Reprogramming of host glutamine metabolism during *Chlamydia trachomatis* infection and its key role in peptidoglycan synthesis

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Abstract

Obligate intracellular bacteria like *Chlamydia trachomatis* undergo a complex developmental cycle between infectious non-replicative (EBs) and non-infectious replicative (RBs) forms. EBs shortly after entering a host cell transform to RBs, a crucial process in infection, initiating chlamydial replication. As *Chlamydia* fail to replicate outside the host cell it is currently unknown how the replicative part of the developmental cycle is initiated. Here we show in a cell-free approach in axenic media that uptake of glutamine by the bacteria is crucial for peptidoglycan synthesis which has a role in *Chlamydia* replication. The increased requirement for glutamine in infected cells is satisfied by reprogramming the glutamine metabolism in a c-Myc-dependent manner. Glutamine is effectively taken up by the glutamine transporter SLC1A5 and metabolized via glutaminase. Interference with this metabolic reprogramming limited growth of *Chlamydia*. Intriguingly, *Chlamydia* failed to produce progeny in SLC1A5 knockout organoids and mice. Thus, we report on the central role of glutamine for the development of an obligate intracellular pathogenic bacterium and the reprogramming of host glutamine metabolism, which may provide a basis for innovative anti-infective strategies.
Chlamydia trachomatis is an obligate intracellular bacterium and the most frequent cause of bacterial sexually transmitted diseases. In a biphasic developmental cycle, EBs enter the host cell and convert into RBs, which replicate in a membrane-enclosed structure called “the inclusion”. Later, RBs convert back into EBs, which can readily infect neighbouring cells. For a long time, it was thought that Chlamydia do not form peptidoglycan until peptidoglycan ring-like structures were recently discovered exclusively in the septa of dividing RBs.

Due to its obligate intracellular lifestyle and an evolutionary reduced genome, Chlamydia requires multiple metabolites from the host cell. Nevertheless, this pathogen lacks the enzyme hexokinase but encodes the enzymes required to utilize D-glucose-6-phosphate (G6P) and to convert it into pyruvate via glycolysis. Thus, the bacteria depend on G6P from the host cell. In EBs, ATP is generated from G6P either via glycolysis or oxidative phosphorylation, whereas in RBs ATP is provided by the host cell, while G6P is nearly exclusively used for cell wall biosynthesis. The TCA cycle of Chlamydia is incomplete due to the absence of three key enzymes, citrate synthase, aconitase and isocitrate dehydrogenase. This incomplete metabolic pathway must be complemented by the constant metabolic supply from the host. We have previously shown that C. trachomatis takes up host-derived malate to feed the partial TCA cycle. In addition, amino acids have to be acquired from the host cells due to the limited capacity of Chlamydia to synthesize amino acids. Despite the advancements in the knowledge about chlamydial metabolism, the processes initiating the transition of EBs to RBs after entry into cells are still unknown.

Results

Glutamine metabolism in Chlamydia: A pathway to peptidoglycan biosynthesis

In our attempts to further define the metabolic interaction of Chlamydia and host cells, we performed metabolite uptake assays in axenic culture. Since glutamine (Gln), glutamate (Glu) and α-ketoglutarate (α-KG) were previously predicted to feed the partial chlamydial TCA cycle, we investigated whether these metabolites are directly taken up by Chlamydia (Fig. 1a,b).
Whereas the amount of Gln decreased rapidly in the supernatant, neither Glu nor α-KG were significantly consumed (Fig. 1c). Using isotopologue profiling with Gln containing only $^{13}$C instead of $^{12}$C atoms ([U-$^{13}$C$_5$]Gln) added to the axenic culture, GC-MS analyses of chlamydial extracts revealed that the $^{13}$C label is efficiently transferred to Glu, Asp and intermediates of the partial TCA cycle, with the $^{13}$C-labelled fractions ranging from 80% in Gln to less than 3% in pyruvate (Fig. 1d). Substantial fractions of $^{13}$C-labeling in Glu (60%), Asp (50%), succinate (20%), fumarate (35%), and malate (8%) (Fig. 1d) indicated that exogenous Gln is metabolized via glutaminolysis and the TCA cycle to generate Glu, α-KG, succinate, fumarate, malate, oxaloacetate and aspartate. Consistently, the amount of chlamydial NADH probably formed by the conversion of α-KG into succinate and malate into oxaloacetate within the truncated chlamydial TCA cycle (Fig. 1a) is significantly increased when exogenous Gln is added to the axenic medium (Extended data Fig. 1a).

