Julius-Maximilians-Universität Würzburg

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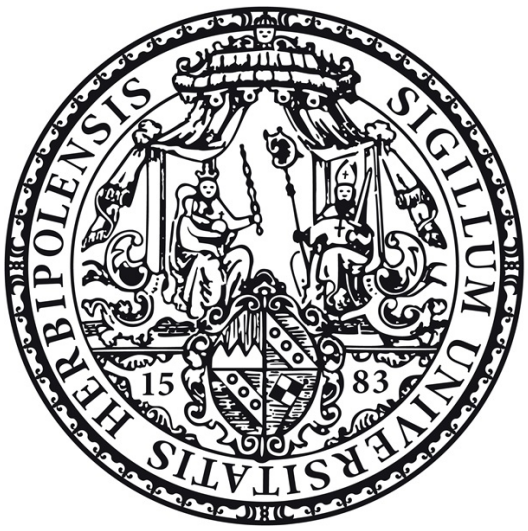


# Role of the proto-oncogene c-Myc in the development of *Chlamydia trachomatis*

# Die Rolle des proto-onkogenes c-Myc in der Entwicklung von *Chlamydia trachomatis*

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Section Infection and Immunity

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# Contents

# Abstract

*Chlamydia trachomatis*, an obligate intracellular human pathogen, is the world's leading cause of infection related blindness and the most common, bacterial sexually transmitted disease. In order to establish an optimal replicative niche, the pathogen extensively interferes with the physiology of the host cell. *Chlamydia* switches in its complex developmental cycle between the infectious non-replicative elementary bodies (EBs) and the non-infectious replicative reticulate bodies (RBs). The transformation to RBs, shortly after entering a host cell, is a crucial process in infection to start chlamydial replication. Currently it is unknown how the transition from EBs to RBs is initiated. In this thesis, we could show that, in an axenic media approach, L-glutamine uptake by the pathogen is crucial to initiate the EB to RB transition. L-glutamine is converted to amino acids which are used by the bacteria to synthesize peptidoglycan. Peptidoglycan inturn is believed to function in separating dividing *Chlamydia*. The glutamine metabolism is reprogrammed in infected cells in a c-Myc-dependent manner, in order to accomplish the increased requirement for L-glutamine. Upon a chlamydial infection, the proto-oncogene c-Myc gets upregulated to promote host cell glutaminolysis via glutaminase GLS1 and the L-glutamine transporter SLC1A5/ASCT2. Interference with this metabolic reprogramming leads to limited growth of *C. trachomatis*.

Besides the active infection, *Chlamydia* can persist over a long period of time within the host cell whereby chronic and recurrent infections establish. *C. trachomatis* acquire a persistent state during an immune attack in response to elevated interferon-$\gamma$ (IFN-$\gamma$) levels. It has been shown that IFN-$\gamma$ activates the catabolic depletion of L-tryptophan via indoleamine 2,3-dioxygenase (IDO), resulting in the formation of non-infectious atypical chlamydial forms. In this thesis, we could show that IFN-$\gamma$ depletes the key metabolic regulator c-Myc, which

has been demonstrated to be a prerequisite for chlamydial development and growth, in a STAT1-dependent manner. Moreover, metabolic analyses revealed that the pathogen derouts the host cell TCA cycle to enrich pyrimidine biosynthesis. Supplementing pyrimidines or a-ketoglutarate helps the bacteria to partially overcome the persistent state.

Together, the results indicate a central role of c-Myc induced host glutamine metabolism reprogramming and L-glutamine for the development of *C. trachomatis*, which may provide a basis for anti-infectious strategies. Furthermore, they challenge the longstanding hypothesis of L-tryptophan shortage as the sole reason for IFN-$\gamma$ induced persistence and suggest a pivotal role of c-Myc in the control of the *C. trachomatis* dormancy.

# Zusammenfassung

*Chlamydia trachomatis*, ein obligat intrazelluläres humanes Pathogen, ist weltweit führende Ursache für infektionsbedingte Erblindung und die häufigste, bakterielle sexuell übertragbare Krankheit. Um eine optimale Replikationsnische zu etablieren, interagiert das Pathogen intensiv mit der Physiologie der Wirtszelle. Chlamydien wechseln in ihrem komplexen Entwicklungszyklus zwischen den infektiösen nicht replizierenden Elementarkörperchen (EBs) und den nicht infektiösen replizierenden Retikularkörperchen (RBs), und diese Umwandlung in RBs kurz nach dem Eintritt in die Wirtszelle ist ein entscheidender Prozess in der Infektion, um die Replikation des Bakteriums einzuleiten. Derzeit ist noch nicht bekannt, wodurch diese Transformation von EBs zu RBs eingeleitet wird. In dieser Arbeit konnte gezeigt werden, dass bei einer zellfreien Kultivierung des Pathogens die Aufnahme von Glutamin durch den Erreger entscheidend ist, um den Übergang von EB zu RB zu initiieren. Vor kurzem wurde Peptidoglykan in den Septen von sich replizierenden Chlamydien nachgewiesen. Für die Synthese des Peptidoglykans nutzen die Bakterien das aufgenommene Glutamin. Der Glutaminmetabolismus wird in infizierten Zellen c-Myc abhängig umprogrammiert, um den erhöhten Bedarf an Glutamin zu bewältigen. Bei einer Chlamydieninfektion wird das Proto-Onkogen c-Myc zur Förderung der Glutaminolyse der Wirtszelle über die Glutaminase GLS1 und den Glutamin Transporter SLC1A5/ASCT2 hochreguliert. Ein Eingreifen in diese metabolische Neuprogrammierung führt zu einem reduzierten Wachstum von *C. trachomatis*.

Neben der aktiven Infektion können Chlamydien über einen sehr langen Zeitraum in der Wirtszelle persistieren, wodurch es zur Etablierung von chronischen und wiederkehrenden Infektionen kommt. *C. trachomatis* verfällt bei einem Immunangriff in Persistenz, wenn sie auf das freigesetzte Interferon-$\gamma$ treffen. Es ist bekannt, dass Interferon-$\gamma$ den Katabolismus

von Tryptophan mittels indoleamine 2,3-dioxygenase (IDO) aktiviert, was zur Bildung von nicht infektiösen atypischen Chlamydienformen führt. In dieser Arbeit konnte gezeigt werden, dass Interferon-$\gamma$ den zentralen Stoffwechselregulator c-Myc, der sich für die Entwicklung und das Wachstum von Chlamydien als essentiell erwiesen hat, in Abhängigkeit von STAT1 herunter reguliert. Darüber hinaus zeigte die Analyse des Metabolismus, dass das Pathogen den TCA Zyklus der Wirtszelle umleitet, um die Pyrimidinbiosynthese zu unterstützen. Die Zugabe von Pyrimidinen oder $\alpha$-Ketoglutarat hilft den Bakterien den Status der Persistenz teilweise zu überwinden.

Zusammengenommen deuten die Ergebnisse auf eine zentrale Rolle der c-Myc induzierten Umprogrammierung des Glutaminmetabolismus und des Glutamins selbst für die Entwicklung von *C. trachomatis* hin. Diese Befunde könnten eine Basis für Strategien gegen eine Infektion darstellen. Weiterhin stellen sie die seit langem bestehende Hypothese des Trypotphanmangels als alleiniger Grund für die von Interferon-$\gamma$ induzierte Persistenz in Frage und legen eine zentrale Rolle von c-Myc bei der Kontrolle der *C. trachomatis* Dormanz nahe.

# Abbreviations

| | |
|---|---|
| A | Adenosine |
| AB | Aberrant body |
| AHT | Anhydrotetracycline |
| $\alpha$KG | $\alpha$-ketoglutarate |
| AKT | Serine/threonine-specific protein kinase |
| AMP/ADP/ATP | Adenosine mono/di/triphosphate |
| APS | Ammonium persulfate |
| BAD | BCL2 associated agonist of cell death |
| BAG | Bcl-2 associated athanogene |
| Bcl-2 | B-cell lymphoma 2 |
| bp | Base pair(s) |
| BPTES | Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulphide |
| BSA | Bovine serum albumin |
| C | Cytidine |
| CAD | Carbamoyl aspartase dehydratase |
| Cam | Chloramphenicol |
| cHSP60 | Chlamydial heat shock protein 60 |
| CHX | Cycloheximide |
| CMP/CDP/CTP | Cytidine mono/di/triphosphate |
| cPLA2 | Cytosolic phospholipase A2 |
| CREB | cAMP response element-binding protein |

| | |
|---|---|
| *Ctr* | *Chlamydia trachomatis* |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMKG | Dimethyl $\alpha$-ketoglutarate |
| DMSO | Dimethyl sulfoxide |
| Dox | Doxycycline |
| dpi | Days post infection |
| DTT | Dithiothreitol |
| EB | Elementary body |
| E-box | Enhancer-box |
| EDTA | Ethylenediaminetetraacetic acid |
| EGFR | Epidermal growth factor |
| ERK | Extracellular signal-regulated kinase |
| FBS/FCS | Fetal bovine/ calf serum |
| FGFR | Fibroblast growth factor receptor |
| G | Guanosine |
| GAS | Interferon-$\gamma$ activated sequence |
| GC-MS | Gas chromatography–mass spectrometry |
| GDH | Dehydrogenase |
| Gln | L-glutamine |
| Glu | L-glutamate |
| GLS1 | Glutaminase |
| GMP/GDP/GTP | Guanosine mono/di/triphosphate |
| GPCR | G protein coupled receptor |
| GPNA | $\gamma$-L-glutamyl-(p-nitroanilide) |
| GSEA | Gene set enrichment analysis |
| GSK3 | Glycogen synthase kinase 3 |
| HIF | Hypoxia induced transcription factor |
| hpi | Hours post infection |
| HRP | Horseradish peroxidase |

| | |
|---|---|
| IB | Intermediate body |
| IDO | Indoleamine 2,3-dioxygenase |
| IFN-$\gamma$ | Interferon-$\gamma$ |
| IFNGR | Interferon-$\gamma$ receptor |
| IL | Interleukin |
| Inc | Inclusion protein |
| Inh | Inhibitor |
| Jak | Janus kinase |
| KD | Knock down |
| LC/MS | Liquid chromatography–mass spectrometry |
| LGV | Lymphogranuloma venereum |
| MAPK | Mitogen-activated protein kinase |
| MAX | Myc-associated factor X |
| mDAP | meso-diaminopimelic acid |
| MOI | Multiplicity of infection |
| MOMP | Major outer membrane protein |
| mTOR | Mechanistic target of rapamycin |
| Mxi | Max interacting protein |
| NAD(P)(H) | Nicotinamide adenine dinucleotide (phosphate) |
| OmpA | Outer membrane protein |
| PB | Persistent body |
| PBS | Phosphate-buffered saline |
| PDGFR | Platelet derived growth factor receptor |
| PDK1 | 3-phosphoinositide-dependent protein kinase-1 |
| PEP | Phosphoenolpyruvate |
| pRB | Retinoblastom-Protein |
| PTEN | Phosphatase and tensin homolog |
| PFA | Paraformaldehyde |
| PI3K | Phosphatidylinositol-3-kinase |

| | |
|---|---|
| PIP2 | Phosphatidylinositol-4,5-biphosphate |
| PIP3 | Phosphatidylinositol-3,4,5-triphosphate |
| PPP | Pentose phosphate pathway |
| PP2A | Protein phosphatase 2A |
| PRPS2 | Phosphoribosylpyrophosphate synthase |
| PTK | Receptor tyrosine kinase |
| PVDF | Polyvinylidene fluoride |
| Pyr | Pyruvate |
| Raf | Rapidly growing fibrosarcoma |
| Ras | Rat sarcoma |
| RB | Reticulate body |
| ROS | Reactive oxygen species |
| RTK | Receptor tyrosine kinase |
| SAFE | Surgery for trichiasis, antibiotics, facial cleanliness and environmental improvement |
| SDS | Sodium dodecyl sulfate |
| shRNA | Small hairpin RNA |
| siRNA | Small interfering RNA |
| SLC | Solute carrier |
| STAT1 | Signal transduction and activator of transcription |
| STD | Sexually transmitted diseases |
| TCA | Tricarboxylic acid |
| TEM | Transmission electron microscopy |
| TEMED | Tetramethylethylenediamine |
| TTSS | Type III secretion system |
| U | Uridine |
| UMP/UDP/UTP | Uridine mono/di/triphosphate |
| W | L-tryptophan |
| WHO | World Health Organization |

# 1 Introduction

## 1.1 *Chlamydia* and the taxonomy of *Chlamydiales*

*Chlamydia* have been first described in 1907 by the German dermatologist and radiologist Ludwig Halberstädter and the Austrian bacteriologist Stanislaus von Prowazek [1] [2]. They were named after the ancient Greek term (Greek chlamys: mantel/cloak) for a short cloak worn by Greek military man, due to the incorrect conclusion that *Chlamydia* are intracellular protozoan which appear to cloak the nucleus of infected cells [1] [2] [3]. For a long time, it was assumed that *Chlamydia* are a virus, based on their ability to pass bacterial filters and their strict requirement of cells for cultivation [1] [4]. Only in 1966 it became clear that *Chlamydia* belong to the bacteria, given their morphological integrity throughout the developmental cycle, their multiplication by binary fission, and the presence of ribosomes [4] [5]. This allocation was later confirmed by 16S rRNA (ribosomal ribonucleic acid) sequencing [6] [7]. Today it is known that *Chlamydia* is a prokaryotic organism and the cloak is a cytoplasmic vesicle containing numerous individual organism [3].

The order of *Chlamydiales* encompasses Gram negative, obligate intracellular parasites of eukaryotic cells, which exhibit a characteristic biphasic life cycle [1] [8]. Until the 1980s, *Chlamydia* was classified according to morphology, staining properties, sensitivity to certain chemicals, developmental replication and antigenic typing [5] [9]. A general improvement in phylogeny analysis led to the advent and rise of 16S and later 23S rRNA sequencing in the 1980s [7] [10] [11]. However, only in 1999 a rearrangement of the chlamydial taxonomy based on the 16S and 23S rRNA sequencing was proposed [7] [12] [13]. The order *Chlamydiales* was divided into four families (Figure 1.1), namely *Chlamydiaceae, Parachlamydiaceae,*

*Waddliaceae* and *Simkaniaceae* [7]. Certainly not without contradiction, because of a rather arbitrarily chosen sequence identification threshold [14], the family *Chlamydiaceae* was further subdivided into two genera *Chlamydophila* and *Chlamydia*, which comprise, among seven other species, the important human pathogens *Chlamydophila pneumoniae* and *Chlamydia trachomatis* [7] [13]. The final phylogenetic classification is still valid today (Figure 1.1), but recently it was proposed to reunite the two genera *Chlamydophila* and *Chlamydia* to a single genus *Chlamydia* [15]. Until 2013, it was believed *Chlamydiales* were one of the few exceptions without producing peptidoglycan but evidence was found that chlamydial species possess functional peptidoglycan [16].

## 1.2 Pathogenesis of *Chlamydiales*

All the members of the order *Chlamydiales* replicate obligatorily inside eukaryotic hosts. Even if the so-called environmental members of the *Chlamydiales* are well known as symbionts of amoeba (especially *Parachlamydiaceae*), *Chlamydiales* have an astonishingly broad host range and are highly efficient bacterial pathogens of humans and animals [17] [18]. For instance, *Chlamydia suis* and *Chlamydia pecorum* have been shown to cause livestock diseases and *Waddliacea chondrophila* and *Chlamydia abortus* haven been associated with abortion in cattle and sheep [19]. Moreover, *Chlamydia psittaci*, which is naturally an avian pathogen and a zoonotic agent of atypical pneumoniae, has been shown to be transmitted to humans [20]. There is a high potential for zoonotic transmission to humans for many of these organisms [19]. *Simkania negevensis*, which was isolated as a cell culture contaminant, could be linked to community acquired respiratory diseases in humans. The natural host *S. negevensis* prefers is supposed to be amoeba [17], but recent evidence points at *S. negevensis* becoming a potential emerging human pathogen [21]. Among the best known and most important human pathogens of the *Chlamydiales* are *Chlamydia pneumoniae* and *Chlamydia trachomatis* [18].

The transmission by aerosol of *C. pneumoniae* is very effective and causes acute respiratory diseases, such as pneumonia or bronchitis, sinusitis and pharyngitis [22] [23] [24]. Furthermore, *C. pneumoniae* infections have been putatively associated with chronic diseases

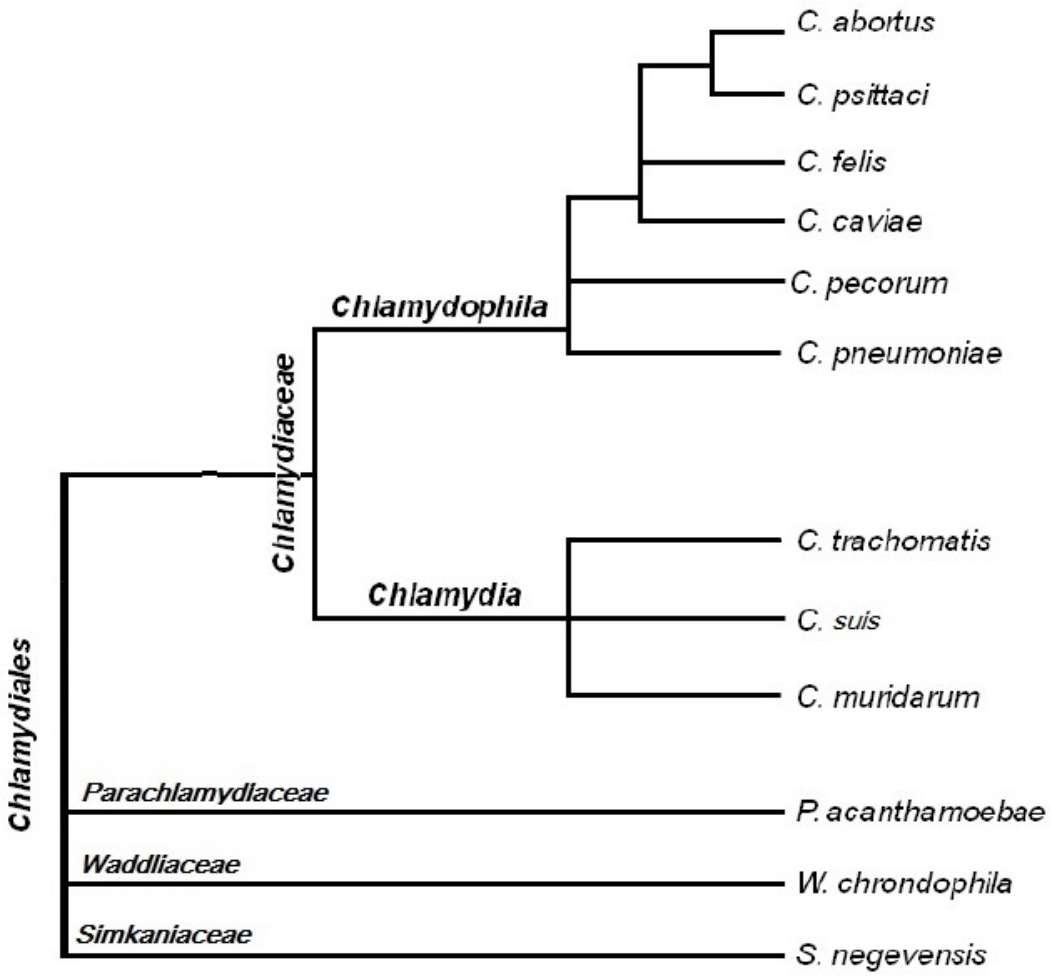


**Fig. 1.1: Taxonomy of the order *Chlamydiales*.** Based on the 16S and 23S rRNA, classification of the *Chlamydiales*. The phylogeny of the order *Chlamydiales* comprises four families, *Chlamydiaceae, Parachlamydiaceae, Waddliaceae* and *Simkaniaceae*. Further the *Chlamydiaceae* family is subdivided into the two genera *Chlamydophila* and *Chlamydia*, however this separation is under debate. Together, they consist of several important human pathogens, such as *C. pneumoniae, C. psittaci* and *C. trachomatis*. The distance of the drawn lines do not correlate to the phylogenetic distance. Modified after Bush and Everett, 2001 [13].

like atherosclerosis [25], asthma [26] and Alzheimer disease [27].

*C. trachomatis* is the most common bacterial cause of sexually transmitted diseases worldwide and the leading cause of infection acquired blindness. The kind of disease *C. trachomatis* provokes upon infection depends on the serovar, based on serological and genomic variations of the chlamydial major outer membrane protein (MOMP) [28] [29] [30]. Originally 15 serovars of *C. trachomatis* have been described [31], which can be subdivided into three biovars.

The causative agents of ocular trachoma, an inflammation in the conjunctiva, are serovars A to C. Chronic or reiterating infections may result in irreversible trachoma blindness if not treated, through scarring of the eyelid and cornea [32]. Trachoma infection is promoted by poverty and low hygiene levels; therefore, the endemic regions are predominantly located within the developing world like Africa and Middle East [32]. Worldwide, an estimated number of 2.2 million people are visually impaired due to trachoma, of whom 1.2 million are blind (World Health Organization (WHO), data from June 2019). The SAFE program has been implemented for its prevention. On the one hand, this includes treatment of affected people by comprising as ultimate ratio surgery and antibiotic therapy with Azithromycin as antibiotic of choice. Along with promoting facial cleanliness and access to sanitation [33].

Serovars D to K are sexually transmitted causing infections of the urogenital tract, leading to urethritis, prostatitis and pelvic inflammatory disease. In women, infection can induce salpingitis, infertility and ectopic pregnancies [23] [34] [35]. Moreover, untreated chlamydial infections increase the risk of HIV in both sexes [36] [37]. Its worldwide spread and, the often asymptomatic nature of the infection, considerably contributes to its position as most common bacterial sexually transmitted disease [38] [39], with about 106 million new cases in 2016, for instance [40]. Interestingly, *C. trachomatis* joins the growing list of pathogens related to cancer development [41], since chlamydial infections have recently been linked to the development of cervical and ovarian cancer [42] [43] [44] [45]. Invasive lymphogranuloma venereum (LGV) caused by the serovars L1 to L3 is likewise sexually transmitted. The infection of monocytes and macrophages by the pathogen results in the spread to the lymphatic system. After infection, the genital ulcer formation might stay unnoticed, but the inflammation of the lymphatic tissue and the lymph node enlargement are commonly painful and if untreated

lead to the irreversible destruction of the tissue [29] [46].

In general, due to the rare development of antibiotic resistance so far [47], chlamydial infections can be efficiently treated with antibiotics, especially macrolides and tetracyclines (WHO guidelines for the treatment of *C. trachomatis*, 2016). Additionally, efforts are made to develop a vaccine against *C. trachomatis* [48] [49] [50].

## 1.3 The development cycle of *Chlamydiales*

*Chlamydiales* have successfully established an intracellular life style, in which they overcome obstacles like entry, living and replication inside a host cell as well as surviving while transitioning to a new host. In order to vanquish the obstructions, *Chlamydiales* developed a unique, biphasic life cycle, alternating between two morphologically and physiologically distinct forms (Figure 1.2) [51]. The chlamydial developmental stage necessary to survive the harsh extracellular conditions represents the electron dense, infectious elementary body (EB), with a mean diameter of 0.3 $\mu$m [52] and a severely reduced metabolic activity [53]. Within the eukaryotic host cell, the predominant developmental stage is the much bigger reticulate body (RB) with a mean diameter of 1 $\mu$m, which is metabolically active [17] [54] and replicates by a polarized cell division process called budding [55].

Several chlamydial ligands and host surface proteins mediate the attachment of the EBs to the host cell [57], such as a reversible electrostatic interaction with heparan sulfate moieties and a subsequent high affinity binding [58]. Due to this irreversible binding step chlamydial effectors get injected into the host cytoplasm via a type III secretion system (TTSS). Thus, the chlamydial effector TarP induces the remodelling of actin and, among others, the uptake of the EB by receptor mediated endocytosis [59] [60]. Internalized EBs differentiate to RBs. The nascent vacuole becomes heavily modified, due to type III secreted inclusion proteins (Inc), in order to ensure a microtubule-based transport to a near Golgi, prenuclear position [61] and to avoid lysosomal fusion [62]. *C. pneumoniae* infected cells contain several inclusions, while *C. trachomatis*, for instance, mediates homotypic fusion of several *Chlamydia* containing vacuoles via IncA [63] [64]. Within the resulting vacuole, the so called inclusion, RBs start

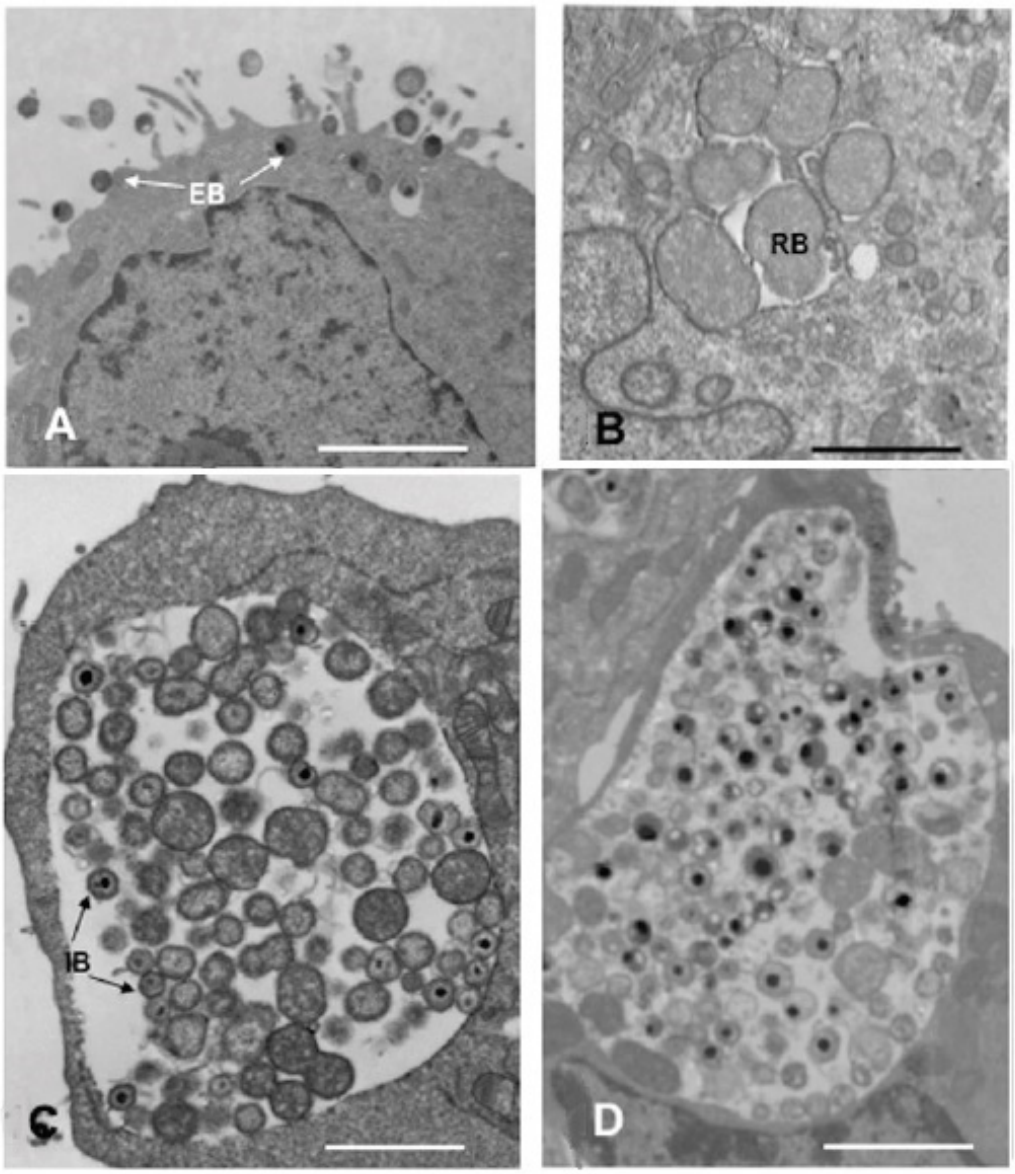


**Fig. 1.2: Stages of *C. trachomatis*.** Different stages of the genital *C. trachomatis* developmental cycle in human endometrial epithelial cells. **A.** Attachment and entry of infectious EBs (elementary bodies), 1 hour after infection. **B.** Inclusion around 8-12 hours of infection with RBs (reticulate bodies). **C.** Inclusion at mid-developmental cycle containing RBs and IBs (intermediate bodies). **D.** Mature inclusion containing more EBs than RBs. Bar = 2 $\mu$m. Modified after Wyrick, 2010 [56].

to multiply by an unipolar growth and FtsZ-independent fission, which is a so called budding process [55]. The interaction of the chlamydial inclusion with other cell compartments provides optimal conditions for nutrient acquisition and replication. Mitochondria, smooth endoplasmic reticulum, peroxisomes and lipid droplets have been shown to be in close contact with the inclusion [57] [65], and the fragmentation of the Golgi apparatus is speculated to supply *Chlamydia* with lipids [66]. After several rounds of fission and depletion of the nutrients and adenosine triphosphate (ATP), the non-infectious RBs re-convert asynchronously into infectious EBs. However, the exact stimulus is currently unknown [56] [58]. A recent finding supports the theory in which RB size controls the timing of RB to EB conversion without the need of an external signal [67]. Thereafter, EBs are released either by host cell lysis or by extrusion, an exocytosis like mechanism [68]. To spread infection, released EBs are then able to infect neighbouring cells in the infected tissue. The life cycle takes between 48 and 96 hours, depending on the species (Figure 1.3).

