled cell growth in response to DNA damage or oncogene activation. Thus, it is strongly involved in the regulation of apoptosis, senescence and cell cycle arrest. Aside from its relevance as a tumour suppressor, p53 was implicated to play a major role in regulation of metabolism. Its critical role in regulating glycolysis, the PPP and mitochondrial respiration becomes apparent considering the consequences of mutant p53 in tumour cells. Increased glucose uptake, aerobic glycolysis, enhanced flux of the PPP and altered fatty acid and glutamine metabolism are characteristic for cancer cells. In line with the dependence on host energy, *Chlamydia* was reported to induce increased expression of glucose transporters and enhanced glucose consumption (Ojcius *et al.*, 1998). As negative regulation of glucose transporters by p53 was demonstrated previously, infection-induced degradation of the tumour suppressor and subsequent alterations in metabolism could thus be important for chlamydial growth.

4.3.1 Importance of glycolysis and PPP for *Chlamydia*

p53 promotes negative regulation of glycolysis and PPP and promotes oxidative phosphorylation to ensure efficient glucose consumption and energy production. Glycolysis is inhibited by repression of Glut 1, 3 and 4 and negative regulation of protein stability of the glycolytic enzyme PGM. In addition, TIGAR, a negative regulator of glycolysis, is transcriptionally induced by the tumour suppressor. The repression of glycolysis on the one hand promotes flux through the PPP, on the other hand p53 directly blocks activity of G6PD, as increased activity of the PPP is advantageous for cancer development. To investigate if p53-mediated negative regulation of these metabolic pathways induces defects in chlamydial inclusion formation and infectivity, two common inhibitors of glycolysis and PPP were applied (Pelicano *et al.*, 2006). 6-aminonicotinamide (6-AN) blocks the PPP at the level of 6-phosphogluconate dehydrogenase (Ki = 0.46 M). The enzyme catalyses the formation of 6-aminonicotinamide adenine dinucleotide phosphate (6-ANADP) which leads to a decrease in NADPH production (Koutcher *et al.*, 1996). 2-deoxyglucose (2-DG) acts as a competitive inhibitor of glycolysis and is phosphorylated by hexokinase (Ki = 0.3 mM) to 2-deoxyglucose-phosphate (2-DG-P) which cannot be further metabolized by phosphoglucose isomerase (PGI) to fructose-6-phosphate. The accumulating glucose-6-phosphate is channelled through the PPP, demonstrated by increased concentrations of PPP metabolites (Urakami *et al.*, 2013).

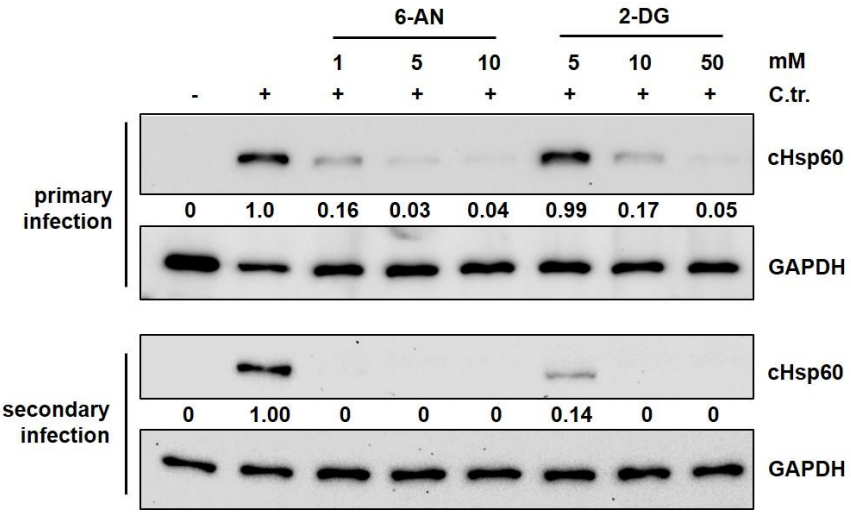


Figure 30 Huvecs were treated for 1 h with 6-aminonicotinamide (6-AN) and 2-deoxyglucose (2-DG) in different concentrations followed by *C. trachomatis* L2 infection (MOI 1, 48 h). An infectivity assay was performed and chlamydial development was monitored in secondary infection. Samples were subjected to SDS-PAGE and western blot analysis and chlamydial infectivity was determined using antibodies against chlamydial Hsp60. C.tr.: *C. trachomatis*.

Huvecs were treated with 6-AN and 2-DG in different concentrations followed by infection with *C. trachomatis* for 48 h. Progeny was again quantified by an infectivity assay. As shown in Figure 30, blocking of PPP results in severe complications for chlamydial growth in the primary infection. At low concentrations of the inhibitor, formation of small inclusions with actively replicating bacteria was still possible while higher concentrations led to chlamydial persistence. These observations are reflected in the secondary infection: Despite the presence of infectious chlamydial particles after treatment with 1 mM 6-AN, formation of progeny fell below the detection limit. Persistence also led to a loss of infectivity. Treatment of 2-DG allowed chlamydial growth at low concentrations (Figure 30). Even though the inhibitor constant of 2-DG is much lower than that of 6-AN, higher levels of the inhibitor are needed to achieve blocking of chlamydial growth, emphasising the more important role of the PPP. Nonetheless, inhibition of glycolysis with higher concentrations of 2-DG resulted in complete loss of chlamydial growth in the secondary infection.

In tumorigenic cells, reprogramming of metabolism and higher rates of glycolysis ensure sufficient energy production. This is primarily caused my mutations or loss of the gene encoding p53. The tumour suppressor was reported to downregulate Glut 1 and Glut 4 (Schwartzenberg-Bar-Yoseph *et al.*, 2004) and indirectly Glut 3, through regulation of the IKK-NFκB-pathway (Kawauchi *et al.*, 2008). As *Chlamydia* are dependent on host energy and mainly glucose-6-phosphate as carbon source, we analysed expression of Glut 1 and Glut 4 during *Chlamydia* infection and in the presence of the p53-activating reagent etoposide.

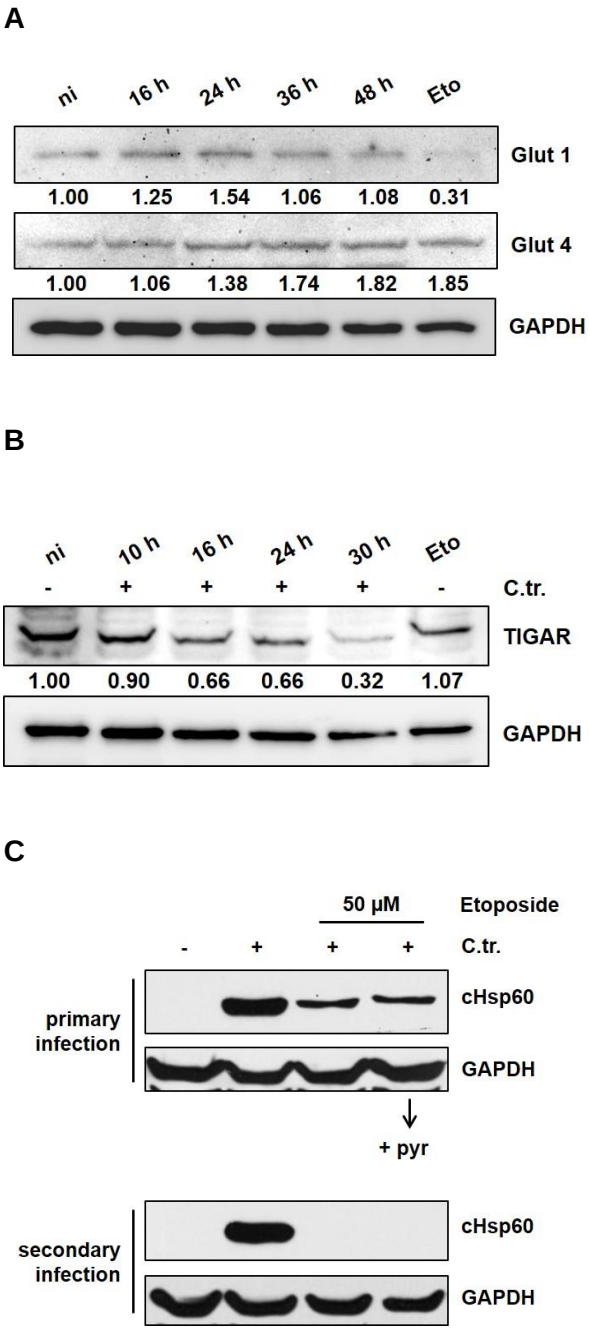


Figure 31 (A) Regulation of glucose transporters. Huvecs were infected with *C. trachomatis* (MOI 1) for several time points. Control cells were treated with etoposide (50 µM, 6 h). Glut 1 and Glut 4 were detected by immunoblotting. (B) TIGAR is downregulated during *Chlamydia* infection. Protein amount of TIGAR was monitored in Huvecs infected for different time points. (C) Supplementation with the glycolytic metabolite pyruvate does not rescue chlamydial growth. Huvecs were pre-treated with etoposide (50 µM, 6 h) and supplemented with 100 µM pyruvate (pyr). Subsequently, cells were infected with *C. trachomatis* (MOI 1, 48 h) and an infectivity assay was performed. Samples were analysed by SDS-PAGE and western blotting. GAPDH was used as loading control. ni: not infected; C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

Glut 1 expression was increased in *Chlamydia* infected cells after 24 h by 54% compared to uninfected cells (Figure 31A). However, levels dropped down to that of uninfected control cells during late stages of infection. Glut 4 expression was increased after *Chlamydia* infection, as well. After etoposide treatment Glut 1 expression was strongly decreased. In contrast to this, protein level of Glut 4 was increased after etoposide treatment (Figure 31A).

Aside from glucose transporters, p53 regulates expression of TIGAR to limit glucose flow through glycolysis. TIGAR negatively influences glycolysis by lowering levels of fructose-2,6-bisphosphate. During *Chlamydia* infections, we observed strong downregulation of TIGAR (Figure 31B). As TIGAR expression is directly dependent on transcriptional activity of p53, this result is in line with infection-induced degradation of the tumour suppressor. As expected, we observed an increase of TIGAR expression after etoposide treatment. However, cells expressing TIGAR display increased metabolic flow through the PPP, higher NADPH levels and are able to reduce oxidative stress efficiently (Bensaad *et al.*, 2006). As these conditions are assumed to be beneficial for chlamydial growth, downregulation of TIGAR is possibly a side-effect of infection and *Chlamydia*-induced degradation of p53. In addition, downregulation of TIGAR leads to enhanced glycolytic flow. To assess if glycolysis is the metabolic pathway preferred by *Chlamydia* and responsible for the growth inhibition after p53-activation, we supplemented infected cells with the glycolytic end product pyruvate. Thus, after inducing DNA damage with etoposide and adding 100 µM pyruvate, we infected cells with *C. trachomatis* and performed an infectivity assay after 48 h of infection. Primary infection showed a reduction of chlamydial growth after etoposide treatment (Figure 31C). Supplementation of pyruvate did not improve bacterial growth. As expected, there was no chlamydial progeny formation in the secondary infection. Consequently, compensation of reduced glycolytic flow caused by activated p53 was not enough to restore chlamydial growth and infectivity.

4.3.2 Cancer-associated p53 mutants do not inhibit chlamydial growth

Another target of p53-mediated metabolic control is the pentose phosphate pathway (PPP). Its inhibitory effect is concentrated on the first enzyme of the PPP, the glucose-6-P-dehydrogenase (G6PD). The PPP diverges glucose from glycolysis for the production of NADPH and ribose-5-phosphate. NADPH is necessary for the reduction of glutathione, scavenging of free oxygen radicals during DNA damage and for lipid biosynthesis. Ribose-5-phosphate on the other hand is the precursor of nucleotides particularly important for the cellular DDR or proliferating cells (Gottlieb, 2011). Direct protein-protein interactions between p53 and the PPP-enzyme G6PD are responsible for its inhibition. Jiang *et al.* demonstrated that p53 induces a conformational change in G6PD that prevents formation of an active dimer even after removal of the tumour suppressor (Jiang *et al.*, 2011).