Interestingly, significant $^{13}$C-labelling was also detected in Ala (17%) and the peptidoglycan precursor, diaminopimelate (DAP) (41%) (Fig. 1d). Whereas Ala contained a high fraction of the M+3 isotopologue (i.e. a molecule with three $^{13}$C-labelled carbon atoms), DAP contained M+4 as main compound, accompanied by M+2, M+3, and M+7 isotopologues (Fig. 1d). The detected isotopologue composition of Ala can be explained by its formation from pyruvate derived from oxaloacetate via PEP catalysed by PEP carboxykinase and pyruvate kinase; an alternative could be the decarboxylation of Asp, although no gene with the required enzymatic activity is annotated in the genome of Chlamydia (scheme in Extended data Fig. 1b). The labelling pattern in DAP points at its formation from Asp and pyruvate contributing four and three labelled carbon atoms, respectively, and resulting in the detected M+3, M+4 and M+7 isotopologues species (Fig. 1d, Extended data Fig. 1b). Minor isotopologues especially M+2 in aspartate and DAP can be explained by metabolic cycling via gluconeogenesis and the PPP, which both are active in Chlamydia. To further support the unexpected finding of chlamydial DAP synthesis in axenic media, we performed an approximate quantification of DAP (for details see Methods) and could estimate a value of about 0.04 mg DAP formation per gram cells of Chlamydia in the presence of Gln in axenic medium (Extended data Fig. 1c). We also supplied axenic cultures with Gln that
was either labelled in the amino group or the amido group. The $^{15}$N-label was only transferred from the amino group of Gln (Extended data Fig. 1d). Together, these results indicated that Gln serves as a major carbon source for the fuelling of the partial TCA cycle of *Chlamydia* and as a source of amino groups for the generation of amino acids from alpha-keto acids via transamination, e.g. Glu, Asp and Ala, required for peptidoglycan biosynthesis.

Synthesis of DAP in axenic medium was particularly intriguing since peptidoglycan synthesis is restricted to the replicative phase of *Chlamydia* and this has never been observed for *Chlamydia* outside their host cell ². We therefore analysed EBs from axenic cultures in the presence or absence of Gln by electron microscopy (EM). Surprisingly, incubation of EBs with Gln, but not Glu led to a gradual time-dependent morphological transition to an intermediate between EBs and RBs, so-called intermediate bodies (IBs) (Fig. 1e; Extended data Fig. 1e). The infectivity of EBs incubated with Gln dropped to 20% (Extended data Fig. 1f), supporting the conclusion that Gln induces the developmental transition of EBs to a non-infectious form.

We then synthesized a clickable form of the dipeptide DA-DA to detect peptidoglycan in these bacteria ² (see Methods). Chlamydial particles incubated in the presence of Gln in this cell-free approach produced detectable peptidoglycan which could be quantified by FACS (Fig. 1f, g, Extended data Fig. 1g). Since peptidoglycan synthesis in *Chlamydia* has been shown to be associated with replication in host cells, we also investigated if Gln could initiate transcription in *Chlamydia* in axenic culture. RNA-seq analysis revealed activation of several genes upon exposure to glutamine, including those previously described as early genes ⁹ (Fig. 1h, Extended data Fig. 1h). This finding was also confirmed by quantitative PCR (qPCR) of several genes already reported to be expressed at early time points of EB to RB conversion ¹⁰ (Extended data Fig. 1i). Interestingly, in the presence of Gln, a significant increase in the copy number of chlamydial DNA was detected in axenic media (Fig. 1i), indicating that Gln access initiates limited replication in *Chlamydia*.