Alongside the active infection, *Chlamydia* may also persist over a long period of time within the host cell and establish a chronic infection. The pathogen remains viable and replicates its genome while it exhibits a decreased metabolic activity and inhibited cell division during persistency, which leads to an enlarged pleomorphic aberrant body (AB) [56]. Transition to persistence is a reversible process, which has been shown to be induced in vitro among others by penicillin [70], iron deficiency [71], amino acid starvation [72] and interferon-$\gamma$ (IFN-$\gamma$) [56] [73]. Excessive host cell inflammatory responses during *Chlamydia* infections cause the infection associated pathology. Chlamydial epithelial and myeloid cell infection leads to the production of inflammatory cytokines [74]. The immune regulated cytokine IFN-$\gamma$ induces indoleamine-2,3-dioxygenase (IDO), an enzyme that catalyses the initial step of L-tryptophan degradation to N-formyl kynurenine and kynurenine [75], leading to persistency [73] [76]. Therefore, the transition to persistence might represent an important chlamydial survival mechanism against the immune response of the host [56] [73].

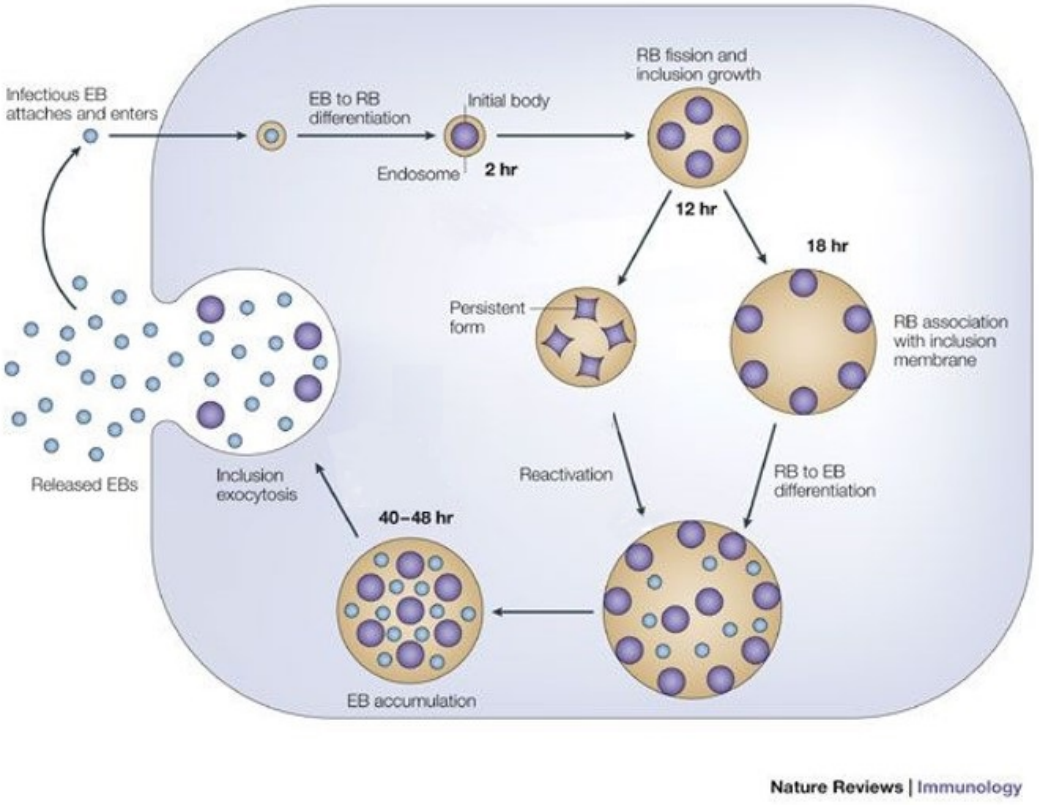


**Fig. 1.3: The chlamydial developmental cycle.** Incorporation via endocytosis of infectious EB (elementary body), which are attached to the host cell. Conversion of the EB into the replicative, metabolically active RB (reticulate body) takes place in the endosome, and the vesicle gets heavily modified to form the so called inclusion. Replication of RBs by a budding process starts and they re-convert to EBs asynchronously towards the end of the developmental cycle. Release of bacterial progenies is either via host cell lysis or by inclusion extrusion Modified after Brunham et al., 2005 [69].

## 1.4 The immune regulated cytokine IFN-$\gamma$ leads to persistency

IFN-$\gamma$ (interferon-$\gamma$) is a type II interferon, which binds the extracellular domain of the interferon-$\gamma$ receptor 1 (IFNGR1). This subunit is associated with the Janus kinase (Jak1), whereas the IFNGR2 subunit is coupled with Jak2. The activation of Jak1 and Jak2 leads to receptor phosphorylation, subsequent recruitment and phosphorylation of the signal transducer and activator of transcription (STAT1). STAT1 gets phosphorylated at the tyrosine 701 and the serine 727 residues, resulting in its homodimerization and nuclear translocation. To regulate the transcription of its target genes, STAT1 homodimers bind to the interferon-$\gamma$ activated sequence (GAS) elements in the promoter region [77] [78] [79] (Figure 1.4). IFN-$\gamma$ functions as a cytokine inhibiting or promoting the growth in a STAT1-dependent manner [77] [80]. STAT1 homodimer occupation of the consensus GAS element in the *c-myc* promoter inhibits its expression transcriptionally [77]. Concurrently, *stat1* expression is a negative target gene of the proto-oncogene c-Myc. Hence, regulation at a transcriptional level takes place in a negative feedback loop of each other [81].

## 1.5 The proto-oncogene c-Myc and its function

The proto-oncogene c-Myc is a transcription factor which is ubiquitously present in eukaryotes, orchestrating manifold cellular processes, such as proliferation, differentiation, cell growth, apoptosis and metabolism. The gene was first discovered as *v-myc* oncogene in avian retrovirus MC29, which is responsible for the tumorigenic effects of the retrovirus [82] [83] [84] [85]. Only in the 1980s, the cellular homolog *c-myc* increasingly gained attention in research. The *myc* oncogene family consists of three highly conserved members, *myc* (c-Myc), *mycl* (L-Myc) and *mycn* (N-Myc), with dissimilar tissue expression patterns during differentiation and development [82] [86]. In a wide range of malignancies the members of the Myc family, especially N-Myc and c-Myc, are deregulated by divers mechanisms [87] [88].

During healthy development, c-Myc is subjected to a tight regulation on a transcriptional and post-transcriptional level, and the activity of the c-Myc protein is additionally modulated by mitogenic signalling via phosphorylation of distinct residues [89] [90]. Furthermore, the 20

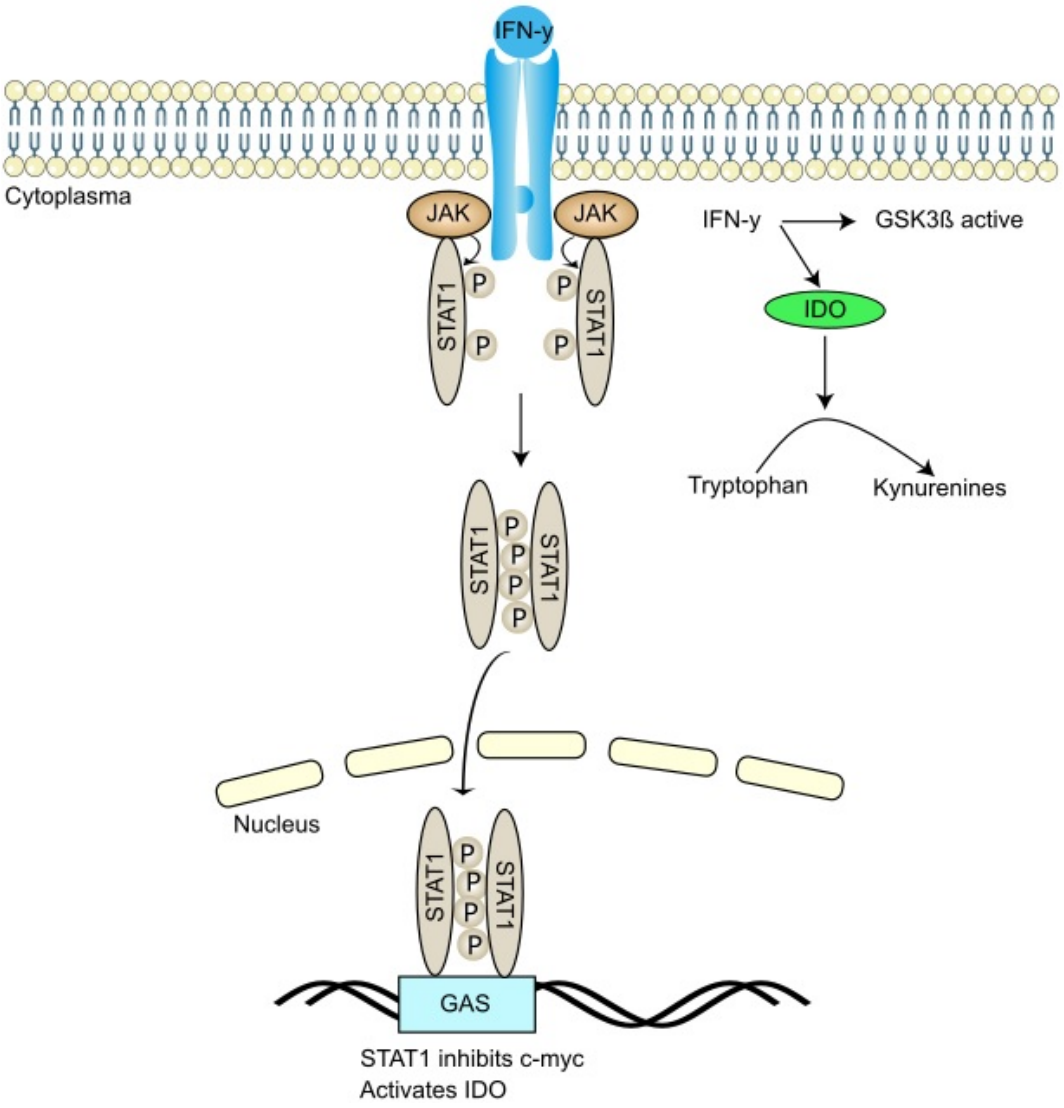


**Fig. 1.4: The Jak-STAT1 pathway.** Interferon-$\gamma$ binds interferon-$\gamma$ receptor, activates Jak and leads to receptor phosphorylation, which recruits and phosphorylates the signal transducer and activator of transcription (STAT1). Phosphorylated STAT1 builds homodimers and get translocated to the nucleus, where it binds the GAS (interferon-$\gamma$ activated sequence) elements to inhibit c-Myc transcription and activate IDO (indoleamine-2,3-dioxygenase). IDO catalyses the initial step of the L-tryptophan degradation. Interferon-$\gamma$ keeps additionally GSK3$\beta$ (glycogen synthase kinase 3) active.

– 30 minutes half-life of both the mRNA and the protein is drastically short [91] [92]. Phosphorylation of serine 62 (Ser62) via the activation of the Ras/Raf/MEK/ERK pathway results in stabilization of c-Myc, while adding a second phosphorylation at threonine 58 (Thr58) leads to ubiquitination, followed by rapid proteasomal degradation. Thr58 phosphorylation is limited through Ras-mediated stimulation of the PI3K/AKT pathway via inhibition of the correspondent kinase, GSK-3$\beta$ [93] [94] (Figure 1.5). Normally c-Myc activity is tightly regulated in primary cells via the mechanisms mentioned before. A huge set of insults interfering with its proper regulation, such as gene amplification, translocation, increased mRNA stability or decreased protein degradation, lead to oncogenic activation of c-Myc [88] [87] [95].

Belonging to the basic helix-loop-helix leucine-zipper (bHLH-LZ) transcription factor family, c-Myc requires a heterodimerization with another bHLH-LZ factor Max to be active and exert its regulatory effect on transcription [96]. An additional layer of c-Myc regulation provides heterodimerization of Max with Mad family members to antagonize c-Myc activity [97]. In order to regulate the transcription of their target genes, the c-Myc/Max complexes bind to the conserved Enhancer-box (E-box) element CACGTG. They act either as a transcription activator or a transcription repressor [88].

A broad range of biological processes are regulated via c-Myc, like cell growth, proliferation, translation and induction of apoptosis [98]. Moreover, c-Myc activity markedly increases the energy production, as well as the anabolic metabolism, promotes the aerobic glycolysis, induces TCA cycle activity and mitochondrial biogenesis. Glutaminolysis is a c-Myc promoted process, in which L-glutamine is converted to L-glutamate via glutaminase. L-glutamate is then further modified by the enzyme glutamate dehydrogenase (GDH) or via several transaminases to the TCA cycle intermediate $\alpha$-ketoglutarate. Hence, energy production raises through the increased glutaminolytic flux. Enhanced TCA cycle intermediates are obtainable for additional production of amino acids, lipids and nucleotides [90]. Thus, the high demand for macromolecular building blocks in rapidly proliferating cells can be satisfied. Nucleotide biosynthesis is also critically controlled via c-Myc by directly regulating the expression of the genes which encode the enzymes involved in the production of precursors of all nucleotides [99] [100]. The cis-regulated element in the 5' UTR of the phosphoribosylpyrophosphate syn-

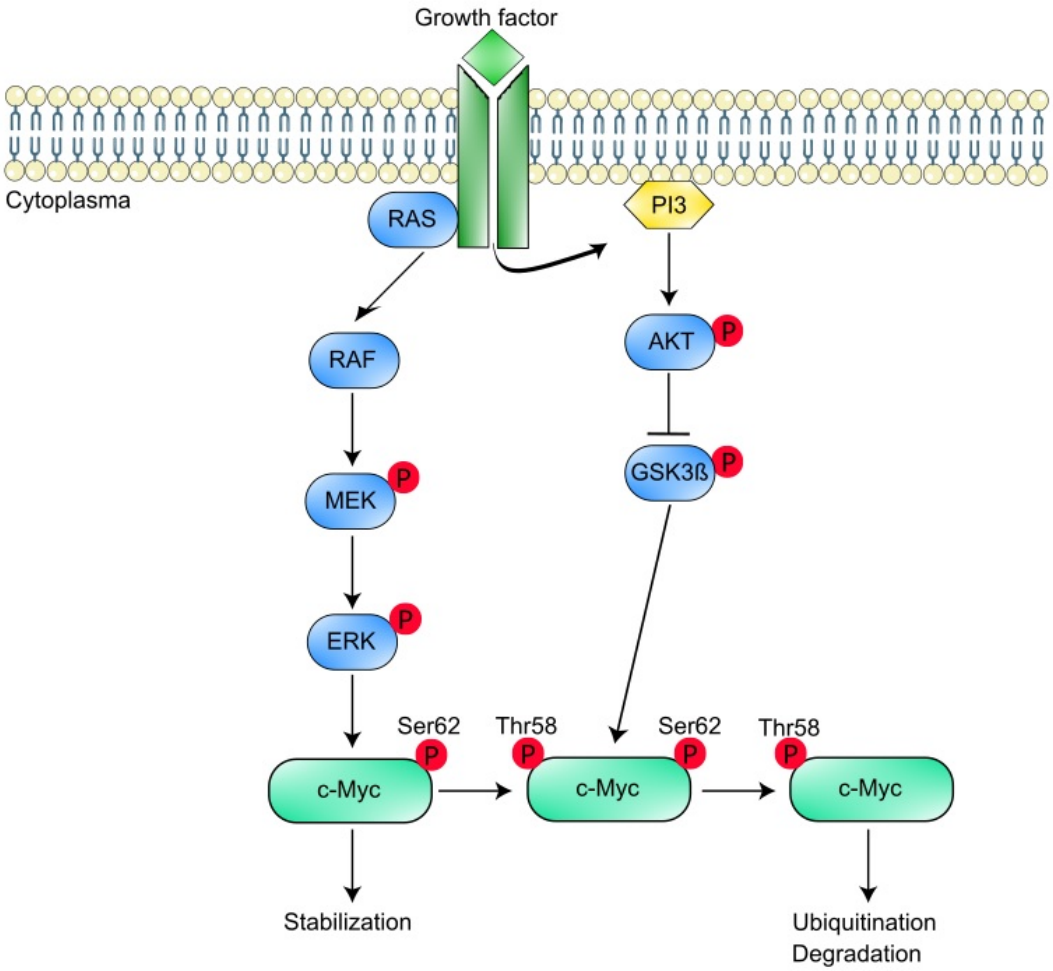


**Fig. 1.5: c-Myc protein modulated by mitogenic signalling via phosphorylation of distinct residues.** Activation of the Ras/Raf/MEK/ERK pathway leads to c-Myc phosphorylation on the serine 62 residue via ERK, which stabilizes the transcription factor. Adding a second phosphorylation at the threonine 58 residue of c-Myc via active GSK3$\beta$ (glycogen synthase kinase 3) leads to ubiquitination and proteasomal degradation of the proto-oncogene. Active PI3K pathway phosphorylates GSK3$\beta$ via AKT, which inhibits the kinase activity and results in stabilization of c-Myc due to no threonine 58 phosphorylation.

thetase (PRPS2), catalysing the first committed step in nucleotide biosynthesis is driven by c-Myc [101]. As a response to growth sensing cues such as estragon receptor/Sp1 [102], Hif $1/2\alpha$ [103] or EGFR/MAP kinase pathway c-Myc regulates carbamoyl aspartase dehydratase (CAD), which has been described as a rate limiting step in the pyrimidine biosynthesis [104].

## 1.6 The energy parasite hypothesis and *Chlamydia*

*Chlamydia* have a remarkable small genome of about 1.06 Mbp, which results from the reductive genome evolution and the intimate dependency on the host cell for nutrient acquisition [105] [106]. Due to the concurrent discussion on a rather viral or bacterial origin of the *Chlamydia*, and the early failure to identify an own chlamydial energy metabolism, the energy parasite hypothesis was proposed [107]. This concept states that the complete absence of own metabolic pathways leads to a strictly dependence of the pathogen on the uptake of ready made host cell derived nutrients and energy equivalents. However, an increasing amount of metabolic reactions was found to be functional in *Chlamydia* in the following years, and its metabolic pathways broadly intersect with and depend on its counterparts in the host cell [108]. The view of an existing but partially limited metabolic capacity of *Chlamydia* was further supported through the advent of genomics [109]. The recent observation that EBs can generate ATP in axenic cultivation from D-glucose-6-phosphate via substrate level phosphorylation, the presence of the glycolytic pathway starting from D-glucose-6-phosphate, a partial unidirectional tricarboxylic acid (TCA) cycle, the pentose phosphate pathway (PPP) and the basal system for oxidative phosphorylation suggested a less strict metabolic dependency on the host as previously thought and questioned the prevailing energy parasite hypothesis [53] [108]. *C. trachomatis* is unable to run gluconeogenesis, while the glycogen synthesis pathway and a functional pentose phosphate pathway are present. A functioning glycolytic pathway starting with D-glucose-6-phosphate produces pyruvate, which *C. trachomatis* is ineligible to feed into its TCA cycle due to the lack of citrate synthase and pyruvate carboxylase, but the pathogen can direct pyruvate towards alanine synthesis by transamination or use it for fatty acid synthesis via acetyl-CoA. The chlamydial TCA cycle lacks citrate synthase, aconitase

and isocitrate dehydrogenase, and has only a unidirectional conversion of $\alpha$-ketoglutarate to succinate, wherefore, it forms only $\alpha$-ketoglutarate to oxaloacetate (Figure 1.6). Then, via transamination oxaloacetate can be converted to aspartate or to phosphoenolpyruvate through decarboxylation. To fuel the bacterial TCA cycle, L-glutamine is degraded to L-glutamate and then further converted to $\alpha$-ketoglutarate [108] [110]. Despite the appearing evidence of certain metabolic capacity, host cell derived nutrients have an important impact on successful replication and are extensively taken up by *Chlamydia*. The primary energy source during RB replication is ATP, which was proven to be taken up and utilized earlier [111] [112]. *Chlamydia* is auxotroph for nucleotides but bacterial transporters have been identified [113] [114]. One of the transporters, the ATP/ADP translocase Npt1 [114] is additionally responsible for the uptake of NAD [115], which is in line with the finding of an accumulation of NAD(P)H at the inclusion membrane and inside the inclusion [116]. Apart from nucleoside import, the active uptake of host glycerophospholipids, cytosolic lipid droplets and fatty acids have been reported [117] [118] [119], and from Golgi derived vesicles Sphingolipids and cholesterol are intercepted [120] [121]. *Chlamydia* lacks hexokinase, therefore the uptake of phosphorylated D-glucose via the chlamydial transporter UhpC [122] [123] is essential for fuelling chlamydial glycolysis. Moreover, *C. trachomatis* accumulates glycogen as an energy store within the inclusion lumen [124]. This is achieved by the uptake of bulk glycogen from the cytosol and intra-luminal synthesis out of imported UDP-glucose [125]. It has been shown that amino acid availability is critically to modulate infection efficiency [126], which is not surprising since *C. trachomatis* is unable to synthesize most amino acids on its own [122] [127] and therefore imports the amino acids from the host cell cytosol [110]. Recently, it has been demonstrated that the pathogen is actively taking up vitamins and TCA cycle derived dicarboxylic acids [110] [128]. With the improvements in understanding the molecular mechanisms and the increasing diversity of imported nutrients needed, the rationale of chlamydial infections in order to use the energy stores provide by the host for a successful infection, becomes more and more evident.

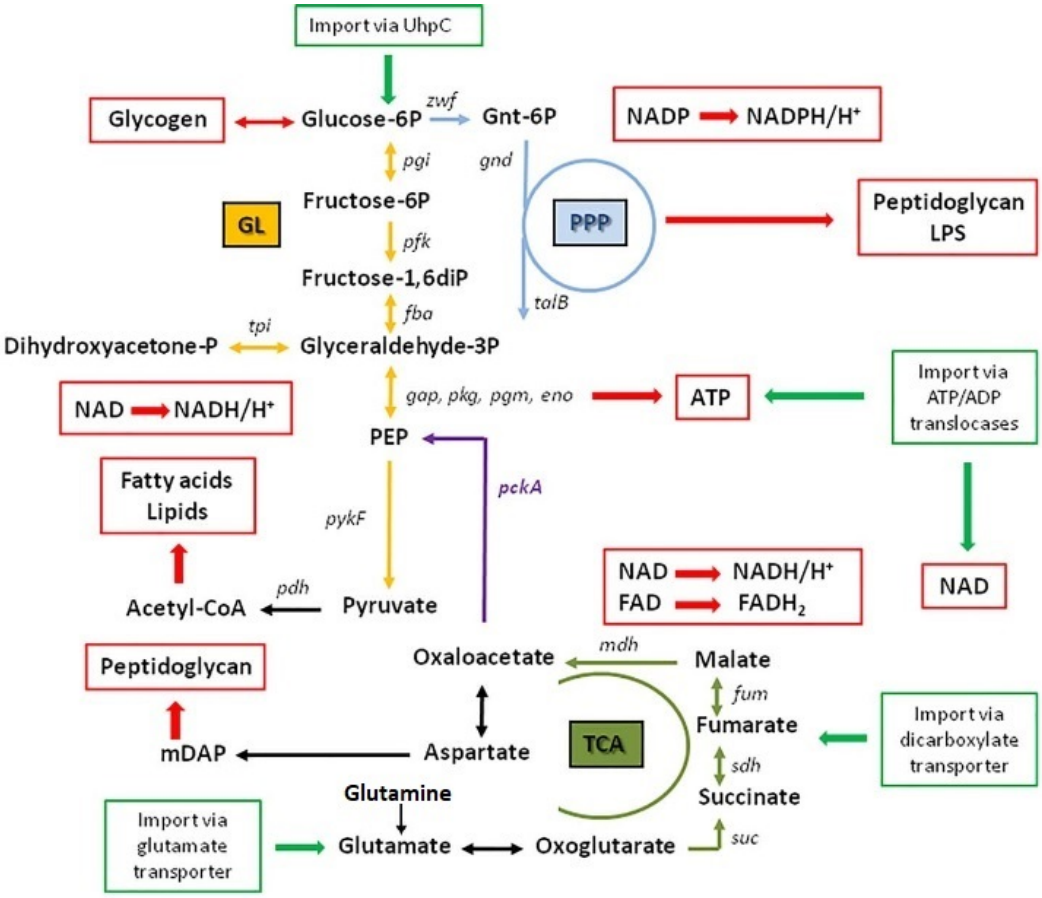


**Fig. 1.6: Chlamydial carbon metabolism.** *C. trachomatis* imports D-glucose-6-phosphate via the UhpC transporter, which can be used for glycogen biosynthesis, or metabolized via glycolysis (GL, yellow) or the pentose phosphate pathway (PPP, blue). Pyruvate, the glycolytic end product, is decarboxylated to acetyl-CoA and further used for chlamydial lipid and fatty acid synthesis. Intermediates of PPP are utilized for LPS and peptidoglycan synthesis. Unidirectional from L-glutamine derived oxoglutarate to oxaloacetate runs the partial TCA cycle (green). Oxaloacetate can be processed to L-aspartate and meso-diaminopimelic acid (mDAP), a substrate for peptidoglycan synthesis, or it can be converted to phosphoenolpyruvate (PEP). Highlighted in green boxes are the transporters. Modified after Mehlitz et al., 2016 [110].

## 1.7 *C. trachomatis* interferes with host cell signalling pathways

In order to establish a flourishing infection, *Chlamydia* extensively interferes with host cell signalling pathways. Underlining the potential link between induction of cervical and ovarian cancer through a chlamydial infection, most of these pathways are implicated in the development of tumours. An important role in invasion and infection regulation plays the receptor tyrosine kinase (RTK) pathway, the platelet derived growth factor receptor (PDGFR), the fibroblast growth factor receptor (FGFR) and the epidermal growth factor receptor (EGFR) [129] [130] [131].

The Ras/Raf/MEK/ERK pathway couples the plethora of extracellular signals, such as growth factors or cytokines, which are bound by cell surface receptors to induce downstream signalling and subsequent transcriptional regulation. If a ligand binds its respective RTK it leads to the activation of the Ras GTPase. Active GTP-loaded Ras recruits Raf to activate its kinase activity, which leads to the phosphorylation of the downstream kinase MEK, phosphorylating and activating its effector kinase extracellular signal regulated kinase (ERK). Among the numerous targets of ERK are proliferative and anti-apoptotic factors, such as pRB, CREB, Bcl-2 and c-Myc [132] [133]. *C. trachomatis* sustainably activates ERK in a Ras/Raf-independent manner [134], utilizing pro-inflammatory signalling via interleukin (IL)-8 production [135], activating cytosolic phospholipase A2 (cPLA2) to facilitate acquisition of host lipids [136], and inducing apoptotic resistance through upregulation and stabilization of the anti-apoptotic Bcl-2 family member Mcl-1 [137] and the Bcl-2 binding protein BAG-1 [138].

The PI3K/AKT pathway is also activated by extracellular stimuli, mostly RTKs but it can also be G protein coupled receptors (GPCR) to which the according ligand binds in order to induce signalling. This receptor stimulation leads to a direct or indirect recruitment and activation of phosphatidylinositol-3-kinase (PI3K), which converts phosphatidylinositol-4,5-bisphosphate (PIP2) into the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3). This reaction is counter balanced by the activity of the phosphatase PTEN. The ac-

tivator kinase PDK1 and the effector kinase AKT are recruited via PIP3, resulting in the full activation of AKT. Active AKT induces apoptosis resistance, cell survival mechanisms, cell growth and proliferation via c-Myc and the nutrient sensor mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) [139] [140] [141]. It has been shown that chlamydial infection leads to AKT-dependent apoptosis resistance in its host cell via phosphorylation and sequestration of pro-apoptotic BAD away from mitochondria [142], and concordant action of AKT and ERK on anti-apoptotic Mcl-1 upregulation [137]. Furthermore, the Ephrin A2 receptor is exploited for invasion as well as AKT signalling induction [143]. Interestingly, unpublished data shows the downregulation of the PI3K counteracting phosphatase PTEN during *C. trachomatis* infection (personal communication Dr. Karthika Rajeeve).

Moreover, the during chlamydial infection known degradation of the cell cycle arrest and apoptosis inducing tumour suppressor p53, via PI3K/AKT-mediated activation of its ubiquitin ligase HDM2, renders host cell resistant to apoptosis and DNA damage-related cell cycle arrest as well as leading to a metabolic reprogramming of the host by upregulating the pentose phosphate pathway (PPP) [144] [145]. In *C. trachomatis* infected cells hypoxia induced transcription factor (HIF)-1$\alpha$ prevents apoptosis via a presumably MAPK-dependent upregulation of anti-apoptotic Mcl-1 [137] [146], while HIF-1$\alpha$ upregulation was found to promote D-glucose uptake in *C. pneumoniae* infected cells [147], suggesting an enhancement of glycolysis which has been proven before in *C. psittaci* infected cells [148]. Known regulators of HIF-1$\alpha$ are both the PI3K/AKT and the MEK/ERK pathways [149], therefore, it is tempting to presume that the stabilization of this transcription factor during chlamydial infection is also achieved by the previously described upregulated pathways.