Increased G6PD activity in p53 mutant cells and after *Chlamydia* infection

Blocking of the PPP reduces NADPH amounts and generation of ribose-5-phosphate for nucleotide synthesis. As *Chlamydia* are not capable of synthesising purine and pyrimidine nucleotides *de novo* (Tipples *et al.*, 1993) and were shown to deplete cellular NADPH pools (Prusty *et al.*, 2012), growth deficits as observed in our experiments are not unexpected. To further analyse this metabolic pathway, activity of the PPP-enzyme G6PD was measured during *Chlamydia* infection in wild type and mutant p53 cells. Moreover, implications of activated p53 on G6PD were investigated. For this, $p53^{-/-}$ H1299, H1299 transfected with HA-tagged p53 and mutant p53 H1299 cells were infected with *C. trachomatis* for 24 h (Muller *et al.*, 2009). Samples were prepared and G6PD activity was measured as described previously.

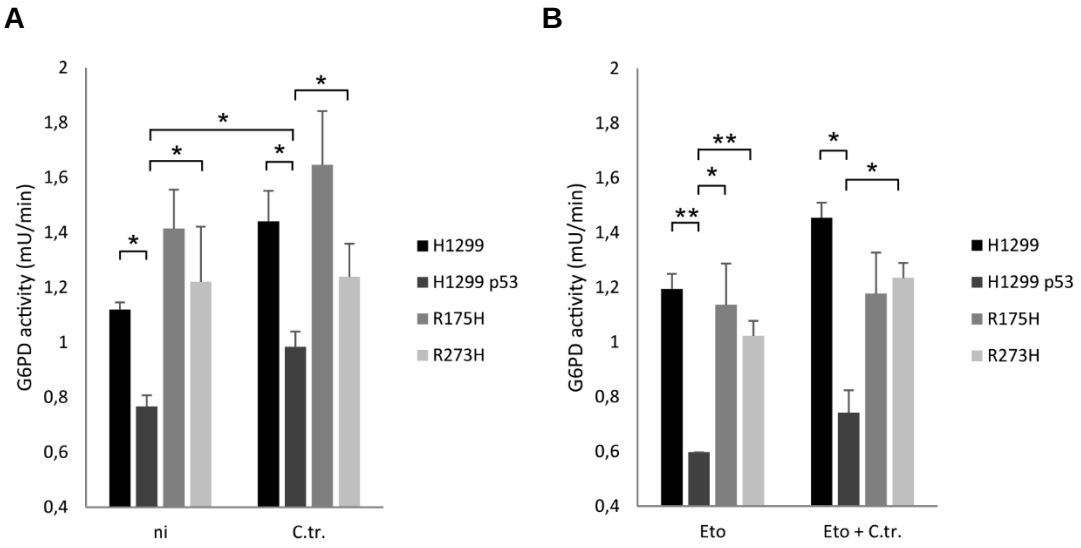


Figure 32 G6PD activity after *Chlamydia* infection and etoposide treatment in $p53^{+/+}$, $p53^{-/-}$ and p53 mutant cells. (A) H1299, H1299 transfected with HA-tagged p53 and p53 R175H and R273H H1299 cells were infected with *C. trachomatis* L2 (MOI 1, 24 h) or (B) pre-treated with etoposide (50 µM) for 6 h followed by *Chlamydia* infection. Samples were processed and G6PD activity was measured as described above. The graph shows mean values ± SEM of two experiments performed in triplicates. p < 0.05: *; p < 0.01: **. ni: not infected; C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

p53 deficiency resulted in a significant increase in G6PD activity in non-infected H1299 cells compared to cells overexpressing wild type p53 (Figure 32A). G6PD activity in H1299 cells was increased by 46%. Mutant p53 led to similar results: p53 R175H cells showed an increase of 84% enzyme activity, p53 R273H cells an increase of 59%. Intriguingly, *Chlamydia* infection led to a significant increase of G6PD activity in p53 overexpressing cells. Treatment of p53 overexpressing cells with etoposide caused 22% reduction of enzyme activity (Figure 32B), compared to non-infected cells, in p53 deficient and mutant cell lines enzyme activity was not

significantly altered. The increase in enzyme activity observed in non-infected cells is even more pronounced in etoposide-treated cells. p53 overexpressing H1299 display 50% less enzyme activity than the p53 deficient cell line (Figure 32B).

Overexpression of p53 impairs chlamydial infectivity

Analysis of infection in $p53^{-/-}$ cells strengthened our discovery of the crucial role of the tumour suppressor during chlamydial growth. To further support these findings, HA-tagged wild type p53 was transiently overexpressed in $p53^{-/-}$ H1299 cells and chlamydial growth was monitored. Depending on the DNA concentration used for transfection, a strong inhibitory effect of chlamydial growth could be observed (Figure 33). While transfection of 0.1 µg of HA-tagged p53 allowed formation of small inclusions and led to a moderate reduction of 15% in the secondary infection, 1.0 and 1.5 µg DNA almost completely abrogated chlamydial infectivity. Surprisingly *Chlamydia* infection did not induce degradation of overexpressed p53. Taken together, in addition to inhibitor treatment leading to activation of the tumour suppressor overexpression of wild type p53 provided further evidence for its direct role in the suppression of chlamydial growth.

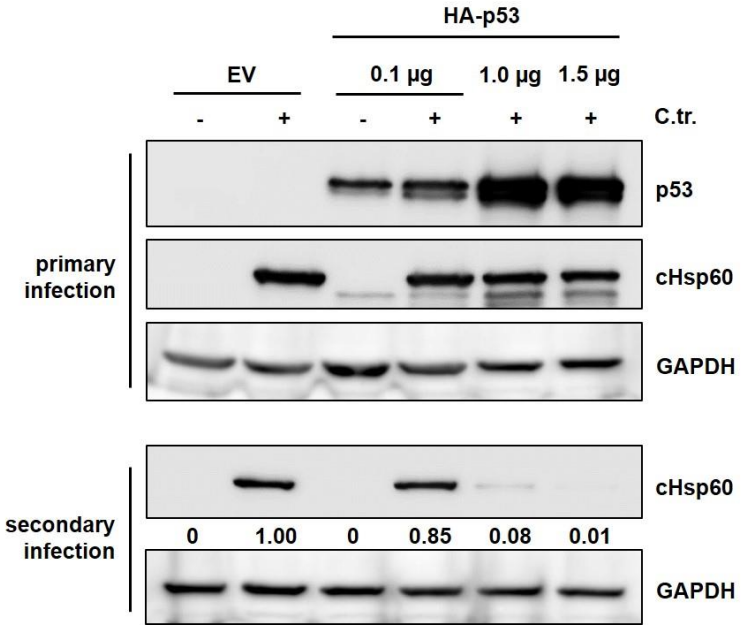


Figure 33 Overexpression of p53 inhibits chlamydial development. H1299 cells were transiently transfected with HA-tagged wild type p53 construct. After 24 h, cells were infected with *C. trachomatis* (MOI 1, 48 h). Chlamydial development was monitored in primary and secondary infection by quantification of chlamydial Hsp60. C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

Cancer-associated p53 mutants do not inhibit chlamydial growth

Two tumour-associated p53 mutants, R175H and R273H, were studied to further investigate the inhibitory effect of the tumour suppressor on *Chlamydia* (Petitjean *et al.*, 2007). Both hot-spot mutations result in defective DNA binding activity to responsive elements of p53 target genes. Thus, the tumour suppressor activity of the protein is lost. Both variants of p53 are stably expressed in the p53 deficient cell line H1299, originating from a human non-small-cell carcinoma. As published by Jiang *et al*., G6PD activity was not inhibited by mutant p53 in these cell lines, although p53 R175H and R273H were still able to bind the enzyme (Jiang *et al.*, 2011).

Infection of $p53^{-/-}$ H1299 cells, with HA-tagged p53 transfected H1299 and both mutant p53 H1299 cell lines was carried out after pre-incubation of cell lines with etoposide for 6 h. Interestingly, infection of $p53^{-/-}$ H1299 resulted in recovery of 25% *Chlamydia* in the secondary infection, p53 mutant H1299 cells allowed 38% and 34% chlamydial growth, respectively (Figure 34). As control, H1299 cells transfected with wild type p53 were treated as mentioned above. Etoposide-induced p53 again led to an almost complete loss of chlamydial infectivity. As already observed after transfection of wild type p53 *Chlamydia* infection did not result in the previously monitored degradation of the tumour suppressor.

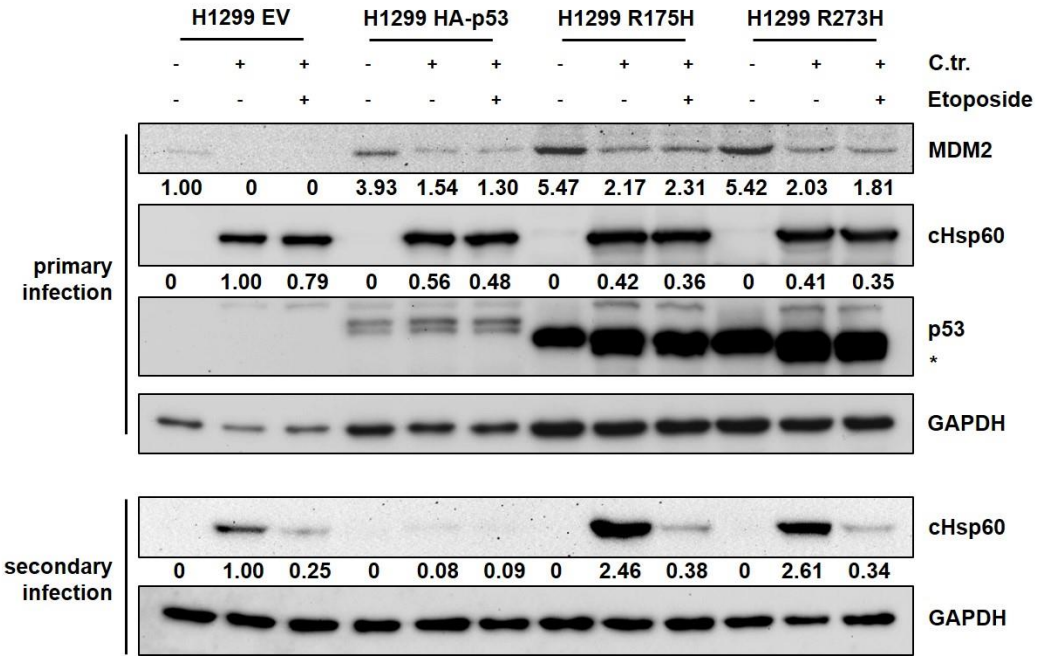


Figure 34 Recovery of chlamydial growth in p53 mutant cells. H1299 ($p53^{-/-}$), H1299 transfected with HA-tagged p53, H1299 R175H and H1299 R273H cells were treated with etoposide (50 µM) for 6 h, followed by *C. trachomatis* infection. Primary and secondary infections were analysed by SDS-PAGE and western blotting. GAPDH was used as loading control. (*) indicates cleavage products. C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

However, opposed to infection of wild type p53 overexpressing cells mutant p53 H1299 cells displayed cleavage products after *Chlamydia* infection. Most p53 mutants are resistant to cellular degradation mechanisms, hot-spot mutations R175H and R273H, however, remained sensitive to HDM2-ubiquitin-mediated degradation (Asher *et al.*, 2003). If degradation was mediated by the proteasome, calpains or by another mechanism remains unclear. The results are in line with experiments using $p53^{-/-}$ HCT116 cells: a p53-deficient background allows chlamydial growth after etoposide-treatment and restores infectivity to some extent. As both p53 mutants lost their transactivation activity as well as their inhibitory function against G6PD in the cytoplasm (Jiang *et al.*, 2011) and as it was already published that silencing of G6PD results in partial induction of chlamydial persistence (Prusty *et al.*, 2012) the influence of the tumour suppressor on metabolic control and its consequences for chlamydial development was further investigated.