**Chlamydia** triggers altered glutamine metabolism in infected cells
Since our data suggested uptake of Gln as a central node for the interaction between host and bacterial metabolism, we investigated how *Chlamydia* infection influences the metabolism of primary human umbilical vein endothelial cells (HUVECs). *Chlamydia* infection induced an increased depletion of most amino acids from the culture medium, indicative of their enhanced uptake by infected cells (Fig. 2a). Gln was one of the most depleted amino acids in the medium (Fig. 2a) and Gln but not Glu uptake was significantly increased in infected cells (Fig. 2b,c).

We then performed isotope labelling experiments by adding \([U^{13}C_5]\)-Gln to the culture medium. HUVECs were then infected with *Chlamydia* and incubated in this medium for 36 hours. LC-MS analysis revealed the labelling pattern of Gln, Glu, Asp and several TCA cycle metabolites (Fig. 2d). We also observed evidence for reductive carboxylation of \(\alpha\)-KG by the host cells as there was a substantial amount of the M+5 isotopologue of citrate. In contrast, Gln-derived labelling of pyruvate was almost absent (Fig. 2d), indicating that the enzymes of gluconeogenic pathway responsible for the conversion of oxaloacetate to pyruvate are inactive under these conditions. Interestingly, while the labelled fraction of Gln did not change upon infection, the M+5 labelled isotopologues of Glu and \(\alpha\)-KG increased, indicating increased glutaminolysis in infected cells (Fig. 2d). Moreover, the M+4 isotopologues of succinate, fumarate, malate and aspartate also increased following *Chlamydia* infection, most likely due to enhanced entry of Gln-derived carbons into the TCA cycle (Fig. 2d).

To analyse if *Chlamydia* obtain building blocks for DAP biosynthesis from the host cell, we performed stable isotope labelling using \([U^{13}C_5]\)-Gln and extracted bacterial cell wall components by acidic hydrolysis. LC-MS detection of DAP confirmed that glutamine-derived carbons are indeed incorporated into bacterial biomolecules essential for *Chlamydia* proliferation (Fig. 2e,f). Moreover, the high proportion of the M+4 isotopologue in DAP suggests that *Chlamydia* either takes up glutamine directly from the host cells and uses it to produce Asp via its truncated TCA cycle, or that glutamine is first converted by the host cell into metabolic intermediates that contain four \(^{13}\text{C}\) carbon atoms (i.e. succinate, fumarate, malate, oxaloacetate or Asp), which are then taken up by *Chlamydia* and used for the synthesis of DAP (Fig. 2d-f, diagram in Fig. 2e). Moreover, the high abundance of the M+1, M+2 and M+3 isotopologues in
DAP also indicates that *Chlamydia* uses metabolic intermediates that are formed by the complete TCA cycle of the host cell. Production of these isotopologues depends on the second and third round of the TCA cycle, which requires the activity of citrate synthase, aconitase and isocitrate dehydrogenase present only in the host metabolism (see diagram in Extended data Fig. 2). Together, these results clearly demonstrate that *Chlamydia*-infected cells increase uptake and metabolism of several amino acids, particularly Gln which provides the metabolic intermediates required for bacterial peptidoglycan production.

**Chlamydia infection increases c-Myc levels**

We next focused on how infected cells compensate for the increased Gln demand. RNA-seq analysis and Gene Set Enrichment Analysis (GSEA, see Methods) including subsequent validation by qPCR revealed upregulation of MYC target genes (Hallmark_MYC_Targets_V2) in cells infected with *Chlamydia* (Fig. 3a; Extended data Fig. 3a). The proto-oncogene c-MYC is also known as a ‘master regulator’ of cellular metabolism, in particular mitochondrial glutamine metabolism. In agreement with previous data, c-Myc levels were strongly increased already 12 hours post infection (hpi) and remained elevated up to 36 hpi in primary epithelial cells from human or mouse fimbriae (Fimb cells) (Extended data Fig. 3b,c), and human osteosarcoma U2OS cells (Extended data Fig. 3d). Similar results were obtained upon infection with different *Chlamydia* species (Extended data Fig. 3e-g).