Taken together, *Chlamydiae* interfere with their host cells to promote two decisive cellular programs, activating anti-apoptotic mechanisms to ensure successful completion of the chlamydial life cycle and creating a unique metabolic niche, which is highly supportive for chlamydial growth, within the host cell via proliferative or pro-survival signalling.

## 1.8 The cancer connection

After showing infections can induce tumour formation more than hundred years ago [150], the motion of malignant transformation induced by virus is commonly accepted. Since then an increasing number of tumour inducing viruses have been identified [151]. There are different ways oncogenic viruses have been demonstrated to promote transformation. A subset of viruses encodes effectors to activate cellular proto-oncogenes, while other viruses contain a transforming oncogene, which is often a viral homologue of a human proto-oncogene. Among others, deregulation of proliferation and growth may result in transformation and, in case of limited immune surveillance, finally result in tumour development [152] [153].

Far less studied is the tumour induction by bacterial pathogens. Up to date only *Helicobacter pylori* is predisposing infected people to gastric cancer and has been classified as a class I carcinogen by the WHO [154]. Responsible for *Helicobacter pylori* induced transformation is the cooperative effect of proliferation promotion, DNA damage, chronic inflammation, apoptosis interference and several bacterial toxins. However, it is also dependent on the immune repertoire of the individual [155].

Despite, evidence indicating a more common link between bacterial infection and cancer predisposition is growing. Common aspects of the predisposing factors discussed which favour transformation are apoptosis inhibition, sustained proliferative signalling and chronic inflammation. Furthermore, accumulation of mutations and genomic instability promoted by genotoxic stress, either directly through bacterial toxins or indirectly via increased ROS production and imposed DNA damage response might lead to deregulated expression of tumour suppressors and proto-oncogenes [156].

First evidence to associate *C. trachomatis* to cancer have been based on an epidemiological correlation between *Chlamydia* reactive serum antibodies and the presence of cervical cancer [43] [44]. Additionally, occurrence of typical transformation predisposing features during chlamydial infection have been demonstrated in recent work. It has been shown that *Chlamydia* induces proliferative signalling, confer apoptosis resistance [137], promote DNA damage [157] and establishes severe inflammation in the infected tissue [135]. Taken together it seems

likely that a *C. trachomatis* infection represents an important risk factor for the development of cancer.

## 1.9 Aim of the study

Previous work has shown that infection of both immortalized and primary cell lines with *C. trachomatis* leads to a pronounced upregulation of the proto-oncogene c-Myc [158] [159]. Furthermore, the activation of the PI3K/AKT and the Ras/Raf/MEK/ERK pathways have been described to stabilize c-Myc in a phosphorylation dependent manner upon chlamydial infection. In line with this, impairment of c-Myc pharmacological and RNAi-based markedly reduced the growth of *C. trachomatis* in infected cells [159]. *Chlamydia* being an energy parasite, lead to the speculation that the pathogen establishes an intracellular growth supportive metabolic niche via its interference with the host cell signalling pathways. Especially since it stabilizes the transcription factor c-Myc, which is involved in the regulation of a plethora of metabolic pathways. For the tumour suppressor p53 and the NADPH-, ribose-generating pentose phosphate pathway a comparable connection has previously been proven [145]. As mentioned before c-Myc increases glutaminolysis and according to previous publications L-glutamine, among other amino acids, is important for chlamydial replication [72]. Moreover, the depletion of tryptophan due to the expression of IDO has been described as sole reason for IFN-$\gamma$ induced persistency in *Chlamydia*. However the STAT1-dependent depletion of c-Myc induced by interferon-$\gamma$, which results in deregulation of the host cell metabolic pathways, driving *Chlamydia* into its persistent form has not been considered. Therefore, we investigate the potential role of L-glutamine during a *C. trachomatis* infection and the impact of IFN-$\gamma$ on the c-Myc levels, disturbing metabolic control and serving as a crucial cause of chlamydial dormancy.

# 2 Results

## 2.1 *C. trachomatis* infection upregulates and stabilizes the proto-oncogene c-Myc

The rigorously regulated c-Myc is known as a master regulator of cellular metabolism [160], particularly the mitochondrial glutamine metabolism [161] [162] inter alia, and in *Chlamydia* infected cells it also plays a role in apoptosis inhibition [158]. Therefore, the levels of c-Myc were examined after infection with a variety of *Chlamydia* species in human and mouse cell lines. In all tested cell lines, c-Myc protein levels were strongly upregulated in a time-dependent manner during infection, starting already at 12 hours post infection (hpi) and remaining elevated up to 48 hpi (Figure 2.1 and Figure 2.2). A similar effect was observed upon infection of HeLa 229 cells with different *Chlamydia* species (Figure 2.2 B and C). Moreover, in line with its role as an active transcription factor, c-Myc is translocated from the cytosol into the nucleus in HUVEC cells after infection with *C. trachomatis* (Figure 2.2 D). These observations are consistent with the previously perceived activation and stabilization of c-Myc upon chlamydial infection [159] [163] [164].

It was interesting to investigate next the potential mechanism, which could be involved in the detected induction of the transcription factor in response to an infection with the pathogen. Hence, in *Chlamydia* infected HeLa 229 cells real-time PCR was performed, revealing a transient increase of c-Myc mRNA during infection (Figure 2.3 A). In addition to the transcriptional regulation, it has been demonstrated that in response to mitogenic signalling, the conserved residues serine 62 (Ser62) and threonine 58 (Thr58) become phosphorylated to regulate c-Myc protein stability [93]. Serine 62 phosphorylation stabilizes c-Myc whereas the

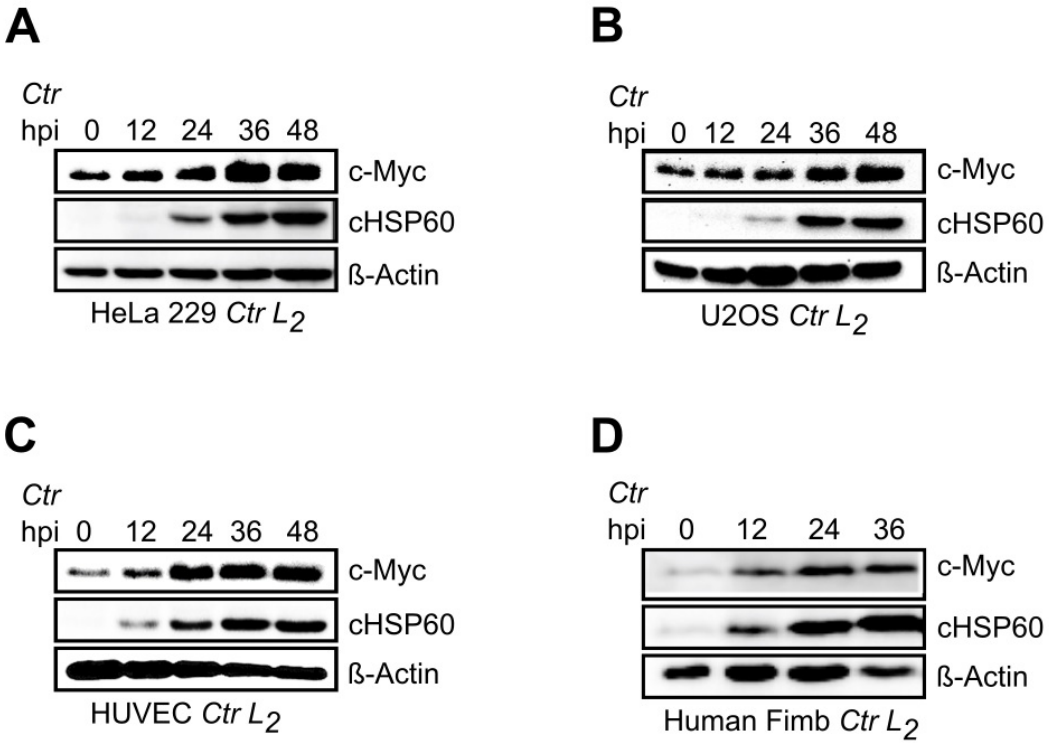


**Fig. 2.1:** ***C. trachomatis*** **infection upregulates the proto-oncogene c-Myc.** Different cell lines were infected with *Chlamydia*, lysed and analysed for c-Myc levels via Western blot. cHSP60 served as infection control and $\beta$-Actin as loading control. For all experiments n=3. One representative blot is shown. **A.** HeLa 229 cells were infected with *C. trachomatis* $L_2$ at MOI 1 for different time points. **B.** U2OS cells were infected with *C. trachomatis* $L_2$ at MOI 1 for different time points. **C.** HUVEC cells were infected with *C. trachomatis* $L_2$ at MOI 1 for different time points. **D.** Human Fimb cells were infected with *C. trachomatis* $L_2$ at MOI 1 for different time points ([159]).

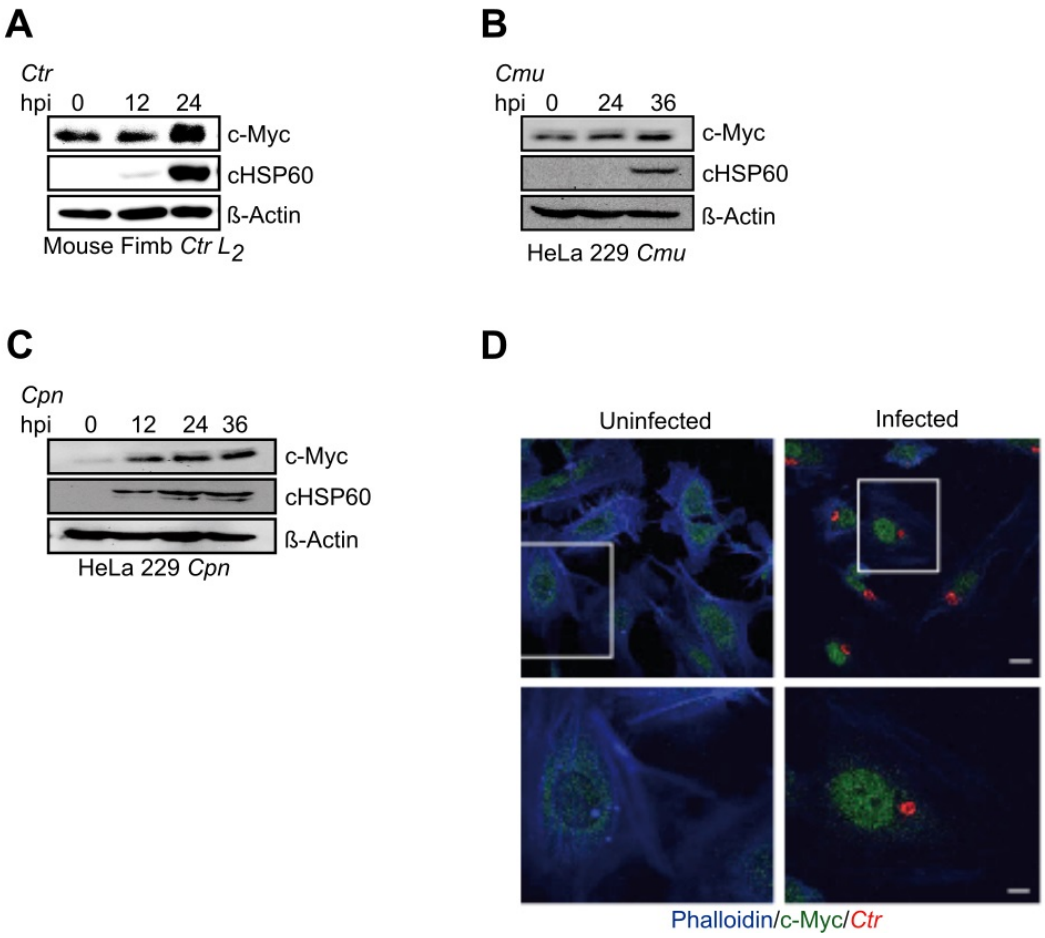


**Fig. 2.2:** ***C. trachomatis*** **infection stabilizes the proto-oncogene c-Myc.** Different cell lines were infected with *Chlamydia*, lysed and analysed for c-Myc levels via Western blot. cHSP60 served as infection control and $\beta$-Actin as loading control. For all experiments n=3. One representative blot is shown. **A.** Mouse Fimb were infected with *C. trachomatis* $L_2$ at MOI 1 for different time points. **B.** HeLa 229 cells were infected with *Chlamydia muridarum (Cmu)* at MOI 1 for different time points. **C.** HeLa 229 cells were infected with *Chlamydophila pneumoniae (Cpn)* at MOI 1 for different time points. **D.** In order to examine subcellular c-Myc localization, HUVEC cells were infected with *C. trachomatis* $L_2$ at MOI 1, fixed with PFA after 18 hours and immunostained for c-Myc (green), *C. trachomatis* (cHSP60, red) and phalloidine (blue). (Thomas Wulff (master student department of microbiology, Würzburg) provided the immunofluorescence image shown in **D** [164]. *C. muridarum* and *C. pneumoniae* experiments were performed by Dr. Karthika Rajeeve (department of microbiology, Würzburg) [159].)

threonine 58 phosphorylation spawns the dephosphorylation of Ser62 via protein phosphatase 2A (PP2A), leading to ubiquitination by the SCF-Fwb7 E3 ligase and proteasomal degradation [165]. It could be observed that *Chlamydia* infection induces the Ser62 phosphorylation but not the one at Thr58 of c-Myc (Figure 2.3 B). Interestingly, in *C. trachomatis* infected cells, ubiquitinated c-Myc levels were markedly decreased (Figure 2.3 C). Consequently, it was determined if the pathogen alters the stability of the c-Myc protein. In order to check this, HeLa 229 cells were infected at different time points prior the addition of cycloheximide, a translation inhibitor. *Chlamydia* infected cells preserved c-Myc for extended periods of time, while in non-infected control cells the c-Myc levels rapidly dropped upon inhibition of translation (Figure 2.3 D), indicating a c-Myc stabilization upon infection [159] [163].

## 2.2 MAPK and PI3K signalling pathways stabilize c-Myc in *C. trachomatis*-infected cells

It is well known that *C. trachomatis* activates the MAPK and the PI3K pathways, which are critical for the growth and the proliferation of the pathogen [137] [143] [166]. Furthermore, activated Ras has been shown to induce Ser62 phosphorylation of c-Myc via the MAPK/ERK pathway in order to stabilize it. Phosphatidylinositol 3-kinase (PI3K) signalling keeps AKT inactive, which prevents the phosphorylation on Thr58 of c-Myc (Figure 1.5, Figure 2.4 A) [167]. Indeed, using specific inhibitors for the MAPK or the PI3K pathway, restricted the propagation of the pathogen and prevented the increase of the c-Myc protein in infected HeLa 229 cells (Figure 2.4 B and C). To show that impairment of chlamydial development via the inhibitors UO126 and LY294002 is mediated through the downregulation of c-Myc, the growth of *Chlamydia* was rescued upon anhydrous tetracycline (AHT)-induced c-Myc overexpression in the U2OS $^{Tet-On}$ cell line (Figure 2.5 A). More than that, under these conditions infectious progenies were obtained (Figure 2.4 D). Supportive for c-Myc playing a central role during chlamydial development were the experiments in HCT116 and HeLa 229 cells depleting c-Myc (Figure 2.5 C), and using the chemical inhibitor 10058-F4 (Figure 2.5 B), a cell permeable thiazolidinone specifically inhibiting transactivation of c-Myc target gene expression [159]

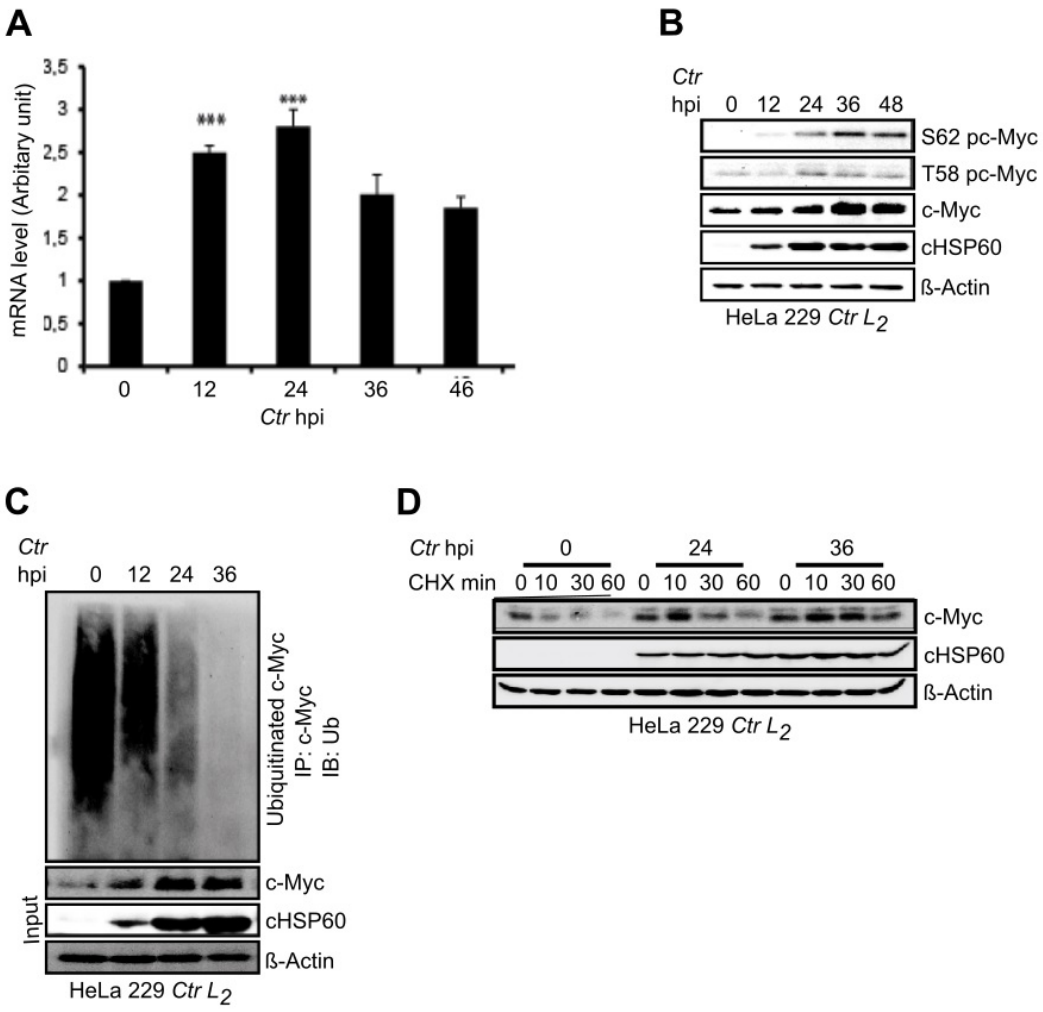


**Fig. 2.3: Potential mechanism of c-Myc stabilization during infection.** cHSP60 served as infection control and $\beta$-Actin as loading control. For all experiments n=3. One representative blot is shown. **A.** HeLa 229 cells were infected with *C. trachomatis* $L_2$ at MOI 1 for different time points. Total RNA was isolated and the c-Myc mRNA levels were detected via RT-PCR. *** $p<0.001$. **B.** HeLa 229 cells were infected with *C. trachomatis* $L_2$ at MOI 1 for different time points and analysed for Serine 62 and Threonine 58 phosphorylation of c-Myc. **C.** From HeLa 229 cells infected with *C. trachomatis* $L_2$ at MOI 1 for different time points, c-Myc was immunoprecipitated and ubiquitin was detected in the precipitate using Western blot. The input of the experiment was probed against c-Myc. **D.** HeLa 229 cells were infected with *C. trachomatis* $L_2$ at MOI 1 for 24 or 36 hours or left uninfected. The cells were treated with CHX (cycloheximide), a translation inhibitor, for 10, 30 or 60 minutes, then lysed and analysed by Western blot for c-Myc stabilization. (Dr. Karthika Rajeeve (department of microbiology, Würzburg) provided the mRNA analysis shown in **A** and performed the immunoprecipitation experiment [159].)

[163].

## 2.3 Reprogramming host glutamine metabolism is essential for *C. trachomatis*

High throughput RNA sequencing on control and *Chlamydia*-infected HUVEC cells was performed to compare gene expression profiles. Gene set enrichment analysis (GSEA) on the data revealed an upregulation of c-Myc target genes in the cell which were infected with *C. trachomatis*. Excitingly, the analysis of the infected cells showed a strong influence of *Chlamydia* on the host glutamine metabolic pathway and the host metabolite transporters [159]. Hence, the importance of L-glutamine (Gln) for the intracellular growth of *Chlamydia* was studied using L-glutamine depleted medium. The pathogen failed to replicate and produce infectious progenies in L-glutamine deficient medium, in HeLa 229 as well as in primary Human Fimb cells. Additionally, the infected cells were also not able to induce c-Myc expression [159] [164]. Recently, it had been shown that depletion of L-glutamine results in a rapid reduction of c-Myc, depending on the 3' UTR of the gene [168]. A HCT116 cell line allowing the restoration of c-Myc expression during glutamine starvation was used to rule out that chlamydial growth defect is an indirect L-glutamine effect due to c-Myc downregulation. Nevertheless, amino acid depletion attenuated the development of *C. trachomatis*, hinting that in the absence of L-glutamine, c-Myc cannot rescue chlamydial growth [159].

Previously, it has been predicted that *C. trachomatis* uses L-glutamine, L-glutamate (Glu) and $\alpha$-ketoglutarate ($\alpha$-KG) to feed their partial TCA cycle [109] [110] [169]. To further define if the pathogen takes these metabolites up directly, uptake assays in axenic culture were performed. GC-MS analysis of the supernatant showed neither a L-glutamate nor $\alpha$-ketoglutarate but a significant depletion of L-glutamine (Figure 2.6 A). Isotopologue profiling uses stable isotopes, which are incorporated into multiple metabolites in order to detected specific isotopologue compositions that reflect the biosynthetic routes and metabolic fluxes from the precursors to the products. Therefore, the axenic medium was supplemented with [U-$^{13}C_5$] glutamine and the chlamydial extracts were analysed for the distribution of the isotope.

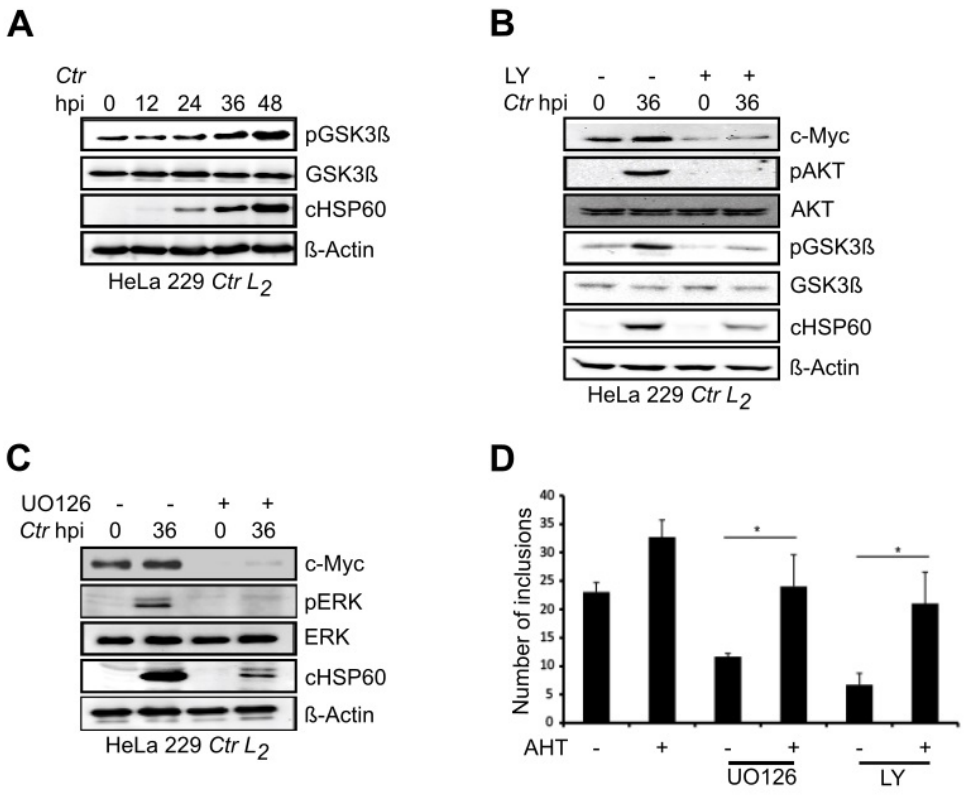


**Fig. 2.4:** ***Chlamydia*** **stabilizes c-Myc via the PI3K and MAPK pathway.** cHSP60 served as infection control and $\beta$-Actin as loading control. For all experiments n=3. One representative blot is shown. **A.** HeLa 229 cells were infected with *C. trachomatis* $L_2$ at MOI 1 for different time points and analysed for pGSK3$\beta$. **B. and C.** HeLa 229 cells were treated with either (**B**) LY294002 (30 $\mu$M), a PI3K inhibitor, or (**C**) UO126 (30 $\mu$M), a MAPK inhibitor, for 12 hours and then infected with *C. trachomatis* $L_2$ at MOI 1 for 36 hours. The samples were analysed for c-Myc, pAKT and pERK. **D.** U2OS $^{Tet-On}$ cells were induced with AHT (100 ng) for 12 hours, treated with UO126 or LY294002 inhibitor or left untreated and infected with *C. trachomatis* $L_2$ at MOI 1. From this experiment an infectivity assay was performed and the number of inclusions was counted to plot the shown graph. * $p<0.05$. (Analysis shown in **D** was performed by Dr. Karthika Rajeeve (department of microbiology, Würzburg) [159].)

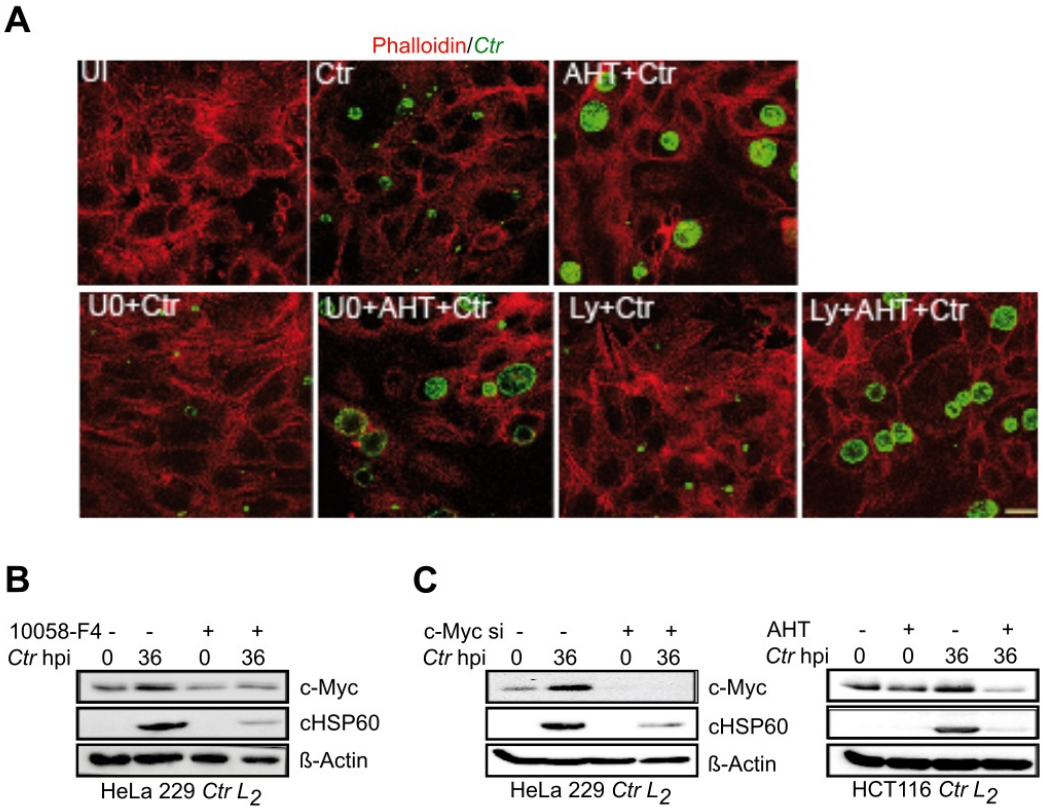


**Fig. 2.5: c-Myc plays a central role in *C. trachomatis* infection.** cHSP60 served as infection control and $\beta$-Actin as loading control. For all experiments n=3. One representative blot is shown. **A**. U2OS $^{Tet-On}$ cells were induced with AHT (100 ng) for 12 hours, treated with UO126 (UO) or LY294002 (Ly) inhibitor or left untreated and infected with *C. trachomatis* $L_2$ at MOI 1 for 30 hours (UI uninfected). The cells were fixed with PFA and immunostained for *C. trachomatis* (cHSP60, green) and phalloidine (red), scale is 15 $\mu$M. **B.** HeLa 229 cells were treated with the 10058-F4 c-Myc inhibitor for 12 hours and infected with *C. trachomatis* $L_2$ at MOI 1 for 36 hours. The cells were lysed and chlamydial infection (cHSP60) was detected by Western blot. **C.** To knock down c-Myc, siRNA pool was used (left) or HCT116 cells expressing a shRNA to silence c-Myc expression. After 48 hours of tansfection, the HeLa 229 cells were infected with *C. trachomatis* $L_2$ for 36 hours. HCT116 cells were treated with AHT (1 $\mu$g/mL) for 48 hours and then infected with *C. trachomatis* $L_2$ for 36 hours. (Immunostaining was performed by Dr. Karthika Rajeeve (department of microbiology, Würzburg) [159].)