4.3.3 Overexpression of G6PD rescues chlamydial growth

G6PD-overexpressing cells display significantly increased levels of reduced glutathione (GSH) and decreased levels of ROS. In addition, cells are strongly protected against oxidants-mediated cell death (Salvemini *et al.*, 1999). An increased enzyme activity of G6PD was observed during *C. trachomatis* infection in our experiments. Moreover, inhibition of PPP abrogated chlamydial growth. To assess the rescuing potential of overexpressed G6PD, HeLa229 were transiently transfected with the construct pCMV6-Myc-DDK-G6PD and empty vector control. Activation of p53 was again achieved by treatment with etoposide for 6 h. As assessed by an infectivity assay chlamydial growth could be recovered by G6PD-overexpression. Transfection of 0.5 µg pCMV6-Myc-DDK-G6PD did not result in rescue of chlamydial growth, yet. However, 1 µg of the construct lead to increased chlamydial growth in the primary infection and 16% growth recovery in the secondary infection (Figure 35A).

Similar experiments were conducted in Huvecs using nutlin-3 to stabilise p53. Chlamydial growth and infectivity was assessed by infectivity assay and western blot analysis. Growth recovery of 20% after transfection of 0.5 µg and 60% after transfection of 1.0 µg G6PD was monitored when compared to empty vector control (Figure 35B). In accordance with previous results G6PD overexpression substantially rescued chlamydial growth. Abundance of G6PD is thought to overcome the inhibitory function of p53 on the PPP. Chlamydial growth however was not entirely recovered suggesting secondary effects of p53-activation or p53-independent implications. To test this hypothesis, overexpression of G6PD was conducted in the $p53^{-/-}$ cell line H1299. In concordance with previous observations p53 deficiency partially allowed chlamydial growth in cells stimulated with the DNA-damaging agent etoposide. Intriguingly, overexpression of G6PD triggered a surge in chlamydial growth and subsequent infectivity.

Thus, the p53 null background rescued 20% of infectious *Chlamydia* compared to control infection and G6PD overexpression further increased it to 67% (Figure 36).

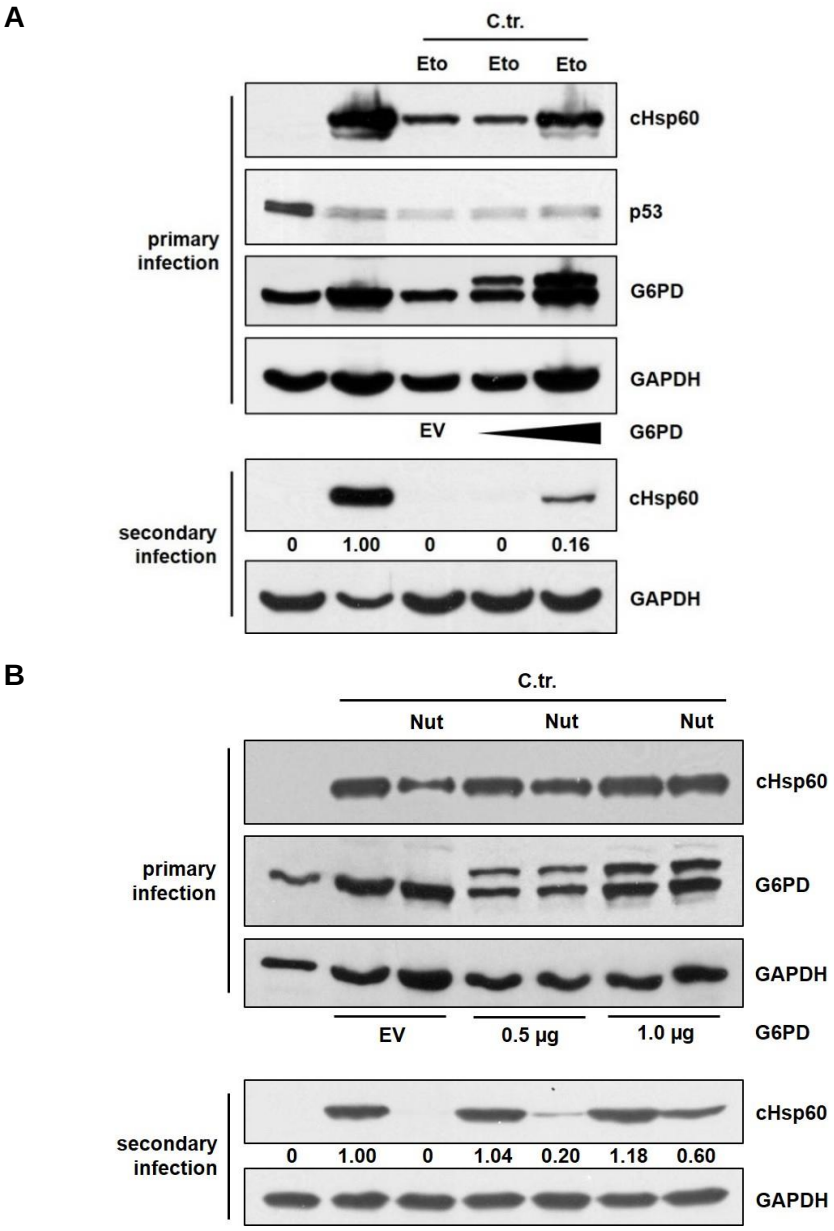


Figure 35 (A) G6PD overexpression rescues chlamydial infectivity in etoposide-treated HeLa229 cells. HeLa229 cells were transiently transfected with a Myc-DDK-tagged G6PD construct. After 24 h cells were treated with etoposide (50 µM) for 6 h and infected with *C. trachomatis* followed by an infectivity assay. (B) G6PD overexpression rescues chlamydial infectivity in nutlin-3-treated Huvecs. Huvecs were transfected with Myc-DDK-tagged G6PD construct. After 24 h cells were treated with nutlin-3 (10 µM) and infected with *C. trachomatis* for 48 h. G6PD overexpression was verified using an antibody against G6PD. Chlamydial infectivity was quantified using chlamydial Hsp60 antibody. GAPDH was used as loading control. C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

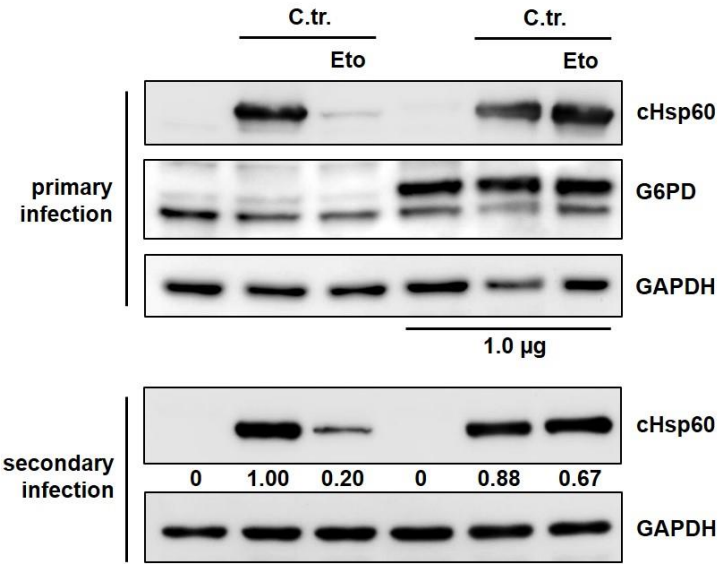


Figure 36 G6PD overexpression rescues chlamydial infectivity in etoposide-treated H1299 cells. H1299 cells were transiently transfected with Myc-DDK-tagged G6PD, treated with etoposide and infected with *C. trachomatis* (MOI 1, 48 h) and subsequently analysed for chlamydial infectivity. C.tr.: *C. trachomatis*. This figure was first published in Siegl *et al*., 2014.

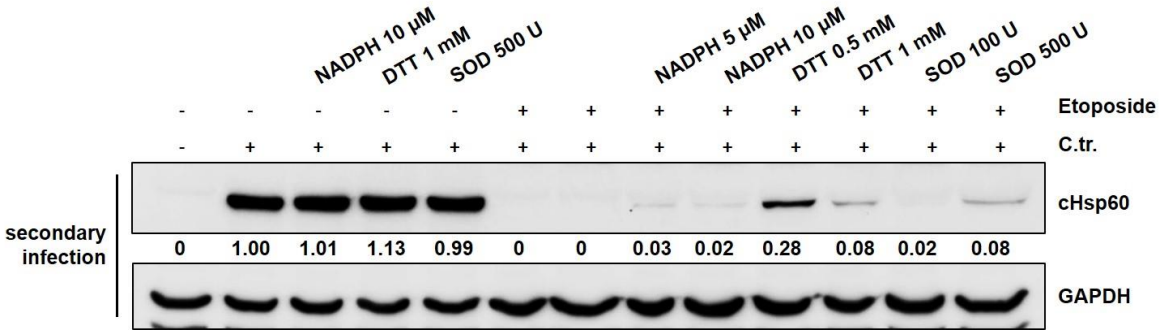
4.3.4 Relevance of oxidative stress and senescence-induction

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, like hydroxyl radicals (·OH), hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-). ROS formation occurs during oxidative phosphorylation in mitochondria and is essential for cell signalling and homeostasis. After UV irradiation or heat exposure, strong elevation of ROS levels potentially results in oxidative stress for the cell. Enzymes like catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) catalyse removal of ROS. As already mentioned, NADPH, mainly generated in the PPP, acts as an important cofactor for glutathione reductase, the enzyme responsible for generating monomeric reduced glutathione (GSH) which is in turn necessary for the conversion of H_2O_2 into H_2O.

The DNA DSB-inducing agent etoposide was described to cause production of ROS (Dumay *et al.*, 2006) triggering p53-mediated loss of mitochondrial membrane potential and apoptosis. For nutlin-3 varying reports are published: evidence was given that nutlin-3 mediates ROS production dependent on mitochondrial localization of p53 (Lee *et al.*, 2013). Nelson *et al.* demonstrated a lack of ROS generation after activation of p53 by nutlin-3 (Nelson *et al.*, 2012). Contribution of cellular ROS to chlamydial growth inhibition was evaluated by removing the toxic molecules with common anti-oxidants and inhibitors of NADPH oxidase, as well as supplementation with the reducing agent NADPH. During initial stages of *Chlamydia* infection low ROS levels are required for bacterial growth (Boncompain *et al.*, 2010). To exclude growth

inhibiting side-effects ROS-scavenging compounds were added to *Chlamydia* control infections. p53-activation was achieved by either etoposide (Figure 37A) or nutlin-3 (Figure 37B). Chlamydial recovery was assessed by performing infectivity assays. Supplementation of cell media with NADPH (10 µM), DTT (1 mM) or SOD (500U) did not delay or impair chlamydial growth. Addition of NADPH and SOD did not improve inclusion formation and infectivity. 0.5 mM DTT on the other hand, being a strong reducing agent protecting thiol groups from oxidation, rescued 28% of *Chlamydia*.