Further investigation unveiled a transient upregulation of c-Myc mRNA expression in response to *Chlamydia* infection (Extended data Fig. 3h) and the accumulation of c-Myc protein in the nucleus (Extended data Fig. 3i,j). In addition, c-Myc was phosphorylated at the conserved residues serine 62 (S62) and threonine 58 (T58) (Fig. 3b) involved in the control of c-Myc protein stability in response to mitogenic signalling and *Chlamydia* infection. Indeed, we found markedly reduced levels of ubiquitinated c-Myc (Fig. 3c) and stabilized c-Myc in infected cells with inhibited translation (Fig. 3d), demonstrating that c-Myc is really stabilized upon infection.

**c-Myc is stabilized in Chlamydia-infected cells via MAPK and PI3K signalling pathways**
MAPK and PI3K pathways activated during infection are both critical for chlamydial development. The same pathways have been demonstrated to function in the stabilization of c-Myc (Fig. 3e). Inhibition of MAPK or PI3K pathways using specific inhibitors indeed prevented the up-regulation of c-Myc protein levels and attenuated the propagation of the bacteria in infected cells (Fig. 3f,g). Interestingly, the downstream effector GSK3ß (Fig. 3e) was phosphorylated at S9 in infected cells (Fig. 3h,i), a modification known to inhibit glycogen synthesis and to increase free glucose in the cell. Intriguingly, Chlamydia primary infection (Fig. 4a-c; AHT control in Extended data Fig. 4a) and infectivity (Extended data Fig. 4b,c) was rescued in U2OS Tet-On cells upon anhydrous tetracycline (AHT)-induced expression of c-Myc despite inhibition of the MAPK or PI3K pathway, indicating that the anti-chlamydial activity of U0126 and Ly294002 is mediated by the down-regulation of c-Myc. The central role of c-Myc for chlamydial growth was further supported by experiments with the chemical c-Myc inhibitor 10058-F4 (Fig. 4d, Extended data Fig. 4d) and the silencing of c-Myc expression (Fig. 4e,f; Extended data Fig. 4e,f).

Chlamydia depends on host cell Gln uptake

GSEA of the RNA-seq data also revealed a strong influence of Chlamydia infection on the host metabolite transporter and cellular amino acid and amine metabolic pathway (Extended data Fig. 5a-c). Gln turned out to be essential for the intracellular growth of Chlamydia, since they failed to form inclusions and replicate in host cells cultured in medium without Gln (Fig. 5a,b and Extended data Fig. 5d). Bacteria from these cultures did not initiate infections in fresh cells in the presence of Gln, demonstrating that no infectious progeny were produced in the absence of Gln (Fig. 5c,d, Extended data Fig. 5e). To investigate if Gln plays a role in EB to RB conversion in host cells, we used the EB-RB reporter strain Ct mCh(GroL2) GFP(OmcA2). In the absence of Gln, Chlamydia, did not convert into the actively replicating RB form, with low GFP expression and constant mCherry fluorescence (Fig. 5e).

c-Myc can function in a glutamine sensing pathway via a mechanism dependent on the 3' UTR of the gene. We used HCT116 cells expressing the MYC-ER fusion protein from a construct...
lacking the 3’UTR, which allows the restoration of c-Myc expression in Gln-deprived cells. However, Gln depletion severely attenuated the growth and development of *Chlamydia* (Fig. 5f,g), and even complementing with downstream metabolites like nucleosides did not compensate for the lack of Gln (Extended data Fig. 5f), indicating that c-Myc cannot rescue chlamydial growth in the absence of Gln.