The samples revealed an efficient transfer of the $^{13}$C label to glutamate (60%), aspartate (50%) and intermediates of the truncated TCA cycle, succinate (20%), fumarate (35%) and malate (8%) (Figure 2.6 C), indicating the utilization of exogenous L-glutamine to metabolize it via glutaminolysis and fuelling the pathogens' partial TCA cycle. Adding exogenous L-glutamine also resulted in a significantly increased amount of chlamydial NADH, which is most likely formed by the conversion of $\alpha$-ketoglutarate into succinate and malate into oxaloacetate in the partial TCA cycle of *C. trachomatis* [159]. Intriguingly, the peptidoglycan precursor diaminopimelate (DAP) (41%) and alanine (Ala) (17%) were also detected with $^{13}$C-labelling (Figure 2.6 C). Moreover, in another experiment, L-glutamine either labelled at the amino or the amido group was added to the axenic culture, showing that the $^{15}$N-label was only transferred from the amino group of L-glutamine to alanine (Figure 2.6 B). Together, these results point out L-glutamine to serve as a major carbon source for *Chlamydia* to fuel their partial TCA cycle and as a nitrogen source for generating amino acids to build peptidoglycan [159].

Since it has been reported that peptidoglycan synthesis is restricted to the replicative phase of the pathogen, which was never observed so far outside the host cell [16], DAP synthesis in axenic culture was particularly interesting. Thus, electron microscopy (TEM) pictures were taken of the EBs from the axenic culture either in the absence or the presence of L-glutamine. EBs incubated with L-glutamine did not show a complete transition to RBs but, interestingly, a conversion to so called intermediate bodies (IBs) [170], which is a form between EBs and RBs (Figure 2.7 B). An infectivity assay was performed with the L-glutamine treated EBs to check their capacity of infecting HeLa 229 cells, because only EBs are infectious. Compared to the control EBs, the infectivity of the L-glutamine treated EBs dropped to 20% (Figure 2.7 A), supporting that L-glutamine induces the transition of EBs to RBs [159].

In axenic culture, L-glutamine being exclusively taken up by *Chlamydia* insinuated that L-glutamine is also a central amino acid in the host for the pathogen in order to complete its partial TCA cycle and produce peptidoglycan. The question therefore was, how the infected cells manage the increased levels of L-glutamine required through fast replicating *Chlamydia*. According to GSEA a set of glutamine transporter genes including SLC1A5/ASCT2 [171]

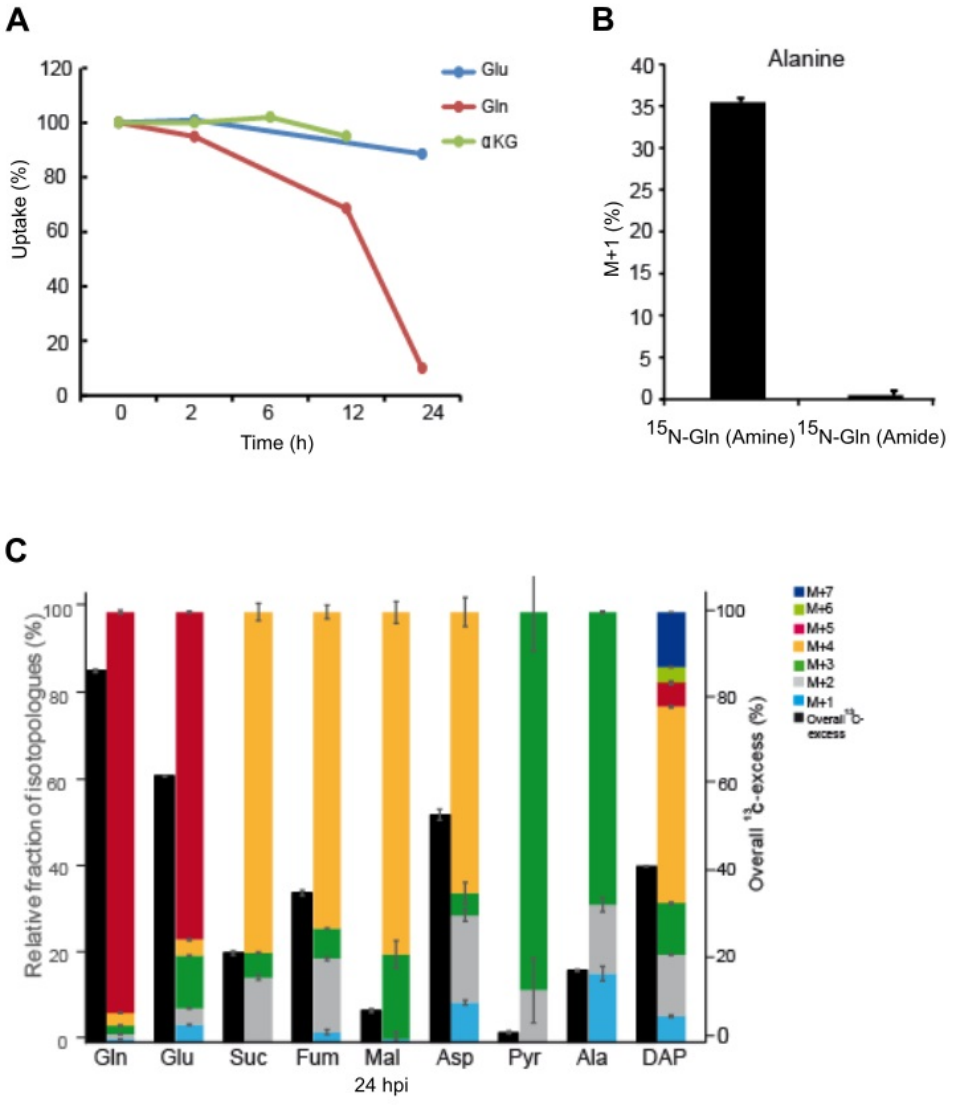


**Fig. 2.6:** ***C. trachomatis*** **imports L-glutamine in axenic medium.** HeLa 229 cells were infected with *C. trachomatis* $L_2$ at MOI 1, and bacteria were isolated and purified at 48 hpi. *Chlamydia* were then kept for 2, 6, 12 or 24 hours in axenic medium, consisting of basic formulation DMEM with 0.5 mM glucose-6-phosphate and 1 mM of the metabolite to be tested. For all experiments n=3. **A.** Supernatants were collected, from the above described axenic cultivation at different time points of incubation and the relative amounts of the indicated metabolites were determined by GC/MS using internal standards. **B.** Purified EBs were cultivated in axenic medium containing $^{15}$N-labelled glutamine and analysed via GC/MS. **C.** In axenic medium with $^{13}C_5$-glutamine, purified EBs were incubated for 24 hours and analysed for $^{13}$C enrichments and isotopologue distribution of selected metabolites. (GC/MS was performed and analysed by the group of Prof. Dr. Wolfgang Eisenreich (TU München) [159].)

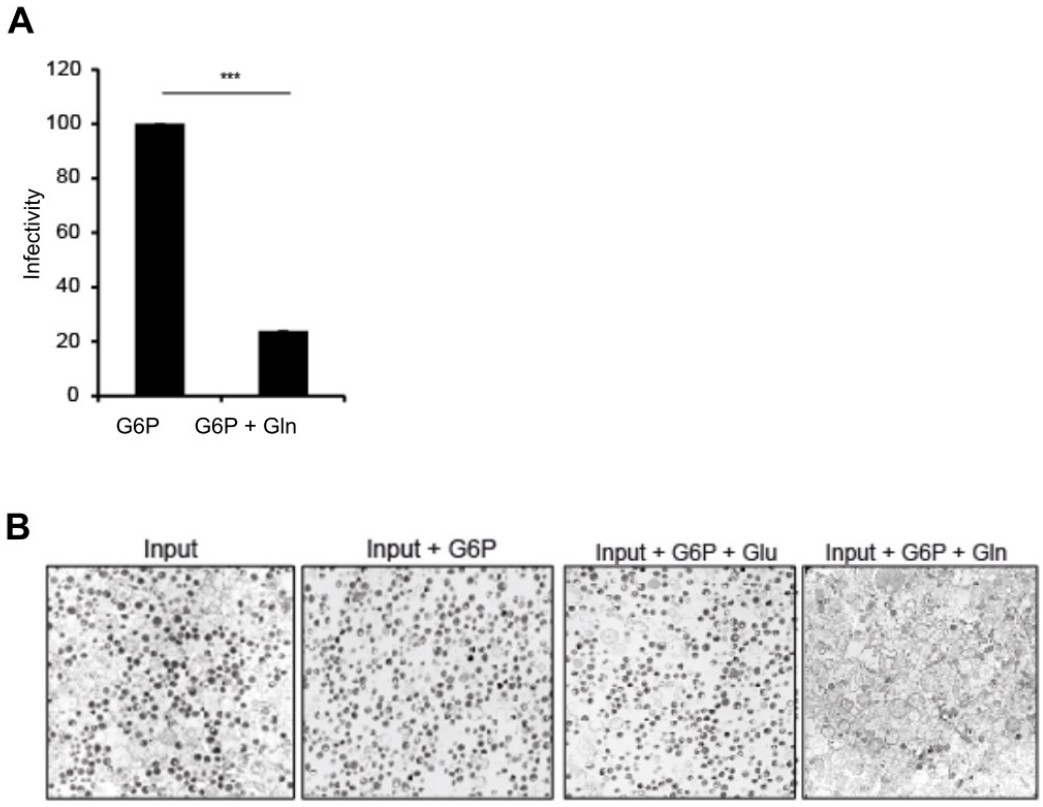


**Fig. 2.7: L-glutamine triggers conversion in axenic medium.** HeLa 229 cells were infected with *C. trachomatis* $L_2$ at MOI 1, and bacteria were isolated and purified at 48 hpi. *Chlamydia* were then kept for 2, 6, 12 or 24 hours in axenic medium, consisting of basic formulation DMEM with 0.5 mM glucose-6-phosphate and 1 mM of the metabolite to be tested. For all experiments n=3. **A.** Purified EBs were incubated in axenic medium either with glucose-6-phosphate or glucose-6-phosphate and L-glutamine for 2 hours. Then the bacteria were used to infect plated HeLa 229 cells. Inclusion forming units were calculated and plotted 24 hpi. *** $p<0.001$. **B.** Purified EBs were incubated in axenic medium either containing glucose-6-phosphate or glucose-6-phosphate and L-glutamine or L-glutamate.The bacteria were pelleted and analysed via transmission electron microscopy after 2 hours of incubation. Scale is 2 $\mu$M. (Dr. Karthika Rajeeve (department of microbiology, Würzburg) provided the infectivity analysis shown in **A** [159].)

and the mRNA for glutaminase (GLS1), an enzyme converting L-glutamine to L-glutamate, were induced upon *C. trachomatis* infection [159]. Interestingly, both SLC1A5/ASCT2 and GLS1 are target genes of c-Myc and have been shown to be induced in cancer cells to enhance catabolism and L-glutamine uptake [162] [172]. Protein analysis via western blot revealed an upregulation of GLS1 and SLC1A5/ASCT2 (Figure 2.8 A). More than that, replication of *Chlamydia* was drastically reduced after blocking the activity of GLS1 or SLC1A5/ASCT2 using specific chemical inhibitors BPTES and GPNA (Figure 2.8 B and C), or depleting GLS1 or SLC1A5/ASCT2 via siRNA (short interfering RNA) (Figure 2.8 D) or shRNA (small hairpin RNA) [159] [164]. Due to the fact that GLS1 and SLC1A5/ASCT2 are targets of c-Myc, a knock down of c-Myc was performed leading to a strong depletion of both proteins, which resulted in decreased chlamydial growth [159] [164]. The overexpression of SLC1A5/ASCT2 retained the growth of the pathogen, even when c-Myc was depleted in the cells [159], but *Chlamydia* failed to produce infectious progenies [159] in this setup, supporting the importance of L-glutamine in establishing the primary infection and triggering the transition of EBs to RBs. Nonetheless, c-Myc, as a regulator of many host cell functions, might be essential for *Chlamydia* to develop completely [159] [164].

## 2.4 c-Myc depletion via interferon-$\gamma$ impairs the development of *C. trachomatis*

Since c-Myc and its target genes are indispensable for the intracellular development of *Chlamydia* [159] [163] [164] (Figure 2.4 and Figure 2.5), it was interesting to investigate the c-Myc levels during chlamydial persistency. In the field of *Chlamydia* research, the term persistence is used to describe an impairment of the pathogen in its development during primary infection and therefore no infectious progenies are produced for the secondary infection. Penicillin treatment had no effect on the pronounced increase of c-Myc (Figure 2.9 A). The c-Myc amounts were even comparable to amounts of normal infection (Figure 2.9 A). Penicillin acts on the levels of bacteria, while IFN-$\gamma$ induced an altered c-Myc amount (Figure 2.9 A). Furthermore, no phosphorylation at serine 62 of c-Myc could be detected upon IFN-$\gamma$ treatment

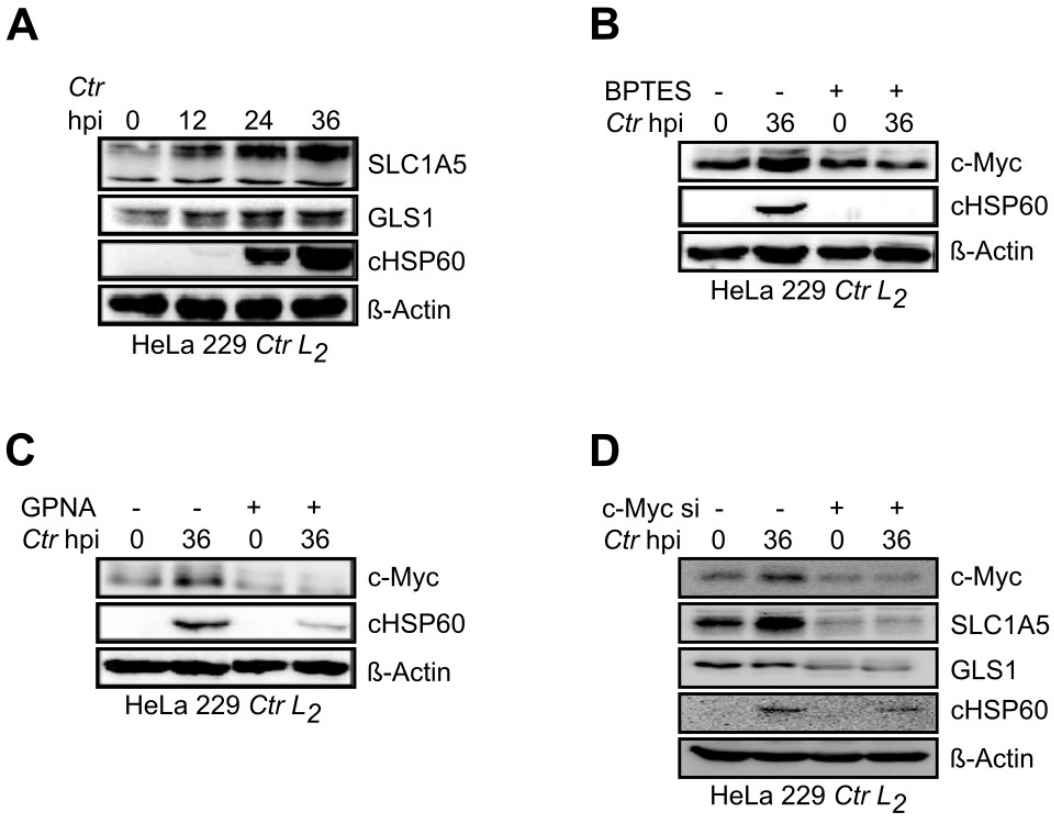


**Fig. 2.8:** ***C. trachomatis*** **regulates glutaminase and SLC1A5/ASCT2.** HeLa 229 cells were infected with *C. trachomatis* $L_2$ at MOI 1 for different time points and lysed for analysis via Western blot. cHSP60 served as infection control and $\beta$-Actin as loading control. For all experiments n=3. One representative blot is shown. **A.** Amounts of SLC1A5/ASCT2 and glutaminase (GLS1) during an infection time course were detected. **B. and C.** Prior chlamydial infection, HeLa 229 cells were treated with either **(B)** BPTES, a glutaminase inhibitor, or **(C)** GPNA, a specific SLC1A5/ASCT2 inhibitor, for 4 hours. c-Myc levels and infection efficiency were examined. **D.** 48 hours prior infection, HeLa 229 cells were transfected with siRNA against c-Myc. SLC1A5/ASCT2 and glutaminase levels were analysed.(Thomas Wulff (master student department of microbiology, Würzburg) provided the Western blots shown in **A**, **B** and **C**[159] [164].)

(Figure 2.9 C) that would prevent the ubiquitination and proteasomal degradation of c-Myc. Differences in the c-Myc levels might serve as another rout of causing persistence. In HeLa 229 cells 10 ng/ml of interferon-$\gamma$ was sufficient to deplete c-Myc (Figure 2.10 A), whereas in primary cells this effect was evident with 50 ng/ml of IFN-$\gamma$ (Figure 2.9 D). The depletion of c-Myc upon interferon-$\gamma$ treatment was also tested on the clinically relevant strain *C. trachomatis* serovar D (Figure 2.10 B). Both kind of treatments prevented formation of infectious progenies (Figure 2.9 B). Therefore, the effects of interferon-$\gamma$ on the host cell signalling were investigated. According to literature [77], IFN-$\gamma$ signals through the JAK-STAT pathway (Figure 1.4). Consequently, the phosphorylation statues of STAT1 was analysed. Addition of interferon-$\gamma$ led to the phosphorylation of serine 727 and tyrosine 701 of STAT1 (Figure 2.10 C).

Due to the key role c-Myc seems to play in chlamydial development and persistency, we tested if increasing c-Myc levels could rescue from persistence. The U2OS $^{Tet-On}$ cell line which overexpresses c-Myc upon anhydrous tetracycline (AHT) induction was used to address this question. To study bacterial replication efficiency and the ability to produce infectious offspring, the cells were left untreated or induced with AHT, and/or treated with interferon-$\gamma$, and/or infected with *C. trachomatis* and analysed via western blot. Overexpression of c-Myc rescued *C. trachomatis* from persistency (Figure 2.11 A and B) and gave rise to infective progenies (Figure 2.11 C and Figure 2.12 A).

## 2.5 L-tryptophan and c-Myc are required to rescue *C. trachomatis* from persistency

It is known that adding exogenous L-tryptophan releases *Chlamydia* from IFN-$\gamma$ mediated persistency [73]. HeLa 229 and Human Fimb cells were treated with interferon-$\gamma$, infected with the pathogen and replenished with exogenous L-tryptophan, in order to examine the role of c-Myc in this scenario. As anticipated, chlamydial growth was rescued by addition of L-tryptophan (Figure 2.12 A and C) and led to infectious progenies (Figure 2.12 C). Interestingly, even without infection c-Myc was stabilized in the L-tryptophan treated samples (Figure

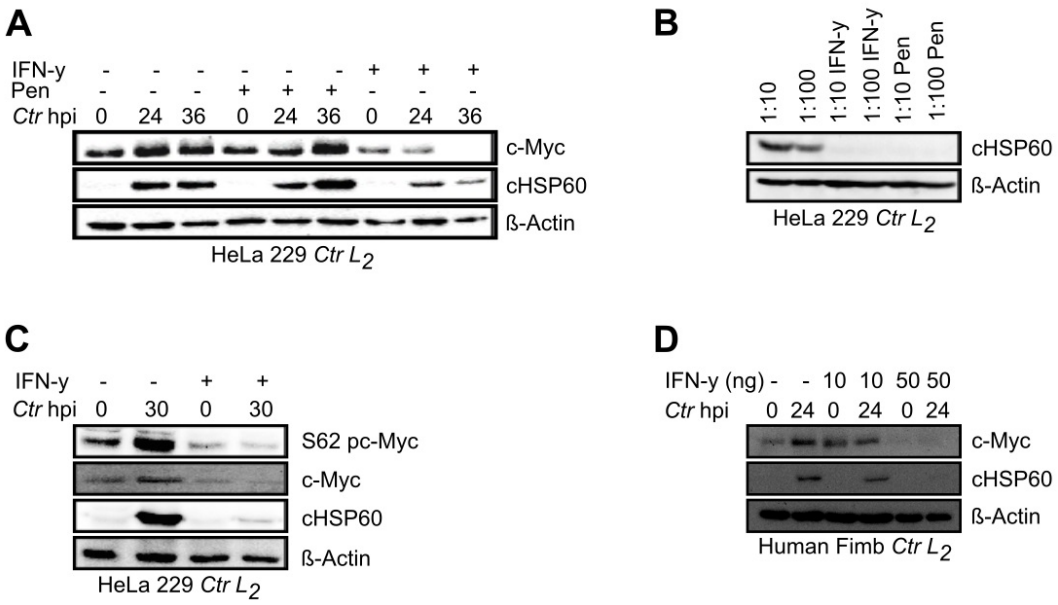


**Fig. 2.9: Interferon-$\gamma$ leads to impairment of chlamydial growth.** $\beta$-Actin served as loading control and cHSP60 as infection control for all the Western blot analysis. For all experiments n=3. One representative blot is shown. **A.** HeLa 229 cells were either untreated or pretreated for 2 hours with penicillin (1U) or interferon-$\gamma$ (IFN-$\gamma$) (10 ng/mL), infected with *C. trachomatis* $L_2$ at MOI 1 at different time points and analysed for c-Myc. **B.** For the experiment **A** an infectivity assay was preformed. Progenies (either diluted 1:10 or 1:100) were added to freshly plated HeLa 229 cells and infection efficiency was examined. **C.** HeLa 229 cells were treated with IFN-$\gamma$ (10 ng/mL) for 2 hours, infected with *C. trachomatis* $L_2$ at MOI 1 and lysed 30 hpi to detect serine 62 phosphorylation status of c-Myc. **D.** Human Fimb cells were treated with 10 or 50 ng/mL of IFN-$\gamma$ for 2 hours and then infected with *C. trachomatis* $L_2$ at MOI 1 for 24 hours. c-Myc levels were analysed.

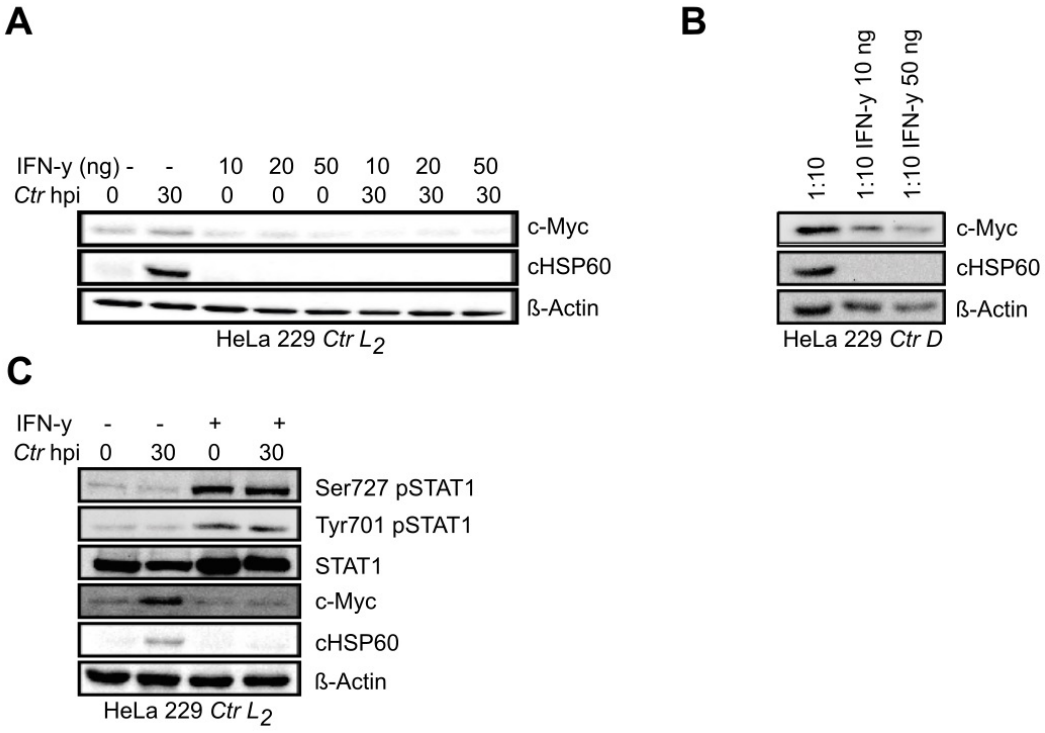


**Fig. 2.10: Interferon-$\gamma$ downregulates c-Myc.** $\beta$-Actin served as loading control and cHSP60 as infection control for all the Western blot analysis. For all experiments n=3. One representative blot is shown. **A.** HeLa 229 cells were treated with different amounts of IFN-$\gamma$. After 2 hours, cells were infected with *C. trachomatis* $L_2$ at MOI 1. 24 hpi, cells were lysed and samples were blotted for c-Myc. **B.** In HeLa 229 cells an infectivity assay with IFN-$\gamma$ incubated *C. trachomatis* serovar D was performed. Progenies were added 1:10 diluted. Lysates were taken 24 hpi to investigate infectivity. **C.** HeLa 229 cells were treated with IFN-$\gamma$ (10 ng/mL) for 2 hours, infected with *C. trachomatis* $L_2$ at MOI 1 and lysed 30 hpi to detect the phosphorylation status of STAT1.

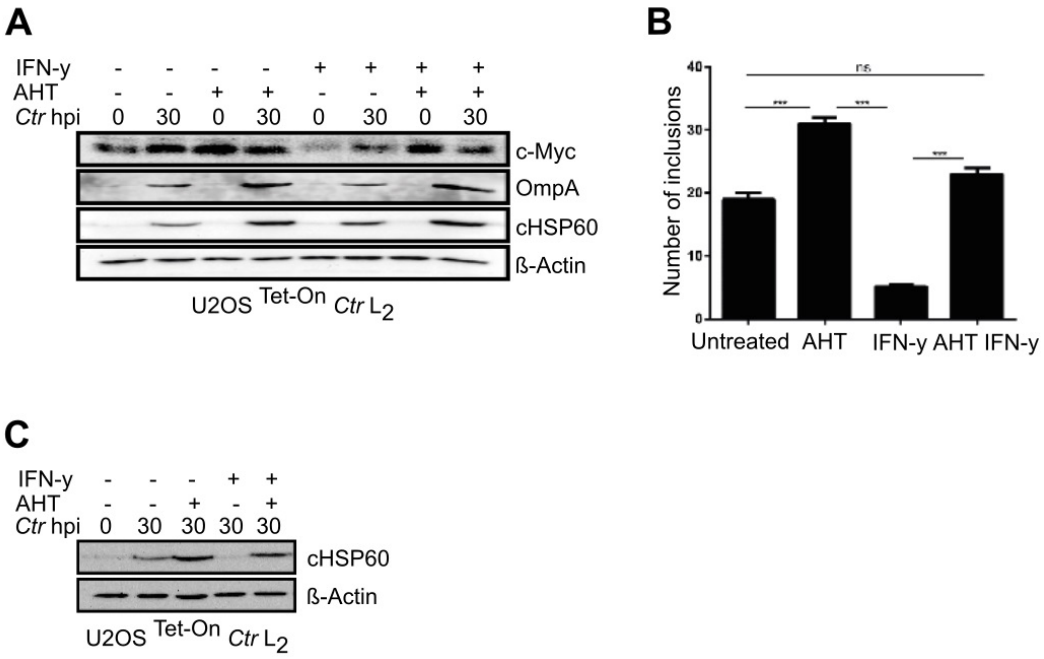


**Fig. 2.11: Overexpression of c-Myc overcomes persistency.** OmpA or cHSP60 served as infection control and $\beta$-Actin as loading control for the Western blot analysis. For all experiments n=3. One representative blot is shown. **A.** For 2 hours, U2OS $^{Tet-On}$ cells were induced with 100 ng/mL AHT, treated with 10 ng/mL IFN-$\gamma$ (interferon-$\gamma$) and infected with *C. trahcomatis* $L_2$ at MOI 1. Samples were lysed 30 hpi to detect infection efficiency. **B.** Numbers of inclusions were counted under the different conditions used in **A** to plot the shown graph. *** $p<0.001$, ns not significant. **C.** For the experiment **A** an infectivity assay was performed by adding the progenies onto fresh U2OS $^{Tet-On}$ cells. U2OS $^{Tet-On}$ cells were examined 30 hpi for infectious progenies. (Dr. Karthika Rejeeve (department of microbiology, Würzburg) provided the images shown in **A**, **B** and **C**.)