A



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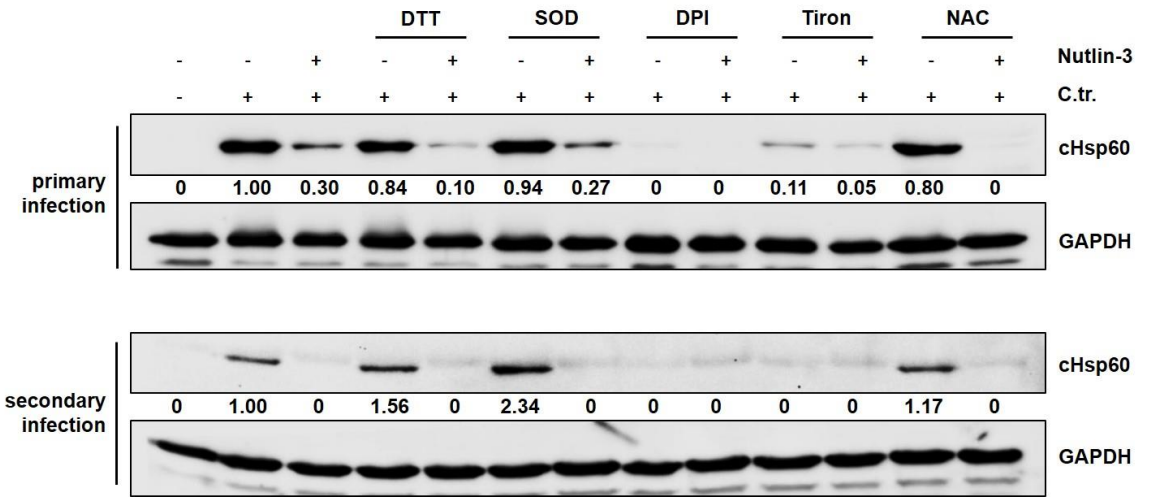

Figure 37 Scavenging of reactive oxygen species (ROS) partially rescues chlamydial infectivity. (A) HeLa229 were treated with etoposide for 6 h. Subsequently, the ROS removal agents NADPH, DTT and SOD were added and cells were infected with *C. trachomatis* for 48 h, followed by an infectivity assay. (B) HeLa229 were treated with nutlin-3 for 1 h. Subsequently, the ROS removal agents DTT, SOD, DPI, Tiron and NAC were added and further processed as mentioned above. All samples were analysed by SDS-PAGE and western blotting. C.tr.: *C. trachomatis*. Parts of this figure were first published in Siegl *et al*., 2014.

The ROS scavenger DPI (diphenyleneiodonium) applied by Chumduri *et al*. for removal of *Chlamydia*-induced ROS (Chumduri *et al.*, 2013) had a strong toxic effect on *Chlamydia* in the primary infection. DPI is generally applied as an inhibitor against NADPH oxidase an important cellular ROS generator. Li *et al*. described a general block of flavoenzymes (Li *et al.*, 1998). DPI was also demonstrated to activate p53 and induce its expression (Park *et al.*, 2007). Tiron and the free radical scavenger NAC (N-Acetyl-L-cysteine) had similar growth limiting effects on *Chlamydia*. With the exception of DTT anti-oxidant treatment did not significantly enhance chlamydial infectivity.

DNA damage and formation of ROS observed after *Chlamydia* infection could initiate induction of cellular senescence. Depending on the severity of damage, p53 triggered by the cellular DDR transcriptionally activates p21, one of the main mediators of cellular arrest. Nutlin-3 and etoposide treatment cause p53-mediated induction of cellular senescence (te Poele *et al.*, 2002, Efeyan *et al.*, 2007). Induction of cellular senescence was investigated by performing senescence-associated β-galactosidase staining of *C. trachomatis*-infected cells. Additionally, senescence-induction after G6PD overexpression and after activation of p53 was of interest. *Chlamydia* infected cells were negative for SA-β-gal staining as well as uninfected control cells. No difference was apparent in G6PD-overexpressing infected cells compared to control cells. Activation of p53 with etoposide and nutlin-3 and infection with *C. trachomatis* however resulted in strong positive β-gal staining in empty vector control cells. Overexpression of G6PD efficiently prevented induction of cellular senescence (Figure 38).

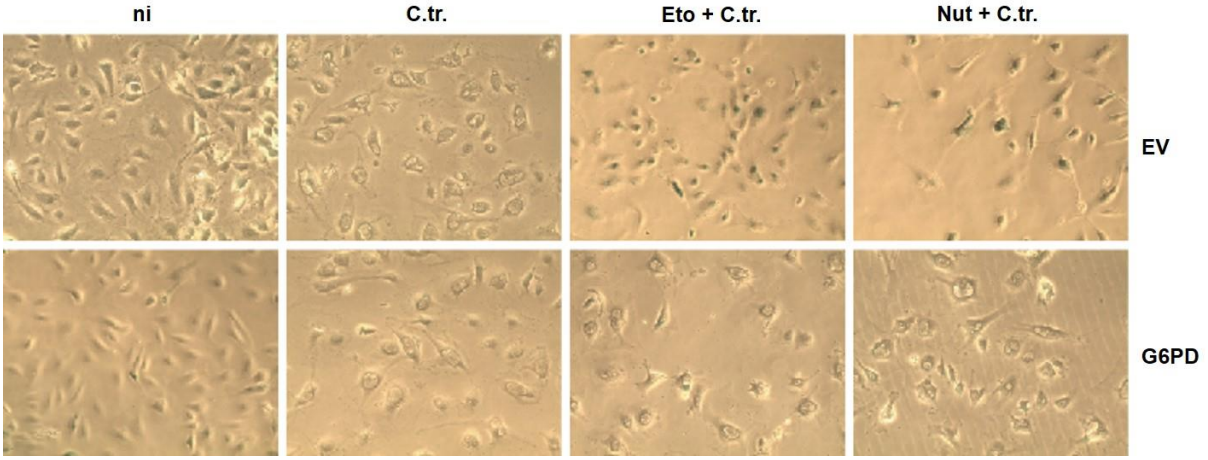

Figure 38 Senescence-associated β-galactosidase staining of *Chlamydia*-infected cells. Huvecs were transiently transfected with Myc-DDK-tagged G6PD or empty vector. After 24 h, cellular p53 was activated using 50 µM etoposide and 10 µM nutlin-3, respectively, followed by *C. trachomatis* infection (MOI 1, 24 h). Cells were subsequently fixed and stained as described in the methods section. For analysis of SA-β-gal staining light microscopy was performed. ni: not infected; C.tr.: *C. trachomatis*.

5 Discussion

Bacterial infections of humans are diverse and are caused by extracellular, facultative intracellular or obligate intracellular pathogens. To overcome both innate and acquired immunity extracellular and intracellular pathogens apply a number of regulatory mechanisms effectively mounting an anti-immunity response in the host. Bacterial capsules or secretion of virulence factors of extracellular pathogens are effective methods avoiding phagocytosis or activation of complement. Intracellular organisms have developed the capacity to invade and replicate in host cells such as endothelial and epithelial cells concealed from the immune system. The host cell provides a protected environment suitable for multiplication as well as constant supply of metabolites and energy. Thereby the pathogen has to circumvent the immune system and avoid induction of apoptosis of the host cell.

Chlamydia infection is the most common bacterial STD in the world. The pathogen efficiently protects its host from apoptosis in response to intracellular stress and prevents recognition of infected cells by lymphocytes (Zhong *et al.*, 1999, Zhong *et al.*, 2000). Even though the immune system recognises *Chlamydia* infection and controls it through IFNγ secretion, induction of persistence and inefficient clearance often lead to chronic infections and long-term damage. Like other intracellular living bacteria *Chlamydia* depends on successful completion of its life cycle. Due to the strong reduction of the genome the pathogen is dependent on nutritional supply and energy of the host. In addition, recently published data presented evidence that *Chlamydia* belongs to the group of genotoxicity-inducing pathogens (Chumduri *et al.*, 2013). These findings were previously observed in microorganisms like *Shigella*, *Neisseria* and *Helicobacter* which were reported to induce DNA damage triggered by increased ROS production (Wei *et al.*, 2010, Vielfort *et al.*, 2013, Bergounioux *et al.*, 2012). As a consequence of oxidative stress, cells exhibit DNA fragmentation, DSBs or telomere shortening (Evans *et al.*, 2004, Kawanishi *et al.*, 2004). As irreparable DNA damage is recognized and triggers signalling pathways leading to cell cycle arrest, senescence or apoptosis, *Chlamydia* employs several strategies to manipulate the host cell with the aim of promoting growth and suppressing host cell defence mechanisms. Apoptosis is blocked by *Chlamydia* at the level of mitochondria through degradation of BH3-only proteins and upregulation of anti-apoptotic proteins like Mcl-1 and cIAP-2 (Fan *et al.*, 1998, Fischer *et al.*, 2004, Rajalingam *et al.*, 2006, Rajalingam *et al.*, 2008). In addition, *Chlamydia* induces pro-survival pathways like PI3K- and ERK-signalling (Rajalingam *et al.*, 2008, Verbeke *et al.*, 2006).

Within this thesis differences between *Chlamydia* infections of human and mouse cells were revealed. Strong cytotoxicity prevented efficient infection of mouse cells. In the course of these experiments a central role of the tumour suppressor p53 was disclosed. *C. trachomatis* and

other chlamydial species initiated degradation of p53 by activation of the PI3K-Akt signalling cascade in human cells (Figure 39, left panel). Moreover, a strong anti-bacterial effect of the tumour suppressor was detected. Activation of p53 by pharmacological inhibitors and overexpression of the tumour suppressor led to severe growth inhibition of *Chlamydia* characterised by defective inclusion formation and loss of infectivity. These results were further strengthened by partial recovery of chlamydial growth in p53 knockout and mutant cells. Analysis of glycolytic and pentose phosphate pathway was conducted to investigate the growth inhibition. A potential correlation between activation of p53 and metabolic inhibition causing severe growth defects for *Chlamydia* was subsequently proposed (Figure 39, right panel). Overexpression of G6PD, the rate-limiting enzyme of the PPP, resulted in significant recovery of chlamydial growth.

Degradation of the tumour suppressor p53 is beneficial for *Chlamydia* in many ways. Continued activation of the PI3K-Akt pathway is ensured as the endogenous inhibitor of this pathway, the oncogene PTEN, is a direct target of p53 transcriptional activity (Stambolic *et al.*, 2001). Activation of the PI3K-Akt pathway promotes host cell survival by induction of several anti-apoptotic mechanisms (Kennedy *et al.*, 1997). Due to the early onset of p53 degradation *Chlamydia*-induced DNA damage does not trigger cellular DDR and subsequent cell cycle arrest, senescence or apoptosis induction. As DNA damage and removal of p53 were previously detected during infections of *Neisseria*, *Shigella* and *Helicobacter*, degradation of the tumour suppressor could conceivably be a precautionary measure during certain bacterial infections. Moreover, degradation of p53 prevents inhibition of G6PD. Thus, resulting effects like decreased nucleotide synthesis and restricted provision of the reducing agent NADPH are avoided. Reducing agents and nucleotides are not only necessary for chlamydial growth but also for cellular detoxification of ROS and DNA repair.