**Chlamydia** infection reprograms host cell metabolism by inducing glutamine uptake and catabolism

The data obtained so far suggested that Gln is also a central host-derived amino acid metabolised by fast replicating *Chlamydia*. We therefore investigated the regulation of genes related to glutamine uptake and catabolism. These analyses revealed SLC1A5/ASCT2 among the top upregulated genes during infection (Fig. 6a). SLC1A5/ASCT2 and glutaminase (GLS1), one of the enzymes converting Gln to Glu (Fig. 6b), have been shown to be c-Myc target genes induced to enhance glutamine uptake and catabolism in cancer cells. Western blots of control and *Chlamydia*-infected samples revealed a 5.2-fold up-regulation of SLC1A5 and a 1.8-fold up-regulation of GLS1 (Fig. 6c). Induction of SLC1A5 depended on viable bacteria since heat-killed *Chlamydia* neither induced c-Myc nor SLC1A5 (Extended data Fig. 6a). Furthermore, depletion of SLC1A5 or GLS1 (Fig. 6d,e) or chemical inhibition of SLC1A5 or GLS1 using GPNA and BPTES (Fig. 6f,g) drastically reduced chlamydial replication. Since SLC1A5 and GLS1 are transcriptional targets of c-Myc, we knocked down c-Myc with siRNA, which resulted in strong depletion of both proteins and also blocked chlamydial growth (Fig. 6h).

Most interestingly, when we depleted c-Myc in cells overexpressing SLC1A5 we could retain *Chlamydia* growth during the primary infection, but the progeny was not infectious (Fig. 6i and Extended data Fig. 6b), in line with a role of Gln in establishing the primary infection (Fig. 1e). However, other c-Myc regulated host cell functions, for example glutaminolysis and the production of TCA cycle intermediates, may be essential for the full development of *Chlamydia*.
Taken together, these data demonstrate the importance of an infection-dependent regulation of c-Myc and its targets SLC1A5 and GLS1 in the reprogramming of the Gln metabolism in host cells to promote *Chlamydia* replication and development. Recently, *Slc1a5* knockout mice have been generated which show no major phenotype. Mass spectrometric analysis revealed that organoids generated from fallopian tube tissue from these mice contain almost 50% lower levels of Gln and the downstream metabolites Glu, Asp and Ala compared to organoids from wild type mice (Extended data Fig. 6c). Interestingly, trans-cervical infection of these mice revealed a significant growth defect of *C. trachomatis* and the mouse pathogenic strain *C. muridarum* in the absence of *Slc1a5* (Fig. 6j,k). Moreover, infection and progeny formation were considerably reduced in fallopian tube organoids developed from *Slc1a5* KO mice (Fig. 6l, Extended data Fig. 6d), demonstrating that cells of *Slc1a5* KO mice have reduced capacity to allow chlamydial replication. Further experiments are required to demonstrate that the strong phenotype observed on chlamydial growth in these organoids depends on disturbed reprogramming of glutamine metabolism. These data suggest that limiting the uptake of the non-essential amino acid glutamine could serve as a therapeutic approach against *Chlamydia* infection.