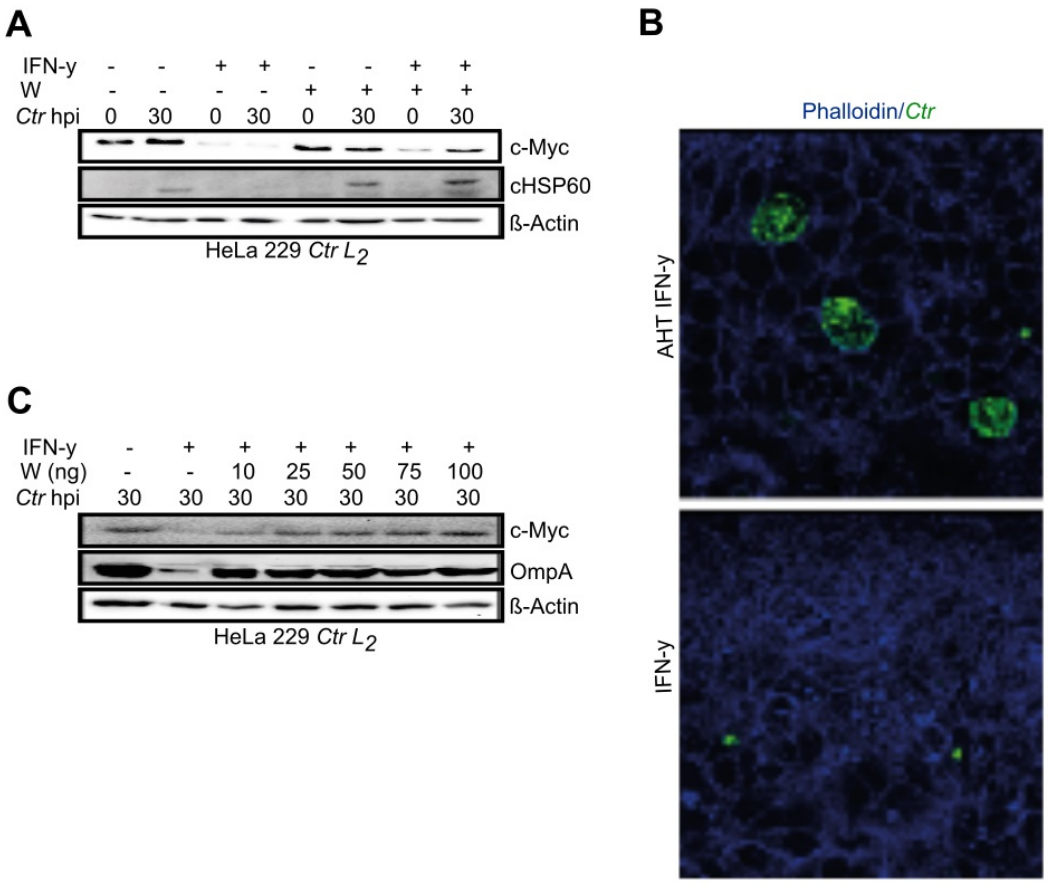


**Fig. 2.12: Exogenous L-tryptophan as well as c-Myc overexpression rescue *Chlamydia* from persistency.** OmpA or cHSP60 served as infection control and $\beta$-Actin as loading control for the Western blot analysis. For all experiments n=3. One representative blot is shown. **A.** HeLa 229 cells were left untreated or were pretreated with IFN-$\gamma$ (10 ng/mL) or L-tryptophan (100 ng/mL) for 2 hours, infected with *C. trahcomatis* $L_2$ at MOI 1 and lysed 30 hpi to examine c-Myc levels and infection efficiency. **B.** For the infectivity assay in Figure 2.11 C an immunofluorescence analysis was performed. Samples were fixed with PFA 30 hpi and immunostained for *Chlamydia* (cHSP60, green) and phalloidine (blue). **C.** An infectivity assay was performed of primary *C. trachomatis* $L_2$ infection treated with IFN-$\gamma$ and addition of different amounts of exogenous L-tryptophan (W). Infectious progenies were detected. (Dr. Karthika Rejeeve (department of microbiology, Würzburg) provided the images shown in **B**.)

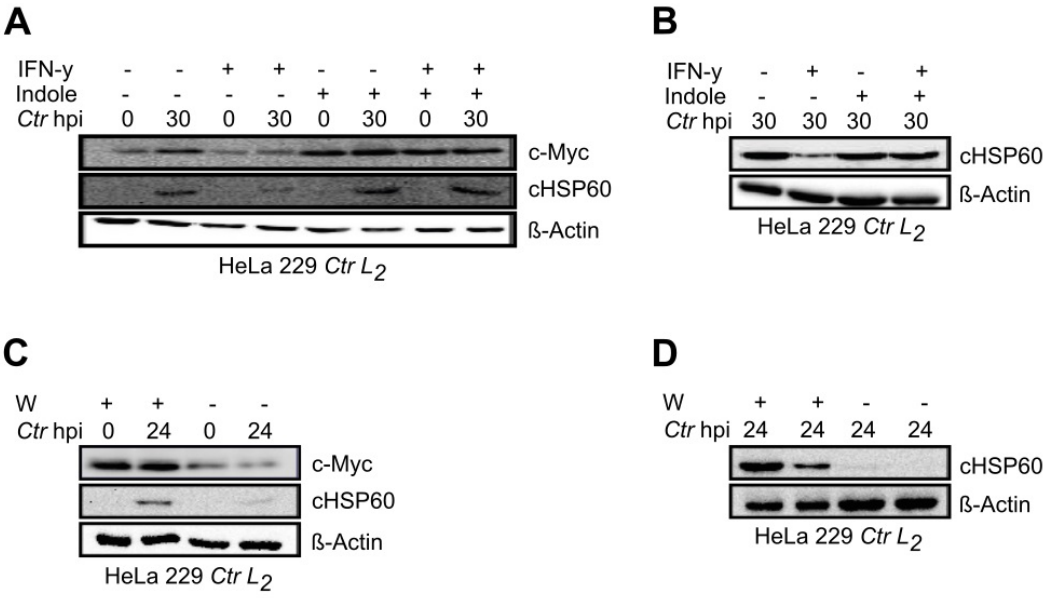


**Fig. 2.13: L-tryptophan and c-Myc are important to overcome persistency.** cHSP60 served as infection control and $\beta$-Actin as loading control for Western blot analysis. For all experiments n=3. One representative blot is shown. **A.** HeLa 229 cells were either left untreated or were pretreated for 2 hours with IFN-$\gamma$ (10 ng/mL) or 50 $\mu$M indole (an intermediate of tryptophan synthesis) and infected with *C. trachomatis* $L_2$ at MOI 1. At 30 hpi, c-Myc levels and infection efficiency were analysed. **B.** An infectivity assay was performed of experiment **A**. HeLa 229 cells were lysed 30 hpi and progeny infectivity was examined. **C.** HeLa 229 cells were grown in L-tryptophan-free medium or medium supplemented with 5 mg/L L-tryptophan (W), infected with *C. trachomatis* $L_2$ at MOI 1. Amounts of c-Myc were detected at 24 hpi. **D.** With the same samples as in **C** an infectivity assay was performed, and 24 hpi the infection of the progenies were examined.

2.12 B). All the *C. trachomatis* strains are auxotroph for L-tryptophan but the genital strains are capable of using exogenous indole, which the microflora in the genital tract provides, as a substrate since they retained a tryptophan synthesis gene (*trpB*) [76] [173] [174]. Indole is an intermediate in the tryptophan biosynthesis. Therefore, it was examined if chlamydial growth is recovered with exogenous indole. Interestingly, indole was sufficient to overcome persistence (Figure 2.13 A), resulting in formation of infectious progenies (Figure 2.13 B) and it led in both infected and non-infected samples to a stabilization of c-Myc (Figure 2.13 A). Hence, these data support the role of c-Myc in the rescue of chlamydial persistence mediated via L-tryptophan or indole.

Since c-Myc seems to be the key player, it was tested whether the overexpression of the transcription factor in a L-tryptophan free environment would rescue chlamydial growth. Thus, the U2OS $^{Tet-On}$ cell line was cultivated in L-tryptophan free medium, treated with IFN-$\gamma$, induced with AHT and infected with *C. trachomatis*, to test the replication efficiency and the ability to produce infectious offspring via western blot. Intriguingly, neither a primary infection (Figure 2.14 A) nor a secondary infection could be detected. Without L-tryptophan in the medium, c-Myc was also not upregulated upon chlamydial infection (Figure 2.14 A). The influence of L-tryptophan on the c-Myc levels were investigated. Exogenous L-tryptophan increased c-Myc levels in the absence of *Chlamydia* (Figure 2.14 B). As a next step, it was examined if the pathogen develops without c-Myc being present but the addition of endogenous L-tryptophan. Therefore, c-Myc was depleted via AHT-inducible shRNA mediated gene silencing in HeLa 229 cells, and grown in the presence of additional L-tryptophan. *Chlamydia* was unable to grow in the presence of exogenous L-tryptophan with reduced levels of c-Myc (Figure 2.15 A), and were incapable of establishing a secondary infection (Figure 2.15 B). These data indicated that to overcome the state of persistency both c-Myc and L-tryptophan are needed.

According to the RNA sequencing data, the cells infected with *C. trachomatis* show an upregulation of the L-amino acid transporter solute carrier family 7 member 5 (LAT1/SLC7A5), which is a system L-amino acid transporter with high affinity for branched chain amino acids and bulky amino acids like L-tryptophan [175]. Moreover, there are binding sites for c-Myc on the LAT1/SLC7A5 promotor and the overexpression of c-Myc increases the expression of the transporter [175]. For that reason, the protein levels of LAT1/SLC7A5 upon interferon-$\gamma$ treatment and during a *Chlamydia* infection were analysed by western blot. In infected HeLa 229 cells LAT1/SLC7A5 was stabilized in a time-dependent manner (Figure 2.15 C), and irrespective of infection, in the IFN-$\gamma$ treated samples LAT1/SLC7A5 protein levels reduced (Figure 2.15 C). Subsequently, the corresponding intracellular levels of L-tryptophan were measured via LC-MS. The measurement indicated increased intracellular amount of L-tryptophan during chlamydial infection, which is depleted through the addition of interferon-$\gamma$, and partially rescued by the overexpression of c-Myc (Figure 2.15 D).

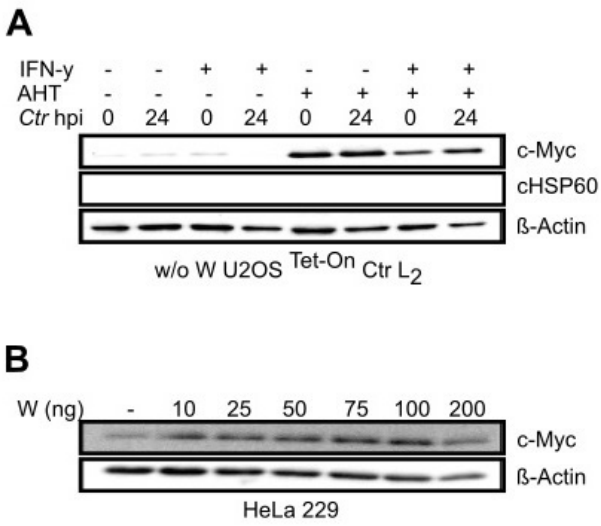


**Fig. 2.14: L-tryptophan stabilizes c-Myc.** cHSP60 served as infection control and $\beta$-Actin as loading control for Western blot analysis. For all experiments n=3. One representative blot is shown. **A.** U2OS $^{Tet-On}$ cells grown in L-tryptophan-free medium were either untreated or treated with IFN-$\gamma$ (10 ng/mL) or AHT (100 ng/mL) and infected with *C. trachomatis* $L_2$ at MOI 1. Cells were lysed to analyse infection efficiency. **B.** HeLa 229 cells were supplemented with increasing amounts of L-tryptophan (W) and lysed after an incubation of 30 hours to examine c-Myc levels.

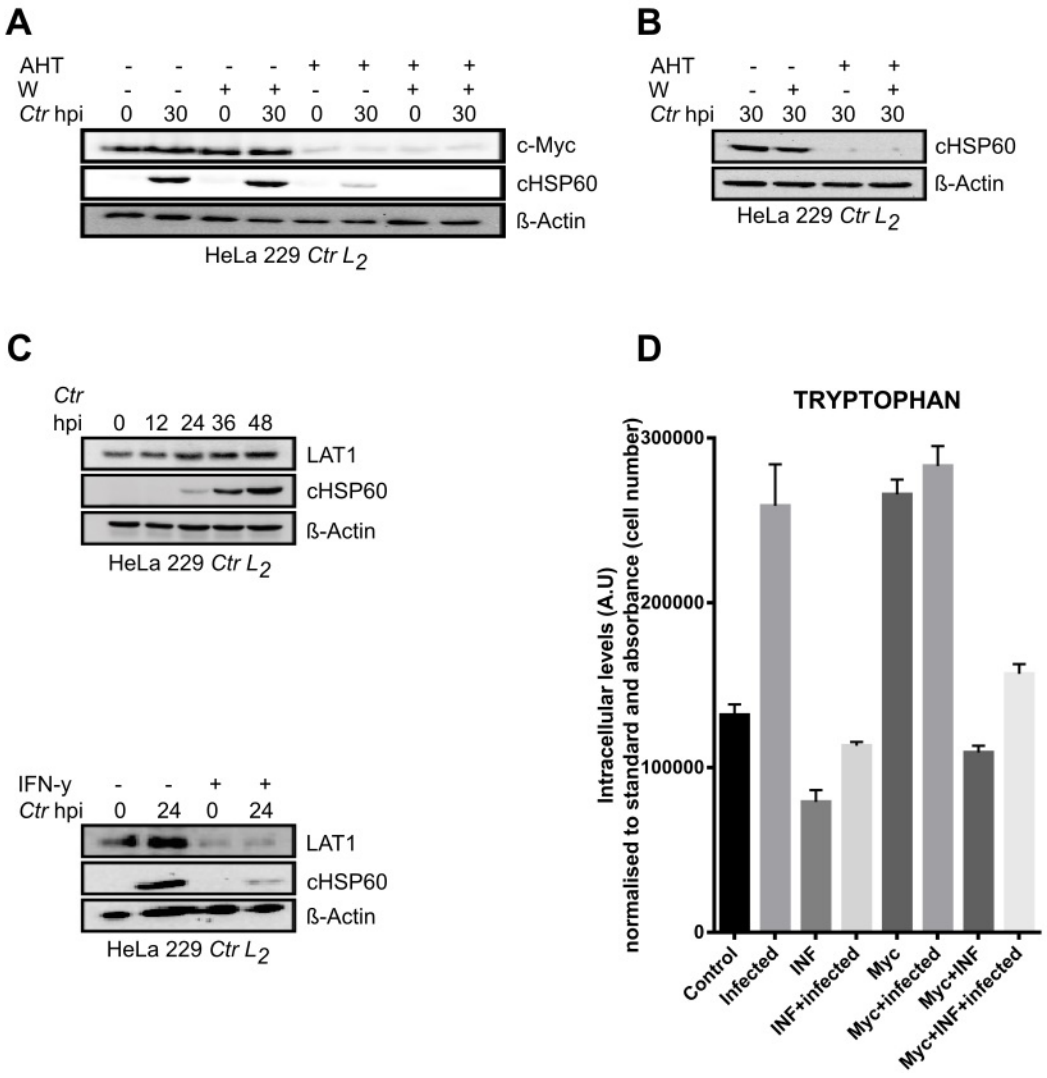


**Fig. 2.15: L-tryptophan and c-Myc are important to overcome persistency.** cHSP60 served as infection control and $\beta$-Actin as loading control for Western blot analysis. For all experiments n=3. One representative blot is shown. **A.** HeLa 229 cells expressing a shRNA to silence c-Myc expression were treated with AHT (1 $\mu$g/mL) 48 hours and then pretreated with L-tryptophan (W) (100 ng/mL) for 2 hours and infected with *C. trachomatis* $L_2$ for 30 hours. The samples were analysed for c-Myc levels. **B.** Infectivity assay of experiment **A** was performed to detect infection efficiency. **C.** HeLa 229 cells were pretreated for 2 hours with 10 ng/mL IFN-$\gamma$ or left untreated, infected with *C. trachomatis* $L_2$ at MOI 1 for different time points, and analysed for the L-tryptophan transporter LAT1/SLC7A5. **D.** Supernatants were collected, of U2OS $^{Tet-On}$ cells either untreated or treated with IFN-$\gamma$ (10 ng/mL) or AHT (100 ng/mL) and infected with *C. trachomatis* $L_2$ at MOI 1, at 36 hpi and the relative amount of L-tryptophan was determined by LC/MS. Data are presented as mean $\pm$ SEM of triplicates. (Sudha Janaki-Raman (department of biochemistry and molecular biology, Würzburg) provided the analysis shown in **D**.)

## 2.6 Chlamydial infection is rescued by L-tryptophan via activation of the pGSK3$\beta$-c-Myc axis

HeLa 229 cells were cultivated in medium with or without additional L-tryptophan and infected with *C. trachomatis* to explore how L-tryptophan signals to alter levels of c-Myc. As anticipated, reduced chlamydial infection and no production of infectious offspring were detected without the availability of L-tryptophan (Figure 2.13 C). However, the addition of exogenous L-tryptophan increased the replication and led to the formation of infective progenies, as well as the recovery of the levels of c-Myc (Figure 2.13 C and D). It is known that interferon-$\gamma$ activates PI3K and AKT by binding to its receptor but in order to have it in its active form dephosphorylates glycogen synthase kinase-3$\beta$ (GSK3$\beta$) [176]. This dephosphorylation of GSK3$\beta$ results in the phosphorylation at threonine 58 of c-Myc, which is followed by ubiquitination and proteasomal degradation [177]. Thus, in HeLa 229 and Human Fimb cells infected with *Chlamydia*, treated with IFN-$\gamma$ and or L-tryptophan, the phosphorylation status of GSK3$\beta$ and AKT was examined via western blot. As expected, AKT was phosphorylated in the presence of L-tryptophan independent of infection (Figure 2.16 A and C). Treatment with interferon-$\gamma$ dephosphorylated GSK3$\beta$ and lead to a reduction of c-Myc (Figure 2.16 A and C), in line with the literature [79]. Intriguingly, the samples with additional L-tryptophan showed an increased GSK3$\beta$ phosphorylation and an upregulation of c-Myc levels (Figure 2.16 A and C), indicating that the activation of the pGSK3$\beta$-c-Myc axis rescues *Chlamydia* from persistency.

## 2.7 Addition of alpha-ketoglutarate and nucleosides rescues *C. trachomatis* from interferon-$\gamma$ induced persistence

Metabolite analysis was performed to understand the importance of c-Myc during the rescue of *C. trachomatis* from persistency. The U2OS $^{Tet-On}$ cell line with inducible c-Myc overexpression was used, either induced or not induced with AHT, and/or treated with IFN-$\gamma$ and infected with *Chlamydia* and then analysed by mass spectrometry. To identify the

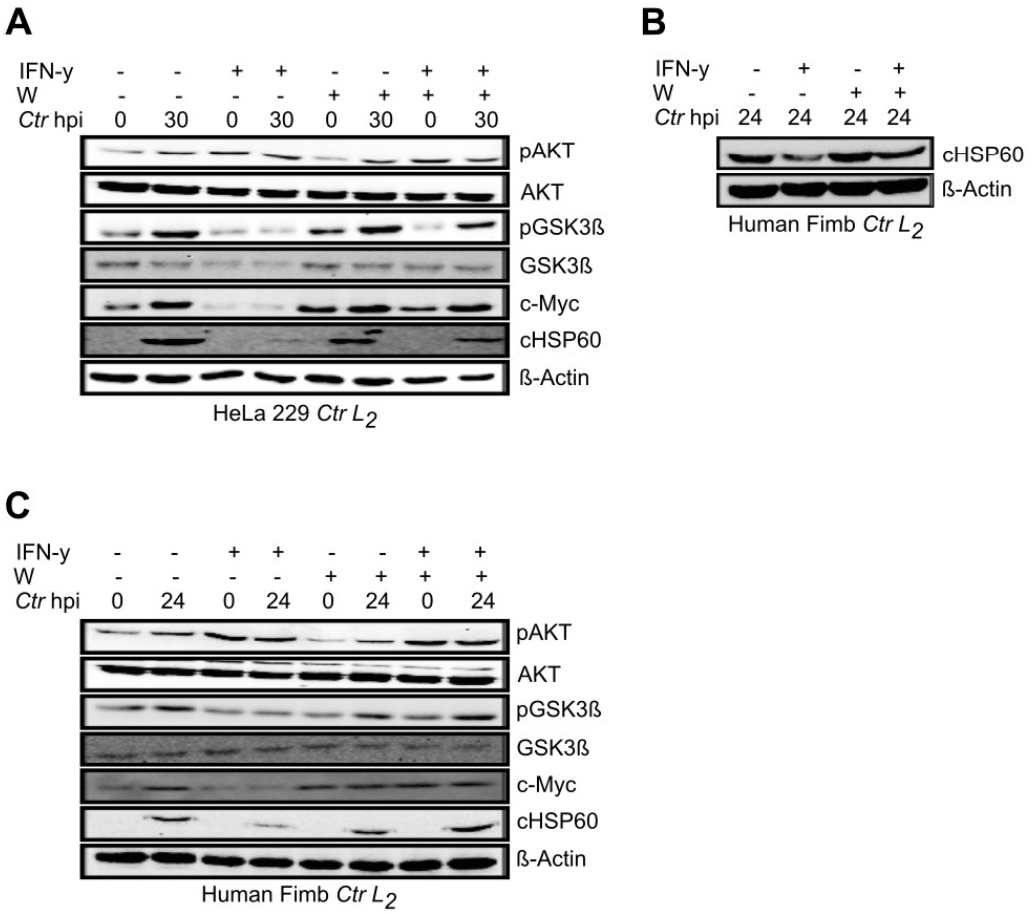


**Fig. 2.16: L-tryptophan activates the pGSK3$\beta$-c-Myc axis to rescue chlamydial infection.** cHSP60 served as infection control and $\beta$-Actin as loading control for Western blot analysis. For all experiments n=3. One representative blot is shown. **A. and C.** HeLa 229 cells (**A**) and Human Fimb cells (**C**) were either left untreated or were pretreated with IFN-$\gamma$ (10 ng/mL or 50 ng/mL) or L-tryptophan (100 ng/mL) for 2 hours, infected with *C. trachomatis* $L_2$ at MOI 1 and lysed 30 hpi or 24 hpi to detect the amounts of pAKT, pGSK3$\beta$ and c-Myc. **B.** Infectivity assay of Human Fimb cells treated with IFN-$\gamma$ and L-tryptophan (W), in order to analyse infection efficiency.

metabolites that might be involved in overcoming persistence, the metabolites which were affected by interferon-$\gamma$ treatment and the ones that were rescued via c-Myc overexpression were determined. Astonishingly, all metabolites in the urea cycle, the TCA cycle and the levels of L-glutamine and L-glutamate were reduced in the IFN-$\gamma$ treated samples (Figure 2.17). Remarkably, only some urea cycle metabolites and few TCA cycle metabolites like L-glutamine, L-glutamate, $\alpha$-ketoglutarate and citrate were restored upon overexpression of c-Myc (Figure 2.17). In the infected samples, aspartate, glycine and serine were significantly increased and were depleted after interferon-$\gamma$ treatment (Figure 2.17). Further examination revealed that these metabolites were shuttled into nucleotide biosynthesis, as seen in the amounts of adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanidine triphosphate (GTP) and uridine triphosphate (UTP) (Figure 2.18 B). Interestingly, c-Myc overexpression upon infection reversed the blockage of the uridine monophosphate (UMP) to UTP conversion taking place in the presence of IFN-$\gamma$. Remarkably, all the arginine was completely depleted during chlamydial infection no matter what the other conditions were (Figure 2.19 B). Interferon-$\gamma$ treatment led to a significant reduction of the intermediates of the purine nucleotide catabolism, which in the presence of c-Myc and *Chlamydia* infection was rescued (Figure 2.19 A). Due to the specific pattern pointing towards pyrimidine biosynthesis in the metabolic flux, it was examined if the addition of pyrimidines would help to overcome IFN-$\gamma$ induced persistence. HeLa 229 and U2OS $^{Tet-On}$ cells were infected with *C. trachomatis*, treated with interferon-$\gamma$ and either the cell permeable dimethyl ester of $\alpha$-ketoglutarate or nucleosides were added to monitor chlamydial replication and secondary infection via western blot. Both, the cell permeable dimethyl ester of $\alpha$-ketoglutarate as well as the nucleosides, were sufficient to overcome persistency (Figure 2.20 A and C) and to produce infectious progenies (Figure 2.20 B and D) in both cell lines. Interestingly, $\alpha$-ketoglutarate was more efficient than nucleosides to rescue *Chlamydia* from persistence. Furthermore, uridine and cytidine alone were sufficient for the rescue (Figure 2.20 E), supporting the importance of pyrimidines for *C. trachomatis* development.

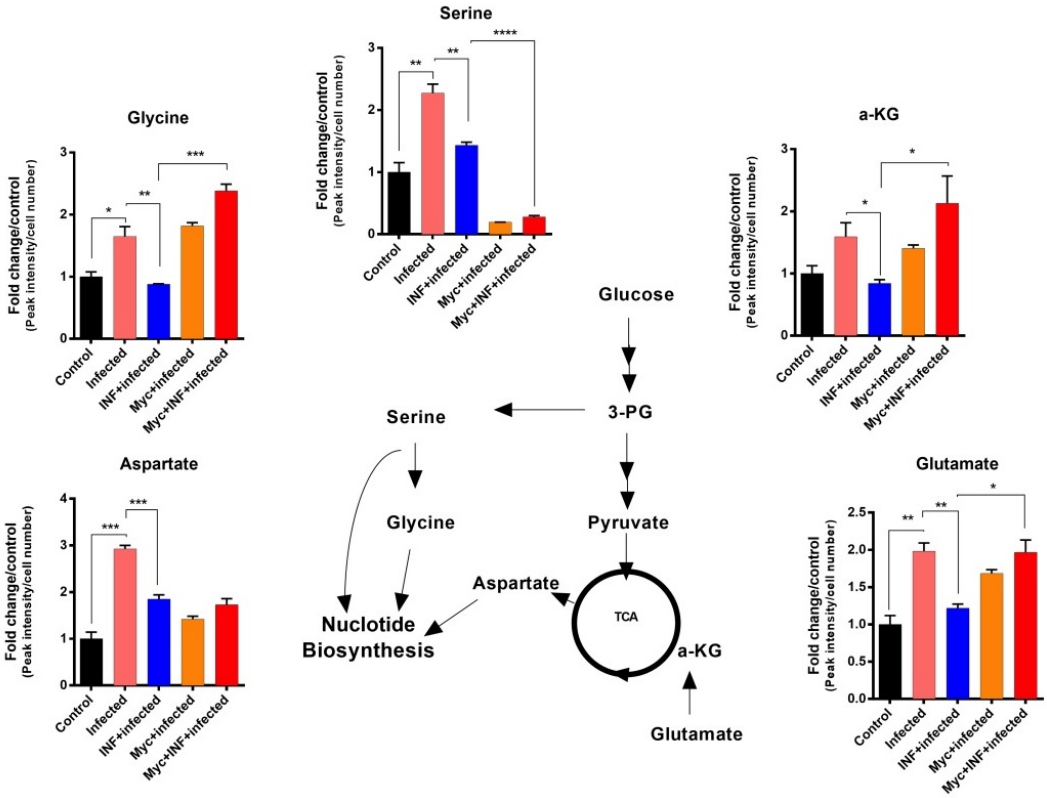


**Fig. 2.17: Metabolic changes upon infection and c-Myc overexpression.** U2OS $^{Tet-On}$ cells were either untreated or treated with IFN-$\gamma$ (10 ng/mL) or AHT (100 ng/mL) and infected with *C. trachomatis* $L_2$ at MOI 1. Metabolites were extracted of the cells and analysed via LC/MS. TCA cycle and metabolites important for nucleoside synthesis. (Sudha Janaki-Raman (department of biochemistry and molecular biology, Würzburg) provided the analysis shown here.)