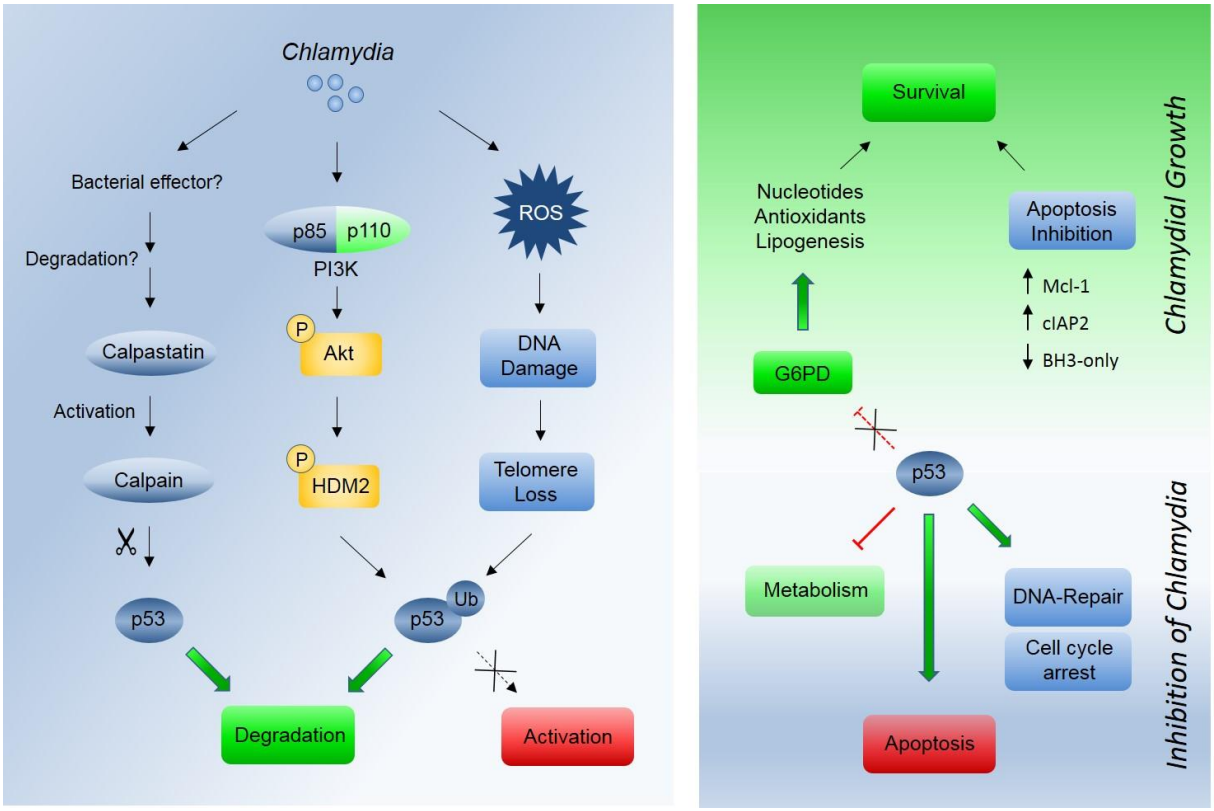

Figure 39 *Chlamydia* infection of primary human cells induces degradation of the tumour suppressor p53 (left panel). *Chlamydia* infection of host cells induces formation of ROS and DNA damage. The tumour suppressor p53 is strongly activated by genotoxic and oxidative stress and transcriptionally initiates expression of cellular genes of DNA damage response and signalling pathways inducing cell cycle arrest, senescence or apoptosis. *Chlamydia* infection was previously shown to strongly activate PI3K-Akt signalling in order to induce anti-apoptotic signalling by up-regulation of the anti-apoptotic proteins Mcl-1 and cIAP2 and degradation of pro-apoptotic BH3-only proteins. This study revealed a new mechanism employed by *Chlamydia* to ensure host cell survival: PI3K-Akt signalling activates the E3-ubiquitin ligase HDM2 which subsequently mediates proteasomal degradation of p53. In addition, calpain-dependent cleavage of p53 is proposed in this study. After *Chlamydia* infection, the endogenous calpain inhibitor calpastatin was found to be downregulated and calpain was demonstrated to contribute to degradation of the tumour suppressor. Moreover, a growth-inhibiting effect of activated p53 resulting in loss of chlamydial infectivity was described (right panel). Degradation of p53 is thought to release inhibition of the pentose phosphate pathway, thus facilitating supply of nucleotides for chlamydial growth and cellular DDR, as well as NADPH for control of oxidative stress.

5.1 *Chlamydia* activates PI3K-Akt-signalling to initiate degradation of p53

Etoposide treatment of cells induces DSBs and subsequently induction of G2/M arrest leading to programmed cell death despite repair of DSBs through non-homologous end-joining (NHEJ) and homologous recombination (HR). According to Karpinich *et al.,* etoposide induces phosphorylation of p53 through DNA-PK. p53 in turn enhances expression of the pro-apoptotic protein Bax which translocates to mitochondria and induces MPT and apoptosis (Karpinich *et al.*, 2002). However, Yoo *et al.* found evidence that etoposide concomitantly induces autophagic cell death and apoptosis (Yoo *et al.*, 2012).

Chlamydial infections confer resistance against etoposide-induced apoptosis (Fan *et al.*, 1998, Dean *et al.*, 2001). These results were confirmed in our experiments by infecting cells with *C. trachomatis* and subsequently inducing strong DNA damage with etoposide. While non-infected cells died after several hours *Chlamydia* infected cells were protected against apoptosis. This is in line with other publications showing apoptosis resistance of infected cells against several cell death-inducing agents (Fischer *et al.*, 2004, Rajalingam *et al.*, 2008). In the presence of *Chlamydia* infection etoposide treatment induced DSBs indicated by the enhanced phosphorylation status of H2Ax. Thus, manipulation of signalling pathways was thought to occur downstream. Due to the involvement of p53 in etoposide-mediated apoptosis, influence of *Chlamydia* infections on expression level and activity of the tumour suppressor was investigated. To ensure wild type p53 status in host cells primary human umbilical vein endothelial cells (Huvecs) were used for experiments. Cancer cell lines often harbour mutations in the *tp53* gene potentially leading to invalid experimental interpretations. Infection of Huvecs with *Chlamydia* resulted in downregulation of p53 protein levels in a time dependent manner with a maximum of protein decrease after 12 to 16 h. Degradation could be confirmed by infecting cells with different chlamydial serovars including a mouse-specific strain. Infection of isolated primary endothelial cells of murine fimbriae and mouse embryonic fibroblasts (MEFs) did not result in p53 downregulation suggesting a species-specific occurrence.

Several pathways are responsible for turnover of p53, the most common being the ubiquitin-dependent proteasomal degradation (Ogawara *et al.*, 2002). To analyse if *Chlamydia* infection induces p53 degradation via the PI3K-Akt-HDM2 pathway, phosphorylation level of Akt was verified. A time- and MOI-dependent activation of the kinase was observed. This is in line with previous publications demonstrating Akt-phosphorylation during *Chlamydia* infections. Wang *et al.* reported activation of Akt after *C. pneumoniae* infection in vascular smooth muscle cells (Wang *et al.*, 2013), *C. trachomatis* infection also induced phosphorylation of Akt (Verbeke *et al.*, 2006, Rajalingam *et al.*, 2008). In addition PI3K pathway activation was shown to be essential for apoptosis resistance during *Chlamydia* infections (Verbeke *et al.*, 2006).

Activation of PI3K signalling initiates phosphorylation of Bad and recruitment of the pro-apoptotic protein to the chlamydial inclusion membrane. In agreement with Ogawara *et al.*, we detected phosphorylation of the E3 ubiquitin ligase HDM2 responsible for marking p53 with polyubiquitin chains for proteasomal degradation (Ogawara *et al.*, 2002). Pharmacological inhibition of HDM2 or gene silencing resulted in stabilisation of p53. Consistent with our expectations, inhibition of the proteasome prevented degradation of the transcription factor. Our findings are in line with published data demonstrating impaired DDR and lack of DNA repair protein recruitment during *Chlamydia* infections (Chumduri *et al.*, 2013). The mechanism underlying activation of the PI3K pathway by *Chlamydia* is still unknown. An initial short activation phase 5 to 30 min after *Chlamydia* infection was reported by Rajalingam *et al.*, followed by a longer activation period starting 12 to 16 h after infection (Rajalingam *et al.*, 2008, Verbeke *et al.*, 2006). Mechanical stress triggered by the growing chlamydial inclusion and an ensuing cellular stress response (Kippenberger *et al.*, 2005) as well as oxidative stress caused by the infection (Zhuang *et al.*, 2003) were proposed to be responsible for activation of PI3K and subsequent Akt phosphorylation. Additionally, *Chlamydia* was reported to activate Ras and subsequently the MAPK-pathway (Su *et al.*, 2004) which in turn could switch on PI3K signalling. The *C. trachomatis* effector protein Tarp (translocated actin-recruiting phosphoprotein) rapidly being tyrosine phosphorylated by unknown host kinases upon cell entry was also suggested to be responsible for PI3K activation by recruiting the Rac guanine nucleotide exchange factor Vav2 and the regulatory subunit of PI3K (Lane *et al.*, 2008).

In addition to the ubiquitin-dependent degradation of p53 the alternative pathway of cytoplasmic calpain-mediated p53 cleavage was analysed (Kubbutat *et al.*, 1997). *Chlamydia* infected cells treated with calpain inhibitor exhibited marked stabilisation of p53. However, due to the ongoing degradation by the proteasome p53 levels were still lower than in uninfected control cells. Calpain-mediated degradation of p53 was previously reported during *Shigella flexneri* infections of epithelial cells (Bergounioux *et al.*, 2012) initiated by the pathogen's virulence effector VirA. Calpastatin reduction in infected cells suggests a *Chlamydia*-mediated degradation of the endogenous inhibitor of calpains. Bacteria-induced downregulation of calpastatin was demonstrated for the first time during *Shigella* infection of epithelial cells (Bergounioux *et al.*, 2012). Apparently, *Chlamydia*-mediated downregulation of the tumour suppressor is facilitated by two cellular degradation pathways.

Degradation of p53 did not occur in *Chlamydia*-infected mouse cells. Neither human nor mouse strains of *Chlamydia* initiated downregulation of the tumour suppressor. As expected, phosphorylation of Akt was not detected after infection of epithelial cells of mouse fimbriae with *C. trachomatis*. Strong cytotoxicity was observed in infected mouse cells leading to reduced infectivity and loss of bacterial progeny formation. Immortalised MEFs were thought to be defective in terms of p53 regulation, however, it was demonstrated that MEFs immortalised by

serial passaging display downregulation of the tumour suppressor through destabilisation of its mRNA (Kim *et al.*, 2001) than resistance to ubiquitin-mediated degradation. Immortalisation of MEFs with the SV40 virus large T-antigen overcomes p53 and pRB dependent cell cycle arrest. Binding of the large T-antigen to p53 increases its half-life and cellular levels, as p53 is no longer subjected to HDM2-mediated proteasomal degradation (Sladek *et al.*, 2000). For this reason, primary epithelial cells of mouse fimbriae were used for analysis of p53 regulation. Intriguingly, no p53 degradation after *Chlamydia* infection could be observed in these cells. In p53-deficient MEFs less cytotoxicity was observed resulting in increased bacterial load in secondary infections. Flow cytometric analysis revealed that infection-induced cell death was rather necrotic than apoptotic. In line with this, a recent publication demonstrated that loss of p53 function in MEFs as well as human colorectal and human breast cancer cell lines confers resistance to necrotic, PARP-mediated cell death (Montero *et al.*, 2013).

5.2 Activation of p53 inhibits chlamydial growth

Activation of the tumour suppressor qualities of p53 by DNA damaging agents like etoposide, nutlin-3, cisplatin, doxorubicin or camptothecin is often conducted during anti-cancer treatments (Havelka *et al.*, 2007, Verma *et al.*, 2010, Walles *et al.*, 1996). Further means are ionizing radiation or exposure to UV light (Renzing *et al.*, 1996). DNA damage induces enzymes involved in DDR like ATM and DNA-PK leading to activation of p53 in a phosphorylation-acetylation cascade (Sakaguchi *et al.*, 1998). Induction of apoptosis, long-term growth arrest and senescence are all anti-proliferative mechanisms resulting from stabilisation and activation of p53. A large percentage of cancers harbours dysfunctional p53 or displays complete loss of the protein. Many anticancer drugs like nutlin-3 (Supiot *et al.*, 2008) and etoposide (Chiu *et al.*, 2005) sensitise cancer cells for radiation or induction of apoptosis in a p53-independent manner. Beyond that, the tumoricidal drug etoposide induces G2/M arrest and G1 arrest in a p53-dependent way (Attardi *et al.*, 2004) and S-phase accumulation in a p53-independent way (Nam *et al.*, 2010).