**Discussion**

After the completion of the developmental cycle EBs released from the host cell have to avoid the transition to RBs since only EBs are capable of entering uninfected host cells and initiate a new generation of progeny. The longstanding view that EBs are metabolically inactive has recently been revised, since it was found that EBs kept in axenic medium in the presence of G6P, a metabolite available to *Chlamydia* only inside host cells, are able to generate ATP via glycolysis. However, despite this active metabolism, EBs do not initiate the conversion to RBs under these conditions. Here we provide evidence that Gln is the key metabolite that initiates peptidoglycan synthesis and the EB to RB transition. While the concentration of Gln in uterine fluid is as low as 0.13 mM, in the cytoplasm of mammalian cells Gln can reach levels of 2 to 30 mM. The strong differences in Gln concentrations between the outside and the inside of host cells strongly support a function of Gln as a metabolic trigger for EB to RB transition.
Our finding that L-glutamine serves as a crucial amino acid for the replication of *Chlamydia* corroborates previous findings \(^{31,32}\). Many of the glutamine-derived intermediates in *Chlamydia* serve as precursors for peptidoglycan biosynthesis (Fig. 1d and Extended data Fig. 1b) underlining the central role of glutamine for the chlamydial metabolism. Chlamydiae do not form a peptidoglycan sacculus typical for other Gram-negative bacteria but only assemble peptidoglycan rings in the mid-cell of actively dividing RBs. We detected the accumulation of peptidoglycan here also in *Chlamydia* outside of host cells but only in the presence of Gln (Fig. 1f, g), where we also observed a change in the morphology of the EBs (Fig. 1e, Extended data Fig. 1e). Peptidoglycan accumulated in or close to the bacteria and was not assembled as a ring which could indicate that the crosslinking of the peptidoglycan disaccharide pentapeptide did not occur in this setup. The transcriptional activation of early genes and the increase in the copy number of the bacterial genomes (Fig. 1h,i), however, indicated that *Chlamydia* already initiate the start of the replication machinery.

*Chlamydia* and other obligate intracellular bacteria replicate in differentiated cells that run the reduced metabolism of non-dividing cells. Since all metabolites of replicating *Chlamydia* originate from the host cell, the metabolism of the cell must drastically change to meet the requirements of the infection. Glutamine is one of the most abundant amino acids in serum and fast-growing cells take up glutamine to support anabolic metabolism at multiple nodes. We show here that *Chlamydia* depend on the direct uptake of glutamine as well as on glutamine-derived host metabolites and therefore reprograms the host cell by stabilizing the central metabolism regulator c-Myc. The profile of c-Myc regulated genes and the dependence of the replicating *Chlamydia* on glutaminolysis parallels the reprogramming of dormant cells to fast proliferating and in particular tumour cells \(^{33}\). c-Myc promotes glutamine uptake and glutaminolysis by increasing the expression of the glutamine transporters ASCT2/SLC1A5 and SNAT5/SN2 and of glutaminase (GLS) \(^{13}\). We demonstrate here that bacteria induce host cell glutamine uptake and glutaminolysis for their replication. In addition, chlamydial growth was severely affected in genital infections of ASCT2/SLC1A5 knockout mice and is thereby the demonstration of a dominant role of host cell glutamine reprogramming in an *in vivo* infection. Gln is channelled into...
different anabolic and catabolic pathways via glutaminolysis, generating Glu and subsequently \( \alpha \)-KG. While Gln-derived metabolic intermediates have been shown to be essential for the infection and replication of several viruses\(^{34-39}\), the relevance of glutamine metabolism for viral infection still has to be demonstrated \textit{in vivo}.

In addition to glutamine transporters and glutaminase, we found other prominent c-Myc-regulated amino acid transporters like SLC43A1, SLC7A11, and SLC7A1 are also upregulated during infection (Fig. 6a). This global reprogramming of amino acid supply in infected host cells by c-Myc stabilization may explain our intriguing finding that overexpression of SLC1A5 can only partially rescue chlamydial infection in c-Myc-depleted cells. While SLC1A5 restores bacterial replication, it fails to support the production of infectious progeny in the absence of c-MYC. Thus, the provision of glutamine is essential, but not sufficient to permit the complex cycle of chlamydial replication and development.

The central addiction of \textit{Chlamydia} to host glutamine is reminiscent of mammalian cell proliferation, malignant transformation and therefore a hallmark of cancer cells which use extracellular glutamine to fulfil the metabolic demands of producing cell mass. Glutamine addicted tumour cells and, as we show here, \textit{Chlamydia}-infected cells are therefore highly sensitive to pharmacological disruption of glutamine metabolism. Current approaches to target c-Myc or glutamine metabolism as targets for innovative cancer therapy may also prove to be efficient in treating \textit{Chlamydia} infection.