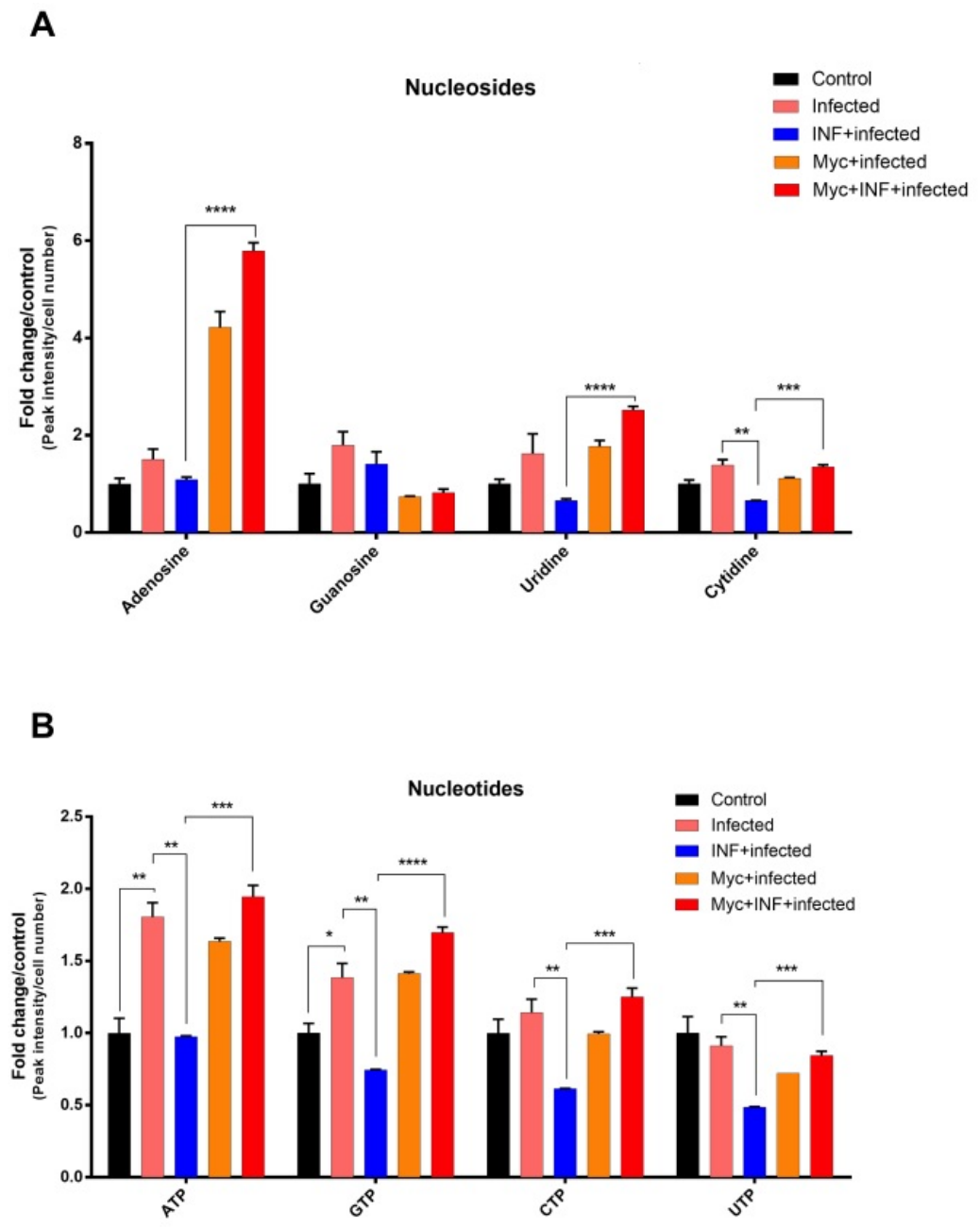


**Fig. 2.18: Metabolic changes upon infection and c-Myc overexpression.** U2OS $^{Tet-On}$ cells were either untreated or treated with IFN-$\gamma$ (10 ng/mL) or AHT (100 ng/mL) and infected with *C. trachomatis* $L_2$ at MOI 1. Metabolites were extracted of the cells and analysed via LC/MS. **A.** Nucleosides. **B.** Nucleotides. (Sudha Janaki-Raman (department of biochemistry and molecular biology, Würzburg) provided the analysis shown here.)

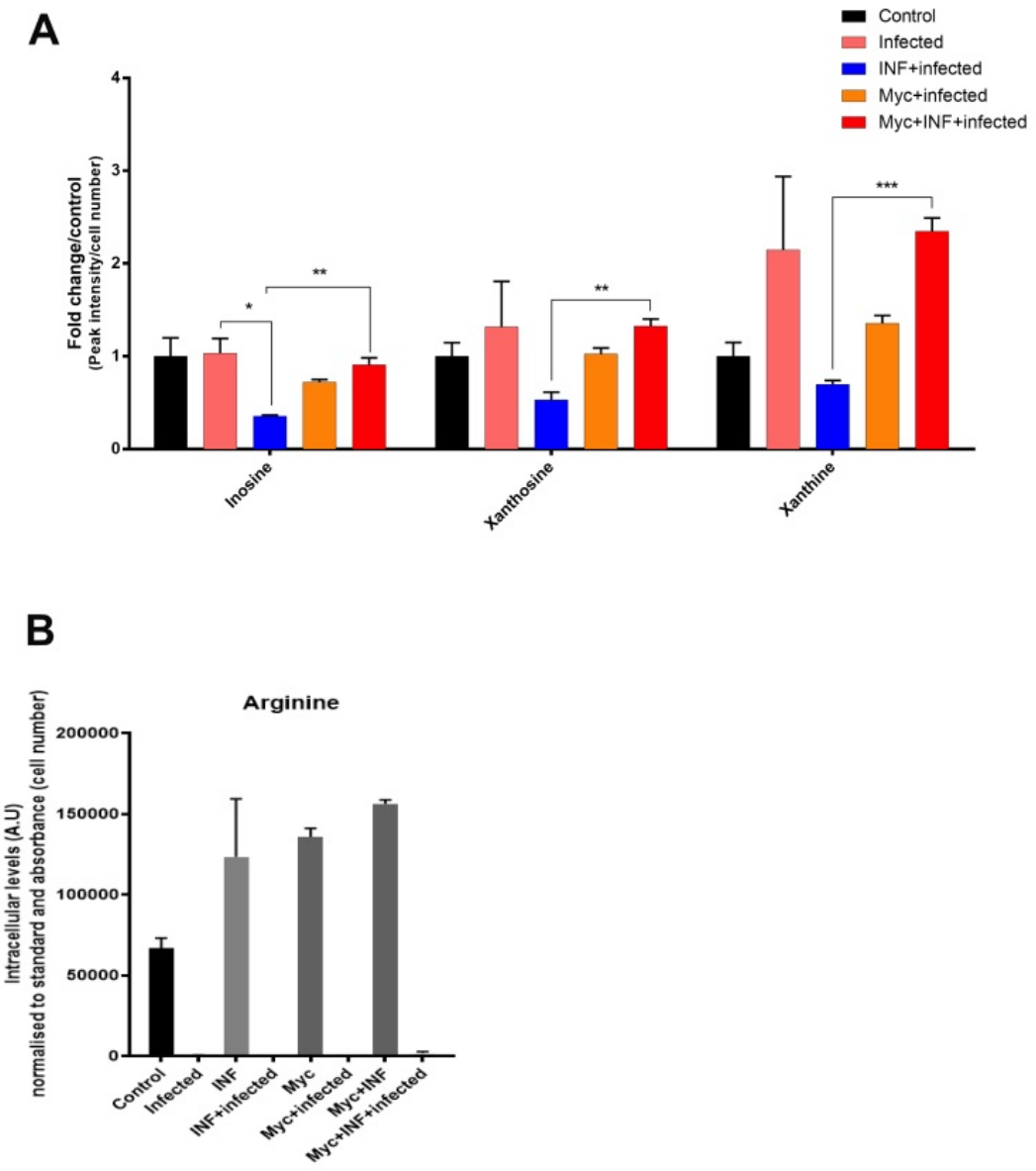


**Fig. 2.19: Metabolic changes upon infection and c-Myc overexpression.** U2OS $^{Tet-On}$ cells were either untreated or treated with IFN-$\gamma$ (10 ng/mL) or AHT (100 ng/mL) and infected with *C. trachomatis* $L_2$ at MOI 1. Metabolites were extracted of the cells and analysed via LC/MS. **A.** Intermediates of purine nucleotide catabolism. **B.** Arginine. (Sudha Janaki-Raman (department of biochemistry and molecular biology, Würzburg) provided the analysis shown here.)

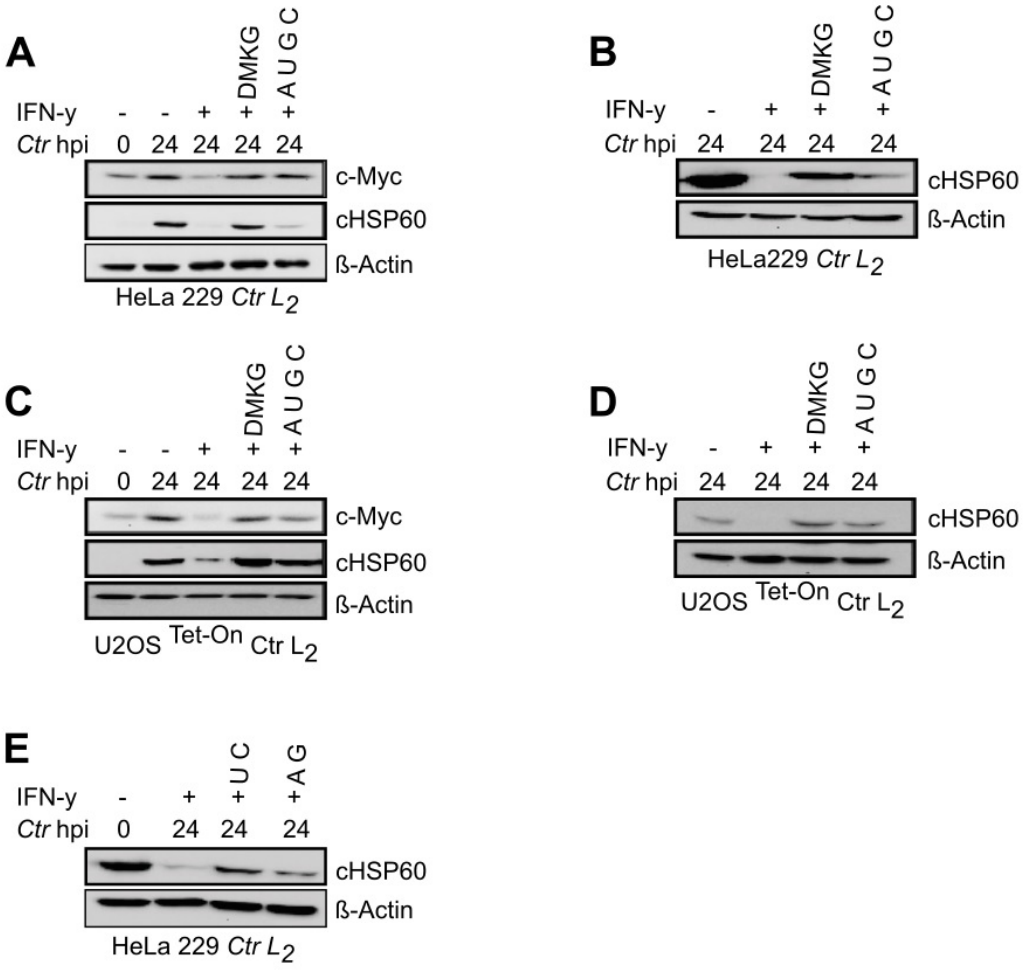


**Fig. 2.20: Alpha-ketoglutarate and nucleosides overcome interferon-$\gamma$ induced persistence.** cHSP60 served as infection control and $\beta$-Actin as loading control for Western blot analysis. For all experiments n=3. One representative blot is shown. **A. and C.** HeLa 229 cells (**A**) and U2OS $^{Tet-On}$ cells (**C**) were treated with IFN-$\gamma$ (10 ng/mL), $\alpha$-ketoglutarate (4 mM, cell permeable dimethyl ester) or nucleosides (adenosine (A), cytidine (C), guanosine (G) and uridine (U)) (100 $\mu$M) for 2 hours, infected with *C. trachomatis* $L_2$ and lysed 24 hpi for analysis of c-Myc amounts. **B. and D.** Infectivity assays of **A and C**. Cells were lysed 24 hpi to examine infectious progenies. **E.** HeLa 229 cells were treated and infected as in **A** but nucleosides were added as pyrimidines and purines separated. An infectivity assay was performed and analysed for infection efficiency.

## 2.8 Interferon-$\gamma$ reduces *C. trachomatis* infection in organoids

In order to get closer to an *in vivo* situation and due to the fact that a suitable animal model is absent, the study was extended in organoids derived from fallopian tube biopsies. IFN-$\gamma$ treated fallopian tube organoids showed a reduced infection compared to the untreated ones (Figure 2.21 and Figure 2.22). The inclusions were not merely smaller in size compared to the control infection but also considerably less upon IFN$\gamma$ treatment (Figure 2.21 A and B). This was the case for organoids derived from three different patient biopsies (Figure 2.21 C left graph). Furthermore, it was examined whether *Chlamydia* were able to produce infectious offspring, which will be able to reinfect the organoids after splitting them. The secondary infection was decreased in the IFN-$\gamma$ treated organoids, whereas the untreated samples showed an assimilable infection as the primary one (Figure 2.21 C right graph). Since the organoids exhibited similar effects as the cell lines upon IFN-$\gamma$ treatment, it was important to investigate wether the same signalling pathway was switched on to reduce c-Myc levels. Addition of IFN-$\gamma$ led to STAT1 phosphorylation at tyrosine 701 and serine 727 in organoids (Figure 2.22 A). Transmission electron microscopy showed persistent inclusions in the organoids treated with IFN-$\gamma$ (Figure 2.22 B). Taken together, these results demonstrate that organoids derived form patient biopsies are a promising infection model.

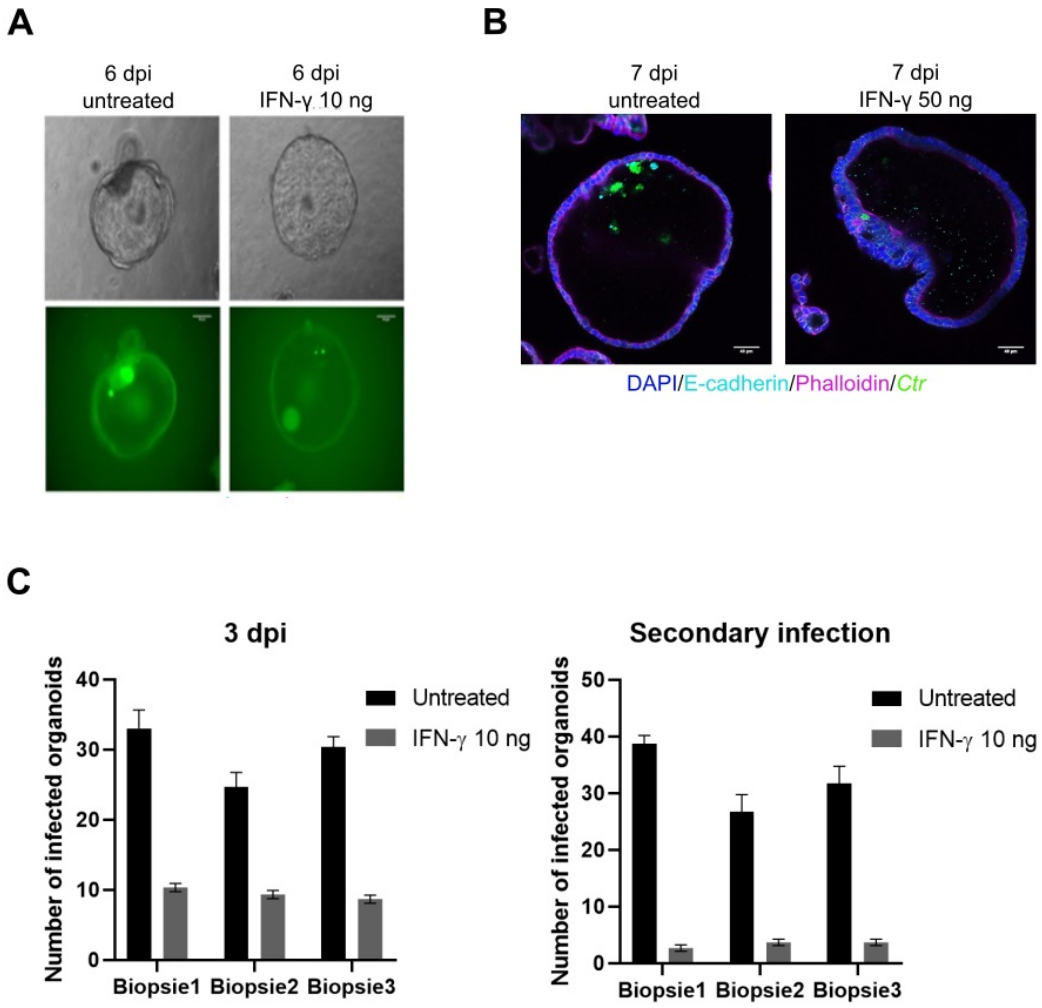


**Fig. 2.21: Organoids infected with *C. trachomatis*.** cHSP60 served as infection control and $\beta$-Actin as loading control for Western blot analysis. For all experiments n=3. Fallopian tube organoids were either untreated or treated with IFN-$\gamma$ (10 or 50 ng/mL) and infected with *C. trachomatis* $L_2$ (CFU 5 x $10^5$). **A.** Light microscopy picture taken 6 days post infection (dpi), *Chlamydia* shown in green (GFP signal). Scale is 40 $\mu$m. **B.** Organoids were fixed with PFA after 7 dpi and immunostained for *C. trachomatis* (GFP, green), E-cadherin (turquoise), phalloidine (magenta) and DAPI (blue). Scale is 40 $\mu$m. **C.** Primary infection (left graph) for 3 days. After 3 dpi, infected organoids were split and checked for reinfection (right graph). (Naziia Kurmasheva (master student department of microbiology, Würzburg) maintained organoids and provided the images shown in **A** and **B**.)

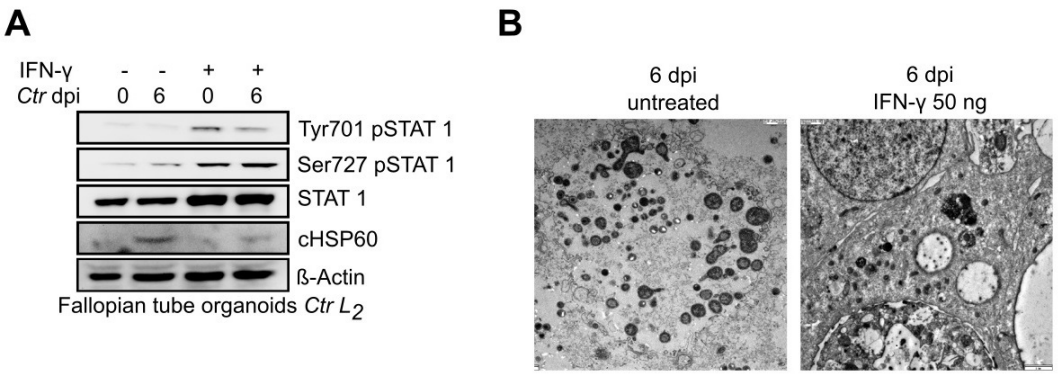


**Fig. 2.22: Organoids infected with *C. trachomatis*.** cHSP60 served as infection control and $\beta$-Actin as loading control for Western blot analysis. For all experiments n=3. One representative blot is shown. Fallopian tube organoids were either untreated or treated with IFN-$\gamma$ (10 or 50 ng/mL) and infected with *C. trachomatis* $L_2$ (CFU 5 x $10^5$). **A.** Activation of Jak-STAT1 pathway upon IFN-$\gamma$ treatment was examined via detection of STAT1 phosphorylation and infection efficiency (cHSP60). **B.** Transmission electron microscopy (JEOL JEM-2100) was performed to detect persistent inclusions in IFN-$\gamma$ treated organoids. Left panel active inclusion, right panel persistent inclusion. (Organoids were maintained by Naziia Kurmasheva (master student department of microbiology, Würzburg).)

# 3 Discussion and Outlook

With respect to nutrition acquisition and the perfect replication niche the obligate intracellular pathogen *C. trachomatis* depends completely on its host cell. Therefore *Chlamydia* takes over the host cell and manipulates various cellular processes such as cell cycle, signalling pathways and metabolism, altering the proteome of the cell [178] [179] [180]. Many metabolites, for example nucleotides or lipids, have to be obtained from the host cell, since *Chlamydia* has an incomplete metabolism [110] [148] [181]. It has been shown that *C. trachomatis* upregulates c-Myc [158], and activates the PI3K/AKT and the Ras/Raf/MEK/ERK pathways. The latter stabilizes c-Myc upon infection [159]. c-Myc regulates a plethora of metabolic pathways [160] [161] [162], which leads to the speculation that the pathogen establishes a supportive intracellular growth niche via its interference with this protein. This work sheds light on the importance of the non-essential amino acid L-glutamine, the involvement of c-Myc in a successful chlamydial infection and the impact of IFN-$\gamma$ on c-Myc causing chlamydial dormancy.

## 3.1 c-Myc and L-glutamine cooperation for efficient chlamydial growth

To attain the importance of c-Myc and the amino acid L-glutamine for the intracellular replication of *C. trachomatis*, the abundance of c-Myc was examined during an infection course. In line with previous findings [158] [159] [163] [164], the c-Myc levels were markedly increased after chlamydial infection (Figure 2.1 and Figure 2.2). Furthermore, a substantial re-localization of c-Myc from the cytosol to the nucleus was observed in infected cells (Figure

2.2 D), as requisite to exert its function as a regulatory transcription factor. Intriguingly, optimal replication of *Chlamydia* was dependent on distinct c-Myc levels, since massive ectopic overexpression of c-Myc in the U2OS $^{Tet-On}$ cell line significantly decreased the growth efficiency of the pathogen [159] [164]. Overexpression levels which led to reduced growth of *C. trachomatis* did not show an cytotoxic effect. In order to have the ideal development of the pathogen, the c-Myc levels had to be balanced. In this system, chlamydial infection appeared to limit massive c-Myc overexpression, since c-Myc protein abundance in induced cells was lower in infected cells than in control cells. Recently, it has been shown that distinct c-Myc levels induce defined transcriptional programs [182]. Therefore, a certain c-Myc level might coincide with a specific host programming, creating advantageous replication conditions for *Chlamydia*. Of note, previously it has been reported that apart from viruses [183] [184] also Salmonella spp. [185] [186] [187] and the eukaryotic parasite *Toxoplasma gondii* [188] alter the c-Myc levels after infection. Consequently, modulation of c-Myc might emerge in infection biology as a new concept of intracellular pathogens.

To meet the requirements of an infected cell, the metabolism has to change drastically, since all metabolites *Chlamydia* requires for replication originate from the host cell. L-glutamine is taken up by fast growing cells to support anabolic metabolism, using L-glutamine as precursor for the synthesis of other amino acids, for protein synthesis, for the production of glutathione, as a substrate for TCA cycle anaplerosis and as a nitrogen donor in nucleotide biosynthesis [189] [190]. *C. trachomatis* stabilizes c-Myc, a central metabolism regulator, to reprogram its host cell, since it depends on the direct uptake of L-glutamine and L-glutamine-derived host metabolites [159] [164]. *Chlamydia* induces this reprogramming profile via c-Myc (Figure 3.1), similar as the reprogramming of fast proliferating tumour cells [189].

Previous findings also demonstrated that L-glutamine is a crucial amino acid for the successful replication of *Chlamydia* [72] [191]. Axenic cultivation demonstrated that *C. trachomatis* efficiently imported L-glutamine (Figure 2.6 A), although neither a chlamydial L-glutamine transporter nor a functional glutaminase has been described. Nevertheless, for the degradation of L-glutamine to L-glutamate the L-glutamine D-fructose-6-phosphate amino-transferase GlmS might be responsible [127]. Meanwhile, fructose-6-phosphate gets converted

into D-glucosamine-6-phosphate, which is a substrate for peptidoglycan synthesis. Many of the L-glutamine derived intermediates found in the isotopologue profile of *Chlamydia* cultivated in axenic medium serve as precursors for the biosynthesis of peptidoglycan (Figure 2.6 B and C), supporting L-glutamine playing the central role in the chlamydial metabolism. Normally, Gram-negative bacteria form a typical peptidoglycan sacculus which is not the case for *Chlamydia*. They only assemble a peptidoglycan ring in the mid cell of actively dividing RBs [192]. Exclusively in the presence of L-glutamine, the accumulation of peptidoglycan was detected in *Chlamydia* outside the host cell [159]. Moreover, under these conditions, a morphological change of the EBs could be observed (Figure 2.7 B) and an increase in the copy number of the bacterial genome [159], indicating the start of the replicating machinery. The stored metabolites may be used by *Chlamydia*, since it is auxotroph for nucleosides, to initiate replication in axenic culture. These findings provide evidence that the key metabolite initiating the EB to RB transition and the peptidoglycan synthesis is glutamine. RB to EB conversion must be avoided outside the host cell, considering that only EBs are capable of entering cells to initiate a new generation of progeny. For long it was believed that EBs are metabolically inactive, but this view has recently been revised by keeping EBs in axenic medium containing glucose-6-phosphate, a metabolite found inside the cells available for *Chlamydia*, generating ATP via glycolysis [53]. However, under this condition, despite the active metabolism, EBs did not initiate the transformation to RBs. Furthermore, in uterine fluid, the glutamine concentration is as low as 0.13 mM [193] and inside the cells glutamine levels reach 2 to 30 mM [194]. Taken together, the pattern of glutamine concentrations inside and outside of cells and the selective uptake of glutamine by *Chlamydia* for peptidoglycan synthesis, substantiate the hypothesis of L-glutamine being the metabolic trigger for EB to RB transition.

In general, it is unknown if *Chlamydia* employs its own type-III-secreted inclusion proteins (Inc) for transmembrane transport activity into and out of the inclusion lumen or predominantly host cell transporters. Hitherto, it has been demonstrated that two host transporters are recruited to the chlamydial inclusion membrane, in order to facilitate vitamins and UDP-glucose transport into the inclusion lumen [125] [128]. GSEA data analysis has brought up

promising L-glutamine transporter candidates, playing a crucial role during chlamydial infection [159]. Excitingly, the upregulated amino acid transporters SLC1A5/ASCT2, SLC3A2, SLC7A1, SLC7A5/LAT1 SLC7A11/xCT, SLC15A4, SLC38A5/SNAT5 and SLC43A1/LAT3 belong to the group of c-Myc regulated target genes [195] [159], as part of c-Myc's central role in reprogramming. *C. trachomatis* promotes L-glutamine uptake and glutaminolysis via c-Myc, by increasing the expression of the L-glutamine transporters SLC1A5/ASCT2 and SLC38A5/SNAT5 and glutaminase (GLS1) [161] for their replication. In genital infections of SLC1A5/ASCT2 knockout mice, chlamydial growth was severely affected, demonstrating a dominant role of host cell glutamine reprogramming in an in vivo infection [159]. The recently generated SLC1A5/ASCT2 knockout mice show no other major phenotype [196]. Since the absence of SLC1A5/ASCT2 revealed a significant growth defect of *C. trachomatis*, a novel therapeutic approach against *Chlamydia* infection could be the limitation of this non-essential amino acid. Increase in the levels of L-glutamine are essential but not sufficient to enable the complex replication cycle and development of *Chlamydia* [159].

## 3.2 c-Myc is a key palyer in interferon-$\gamma$ induced persistent state of *C. trachomatis*

Excessive inflammation and tissue damage in the fallopian tube results in ectopic pregnancy and infertility, due to persistency and recurrent infections [197] [198]. Cytokine secretion is induced by epithelial cells infected with *Chlamydia*, which attracts lymphoid and myeloid cells to the site of infection [199]. The pro-inflammatory cytokine interferon-$\gamma$ is secreted by these cells, leading to a chronic inflammation which was demonstrated to facilitate the development of malignancy like gastrointestinal cancer [200]. To date, the expression of IDO and depletion of the amino acid L-tryptophan is believed to be the reason for chlamydial persistency induced by IFN-$\gamma$ [56] [73], but the overexpression of c-Myc was efficient to rescue *C. trachomatis* of its dormant state (Figure 2.11 A, B and C and Figure 2.12 A). Reduced c-Myc levels have been detrimental for the development of *Chlamydia* (Figure 2.5 B and C) [159] [164], and IFN-$\gamma$ acts via the STAT1-GSK3$\beta$ axis to deplete c-Myc (Figure 2.10 C

and 2.16 A and C). However, neither the rescue of the protein c-Myc alone was sufficient to overcome persistence, but was dependent on the L-tryptophan levels (Figure 2.14 A), nor the presence of exogenous L-tryptophan in a c-Myc knock down scenario was supportive to achieve normal chlamydial growth (Figure 2.15 A). Despite the levels of L-tryptophan, c-Myc is essential to rescue *C. trachomatis* from persistence. Previous findings demonstrating that IFN-$\gamma$ interferes with the production of L-tryptophan rich proteins like major outer membrane protein (MOMP) [201] are in line with the observation that L-tryptophan is still needed to overcome persistence (Figure 2.15 A and Figure 2.14 and Figure 2.16). Genital *C. trachomatis* strains are able to use indole as a precursor for tryptophan synthesis [76] [173] [174]. Remarkably, not only L-tryptophan but also indole addition or depletion have been affecting the levels of c-Myc (Figure 2.13 A). It is still not clear how amino acids like L-tryptophan or its precursor indole influence c-Myc, but it was shown that the deprivation of L-glutamine alters adenosine nucleotide levels which suppresses c-Myc levels, signalling via the 3'UTR [168]. Additional L-tryptophan not only displayed an upregulation of c-Myc but also an increased GSK3$\beta$ phosphorylation (Figure 2.16 A and C). GSK3$\beta$ is inactivated by phosphorylation which also occurs during a normal chlamydial infection (Figure 2.4 A), since active GSK3$\beta$ would lead to destabilization of the c-Myc protein as it phosphorylates the threonine 58 residue of c-Myc resulting in its proteasomal degradation [158] [177]. Taken together, the pGSK3$\beta$-c-Myc axis is activated via L-tryptophan to rescue *C. trachomatis* from persistency, while c-Myc is downstream of interferon-$\gamma$ and has a critical role behind the dormant state of *Chlamydia* (Figure 3.1).