To address the question about the impact of stabilised p53 on chlamydial growth, the two anticancer drugs etoposide and nutlin-3 were applied. While etoposide induces phosphorylation of p53 on key serine residues including Ser6, Ser15, Ser20 and Ser46, nutlin-3 did not activate the tumour suppressor in this way (Thompson *et al.*, 2004). However, it was demonstrated that phosphorylation is not essential for transcriptional activation of p53 (Ashcroft *et al.*, 1999). Thompson *et al*. demonstrated that nutlin-induced p53 shows sequence-specific DNA binding, transactivation of target genes and induction of apoptosis comparable to etoposide- or doxorubicin-activated p53 (Thompson *et al.*, 2004). Treatment of primary human cells with etoposide or nutlin-3 followed by *Chlamydia* infections unexpectedly

lead to severe growth inhibition of the pathogen with a marked deficit in inclusion formation. Microscopic examination revealed the presence of individual *Chlamydia* surrounded by membranes unable to fuse to one single inclusion. Trafficking of *Chlamydia* to the perinuclear region was also extremely delayed and only partially successful at late time points of infection. Bacterial transcription and translation and particularly synthesis of the inclusion protein IncA are necessary for homotypic vesicle fusion (Hackstadt *et al.*, 1999, Van Ooij *et al.*, 1998), however, we could show that bacterial protein synthesis is not abolished in the presence of both etoposide and nutlin-3. Intriguingly, *Chlamydia*-induced degradation of p53, stabilised by etoposide treatment before infection, could be observed. Opposed to our findings etoposide-induced phosphorylation of p53 on Ser20 and Thr18 was shown to inhibit interaction with HDM2 thus preventing its degradation by the proteasome (Craig *et al.*, 1999). Calpain-mediated cleavage of p53 also detected during *Chlamydia* infections and independent of the E3 ligase HDM2 could be responsible for this phenomenon. In addition to the defective inclusion formation, etoposide and nutlin-3 treatment resulted in a general loss of chlamydial infectivity. Although multiplication of the pathogen could be detected by increasing amounts of the chlamydial protein Hsp60, no bacterial progeny could be recovered in infectivity assays. A similar situation was described by Van Ooij *et al*., who incubated *Chlamydia* infected cells at 32°C resulting in impaired inclusion formation due to loss of protein synthesis and IncA expression, and observed a dramatic loss of infectious chlamydial progeny in the secondary infection (Van Ooij *et al.*, 1998). Richards *et al.* found evidence that for effective fusion, inclusions have to be in close proximity and chlamydial trafficking to the minus ends of microtubules has to be possible (Richards *et al.*, 2013). Additionally, successful fusion seemed to be a requirement for strong infectivity and virulence, as chlamydial non-fusing mutant strains were shown to be less virulent (Geisler *et al.*, 2001). However, as we could detect protein expression and chlamydial growth, defective transcription and translation were excluded to be the reason for inhibition of chlamydial growth and inclusion formation.

Analysis of chlamydial morphology by electron microscopy revealed that many RBs were enlarged and aberrant raising the possibility that etoposide treatment at least partially induced persistence in *Chlamydia*. Persistent, non-replicative *Chlamydia* do not finish their life cycle and are unable to differentiate into their infective EB form (Beatty *et al.*, 1994) explaining the complete loss of infectivity. Persistence can be initiated in *Chlamydia* by different stimuli including antibiotics like erythromycin (Clark *et al.*, 1982), penicillin (Matsumoto *et al.*, 1970) and ampicillin (Wolf *et al.*, 2000), as well as cytokines like IFNγ (Beatty *et al.*, 1993). Additionally, depletion of essential nutrients like amino acids, glucose (Harper *et al.*, 2000) or iron (Raulston, 1997) triggers formation of aberrant RB. Recently, Prusty *et al.* demonstrated that co-infection with various human herpes viruses also induces chlamydial persistence through changes in cellular redox balance and increased oxidative stress (Prusty *et al.*, 2012).

Modifications in metabolism or reduction of metabolic support appear to be a common trait of several persistence-inducing stimuli. Thus, p53-mediated regulation of host cell metabolism and reduced metabolic support for the pathogen was thought to contribute to chlamydial growth inhibition.

Van Leeuwen *et al.* convincingly demonstrated that removal of nutlin-3 instantly restored normal cellular levels of p53 (van Leeuwen *et al.*, 2011). In line with our expectations, removal of the stabilising effect on p53 lead to recovery of the chlamydial inclusion and the pathogen's infectivity confirming the crucial role p53 is playing. Quite comparable to re-differentiation of aberrant RBs to EBs after removal of persistence-inducing stimuli (Beatty *et al.*, 1995) removal of p53 gradually allowed chlamydial growth recovery and formation of an inclusion. This observation further strengthened the discovery that p53 was responsible for the inhibition of chlamydial growth. The crucial role of p53 for chlamydial growth inhibition was further confirmed by results obtained after infecting etoposide-treated $p53^{-/-}$ HCT116 cells with *Chlamydia*. A recovery rate of 36% in the secondary infection was observed confirming the inhibitory role of p53. Incomplete recovery was potentially caused by p53-independent aspects, activation of signalling pathways or oxidative stress caused by DNA damage. Other off-target effects of the inhibitor also have to be considered.

5.3 Chlamydial growth is dependent on host cell metabolism

Proliferation state and metabolism of cancerous cells are decidedly different compared to normal, quiescent cells. Consumption of glucose and production of lactate are increased in tumour cells due to accelerated glycolysis known as the Warburg effect (Warburg, 1956). However, this effect is not restricted to mutated cells. It rather depicts a metabolic modulation to support enhanced proliferation, also observed in normal, rapidly proliferating cells (Herrero-Mendez *et al.*, 2009, Almeida *et al.*, 2010). Cell growth and proliferation and a functional transcription and translation machinery are essential for obligate intracellular bacteria. It is still controversial if *Chlamydia* infections are able to enhance cellular proliferation or lead to cell cycle arrest. Increased cell division was observed after *C. trachomatis* infection of human endocervical cells (Chumduri *et al.*, 2013) and *C. pneumoniae* infection of human peripheral blood mononuclear cells and coronary artery smooth muscle cells (Cho *et al.*, 2005, Rupp *et al.*, 2005). However, in contrast to this Hirai *et al.* reported a suppression of cellular division after infection of human acute T cell leukaemia cells with *C. pneumoniae* (Hirai *et al.*, 2013).

Proliferation of cells and especially cancer cells is enhanced by elevated levels of exogenous and endogenous ROS (Gupta *et al.*, 2012). Strongly increased levels of ROS are associated with reduced cellular growth due to induction of cell cycle arrest (Rancourt *et al.*, 1999). During

Chlamydia infection Boncompain *et al.* observed an initial increase of ROS levels within a few hours after cell entry and a return to basal levels approximately 9 h after infection (Boncompain *et al.*, 2010). This return to basal levels was explained by an inhibition of NADPH oxidase activity caused by the relocation of a regulatory subunit of the enzyme to the chlamydial inclusion membrane (Boncompain *et al.*, 2010). The production of low levels of ROS by NADPH oxidase contributes to chlamydial growth as ROS induces the activation of caspase-1 and thus cellular lipid metabolism (Abdul-Sater *et al.*, 2010, Gurcel *et al.*, 2006). Chumduri *et al*. reported an increased synthesis of ROS during *Chlamydia* infections and as a result an upregulation of markers for DNA DSBs and senescence-associated heterochromatin foci (SAHF) (Chumduri *et al.*, 2013). DNA damage is a potent inducer of p53 resulting in the induction of several pathways promoting DNA repair, cell cycle arrest, senescence or apoptosis.

In addition to its role in tumour prevention p53 functions as an important regulator of metabolic pathways. It was shown by Jiang *et al.* that p53 inhibits the PPP and suppresses glucose consumption, NADPH production and biosynthesis of nucleotides (Jiang *et al.*, 2011). p53 directly binds to G6PD, prevents active dimer formation of the enzyme and drastically lowers its activity. The importance of the PPP for *Chlamydia* becomes apparent after silencing of G6PD: As reported by Prusty *et al*., inhibition of the PPP results in partial induction of chlamydial persistence (Prusty *et al.*, 2012). Excessive consumption of NADPH by *Chlamydia* and the importance of GSH for antioxidant defence were also demonstrated (Prusty *et al.*, 2012). To assess the importance of glycolysis and the PPP, chlamydial growth was monitored after blocking the two pathways. *Chlamydia* were strongly inhibited after shutting down the PPP, visible through the formation of aberrant bodies. This is in line with previously published results (Prusty *et al.*, 2012). Application of 2-deoxy-glucose (2-DG), an inhibitor of glucose-6-phosphate formation (Wick *et al.*, 1957), resulted in inhibition of *Chlamydia* as well. Additionally, 2-DG competitively blocks phosphoglucose isomerase (PGI), thus fuelling other metabolic pathways like the PPP. Surprisingly, despite the availability of other glucose metabolising pathways, inhibition of the first step of glycolysis abrogated bacterial growth. This could be due to additional mechanisms of 2-DG action, e.g. protein glycosylation (Kang *et al.*, 2006). Glucose-dependency of *Chlamydia* was also analysed by Szaszák *et al.* under hypoxic conditions (Szaszak *et al.*, 2013). They revealed that progeny formation was impaired after silencing of the glycolytic enzymes and regulators phosphofructokinase (PFK), lactate dehydrogenase (LDH), glycerol-3-phosphate dehydrogenase (GPD2) and forkheadbox O3 (FOXO3) (Szaszak et al., 2013). Additionally, *C. trachomatis* survives and undergoes limited growth with gluconeogenic substrates as the sole carbon source, particularly with glutamate, but also with malate, α-ketoglutarate or oxaloacetate (Iliffe-Lee et al., 1999).

Chlamydia's dependency on glycolytic pathways raised the question whether etoposide- or nutlin-3-mediated growth defects were the result of decreased glucose uptake or consumption. Expression of glucose transporters 1 and 4, both direct targets of p53 (Schwartzenberg-Bar-Yoseph *et al.*, 2004) were analysed. An increase in Glut 1 and Glut 4 protein level after *Chlamydia* infection was detected. This is in line with results from Ojcius *et al.*, who reported an upregulation of Glut 1 after *Chlamydia psittaci* infection of HeLa cells (Ojcius *et al.*, 1998). *Chlamydia* infection increases the energy load inside the cell through induction of glucose uptake, thus compensating for the increased demand for metabolites and energy. Consistent with the findings of Schwartzenberg-Bar-Yoseph *et al.*, Glut 1 was downregulated after activation of p53 with etoposide (Schwartzenberg-Bar-Yoseph *et al.*, 2004). Additionally, protein expression of TIGAR, a direct target of p53 and regulator of glucose breakdown was analysed. In line with the degradation of p53, TIGAR was strongly downregulated during *Chlamydia* infections in p53 wild type cells, suggesting possible benefits for the pathogen. However, TIGAR functions by inhibiting glycolytic flux and directing glucose consumption to the PPP. Thus, the downregulation of TIGAR observed in our experiments rather releases the inhibitory block on glycolysis than promotes the PPP. Nonetheless, the drastic degradation of p53 during *Chlamydia* infection is beneficial for the pathogen as the suppressing effect of p53 on G6PD likely overrides the effect of TIGAR. The predominant p53-mediated inhibition of the PPP was also claimed by Jiang *et al.*, who showed an enhanced PPP flux and NADPH production in p53 deficient cells (Jiang *et al.*, 2011).