\section*{Methods}

\textbf{Chlamydia strains used in the study}

\textit{Chlamydia trachomatis} (serovar L2/434/Bu and D) were used in this study. Some experiments were also performed using \textit{Chlamydia muridarum} and \textit{Chlamydia pneumoniae}. \textit{Chlamydia} were prepared as previously published. Briefly, \textit{Chlamydia} were grown in HeLa229 cells (ATCC\textsuperscript{\textregistered}CCL2.1\textsuperscript{TM}) at an MOI (multiplicity of infection) of 1 for 48 hours (h). The cells were lysed using glass beads (15 mm) for 3 minutes (min) and centrifuged at 2,000 g for 10 min to remove the cell debris. The supernatant containing bacteria was collected and centrifuged at 24,000 g
for 30 min at 4°C. The pellet was washed and resuspended in SPG buffer (0.25 M sucrose/10 mM sodium phosphate/5 mM glutamic acid), aliquoted and stored at -80°C. Chlamydia EBs and cell lines used in the study were verified to be free of Mycoplasma contamination via PCR. The bacteria were titrated and were used at an MOI of 1 in all experiments unless other stated. After 1 hpi the media was replaced with fresh RPMI containing 5% FCS, infected cells were cultured at 37°C and 5% CO₂.

Culture of C. trachomatis in axenic medium

Chlamydia trachomatis L2 was propagated in HeLa229 cells, isolated, purified and incubated in different axenic media as previously described ⁶. In brief, HeLa229 cells were seeded in T175 flasks and infected at an MOI of one. Forty-eight hours post infection, cells were scraped off, disrupted with glass beads and EBs were purified using 60-20% Renografin gradient (Meglumin diatrizoate (Sigma-Aldrich M5266), Sodium diatrizoate hydrate (Sigma-Aldrich S4506), Sodium citrate hydrate (Applichem A4522), EDTA (Servca 11280) add to 50 ml HBSS (Gibco 14025-050) pH-7.4, sterile filtered in 0.2 µm filter and stored at 4°C). EBs were resuspended in axenic media (basic DMEM (Sigma-Aldrich) supplemented with sodium bicarbonate (44 mM), phenol red (42 µM) and glucose-6-phosphate (0.5 mM) and incubated for respective time points at 37°C. According to the experimental set-up, 1mM of L-glutamine / L-glutamate / pyruvate / α-ketoglutarate / [U-¹³C₅] glutamate / [U-¹³C₅] glutamine / [¹⁵N-amine] glutamine or [¹⁵N-amide] glutamine was added to the media. After incubation, samples were centrifuged for 30 min at 21,500 g at 4°C and supernatant was transferred to a new tube. Supernatant and pellets were heat-inactivated (10 min at 90°C) and stored at -80°C for further analysis.

Substrate uptake analysis from axenic culture

a. α-ketoglutarate:

0.2 ml of the supernatant from the axenic medium was spiked with 20 µl of a 5 mM norvaline solution (internal standard) and dried under N₂ flux. The residue was treated with 50 µl methoxyamine in pyridine (20 mg/ml) at 40°C for 90 min, and subsequently with 50 µl N-(tert-butyldimethylsilyl)-N-methyl trifluoroacetamide containing 1% tert-butyldimethylsilyl chloride (MTBSTFA) at 70°C for 30 min. This solution was taken for analysis.
b. Glutamate and glutamine

0.1 ml of the supernatant from the axenic medium containing [U-\(^{13}\)C\(_5\)] glutamate or [U-\(^{13}\)C\(_5\)] glutamine was spiked with 20 µl of a 5 mM non-labelled glutamate or glutamine solution (internal standard) and dried under N\(_2\) flux. The residue was treated with 50 µl acetonitrile and 50 µl MTBSTFA at 70°C for 30 min and taken for analysis.