Since c-Myc is a major metabolic regulator, a metabolomic approach was employed to show an increase in TCA cycle intermediates upon chlamydial infection, which were significantly depleted during IFN-$\gamma$ treatment and otherwise restored when c-Myc was overexpressed (Figure 2.17 and Figure 2.18 and Figure 2.19). Interestingly, the metabolic flux alone was not channelled in any direction to rescue the metabolite levels in the samples with c-Myc expression. Even in IFN-$\gamma$ treated and c-Myc overexpressed cells, *Chlamydia* was required to restore metabolite levels (Figure 2.17 and Figure 2.18 and Figure 2.19). This observation supports the involvement of bacterial proteins in the metabolic reprogramming of the host cell. The

metabolites alanine, aspartate and L-glutamate (Figure 2.17) serve as precursors for cell wall biosynthesis. Therefore they might be beneficial for *C. trachomatis*. Flux analysis demonstrated that citrate levels were increased by L-glutamine, L-glutamate and $\alpha$-ketoglutarate fuelling the reductive TCA cycle. *Chlamydia* itself has an incomplete TCA cycle, which lacks the citrate synthase. The pathogen might take up citrate, since it cannot produced it but citrate is also necessary for fatty acid synthesis. The pathogen scavenges the host saturated fatty acid for de novo synthesis of membrane constituents by actively taking them up [119]. Unexpectedly, arginine levels were drastically reduced in all the conditions with chlamydial infection (Figure 2.19 B). Due to the elevated ornithine levels detected, arginine might be shuttled into the urea cycle to be used for polyamine biosynthesis. Polyamines increase drastically under high cell division conditions like for example tumour growth, pregnancy and cystic fibrosis. They have been shown to induce condensation, to stabilize compact DNA forms and being essential for cell proliferation [202]. To create a favourable environment for their own replication and to keep their host alive, *Chlamydia* might use the polyamines for host DNA stabilization and/or their own DNA condensation and stabilization, as the pathogen produces many progenies in a very short period of time. In the c-Myc overexpressed, IFN-$\gamma$-treated and infected-sample, the TCA cycle products were shuttled into nucleotide biosynthesis and the blockade of UMP to UTP was reversed (Figure 2.18 B). It is known that *C. trachomatis* converts UTP to CTP with its CTP synthase, whereas all the other ribonucleosides or nucleobases have to be taken up from the host. For generating dATP, dCTP and dGTP, *Chlamydia* contains a ribonucleotide diphosphate reductase, but dTTP cannot be produced and thymidine nucleotides cannot be obtained from the host cell [113] [114]. *C. trachomatis* might convert dUMP to dTMP via thymidylate synthase [203].

To rescue *C. trachomatis* from persistency c-Myc, which induces mitochondrial biogenesis and TCA cycle activity, is important. Furthermore, c-Myc promotes the process glutaminolysis, that converts L-glutamine to L-glutamate to further enter the TCA cycle, which provides additional availability of TCA cycle intermediates for enhanced amino acid, lipids and nucleotides production [90]. Cell-permeable $\alpha$-ketoglutarate and nucleoside addition was sufficient to overcome the IFN-$\gamma$-induced dormancy state of *Chlamydia* (Figure 2.20), suggesting

that the increase of c-Myc is required for the pathogen to fine tune the metabolic profile of its host cell to promote enough nutrients for overcoming persistence. c-Myc has an effect on the TCA cycle and the nucleosides, while the TCA itself has an influence on nucleosides and both are important for *C. trachomatis*. Therefore, both nucleoside and $\alpha$-ketoglutarate addition were able to rescue the pathogen from persistence. Upon IFN-$\gamma$ treatment, the essential amino acid L-tryptophan gets depleted, an effect c-Myc overexpression can overcome by upregulating the L-tryptophan transporter LAT1/SLC7A5 levels (Figure 2.15 C) to ensure an effective uptake of the metabolite. The transporter should be blocked in order to show its function and its importance for the rescue of *Chlamydia*.

## 3.3 Host cell metabolism, *Chlamydia* and cancer

It already is known that *Chlamydia* infected cells increase the pentose phosphate pathway (PPP) [145], which c-Myc is an activator of, as well as of nucleotide and NAD(P) biosynthesis with L-glutamine serving as an important substrate [204]. To cope with the induced oxidative stress upon infection, the L-glutamine derived glutathione might help the host [205]. c-Myc has been designated to cooperate in lipid, amino acid and amino sugar synthesis [206], apart from upregulating glutaminolysis and energy production. Here, it could be shown that upon *C. trachomatis* infection c-Myc is stabilized, leading to an upregulation of the host cell glutaminolysis via increasing the glutaminase GLS1 and the L-glutamine transporter SLC1A5/ASCT2 [159] [163] [164]. Furthermore, it could be detected that L-glutamine plays a central role in the initiation of the transition of EB to RB, the peptidoglycan synthesis, the complementation of the host cell and the pathogen TCA cycle and served as a nitrogen source [159] [164].

c-Myc is often deregulated by divers mechanisms in cancer [87] [88], which is the same case in *C. trachomatis*-infected cells. The pathogen upregulates glutaminolysis and reprograms the glutamine metabolism of its host cell through c-Myc controlled metabolic pathways in order to support intracellular lifestyle. As indicated by epidemiologic findings [44], the stabilization of c-Myc by the pathogen contributes to the evolving body of evidence that *C. trachomatis* infec-

tion could favour cervical cancer. Additionally, *Chlamydia* induces proliferating signalling and prevents host cell apoptosis [156]. It stimulates secretion of pro-angiogenic VEGF [207], employs immune evasion mechanism [208] and causes DNA damage [157]. Moreover, *Chlamydia* remarkably alters the metabolism of its host cell to a cancer phenotype [145] [148], using extracellular glutamine to fulfil their metabolic demands to produce cell mass. The points listed above are all aspects of established cancer hallmarks [209]. Cells infected with *Chlamydia*, as well as glutamine addicted tumor cells are highly sensitive to pharmacological interference with the glutamine metabolism [159] [164] (Figure 2.8 B and C). Hence, it would be fascinating to further decipher the host cell metabolic reprogramming role upon an chlamydial infection, and the link to cancer development. Furthermore, the current approaches in innovative cancer therapy to target either c-Myc or glutamine metabolism might prove to be also efficient for treating *C. trachomatis* infections.

## 3.4 Organoids as an infection model

It is common in the *Chlamydia* field to mainly use human cancer cell lines for bacterial propagation and as an infection model but innate immune signalling and metabolic pathways are altered in these cells. Additionally, there is no animal model available, since primary human and primary mouse cells fundamentally differ in the response to an infection with *Chlamydia* [145]. Furthermore, the immunological differences between human and mouse and the given response to IFN-$\gamma$ occur differently in mouse and in human. Hence, another model was needed which is closer to an in vivo scenario. Fallopian tube biopsies were used to derive cells and organoids of them. From the isolated primary cells (FTC cells) and Hec 1B cells, End1 cells and SV-HUC1 cells 3D tissue models were produced, which unfortunately did not polarize and showed no consistency in the results with different patients. Therefore, the study was extended in organoids from the biopsies. *C. trachomatis* could be cultivated for several days in the organoids (Figure 2.21 and Figure 2.22). Furthermore, *Chlamydia* turned persistent upon IFN-$\gamma$ treatment in organoids (Figure 2.22 B). The decrease of infection and the reduction in inclusion size due to IFN-$\gamma$, which could be examined in the cell lines, were also detected

in the organoids (Figure 2.21 A, B and C). More than that, the phosphorylation of STAT1 (Figure 2.22 A) was determined which propounds the activation of the Jak-STAT1 pathway in organoids upon IFN-$\gamma$ treatment. To affirm if IFN-$\gamma$ signals via STAT1 to transcriptionally depleted c-Myc, STAT1 should be inhibited by pharmacological interference with siRNA or an inhibitor against STAT1. Preliminary data showed that LAT1/SLC7A5, which is a c-Myc target, was upregulated upon infection with *C. trachomatis* and reduced in the IFN-$\gamma$ treated organoids. To validate the importance of this L-tryptophan transporter, pharmacological interference with siRNA should be tested.

Taken together, the organoids reflected the same results as the cell lines suggesting organoids derived from biopsies to be a useful model in studying chlamydial infection. The next step would be to create organoids overexpressing c-Myc via a lentiviral transduction, in order to verify if it would also rescue *Chlamydia* of its persistent state in organoids. Moreover, studying the c-Myc levels of the organoids of the different biopsies and if they vary the persistent status of the pathogen in this would help to confirm the role of c-Myc in chlamydial dormancy.

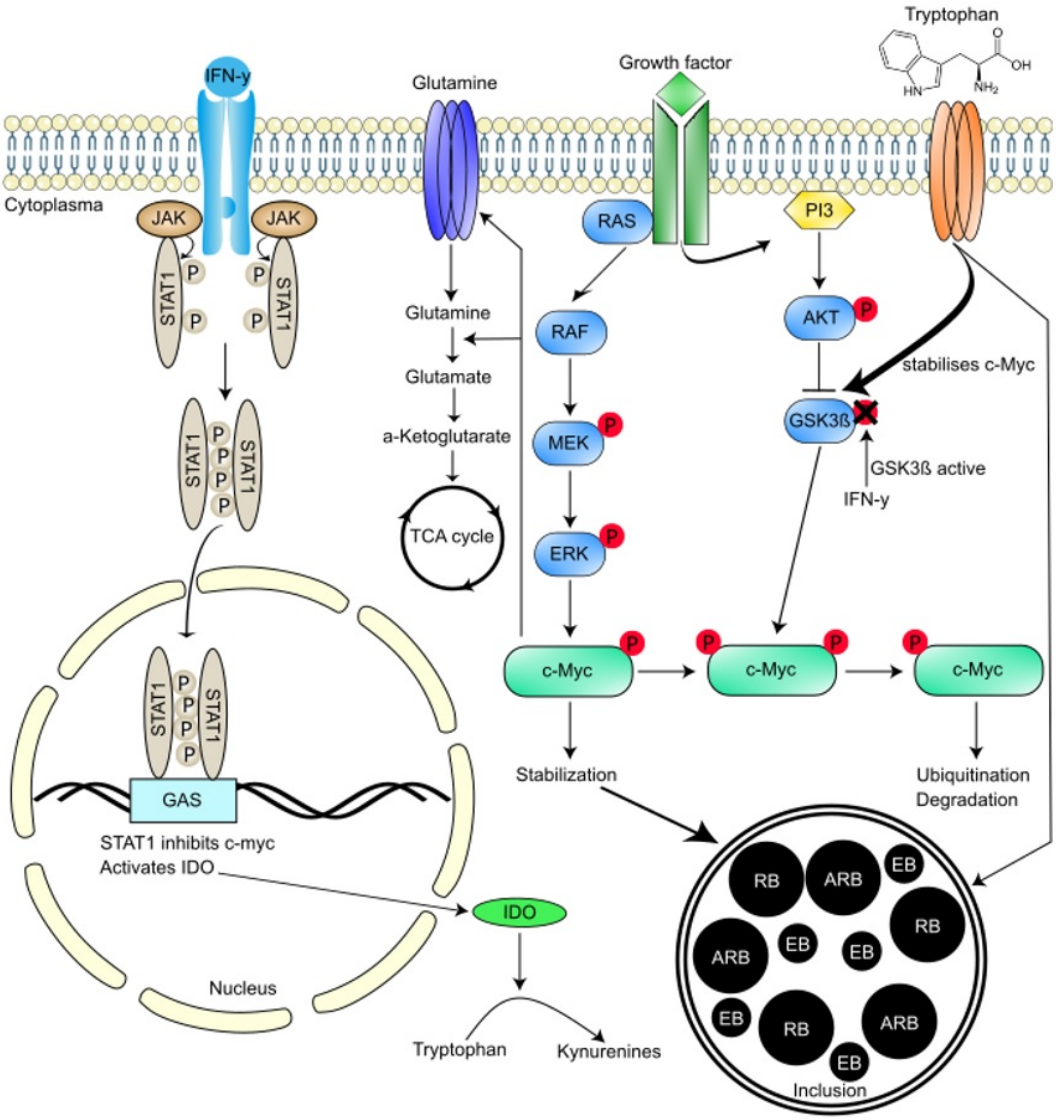
Tryptophan
IFN-y
Glutamine
Growth factor
Cytoplasma
JAK
JAK
STAT1
STAT1
RAS
PI3
Glutamine
RAF
AKT
stabilises c-Myc
Glutamate
a-Ketoglutarate
MEK
GSK3ß
GSK3ß active
IFN-y
TCA cycle
ERK
c-Myc
c-Myc
c-Myc
Stabilization
Ubiquitination
Degradation
GAS
STAT1 inhibits c-myc
Activates IDO
IDO
Nucleus
Tryptophan
Kynurenines
RB
ARB
EB
Inclusion

**Fig. 3.1: Schematic model of reprogramming the host cell upon infection and IFN-$\gamma$ treatment.** The type II interferon-$\gamma$ bind the extracellular domain of its receptor IFNGR1, in order to activate the Janus kinase, which results in receptor phosphorylation. Subsequently, recruitment and phosphorylation of STAT1 takes place. Tyrosine 701 and serine 727 phosphorylation of STAT1 leads to STAT1 homodimers and their translocation to the nucleus. The homodimers in the nucleus bind to GAS elements in the promoter regions of their target genes, in order to regulate their transcription. This causes inhibition of c-Myc transcriptionally and the activation of IDO. The enzyme IDO catalysis the initial step of L-tryptophan degradation to kynurenine. Depletion of the essential amino acid L-tryptophan leads to *C. trachomatis* persistence. Furthermore, IFN-$\gamma$ supports AKT phosphorylation and at the same time GSK3$\beta$ dephosphorylated, which means it is active and phosphorylates threonine 58 of c-Myc, leading to its ubiquitination and proteasomal degradation. The Ras/Raf/MEK/ERK and the PI3K/AKT pathways are activated and a pronounced upregulation of c-Myc takes place upon infection with *C. trachomatis*. Activation of ERK is followed by phosphorylation of serine 62 of c-Myc, leading to its stabilization. Phosphorylation of AKT results in GSK3$\beta$ phosphorylation, which inactivates GSK3$\beta$ and prevents the threonine 58 phosphorylation of c-Myc. Exogenous L-tryptophan rescues *Chlamydia* of the persistent state via increasing GSK3$\beta$ phosphorylation and upregulation of c-Myc levels. Host cell glutaminolysis is increased by the stabilized c-Myc, due to enhanced expression and stabilization of the L-glutamine transporter SLC1A5/ASCT2 and glutaminase. The imported L-glutamine gets converted to L-glutamate by glutaminase and then degraded to $\alpha$-ketoglutarate, which is able to enter the TCA cycle. Moreover,stabilized c-Myc leads to an upregulation of the L-tryptophan transporter LAT1/SLC7A5 and the essential amino acid L-tryptophan is used by *C. trachomatis* to produce their L-tryptophan rich proteins, which is damped upon IFN-$\gamma$ treatment. Host cell reprogramming via c-Myc supports chlamydial intracellular lifestyle by creating a favourable replicating niche.

# 4 Material and Methods

## 4.1 Materials

### 4.1.1 Cell lines and culture media

**Tab. 4.1:** Cell lines used in this study

| Cell line | Properties | Source |
|---|---|---|
| HeLa 229 | Human cervix adenocarcinoma cells | ATCC® CCL-2.1$^{TM}$ |
| U2OS | Human osteosarcoma cells | ATCC® HTB-96$^{TM}$ |
| U2OS $^{Tet-On}$ | U2OS cell line with Dox-inducible Myc overespression | Lorenzin et al., 2016 |
| Human Fimb | Primary human cells, isolated from the fimbriae of the uterine tube | Prof. Dr. Wischhusen, University Hospital of Würzburg |
| HeLa 229 pInducer11 shc-Myc | HeLa 229 cell line with Dox-inducible Myc knock-down | Nadine Vollmuth |
| HUVEC | Human umbilical vein endothelial cells | ATCC® CRL-1730$^{TM}$ |
| Hec 1B | Human uterus adenocarcinoma cells, Endometrium | ATCC® HTB-113$^{TM}$ |
| End1 | Human endocervix cells, endometriosis | ATCC® CRL-2615$^{TM}$ |
| SV-HUC1 | Human SV40 immortalized ureter cells, uroepithelium | ATCC® CRL-9520$^{TM}$ |

| | | |
|---|---|---|
| FTC | Primary human fallopian tube cells, isolated from uterine tube tissue | Prof. Dr. med. Christine Wulff University Hospital of Würzburg |

**Tab. 4.2:** Cell culture media

| **Medium** | **Special remarks** | **Source** |
|---|---|---|
| DMEM, high glucose | Liquid medium containing 4.5 g/L glucose, 4 mM glutamine and 1 mM sodium pyruvate | Sigma-Aldrich (D6429) |
| DMEM, basic formulation | Powder, without glucose, glutamine, sodium pyruvate, phenol red and sodium bicarbonate | Sigma-Aldrich (D5030) |
| RPMI 1640 (1x) + GlutaMAX$^{TM}$ | Standard liquid medium containing 25 mM HEPES | Gibco$^{TM}$ (72400-021) |
| RPMI 1640 w/o W | Liquid medium containing L-glutamine 300 mg/L and 2.0 g/L $NaHCO_3$, without L-tryptophan (W) | PAN Biotech (PO4-17598) |
| Medium 200 | Medium for cultivation of human large vessel endothelial cells | Gibco$^{TM}$ (M200500) |
| F-12 | Ham's F-12 Nutrient Mixture | Gibco$^{TM}$ (31765-027) |
| OptiMEM | Reduced Serum Medium for cell transfection | Gibco$^{TM}$ (51985026) |

**Tab. 4.3:** Media supplements

| **Supplement** | **Stock concentration** | **Source** |
|---|---|---|
| Heat-inactivated Fetal Bovine Serum | -/- | Sigma-Aldrich (F7524) |
| Dialysed Fetal Bovine Serum | -/- | Sigma-Aldrich (F0392) |
| L-tryptophan | 10 $\mu$g/mL in $dH_2O$ | Sigma-Aldrich (TO254) |
| Indole | 50 mM in DMSO | Sigma-Aldrich (13408) |

High-glucose DMEM, F-12 and RPMI media were supplemented with 5% or 10% heat inactivated FBS (v/v). RPMI 1640 w/o W medium was supplemented with 5% dialyzed FBS (v/v) and L-tryptophan as needed. Medium 200 was supplemented with 1x Low Serum Growth. Basic formulation DMEM was supplemented with 3.7 g/L sodium bicarbonate and 0.0159 g/L phenol red, dissolved ad 1 L with $dH_2O$ and steril filtered (0.2 $\mu$m), and supplemented with dialysed FCS and nutrients as needed. Media composition of basic formulation DMEM and RPMI 1640 is given in annex (Table A1). High glucose DMEM was supplemented with WNT (25%, self-made), RSPO (25%, self-made), Noggin (10%, self-made), B27 (2%), Pen/Strep (1%), Nicotinamide (1 mM), human EGF (100 ng/mL), TGF-$\beta$ inhibitor (0.5 $\mu$M), and Rock inhibitor (10 $\mu$M) for organoid cultivation.

**Tab. 4.4:** Recombinant proteins used in this study

| **Recombinant protein** | **Stock concentration** | **Working concentration** | **Source** |
|---|---|---|---|
| Recombinant human interferon-$\gamma$ | 1 mg/mL | 10-50 ng/mL | Gibco$^{TM}$ (PHC4031) |
| Interferon-$\gamma$ | 1 mg/mL | 10-50 ng/mL | Merck Millipore (IF002) |

### 4.1.2 Bacterial strains

**Tab. 4.5:** Bacterial strains

| **Species** | **Source** |
|---|---|
| *Chlamydia trachomatis* serovar L2 (434/Bu) | ATCC® VR-902B$^{TM}$ |
| *Chlamydia trachomatis* serovar D (UW-3/Cx) | ATCC® VR-885$^{TM}$ |

### 4.1.3 Antibodies

**Tab. 4.6:** Primary antibodies

| **Target** | **Molecular weight** | **Origin** | **Dilution** | **Source** |
|---|---|---|---|---|
| $\beta$-Actin | 42 kDa | Mouse, monoclonal | 1 : 10 000 (WB) | Sigma-Aldrich (A5441) |
| AlexaFluor 647 | | | 1 : 200 (IF) | Invitrogen (A22287) |
| Chlamydial HSP60 | 60 kDa | Mouse, monoclonal | 1 : 1 000 (WB) | Santa Cruz (sc-57840) |
| | | | 1 : 500 (IF) | |
| c-Myc | 57 kDa | Rabbit, monoclonal | 1 : 10 000 (WB) | Abcam (ab32072) |
| Anti c-Myc Phospho-T58 | 49 kDa | Rabbit, polyclonal | 1 : 10 000 (WB) | Abcam (ab28842) |

| | | | | |
|---|---|---|---|---|
| Anti c-Myc Phospho-S62 | 49 kDa | Rabbit, polyclonal | 1 : 10 000 (WB) | Abcam (ab51156) |
| DAPI | | | 1 : 1 000 (IF) | Sigma-Aldrich (D9542) |
| Phospho-Akt (Ser473) | 60 kDa | Rabbit, monoclonal | 1 : 1 000 (WB) | Cell signaling (4060) |
| Akt (pan) | 60 kDa | Rabbit, monoclonal | 1 : 1 000 (WB) | Cell signaling (4685) |
| Phospho-Erk | 42, 44 kDa | Mouse, monoclonal | 1 : 1 000 (WB) | Cell signaling (9106) |
| Erk | 42 kDa | Rabbit, polyclonal | 1 : 1 000 (WB) | Cell signaling (9108) |
| Phospho-GSK3$\beta$ (Ser9) | 46 kDa | Rabbit, monoclonal | 1 : 1 000 (WB) | Cell signaling (5558) |
| GSK-3ß (3D10) | 46 kDa | Mouse, monoclonal | 1 : 1 000 (WB) | Cell signaling (9832) |
| Phospho-Stat1 (Ser727) | 91 kDa | Rabbit, polyclonal | 1 : 1 000 (WB) | Cell signaling (9177) |
| Phospho-Stat1 (Tyr701) | 84.91 kDa | Rabbit, monoclonal | 1 : 1 000 (WB) | Cell signaling (9167) |
| OmpA | 42 kDa | Rabbit | 1 : 10 000 (WB) | Self-made antibody |
| Stat1 | 84.91 kDa | Rabbit, polyclonal | 1 : 1 000 (WB) | Cell signaling (9772) |

**Tab. 4.7:** Secondary, conjugated antibodies

| Target | Conjugation | Origin | Dilution | Source |
|---|---|---|---|---|
| Moues IgG | HRP | Goat, polyclonal | 1 : 2 500 (WB) | Santa Cruz (sc-2005) |
| Rabbit IgG | HRP | Goat, polyclonal | 1 : 2 500 (WB) | Santa Cruz (sc-2004) |
| Mouse IgG | Cy2 | Goat, polyclonal | 1 : 150 (IF) | Dianova (115-225-146) |
| Mouse IgG | Cy3 | Goat, polyclonal | 1 : 150 (IF) | Dianova (115-165-146) |
| Mouse IgG | Cy5 | Goat, polyclonal | 1 : 150 (IF) | Dianova (115-175-146) |
| Rabbit IgG | Cy2 | Goat, polyclonal | 1 :150 (IF) | Dianova (111-225-144) |
| Rabbit IgG | Cy3 | Goat, polyclonal | 1 : 150 (IF) | Dianova (111-165-144) |
| Rabbit IgG | Cy5 | Goat, polyclonal | 1 : 150 (IF) | Dianova (111-175-144) |

### 4.1.4 Buffers and Solutions

Buffers and solutions are listed in alphabetical order:

| | |
|---|---|
| ECL substrate solution (1:1 mixture of solution I and II) | <u>Solution I</u><br>5 mL 1 M Tris-HCl (pH 8.5)<br>500 $\mu$L 250 mM luminol in DMSO<br>220 $\mu$L 90 mM coumaric acid in DMSO<br><u>Solution II</u><br>5 mL 1 M Tris-HCl (pH8.5)<br>32 $\mu$L 35% hydrogen peroxide<br>Add $dH_2O$ to a final volume of 50 mL each, store at 4°C in the dark |
| Laemmli lysis buffer (2x) | 10 mL 1.5 M Tris-HCl (pH 6.8) |

| | |
|---|---|
| | 40 mL 10% SDS |
| | 30 mL glycerol |
| | 5 mg bromophenol blue |
| | 1.5 mL $\beta$-mercaptoethanol |
| | Add $dH_2O$ to a final volume of 100 mL, store at $-20°C$ |
| Mowiol solution | 6 glycerol |
| | 2.4 g mowiol |
| | 6 mL $dH_2O$ |
| | 12 mL 0.2 M Tris-HCl (pH 8.5) |
| | Aliquot and store at $-20°C$ |
| PBSTD | 0.3% Triton X-100 |
| | 1% DMSO |
| | In 1x DPBS |
| Phosphate Buffered Saline, Dulbecco's (DPBS) | Gibco$^{TM}$ (14190169) |
| Renografin (60%) | 26g Meglumin diatrizoate |
| | 4 g Sodium diatrizoate hydrate |
| | 0.16 g Sodium citrate hydrate |
| | 0.02 g EDTA |
| | Add 1x HBSS to a final volume of 50 mL and adjust pH to 7.4, steril filter (0.2$\mu$m) and store at 4°C |
| RIPA lysis buffer | 50 mM Tris-HCl pH 7.5 |
| | 150 mM NaCl |
| | 1% Triton X-100 |
| | 1% NP-40 |
| | 0.1% SDS |
| | 10% glycerol |

| | |
|---|---|
| | Complete protease inhibitor cocktail (Roche) |
| | MG-132 protease inhibitor |
| SDS running buffer (10x) | 10 g SDS |
| | 30.3 g Tris |
| | 144.1 g glycine |
| | Add $dH_2O$ to a final volume of 1 L; for use, dilute to 1x with $dH_2O$ |
| Semi-dry transfer buffer (10x) | 77.5 g glycine |
| | 100 mL 10% SDS |
| | 250 mL 1 M Tris (pH 7.5-8) |
| | Add $dH_2O$ to a final volume of 1 L and adjust pH to 8.3; for use, add 200 mL of methanol to 100 mL of 10x Semi-dry transfer buffer and fill up to 1 L with $dH_2O$ |
| Separating polyacrylamide gel buffer (Lower buffer) | 90.85 g Tris |
| | 20 mL 10% SDS |
| | Add $dH_2O$ to a final volume of 500 mL and adjust the pH to 8.8, store at 4°C |
| SPG buffer (1x) | 75 g sucrose |
| | 0.52 g $KH_2PO_4$ |
| | 1.22 g $Na_2HPO_4$ |
| | 0.72 g L-glutamate |
| | Add $dH_2O$ to a final volume of 1 L, adjust pH to 7.4, sterile filter (0.2 $\mu$m) and store at 4°C |

| | |
|---|---|
| Stacking polyacrylamide gel buffer (Upper buffer) | 30.3 g Tris |
| | 20 mL 10% SDS |
| | Add $dH_2O$ to a final volume of 500 mL and adjust pH to 6.8, store at 4°C |
| TBS-T buffer (10x) (Tris-buffered saline with Tween20) | 60.5 g Tris |
| | 87.6 g NaCl |
| | 5 mL Tween20 |
| | Add $dH_2O$ to a final volume of 1 L and adjust pH to 7.5; for use, dilute to 1x with $dH_2O$ |
| Trypsin TrypLE$^{TM}$ Express | Gibco$^{TM}$ (12604039) |