Subsequently, activity of G6PD in p53 wild type and knockout cells, as well as tumour-associated p53 mutants R175H and R273H was analysed. Both are so called hotspot mutations, abolishing the wild type tumour suppressor function of p53 (Hainaut *et al.*, 2000). While mutation R273H is located at the DNA contact area of the protein, mutation R175H lies within a region necessary for conformational stability (Sigal *et al.*, 2000). Both p53 mutants lost their ability to inhibit G6PD (Jiang *et al.*, 2011) even though binding to the enzyme was still possible. Our experiments demonstrated a significant increase of G6PD activity in p53 knockout and mutant cells compared to p53 wild type cells. Similar results were already published (Jiang *et al.*, 2011). Moreover, *Chlamydia* infection led to a significant rise in enzyme activity in both p53 wild type and mutant cells. In p53 wild type cells this phenomenon is explained by the degradation of p53 during *Chlamydia* infections and the resulting loss of its inhibitory activity on G6PD. G6PD activity is promoted after exposure of cells to various extracellular oxidants that lead to decrease in NADPH levels (Kletzien *et al.*, 1994). NADPH consumption by *Chlamydia* and an antioxidant stress response to pathogen-induced DNA damage possibly contributed to enhanced activity of G6PD and the PPP. An explanation for the enhanced enzyme activity in p53 mutant cells could be found in the cellular DNA damage response: After the detection of DSBs the serine/threonine kinase ATM promotes an anti-

oxidant response through induction of G6PD and the pentose phosphate pathway (Cosentino *et al.*, 2011), independent of p53 signalling. Moreover, *Chlamydia* itself encodes a functional G6PD and the increased activity of the enzyme could be the result of an additive effect of bacterial and host enzymes (Iliffe-Lee *et al.*, 1999). In the presence of etoposide, G6PD activity was increased in p53-knockout and –mutant cells both in non-infected as well as *Chlamydia*-infected cells compared to p53 wild type cells. Similar results were shown by Jiang *et al*., who demonstrated reduced G6PD activity in p53 wild type cells after treatment with the DNA damaging agent doxorubicin. This inhibiting effect was not observed in p53 knockout cells (Jiang *et al.*, 2011).

In addition to the activity of G6PD after etoposide treatment and *Chlamydia* infection, chlamydial infectivity was analysed in p53 mutant cell lines. In concurrence with the increased flux of the PPP, a substantial rescue of chlamydial infectivity after etoposide treatment could be observed in p53 knockout and mutant cells, further emphasising the importance of this metabolic pathway for the pathogen's growth. However, chlamydial recovery did not exceed 38% indicating that p53 was not solely responsible for the growth inhibition.

In order to enhance biosynthesis of macromolecules and antioxidant defence, G6PD, the rate-limiting enzyme of the PPP, was transiently overexpressed. Overexpression of G6PD results in decreased accumulation of reactive oxygen species (Leopold *et al.*, 2003), elevated levels of cellular free fatty acids and triglycerides and enhanced cell proliferation (Park *et al.*, 2005). G6PD overexpressing cells were challenged with either etoposide or nutlin-3 and subsequently infected with *C. trachomatis*. Confirming the important role of the PPP, chlamydial growth could be strongly rescued in primary human cells. Part of the rescuing effect can probably be attributed to the restoration of GSH levels and the decrease in ROS production after overexpression of G6PD (Salvemini *et al.*, 1999). As already reported *Chlamydia* infection induces ROS production early during the developmental cycle and thus cause oxidative stress for the cell (Abdul-Sater *et al.*, 2010). At later time points of infection ROS production is rapidly shut down by the pathogen (Boncompain *et al.*, 2010). Consequently, protection against oxidative stress by overexpressed G6PD (Salvemini *et al.*, 1999) is beneficial for the cell and the pathogen. In summary, a functioning pentose phosphate shunt and a resulting strong antioxidant defence are essential for chlamydial growth.

To verify if oxidative stress is causing chlamydial growth inhibition, several ROS removing agents were used after activating p53 by etoposide or nutlin-3. Recovery of chlamydial growth was thought to occur if oxidative stress caused by reduced activity of G6PD is the main reason for the inhibition. Surprisingly, removal of ROS with the exception of DTT treatment resulting in a slight recovery of infectivity did not improve chlamydial growth. It was suggested by Du *et al*. that rescuing the proliferative defects of cells deficient in TAp73, a member of the p53 family

and potent inducer of the PPP, either requires overexpression of G6PD or addition of both ROS removing agents and nucleosides (Du *et al.*, 2013). Removal of ROS alone was not sufficient due to the importance of this pathway for nucleotide biosynthesis (Du *et al.*, 2013). It remains to be determined if supplementing the host cell with nucleosides in addition to scavenging of ROS is sufficient to rescue chlamydial growth in the same way observed after overexpression of G6PD.

Induction of DNA damage and formation of ROS as observed after *Chlamydia* infection are potent inducers of cellular senescence (Parrinello *et al.*, 2003, Dileonardo *et al.*, 1994). The crucial factor determining cellular fate between permanent senescence and transient cell cycle arrest is currently unknown. However, duration of the DNA repair process is important as signalling pathways are either rapidly switched off or long-lasting. p53-induced senescence, as part of its tumour suppressor activity, is primarily triggered by the cellular DDR after extensive damage to DNA or telomeres with p21 being a major transcriptional target of p53 and mediator of permanent cellular arrest (Brown *et al.*, 1997). The p16-pRB tumour suppressor pathway is normally engaged in response to p53-mediated senescence and facilitates a more permanent cellular arrest (Jacobs *et al.*, 2004, Beausejour *et al.*, 2003). During *Chlamydia* infection, Chumduri *et al*. detected the formation of senescence-associated heterochromatin foci (SAHF) induced in an ERK-dependent manner (Chumduri *et al.*, 2013). Despite detection of SAHF infected cells did not go into senescence but continued to proliferate. As we considered senescence induction to possibly cause chlamydial growth inhibition, senescence-associated β-galactosidase staining was performed (Lee *et al.*, 2006). Induction of senescence in *Chlamydia*-infected primary human cells could not be detected confirming the results of Chumduri *et al.* (Chumduri *et al.*, 2013). After challenging cells with etoposide or nutlin-3 strong positive staining for β-galactosidase could be observed. This was also detected by others (Marusyk *et al.*, 2007, Arya *et al.*, 2010). Both nutlin-3 and etoposide treatment result in senescence induction in a strictly p53-dependent way, as p53-deficient cells are either resistant to the drug or are subjected to apoptosis (te Poele *et al.*, 2002, Efeyan *et al.*, 2007). In addition, implications of G6PD overexpression on the induction of permanent cell arrest after etoposide and nutlin-3 treatment were investigated. It was shown before that G6PD activity prevents the onset of senescence (Du *et al.*, 2013) and fibroblasts from G6PD deficient patients display accelerated cellular senescence (Ho *et al.*, 2000). Indeed, increased G6PD activity effectively prevented permanent cell arrest displayed by the lack of SA-β-gal staining. If an active cell cycle reconstitutes chlamydial growth and inclusion formation remains to be clarified. Contradictory opinions are published about the question whether *Chlamydia* promotes proliferation (Rupp *et al.*, 2005, Chumduri *et al.*, 2013) or induces cell cycle arrest (Balsara *et al.*, 2006).

5.4 p53-independent effects as a causative for chlamydial growth inhibition

By overexpression of G6PD chlamydial inclusion formation and infectivity could be rescued in the presence of p53-activating compounds. Increased activity of the PPP was assumed to provide protection against harmful ROS levels and support chlamydial growth through supply of NADPH and nucleotides. Overexpression of the tumour suppressor in p53-deficient cells resulted in the same growth inhibition induced by etoposide and nutlin-3 confirming the decisive role p53 is playing during chlamydial infections. Despite investigating several metabolic pathways including glycolysis, the PPP, formation of ROS and induction of senescence and establishing their importance for successful bacterial growth, the inhibitory effects of the p53-activating substances could not be completely overcome. In addition, low transfection efficiency of G6PD could be responsible for an incomplete rescue of *Chlamydia*.

Extensive studies revealed that both inhibitors aside from their main p53-activating role are causative for a number of side-effects. A p53-indepenent DNA-damaging role was assigned for nutlin-3 indicated by positive γH2Ax staining (Valentine *et al.*, 2011). Furthermore, while inducing a p53-dependent G1/S arrest, the anti-cancer drug led to a p53-independent induction of the G2/M cell cycle checkpoint in p53 deficient cells (Valentine *et al.*, 2011). A study by Wang *et al*. revealed that etoposide mediated induction of the genes for ornithine decarboxylase, TGF beta receptor 2 and metallothionein-2 in a p53-independent way (Wang *et al.*, 1999). In another study it was suggested that etoposide induces a p53-mediated G2/M arrest and a p53-independent S-phase accumulation (Nam *et al.*, 2010). Aside from activation of cell cycle checkpoints, the drug is also known to affect gene expression at different levels, e.g. chromatin remodelling, transcription, and alternative splicing (Jiao *et al.*, 2005, Johnson *et al.*, 2001, Montecucco *et al.*, 2007).

If these side-effects negatively affect growth of the pathogen remains to be investigated. Further experiments are required to fully elucidate the effects of the p53-activating components etoposide and nutlin-3 on the developmental cycle of *C. trachomatis*. Especially in regard to induction of cell cycle arrest, conflicting opinions exist whether *Chlamydia* mediates mitotic arrest or is dependent on a functioning cell cycle: On the one hand *C. trachomatis* infections actively override cell cycle arrest by causing the degradation of cylin B1 and securin (Knowlton *et al.*, 2011), on the other hand the pathogen induces inhibition of host cell cytokinesis (Greene *et al.*, 2003). The facultative intracellular pathogens *N. gonorrhoeae*, (Jones *et al.*, 2007), *S. aureus* (Alekseeva *et al.*, 2013), *Shigella* (Iwai *et al.*, 2007) and *L. monocytogenes* (Leitão *et al.*, 2014) actively induce cell cycle arrest or transition delay.

5.5 Immune response regulation and anti-bacterial function of p53

In recent years, the wide range of functions of the tumour suppressor p53 was further expanded. In addition to the regulation of cell proliferation, apoptosis and senescence, the field of metabolism, but also immune-related processes, especially in connection with cancer, were attributed to p53. The transcription factor is involved in regulation of host defence and inflammation, suppression of NF-κB cytokine induction (Komarova *et al.*, 2005) and cell migration. However, its role during infections is still poorly understood: While upregulation of p53 in response to viral infections was extensively studied (Takaoka *et al.*, 2003, Rivas *et al.*, 2010, Turpin *et al.*, 2005), implications for control of bacterial infections received little attention. Recently, Madenspacher *et al*. demonstrated that mice with deletion or inhibition of p53 exhibit enhanced clearance of both gram-negative and -positive extracellular bacteria after intrapulmonary infection due to an elevated level of alveolar macrophages and polymorphonuclear neutrophils (PMNs) (Madenspacher *et al.*, 2013). Despite efficient clearance of infection, p53-deficient mice display a decrease in survival caused by an overshooting immune response. Fuhrman *et al*. found a novel connection between the p53 homologue CEP-1 and the immune system in *Caenorhabditis elegans*: Proteins of the nucleolus suppress innate immunity against bacteria by preventing the transcriptional activity of the tumour suppressor p53. Mutants for these nucleolar proteins are highly resistant to various bacteria, like *Salmonella*, *Pseudomonas* and *Enterococcus* (Fuhrman *et al*., 2009).

Chlamydial infections *in vivo* normally result in severe inflammations characterized by the infiltration of activated monocytes and macrophages and by an effector cytokine response initiated by Th-1/Th-2 lymphocytes (Hafner *et al.*, 2008). Interestingly, Caspar-Bauguil *et al*. demonstrated a mechanism of immune evasion mediated by *C. pneumoniae*: Infection-induced IL-10 secretion downregulated the expression of MHC class I molecules on the surface of monocytic cells, thereby restricting presentation of bacterial epitopes and decreasing $CD8^+$ T lymphocyte responses (Caspar-Bauguil *et al.*, 2000). Others reported the degradation of host transcription factors required for major histocompatibility complex (MHC) antigen expression by the chlamydial protease CPAF (Zhong *et al.*, 1999). In the meantime, cleavage of these factors was disproved and attributed to off-target effects (Chen *et al.*, 2012). In contrast to immune evasion mechanisms induced by *Chlamydia*, more extensive knowledge could be garnered regarding manipulation of host cells and activation of anti-apoptotic pathways (Sharma *et al.*, 2009). Degradation of p53 was until recently only associated with prevention of apoptosis after DNA damage, occurring during infections of several facultative and obligate intracellular pathogens, including *Chlamydia*. However, DNA damage also links bacterial infections to the host immune system. The DDR, as part of its function as an important barrier against tumour formation (Bartkova *et al.*, 2005), activates several transcription factors:

NF-κB, IRFs (interferon regulatory factors) and the tumour suppressor p53 (Li *et al.*, 2001, Brzostek-Racine *et al.*, 2011) mediate the expression of various genes of the immune system including inflammatory cytokines and chemokines. This intriguing role of the tumour suppressor was so far only described by Madenspacher *et al*. and Fuhrman *et al*. who linked activity of p53 with clearance of bacterial infections, albeit with contradicting functions of the tumour suppressor (Madenspacher *et al.*, 2013, Fuhrman *et al.*, 2009).