### 4.1.5 Chemicals and reagents

**Tab. 4.8:** Chemicals and reagents in alphabetical order

| **Substance** | **Supplier** |
|---|---|
| Acetic acid | Roth |
| Acetonitrile | Merck |
| Ammonium acetate | Merck |
| Ammonium persulfate (APS) | Sigma-Aldrich |
| Anhydrotetracycline hydrochloride (AHT) | Acros |
| Bisacrylamide, Rotiphorese® Gel 30 | Roth |
| Bromophenol blue | Sigma-Aldrich |
| BSA, Albumin Fraction V | Roth |
| B27 | Thermo Scientific$^{TM}$ |

| | |
|---|---|
| Classical PCR Mycoplasma Test kit | Venor®GeM |
| Coumaric acid | Sigma-Aldrich |
| Crystal violet | Merck |
| Dimethyl sulfoxide (DMSO) | Roth |
| Ethanol | Roth |
| Ethylendiamine tetraacetic acid (EDTA) | Sigma-Aldrich |
| Glycerol | Roth |
| Glycine | Sigma-Aldrich |
| G magnetic beads, Dynabeads | Thermo Scientific$^{TM}$ |
| Hank's Balanced Salt Solution (HBSS) | Gibco$^{TM}$ (14025100) |
| Human EGF | Thermo Scientific$^{TM}$ |
| Hydrochloric acid 37% | Merck |
| Indole | Sigma-Aldrich |
| Isopropanol | Roth |
| Lamivudine | Sigma-Aldrich |
| Luminol | Fluka |
| Matrigel | Corning |
| $\beta$-mercaptoethanol | Roth |
| Meglumin diatrizoate | Sigma-Aldrich |
| Methanol | Roth |
| Mowiol 4-88 | Roth |
| Nicotinamide | Sigma-Aldrich |
| Nonfat Dried Milk Powder BC | AppliChem |
| Page Ruler Prestained Protein Ladder | Fermentas |
| Paraformaldehyde (PFA) | Roth |
| Pen/Strep | Invitorgen |
| Quanta SYBER | Quanta Bio |
| Revert Aid First Strand synthesis kit | Fermentas |
| RNA easy kit | Qiagen |

| | |
|---|---|
| Rock inhibitor | AbMole Bioscience |
| siRNA | Santa Cruz |
| D (+)-sucrose | Roth |
| Sodium chloride | VWR |
| Sodium citrate hydrate | Sigma-Aldrich |
| Sodium diatrizoate hydrate | Sigma-Aldrich |
| Sodium dodecyl sulfate (SDS) | Roth |
| Sodium hydroxide | Roth |
| Tetramethylethylendiamine (TEMED) | Sigma-Aldrich |
| TGF-$\beta$ inhibitor | Tocris |
| Tris | Sigma-Aldrich |
| Triton X-100 | Sigma-Aldrich |
| Tween20 | Sigma-Aldrich |
| X-tremeGENE$^{TM}$ HP DNA transfection reagent | Roche |

### 4.1.6 Technical equipment

**Tab. 4.9:** Technical equipment

| **Equipment** | **Manufacturer** |
|---|---|
| AvantiTM J-25I centrifuge | Beckman Coulter |
| Biological safety cabinet Safe 2020 | Thermo Scientific$^{TM}$ |
| Bio-Rad Model 200/2.0 Power Supply | Bio-Rad |
| Chemiluminescence camera system | Intas |
| DUOMAX 1030 Rocking Shaker | Heidolph |
| Electric balance ABS 80-4 | Kern |
| Electric balance EW 1500-2M | Kern |
| HERAcell 150i $CO_2$ incubator | Thermo Scientific$^{TM}$ |

| | |
|---|---|
| Himac CT15RE centrifuge | Hitachi |
| LEICA DM2500 microscope | Leica |
| Light microscope LEICA DM IL LED | Leica |
| Liquid nitrogen tank | Custom BioGenic Systems |
| Megafuge 1.0R centrifuge | Heraeus |
| MiniStar silverline microcentrifuge | VWR |
| PerfectBlue Semi-Dry Blotter | Peqlab |
| PerfectBlue Vertical Double Gel System | Peqlab |
| PerfectSpin Mini centrifuge | VWR |
| Pipetboy accu-jet® pro | Brand |
| Pipettes (pipetman, Eppendorf Research®, Transferpette® S) | Gilson, Eppendorf, Brand |
| Roller mixer SRT9 | Stuart |
| SpeedVac | Thermo Scientific$^{TM}$ |
| Step One Plus device | Applied Biosystems |
| Systec VX-150 autoclave | Systec |
| Taumelmischer | Hartenstein |
| Sonifier | Beanson |
| Vortex | Bender and Hobein AG |
| VWR® Power Source 250V | VWR |
| VWR® Rocking Platform | VWR |

### 4.1.7 Disposable materials

**Tab. 4.10:** Disposables in alphabetical order

| **Material** | **Manufacturer** |
|---|---|
| Cell culture flasks (T25, T75, T175) | Corning |
| Cell scraper | Sarstedt |

| | |
|---|---|
| Cellulose cloth | Wepa |
| Cryo tubes | Sarstedt |
| Falcon tubes (15 mL and 50 mL) | Corning, Greiner |
| Gloves XCEED® nitrile | Microflex® |
| Hollow needles | Braun |
| Low binding tubes | Eppendorf |
| Multiple Well Microplates (6, 12, 96) | Corning |
| Parafilm® M Sealing foil | Hartenstein |
| Pipette tips (20 $\mu$l, 200 $\mu$l, 1000 $\mu$l) | Sarstedt |
| PVDF Western Blotting Membrane | Sigma-Aldrich |
| Reaction tubes (1.5 mL and 2.0 mL) | Sarstedt |
| Serological pipettes (1 mL, 5 mL, 10 mL, 25 mL) | Laborhaus Scheller |
| Starta® C18-E, 55 $\mu$m, 50 mg/1 mL RP18 SPE-Colums | Phenomenex Strata |
| Syringe filter Filtropur S (0.2 $\mu$m) | Sarstedt |
| Syringe (2 mL, 5 mL, 10 mL, 20 mL, 50 mL) | VWR |
| Vacuum Filter/Storage Bottle (0.22 $\mu$m; 250 mL and 500 mL) | VWR |
| Whatman Blotting paper | VWR |

## 4.2 Methods

### 4.2.1 Cell culture

Cell lines and *Chlamydia* stocks were verified to be *Mycoplasma* free via PCR (Venor®GeM Classic PCR Mycoplasmen Test 11-1050).

#### 4.2.1.1 Cell cultivation and passaging

Human Fimb cells, Hec 1B cells, End1 cells, HeLa 229 cells and HeLa 229 pInducer11 shc-Myc cells were cultured in 10% (v/v) heat inactivated FBS RPMI1640 + GlutaMAXTM medium.

FTC cells, U2OS cells and U2OS $^{Tet-On}$ cells were maintained in high-glucose DMEM with 10% (v/v) heat inactivated FBS. SV-HUC1 cells were cultured in 10% (v/v) heat inactivated FBS F-12 medium. All cell lines were grown in a humidified atmosphere containing 5% (v/v) CO2 at 37°C. Dependent on their confluence (75-90%), cells were passaged every three to four days. Therefore, the culture medium was removed and the cells were washed once with DPBS to eliminate cell debris. To detach the adherent cells, the cells were incubated with 1.0 mL TrypLE$^{TM}$ Express Enzyme for 3-5 minutes at 37°C. The cell suspension was diluted in fresh culture medium and seeded in a new flask or well-plates at the desired concentration. After 10 passages, cells were discarded and replaced by fresh ones.

#### 4.2.1.2 Cell culture stocks

For long term storage of cell lines cryostocks were produced and stored in liquid nitrogen. To do so, cells were detached, appropriate culture medium was added and the suspension was centrifuged for 5 minutes at 800 g. A 90% confluent T75 flask cell pellet was resuspended in 3 mL of according cell culture medium supplemented with 10% (v/v) DMSO, 1 mL aliquots were made and gradually cooled down to −80°C in a Cryo box and then transferred and stored at −180°C in a liquid nitrogen tank. In order to re-cultivate long term stored cell lines, cell aliquot was thawed at room temperature, added fresh culture medium and centrifuged for 5 minutes at 800 g. The cell pellet was resuspended in fresh culture medium, transferred to a cell culture flask and incubated at 37°C in 5% (v/v) CO2. Next day, the medium was refreshed.

#### 4.2.1.3 Human organoid formation

Fallopian tube tissue was washed with 1x DPBS, cut and pressed with a glass slide into very small pieces, and centrifuged at 1 000 g for 10 minutes. Pellet was resuspended in matrigel, plated as 50 $\mu$l drops in the centre of the wells of a 24 well-plate, and solidified for 20 minutes at 37°C. Afterwards prewarmed organoid medium (500 $\mu$l per well) was added.

#### 4.2.1.4 Organoid cultivation and passaging

Enlarged organoids were split approximately every seventh day. The matrigel drop was resuspended in cold DMEM medium, centrifuged at 1 000 g for 5 minutes at 4°C, resuspended in matrigel, 50 $\mu$l drops were plated in the centre of the wells of a 24 well-plate, and solidified for 20 minutes at 37°C. Afterwards, prewarmed organoid medium (500 $\mu$l per well) was added. (Naziia Kurmasheva maintained (master student department of microbiology, Würzburg) the organoids and performed the experiments.)

#### 4.2.1.5 Induction and chemicals

Inducible cell line U2OS $^{Tet-On}$ was seeded into well-plates and incubated over night for proper attachment. In order to induce c-Myc overexpression, medium was changed to 10% (v/v) heat inactivated FBS DMEM and 100 ng/mL AHT was added. After 2 hours of induction, cells were infected with *C. trachomatis* and incubated for further 24 to 36 hours. Interferon-$\gamma$, L-tryptophan and Indole were added to cells as needed, 2 hours before infection.

#### 4.2.1.6 Transfection

At a confluence of 60%, cells were transfected with plasmid DNA with X-tremeGENE$^{TM}$ HP DNA transfection reagent and OptiMEM transfection medium. After 5 hours of transfection, the medium was replaced with fresh 5% FCS RPMI medium.

#### 4.2.1.7 Preparation of *C. trachomatis* stocks for infection

*C. trachomatis* were propagated in HeLa 229 cells as previously described (Al-Younes et al., 1999). In brief, HeLa 229 cells were seeded in T75 flasks and infected with an MOI of 1. After 48 hours, cells were scraped off the surface of the flask, transferred to a 50 mL falcon containing glass beads and vortexed for 3 minutes to lyse the cells. The supernatant was transferred into a new tube and centrifuged for 10 minutes at 2 000 g at 4°C to pellet down the cell debris. Again, the supernatant was transferred to special centrifugation tube to pellet *Chlamydia* by centrifugation for 30 minutes at 30 000 g at 4°C. The pellet was washed with

10 mL ice cold 1x SPG buffer and a second time centrifuged for 30 minutes at 30 000 g at 4°C. Depending on the pellet size, the pellet was resuspended in 1-4 mL of ice cold 1x SPG buffer. To disrupt clumps, the suspension was passed first through a G20 needle and then through a G18 needle three times each. Aliquots of the *Chlamydia* suspension were made and stored at −80°C. To determine the titer, a freshly made aliquot was thawed, HeLa 229 cells were infected with different amounts of the aliquot and the inclusion formation was assessed after 24 hours.

### 4.2.2 Infection with *C. trachomatis*

Cells were seeded in well-plates and overnight allowed to grow to a confluence of about 70%. Before infection, the medium was changed to 5% (v/v) FBS. The cells were infected with *C. trachomatis* serovar L2 or *C. trachomatis* serovar D EBs at multiplicity of infection (MOI) 1, by adding directly to the cell culture medium, the appropriate amount of a freshly thawed aliquot. For serovar D, well-plates were centrifuged for 5 minutes at 2 000 g. Infected cells were incubated in a humidified atmosphere with 5% (v/v) CO2 at 35°C.

### 4.2.3 Infectivity assay

For primary infection, cells were infected with *C. trachomatis* at MOI 1 and cultured for 48 hours. To mechanically disrupt infected cells, glass beads were added to the cell culture medium and the well-plate was shaken. Freshly plated cells were infected with various dilutions of the primary infection medium containing released EBs (referred to as secondary infection). Cells of the secondary infection were lysed and analyzed by Western blotting against chlamydial HSP60 to determine the infectivity.

### 4.2.4 *C. trachomatis* in axenic medium

*C. trachomatis* was propagated in HeLa 229 cells, isolated of the cells, purified with Renografin, and incubated in different axenic media (as previously described in Rajeeve et al. [159]). In brief, HeLa 229 cells were seeded in T175 flasks and infected with *Chlamydia* at MOI 1 for 48 hours. Subsequently, the cells were scraped off, disrupted with glass beads, and chlamydial

EBs were purified with a Renografin gradient and resuspended in axenic medium, incubated for indicated time points at 37°C. Axenic medium consists of basic DMEM (Table A1) supplemented with sodium bicarbonate (44 mM), phenol red (42 $\mu$M), glucose-6-phosphate (0.5 mM) and the amino acid (1 mM) according to the experimental setup. After incubation, samples were centrifuged at 21 500 g for 30 minutes at 4°C, supernatant was transferred into a new tube, and both pellets and supernatants were heat-inactivated for 10 minutes at 90°C and stored at −80°C for further analysis.

#### 4.2.4.1 Analysis of substrate uptake

For $\alpha$-ketoglutarate 0.2 mL of the axenic medium supernatant was spiked with 20 $\mu$l 5 mM norvaline solution (internal standard), dried under $N_2$ flux, treated with 50 $\mu$l methoxyamine in pyridine (20 mg/ml) for 90 minutes at 40°C, and subsequently with 50 $\mu$l MTBSTFA for 30 minutes at 70°C. In the case of L-glutamine and L-glutamate 0.1 mL of the axenic medium supernatant were spiked with 20 $\mu$l 5 mM non-labelled L-glutamine or L-glutamate solution (internal standard), dried under $N_2$ flux, and treated with 50 $\mu$l ACN and 50 $\mu$l MTBSTFA for 30 minutes at 70°C. After treatment, samples were taken for analysis. (Analysis was performed by the group of Prof. Dr. Wolfgang Eisenreich (TU München).)

#### 4.2.4.2 Isotopolog profiling

Cell pellets of the axenic incubation were resuspended in 1 mL of methanol, mechanically disrupted with a ribolyser (3x20 sec 6.5 m/s), and centrifuged at 10 000 g for 20 minutes at 4°C. This was performed twice. Then the supernatants were combined, dried under $N_2$ flux, treated with 50 $\mu$l N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide containing 1 % tert-butyldimethylsilylchloride and 50 $\mu$l water free acetonitrile for 30 minutes at 70°C. TBDMS-derivates of amino acids were analysed via GC/MS. Residual cell debris after the centrifugation step was subjected to acidic hydrolysis and protein bound amino acids as well as meso-diaminopimelic acid (mDAP) were analysed as TBDMS derivates.

For GC/MS analysis a GCMS-QP 2010 Ultra spectrometer (Shimadzu, Duisburg Ger-

many) equipped with an Equity TM-5, FUSED SILICA Capillary Column 30 m x 0.25 mm x 0.25 $\mu$m film thickness was used. Data were collected by LabSolution software (Shimadzu). Samples were analysed three times, and the overall $^{13}$C excess (mol-%) and the relative contributions of isotopomers (%) were computed by an Excel based in-house software package by the group of Prof. Dr. Eisenreich (TU München).

For TBDMS-derivates of polar metabolite mixtures, the column was first developed for 2 minutes at 100°C, then a gradient of 3°C min$^{-1}$ to 234°C, 1°C min$^{-1}$ to 237°C, 3°C min$^{-1}$ to 260°C, and finally heated at a gradient of 10°C min$^{-1}$ to a final temperature of 320°C, where it was hold for 2 minutes. To analyse mDAP and TBDMS-amino acids, the column was first developed at 150°C for 3 minutes and then a gradient of 7°C min$^{-1}$ to 280°C, where it was hold for 3 minutes. (Analysis was performed by the group of Prof. Dr. Wolfgang Eisenreich (TU München).)

### 4.2.5 Metabolic profiling

U2OS $^{Tet-On}$ cells were seeded in triplicates, either uninfected or infected with *C. trachomatis* serovar L2 for 36 hours, induced and treated with interferon-$\gamma$. After the respective time, medium was collected, snap frozen in liquid nitrogen, and the cells were washed with ice cold 154mM ammonium acetate (Sigma) and snap frozen in liquid nitrogen. The cells were harvested after adding 480 $\mu$l cold MeOH/H2O (80/20, v/v) (Merck) to each sample containing Lamivudine (Sigma) standard (10 $\mu$M). The cell suspension was collected by centrifugation and transferred to an activated (by elution of 1 mL CH3CN (Merck)) and equilibrated (by elution of 1 mL MeOH/H2O (80/20, v/v)) RP18 SPE-column (Phenomenex). The eluate was collected and evaporated in SpeedVac and was dissolved in 50 $\mu$L CH3CN/5 mM NH4OAc (25/75). Each sample was diluted 1:1 (cells) or 1:5 (medium) in CH3CN. 5 $\mu$l of sample was applied to HILIC column (Acclaim Mixed-Mode HILIC- 1, 3 $\mu$m, 2.1 * 150 mm). Metabolites were separated at 30°C by LC using a DIONEX Ultimate 3000 UPLC system (Solvent A: 5 mM NH4OAc in CH3CN/H2O (5/95), Solvent B: 5 mM NH4OAc in CH3CN/H2O (95/5); Gradient: linear from 100% B to 50% B in 6 min, followed by 15 min const. 40%

B). MS-Analysis was done on a Thermo Scientific QExactive instrument in positive and negative mode. Peak determination and semi-quantitation was performed using TraceFinder™ Software. For mass spectrometry normalization a crystal violet staining and a BCA assay (Thermo Fischer Scientific) was performed. In brief, for the crystal violet staining cells were fixed with 4% PFA/Sucrose (Roth), stained with 0.1% crystal violet (Merck) dissolved in 20% ethanol (Roth), washed with water, dried overnight and the absorbance was measured at 550 nm. The pellet of the cell samples was dried, resuspended in 0.2 M sodium hydroxide (Roth), cooked for 20 minutes at 95°C and absorbance was measured at 550 nm. Prism GraphPad was used for statistical analysis. (Analysis was performed in cooperation with the group of Prof. Dr. Almut Schulze (department of biochemisrty and molecular biology, Würzburg).)

### 4.2.6 Immunodetection of proteins

#### 4.2.6.1 Whole cell lysate

To gain the whole cell lysate, well-plates were kept on ice, the culture medium was removed and 2x Leammli was added to the wells. By scratching the surface of the well with a pipette tip, the cells were lysed and transferred into a 1.5 mL reaction tube. The samples were boiled for 5 minutes at 95°C and stored at −20°C.

#### 4.2.6.2 Denaturing polyacrylamide gel electrophoresis

For molecular weight dependent protein separation, denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Separation was accomplished in a 10% polyacrylamide separation gel, layered by a 3.75% stacking gel.

**Tab. 4.11:** Composition of polyacrylamide gels

| | **Separating gel (10%)** | **Stacking gel (3.75%)** |
|---|---|---|
| Separating polyacrylamide gel buffer (pH 8.8) | 2.5 mL | — |
| Stacking polyacrylamide gel buffer (pH 6.8) | — | 2.5 mL |

| | | |
|---|---|---|
| Milli-Q® $H_2O$ | 4.15 mL | 6.25 mL |
| Rotiphorese® Gel 30 (37.5:1) | 3.35 mL | 1.25 mL |
| 10% APS | 75 $\mu$L | 100 $\mu$L |
| TEMED | 7.5 $\mu$L | 20 $\mu$L |

Gels were casted, loaded with 10 to 25 $\mu$L of whole cell lysate and run in 1x SDS running buffer at 90 volts until the dye front reaches the separating gel, and at 135 volts until sufficient separation. As a separation control and for molecular weight assignment PageRuler$^{TM}$ Prestained Protein Ladder was used.

#### 4.2.6.3 Western Blotting and Immunodetection

Separated proteins were transferred from the separating gel onto a PVDF membrane. In brief, by incubation in methanol PVDF membranes were activated and Whatman paper was soaked in 1x Semi-dry transfer buffer. A sandwich was built, which starting from the anode, consists of one Whatman paper, the activated PVDF membrane, the separating gel and finally two Whatman papers at the cathode. Blotting was completed in 2 hours at 1 mA per $cm^2$. After the transfer, PVDF membranes were blocked in 5% (w/v) Nonfat Dried Milk Powder in 1x TBS-T buffer for 1 hour at room temperature to reduce unspecific antibody binding, and then incubated in the appropriate primary antibody over night at 4°C shaking. Subsequently, PVDF membranes were washed with TBS-T buffer three times for 5 minutes, incubated in the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature, washed again three times for 5 minutes with 1x TBS-T and developed by adding a 1:1 mixture of ECL solution 1 and 2 in an Intas Chemiluminescence Imager. (When needed, some biological replicates were performed by Thomas Wulff (master student department of microbiology, Würzburg) and Dr. Karthika Rajeeve (department of microbiology, Würzburg).)

### 4.2.7 Immunofluorescence analysis

Cells were seeded on cover slips, infected with *C. trachomatis* at MOI 1 for indicated time points, and fixed with 4% PFA/Sucrose. Prior to fixation, cells were washed twice with 1x DPBS. Fixed cells were permeablizied for 30 minutes with 0.2% Triton-X-100 in 1x DPBS, blocked for 45 minutes with 2% FBS in 1x DPBS and incubated with primary antibody for 1 hour at room temperature. Dilutions of primary antibodies were made in 2% FBS in 1x DPBS. Samples were washed, incubated with fluorescence dye conjugated secondary antibodies for 1 hour in the dark at room temperature, and then cover slips were mounted with mowiol onto microscopy slides. The slides were air dried for 24 hours and examined at a LEICA DM2500 fluorescence microscope. Images were analysed with LAS AF program and Image J software. (When needed, some biological replicates were performed by Thomas Wulff (master student department of microbiology, Würzburg).)

Organoids were released of the matrigel, incubated for 45 minutes in recovery solution on ice, resuspended, and centrifuged at 100 g for 1 minute at 4°C. Pellet was fixed with 4% PFA/Sucrose for 20 minutes at room temperature, centrifuged at 100 g for 1 minute at 4°C, permeabilized for 1 hour at room temperature with 500 $\mu$l 1% BSA in PBSTD, and incubated with primary antibody over night at 4°C. Dilutions of primary antibodies were made in 5% FBS in 1x DPBS. Organoids were washed three times with 5% FBS in 1x DPBS, incubated with secondary antibody for 3 hours at room temperature in the dark, the samples were washed, incubated with DAPI for 15 minutes at room temperature in the dark, washed, and incubated with AlexFluor 647 for 1 hour at room temperature in the dark. Samples were washed and were kept in 5% FBS in 1x DPBS until they were examined at a LEICA microscope. Images were analysed with LAS AF program and Image J software. (Imaging was performed together with Naziia Kurmasheva. Organoid staining was performed by Naziia Kurmasheva (master student department of microbiology, Würzburg).)

### 4.2.8 Immunoprecipitation

HeLa 229 cells uninfected or with *C. trachomatis* infected at MOI 1 were lysed by denaturing RIPA lysis buffer to prevent co-precipitation of interacting c-Myc partners. Lysates were incubated with 3 $\mu$g anti-c-Myc antibody for 1 hour at 4°C, and followed by protein G magnetic beads incubation for 2 hours at 4°C. Samples were washed several times, eluted via addition of 2x Laemmli buffer, heated for 5 minutes at 95°C, and then separated by SDS-PAGE and after probing against ubiquitin antibody visualized via immunoblotting. (Immunioprecipitation was performed by Dr. Karthika Rajeeve (department of microbiology, Würzburg).)

### 4.2.9 Real Time PCR

From uninfected or HeLa 229 cells infected with *C. trachomatis* RNA was isolated using the RNA easy kit according to the manufacturer instructions, then reverse transcribed with the Revert Aid First Strand synthesis kit according to the manufacturer instructions, and diluted with RNase free water 1:10. RT-PCR reactions were prepared with Quanta SYBR, and performed on aStep One Plus device. Data analysis was done using $\Delta\Delta$Ct method, Step One Plus software package and Excel. GAPDH was used as endogenous control. (When needed, some biological replicates were performed by Dr. Karthika Rajeeve (department of microbiology, Würzburg).)

### 4.2.10 Statistical analysis

N number of independent experiments were performed and at least three technical replicates were accomplished in all experiments. Prism GraphPad8 Software was used for statistical analysis and edgeR module to calculate p-values, pairwise comparison and Benjamini-Höchberg algorithim to calculate false discovery rates as q-values. Analysis was used for Figure 2.3 A, Figure 2.4 D, Figure 2.7 A, Figure 2.11 B and Figure 2.21 C.

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# List of Figures

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# Affidavit

I hereby confirm that my thesis entitled Role of the proto-oncogene c-Myc in the development of *Chlamydia trachomatis* is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

| | |
|---|---|
| Place, Date | Signature |

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# Annex

**Tab. A1:** Media composition of cell culture media used
Amounts are given in mg/L. If different amino acid or vitamin salts are used, the respective salt is annotated behind the particular value. Inorganic salts are not listed. Basic formulation DMEM, if not indicated otherwise, was supplemented with 1 000 mg/L D-glucose and up to 4.0 mM L-glutamine.

| | **Basic formulation DMEM** (D5030, Sigma-Aldrich) | **RPMI1640 + GlutaMAX$^{TM}$** (72400, Gibco$^{TM}$) |
|---|---|---|
| **Amino acids** | | |
| L-Arginine | 84.0 (Arg-HCl) | 200.0 |
| L-Asparagine | — | 50.0 |
| L-Aspartic acid | — | 20.0 |
| L-Cystine | 62.6 ($Cys_2$-2HCl) | 50.0 |
| L-Glutamic acid | — | 20.0 |
| L-Glutamine | Variable | — |
| L-Alanyl-L-glutamine | — | 446.0 |
| Glycine | 30.0 | 10.0 |
| Histidine | 42.0 (His-HCl-$H_2O$) | 15.0 |
| L-Hydroxyproline | — | 20.0 |
| L-Isoleucine | 105.0 | 50.0 |
| L-Leucine | 105.0 | 50.0 |
| L-Lysine-HCl | 146.0 | 40.0 |
| L-Methionine | 30.0 | 15.0 |

| | | |
|---|---|---|
| L-Phenylalanine | 66.0 | 15.0 |
| L-Proline | — | 20.0 |
| L-Serine | 42.0 | 30.0 |
| L-Threonine | 95.0 | 20.0 |
| L-Tryptophan | 16.0 | 5.0 |
| L-Tyrosine | 103.8 (Tyr-2Na-2$H_2O$) | 20.0 |
| L-Valine | 94.0 | 20.0 |
| **Vitamins** | | |
| Biotine | — | 0.2 |
| Choline chloride | 4.0 | 3.0 |
| Folic acid | 4.0 | 1.0 |
| Inositol | 7.2 | 35.0 |
| Niacinamide | 4.0 | 1.0 |
| D-Pantothenate | 4.0 (hemicalcium) | 0.25 (calcium) |
| Para-aminobenzoic acid | — | 1.0 |
| Pyridoxal-HCl | 4.0 | — |
| Pyrodoxine-HCl | — | 1.0 |
| Riboflavin | 0.4 | 0.2 |
| Thiamine-HCl | 4.0 | 1.0 |
| Vitamin B12 | — | 0.005 |
| **Others** | | |
| D-Glucose | Variable | 2000 |
| Glutathione (reduced) | — | 1.0 |
| HEPES | — | 5958 |
| Phenol Red | 15.9 | 5.0 |
| Pyruvate-Na | — | — |

# Publications and presentations

**Research articles**:

- Karunakaran, K., Subbarayal, P., **Vollmuth, N.** and Rudel, T. (2015)
  Gp96 shedding by Chlamydia prevents re-infection
  Molecular Microbiology, 98: 694-711. doi:10.1111/mmi.13151

- Mehlitz, A., Eylert, E., Huber, C., Lindner, B., **Vollmuth, N.**, Karunakaran, K., Goebel, W., Eisenreich, W. and Rudel, T. (2017)
  Metabolic adaptation of Chlamydia trachomatis to mammalian host cells
  Molecular Microbiology, 103: 1004-1019. doi:10.1111/mmi.13603

**Publications in preparation**:

- Rajeeve, K., **Vollmuth, N.**, Janaki-Raman, S., Wulff, T., Schmalhofer, M., Schmitz, W., Baluapuri, A., Huber, C., Fink, J., Dejure, F., R., Wolf, E., Eisenreich, W., Schulze, A., Seibel, J. and Rudel, T. (2019)
  Chlamydia trachomatis stabilizes the proto-oncogene c-Myc to de-route glutamine metabolism for its survival
  Under review

- **Vollmuth, N.**, Janaki-Raman, S., Schmitz, W., Kurmasheva, N., Schulze, A., Rudel, T. and Rajeeve, K. (2020)
  c-Myc is the key player in IFN-$\gamma$ induced Chlamydia trachomatis dormancy
  In preparation

**Oral presentations**:

- c-Myc plays a crucial role in IFN-$\gamma$ induced Chlamydia trachomatis dormancy. 17. Deutscher Chlamydien Workshop, Georg-August Universität Göttingen, Germany (2019).
- c-Myc plays a crucial role in IFN-$\gamma$ induced Chlamydia trachomatis dormancy. CBRS, Seattle WA, USA (2019).
- c-Myc plays a crucial role in IFN-$\gamma$ induced Chlamydia trachomatis dormancy. 3D Tissue Infection Symposium, Juliusspital Würzburg, Germany (2019.)

**Poster**

- Chlamydia trachomatis stabilizes the proto-oncogene c-Myc to de-route glutamine metabolism for its survival. Rajeeve, K., **Vollmuth, N.**, Janaki-Raman, S., Wulff, T., Schmalhofer, M., Schmitz, W., Baluapuri, A., Huber, C., Fink, J., Dejure, F., R., Wolf, E., Eisenreich, W., Schulze, A., Seibel, J. and Rudel, T. $13^{th}$ International GSLS Symposium, Rudolf-Virchow-Center, Julius-Maximilians Universität Würzburg, Germany (2018).