Through regulation of metabolic processes, p53 maintains the homeostasis of cellular metabolism and redox balance in cells. This contributes significantly to the important role of p53 as a tumour suppressor (Liang *et al.*, 2013). Extensive studies have shown that cancer cells depend upon changes in metabolism for continued tumour growth (Vousden *et al.*, 2009, Heiden *et al.*, 2009). Dependence on amino acids, lipids, nucleotides and energy suggests a similar importance of metabolic alterations for chlamydial growth as for tumorigenic cells. It further highlights the significance of infection-induced breakdown of the tumour suppressor. Degradation of p53 not only prevents induction of apoptosis upon DNA-damage, but rather facilitates complex changes of cellular metabolism necessary for chlamydial growth.

Due to its essential role in prevention of tumour formation, degradation of p53 poses great risks for the integrity of the cell. In contrast to oncogenes which result from the activation of proto-oncogenes, tumour suppressor genes cause cancer after inactivation or degradation. Thereby most of the mutations of tumour suppressors leading to cancer are acquired, not inherited. Inactivation of p53 by degradation or other mechanisms entails accumulation of genetic damage leading to transformation and tumorigenesis. The link between bacterial infections and carcinogenesis is however still controversial. The role-model of pathogen-mediated degradation of p53 is the gastric mucosal coloniser *H. pylori*, which is strongly associated with the development of gastric adenocarcinoma (Uemura *et al.*, 2001). However only a minority of patients develops stomach cancer. Intriguingly, not loss of the tumour suppressor but chronic inflammation is assumed to be the crucial factor in the carcinogenic process (Herrera *et al.*, 2009). Association of *Chlamydia* infection with cancer remains poorly investigated, however, a causal association between lung cancer and *C. pneumoniae* infection (Littman *et al.*, 2005) was made. Moreover, as already mentioned, *C. trachomatis* G was strongly associated with onset of cervical squamous cell carcinoma (SCC) (Anttila *et al.*, 2001). More than 90% of cervical carcinomas are linked to infections with HPV (Walboomers *et al.*, 1999). *C. trachomatis* infections are associated with an increased risk for the development of ICC in both HPV DNA-positive and -negative patients. High-risk HPV E6 proteins were shown to induce cellular E3 ubiquitin ligase to bind p53 and mediate ubiquitin-proteasome degradation. Thus, *Chlamydia* infections mediate the very same aspect as HPV and were not without reason implicated as cofactors for the development of malignancy. Other *C. trachomatis* serovars were linked to development of invasive SCC of the uterine cervix, with

multiple exposures to different serovars increasing the risk of cancer (Smith *et al.*, 2004, Koskela *et al.*, 2000).

5.6 Perspectives

In the scope of this thesis *Chlamydia* was shown to induce degradation of the tumour suppressor p53 by activating PI3K-Akt-HDM2 signalling in human cells. Activation of the PI3K-Akt pathway during *C. trachomatis* and *C. pneumoniae* infections was previously reported by others (Rajalingam *et al.*, 2008, Verbeke *et al.*, 2006), however, only in the context of upregulation of the anti-apoptotic proteins Mcl-1 and cIAP-2 and sequestration of the pro-apoptotic protein Bad. Overall, a new function could be attributed to the pro-survival pathway during *Chlamydia* infections. Inhibition of PI3K activation discloses its relevance, as protection against apoptosis conferred by *Chlamydia* infection is removed. Moreover, stabilisation of p53 caused by PI3K inhibition results in severe restriction of chlamydial growth and defects in inclusion fusion. Nevertheless, several questions remain unanswered concerning activation of this pathway. Secretion of bacterial factors through the type III secretion system were proposed to be responsible for sustained PI3K activation (Verbeke *et al.*, 2006). In addition, host-cell stress responses caused by oxidative or mechanical stress during infection could initiate modulation of host cell signalling (Taylor *et al.*, 2005, Kippenberger *et al.*, 2005).

The relevance of p53 degradation in naturally occurring *Chlamydia* infections of humans remains elusive. Although isolated primary fimbriae cells of the fallopian tube infected with *Chlamydia in vitro* were found positive for p53 downregulation, host cell modulation during infections needs to be investigated *in vivo*. Mice are the most commonly used animals to study chlamydial infections, however, it is not the most suitable model. Inoculation of mice with *C. trachomatis* only leads to mild genital tract infections that are cleared relatively quickly due to innate mouse-specific host defence mechanisms. Thus, infections usually do not ascend to the upper genital tract and development of chronic infections observed in humans is not possible (Darville *et al.*, 1997). In the course of this work, experiments were conducted demonstrating increased cytotoxicity and the absence of p53 degradation in both *C. trachomatis* and *C. muridarum* infected mouse cells, emphasising the inadequacy of a mouse model.

As with *Chlamydia,* degradation of p53 by the pathogens *S. flexneri*, causative agent of bacillary dysentery in humans, and *N. gonorrhoeae* were so far only analysed *in vitro*. Because of the suggested causal relation between *H. pylori* infection and development of chronic gastritis and gastric cancer, pathogen-mediated modulation of p53 was already analysed *in vivo*. During carcinogenesis, *H. pylori* is said to cause mutations in p53-Rb tumour suppressor

pathways and telomerase reactivation. p53 downregulation was demonstrated in Mongolian gerbils (Wei *et al.*, 2010); in contrast to this, biopsy samples of human gastric cancer patients showed significant increase of p53 expression during infection (Salih *et al.*, 2013). Additionally, Murakami *et al.* demonstrated development of p53 point mutations during *H. pylori* infections in humans and Japanese monkeys, but not in Mongolian gerbils (Murakami *et al.*, 2002). However, as already mentioned above, only a small number of patients carrying *H. pylori* suffer from stomach cancer. It remains unclear how *Chlamydia* infections affect tumour suppressor signalling in patients. *In vitro* data presented in this work has to be confirmed in patient samples to further link bacterial infections to carcinogenesis. Downregulation or loss of p53 alleviates and contributes to tumour formation due to the inability of cells to induce apoptosis or senescence after harmful events. However, although p53 knockout mice are likely to develop tumours, metastasis does not occur frequently and tumours do not display invasive pathology (Attardi *et al.*, 1999) as observed in tumours harbouring mutant p53 (Liu *et al.*, 2000, Lang *et al.*, 2004).

Dependence of *Chlamydia* on cellular nutrients and energy and especially metabolic pathways like the PPP raises the question of whether bacteria grow less or not at all in cells deficient for G6PD. In addition, chlamydial infectivity pattern in patients harbouring G6PD deficiency, the most common human enzyme defect, or loss of protein function due to mutations should be determined. Detoxification of ROS, occurring in *Chlamydia* infected cells, is impaired in these cells. On the one hand, this leads to elevated apoptosis activity, on the other hand, enhanced levels of ROS and less nucleotide production could be detrimental for chlamydial growth. Oxidative stress further leads to acute haemolysis as G6PD is especially important in red blood cell metabolism, thus weakening the immune system. In contrast to this, it was reported that in strong cases of G6PD deficiency, with less than 5% enzyme activity, insufficient production of NADPH results in reduced activity of NADPH oxidase and defective oxidative defence against pathogens, e.g. during *S. aureus* infection of neutrophils (Paek *et al.*, 2009, Nkhoma *et al.*, 2009).

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Index of Abbreviations

°C	Degree Celsius
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar

A

AIF	Apoptosis-inducing factor
Akt	Protein kinase B (PKB)
ALDH4	Aldehyde dehydrogenase 4 family, member A1
ANT	Adenine nucleotide transporter
Apaf-1	Apoptotic protease activating factor 1
APS	Ammonium persulfate
ARF	Alternate reading frame
ASPP2	Apoptosis-stimulating protein of p53-2
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia-telangiectasia and Rad3-related

B

BH	Bcl-2 homology domains
BIR	Baculovirus IAP repeat
BSA	Bovine serum albumin

C

C. pneumoniae	*Chlamydophila pneumoniae*
C. trachomatis	*Chlamydia trachomatis*
C. muridarum	*Chlamydia muridarum*
CBP	CREB binding protein
CDK	Cyclin dependent kinase
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
cIAP	Cellular inhibitor of apoptosis
CPAF	Chlamydial protease-like activity factor (CPAF)
CREB	cAMP-response element-binding protein

CTP	Cytidine triphosphate

D

DAPI	4´,6-Diamidino-2-phenylindole
DBD	DNA binding core domain
DD	Death domain
DDR	DNA damage response
DED	Death effector domain
DIABLO	Direct inhibitor of apoptosis protein-binding protein with low PI
DISC	Death initiation signalling complex
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dNTP	Deoxyribonucleotide triphosphate
DSB	Double strand break

E

E. coli	*Escherichia coli*
EB	Elementary body
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal regulated kinase

F

FADD	Fas-associated protein with a DD
FCS	Fetal calf serum

G

G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
Glut 1	Glucose transporter 1
Glut 4	Glucose transporter 4
GPx	Glutathione peroxidase
GTP	Guanosine triphosphate

H

h	Hour
h	Human
HAUSP	Herpes virus-associated ubiquitin-specific protease
HDM2	Human double minute 2
HRP	Horseradish peroxidase
Hsp60	Heat shock protein 60
Huvecs	Human umbilical vein endothelial cells

K

kbp	Kilobase pair
kDa	Kilodalton

L

l	Litre
LB	Lysogeny broth
LFS	Li-Fraumeni syndrome
LGV	lymphogranuloma venereum
LPS	Lipopolysaccharide

M

M	Molar
m	Murine
MAPK	Mitogen-activated protein kinase
Mbp	Megabase pair
MDM2	Murine double minute 2
MEF	Mouse embryonic fibroblast
MEK	Mitogen-activated protein/extracellular signal-regulated kinase kinase
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
MnSOD	Manganese superoxide dismutase
MOMP	Major outer membrane protein
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin

N

NADPH	Nicotinamide adenine dinucleotide phosphate
nm	Nanometre
nM	Nanomolar

P

PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
pCAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PDK1	3'-phosphoinositide-dependent kinase
PFK	Phosphofructokinase
PGM	Phosphoglycerate mutase
PI3K	Phosphatidylinositol 3 kinase
PID	Pelvic inflammatory disease
PIP3	Phosphoinositide-3,4,5-trisphosphate
PPP	Pentose phosphate pathway
PTEN	Phosphatase and tensin homolog
PTPC	Permeability transition pore complex

R

RB	Reticulate body
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription PCR

S

SA-β gal	Senescence-associated β-galactosidase activity
SCO2	Synthesis of cytochrome c oxidase 2
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second
SEM	Standard error of mean

siRNA	Small interfering RNA
SMAC	Second mitochondrial-derived activator of caspase
STD	Sexually transmitted disease

T

TEMED	Tetramethylethylenediamine
TIGAR	TP53-induced glycolysis and apoptosis regulator
TNF	Tumour necrosis factors
TRAIL	TNF-related apoptosis-inducing ligand
Tris	Tris(hydroxymethyl)aminomethane

U

U	Unit
USP	Ubiquitin-specific proteases
UTP	Uridine triphosphate
UV	Ultraviolet

V

VDAC	Voltage dependent anion channel

X

XIAP	X-linked IAP

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Selbstständigkeitserklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Degradation of Tumour Suppressor p53 during Chlamydia trachomatis Infections“ eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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

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

Würzburg, den 27.10.14 _____