Isotopologue profiling with Chlamydia from axenic culture:

Bacterial pellets were suspended in 1 ml of methanol and were mechanically disrupted using a ribolyser (3 x 20 sec 6.5 m/s). Afterwards the solution was centrifuged (10,000 g for 20 min, 4°C). This procedure was performed twice. The supernatants were combined and then dried under N\(_2\) flux. The residue was treated with 50 µl of MTBSTFA and 50 µl of water free acetonitrile at 70°C for 30 min. The tert-butyldimethylsilyl (TBDMS)-derivatives of amino acids and other metabolites were then analysed by GC/MS. The residual cell debris after centrifugation was subjected to acidic hydrolysis as described earlier\(^{40}\) and protein bound amino acids as well as diaminopimelate (DAP; retention time, 24.48 min; m/z 589) were analysed as TBDMS derivatives.

GC/MS conditions

All derivatives mentioned above were analysed by GC-MS using a GCMS-QP 2010 Ultra spectrometer (Shimadzu, Duisburg, Germany) equipped with an EquityTM-5, fused silica capillary column, 30 m x 0.25 mm x 0.25 µm film thickness. All data were collected using LabSolution software (Shimadzu). The samples were analysed three times as technical replicates. The overall \(^{13}\)C excess (mol-%) and the relative contributions of isotopomers (%) were computed by an Excel-based in-house software package according to published procedures\(^{41}\).

TBDMS-derivatives of polar metabolite mixtures

The column was first developed at 100°C for 2 min, then using a gradient of 3°C min\(^{-1}\) to 234°C, followed by 1°C min\(^{-1}\) to 237°C and 3°C min\(^{-1}\) to 260°C. Finally, the column was heated at a gradient of 10°C min\(^{-1}\) to a final temperature of 320°C where it was hold for 2 min.

Analysis of TBDMS-amino acids and DAP
The column was first developed at 150°C for 3 min, then using a gradient of 7°C min\(^{-1}\) to 280°C where it was hold for 5 min. For quantitative DAP analysis in *Chlamydia* DAP DL-2,6-Diaminopimelic acid (Sigma Aldrich No. 92591) was used as standard for the calibration. Chlamydial DAP synthesis in axenic media was performed by an approximate quantification of DAP on the basis of peak integrals of the samples used for isotopologue profiling with \(^{13}\)C\(_5\)-Gln (Fig. 1d). This analysis revealed that the preparation contained approximately 0.1 mg DAP per gram wet weight of *Chlamydia*. The newly built DAP is highly labelled and the overall \(^{13}\)C excess of 40% shown as a mixture of newly built \(^{13}\)C labelled DAP and residual unlabelled DAP from the inoculum. On the basis of 40% newly formed DAP, we could estimate a value of about 0.04 mg DAP formation per gram cells of *Chlamydia* in the presence of Gln in axenic medium (Extended data Fig. 1c).

**Transmission electron microscopy**

Chlamydial EBs were incubated with the axenic medium with G6P and with or without Gln. The bacteria pellet was fixed with 2.5% glutaraldehyde (50 mM sodium cacodylate (pH 7.2), 50 mM KCl, 2.5 mM MgCl\(_2\)) at room temperature. The cells were incubated for 2 h at 4°C with 2% OsO\(_4\) buffered with 50 mM sodium cacodylate (pH 7.2), washed with distilled H\(_2\)O and incubated overnight at 4°C with 0.5% uranyl acetate (in distilled H\(_2\)O). The cells were dehydrated, embedded in Epon812 and ultrathin-sectioned at 50 nm. Sections were stained with 2% uranyl acetate in ethanol followed by staining with lead citrate and analysed in a Zeiss EM10 microscope (Zeiss). Electron micrographs were processed using ImageJ (Fiji).

**Click chemistry and FACS analysis of chlamydial EBs**

Click chemistry was performed as described in \(^2\). Axenic culture with purified EBs from *Chlamydia* was fed with ADA-DA (10 \(\mu\)M). Clickable Alexa Fluor 532-azide and Click-iT® Cell Reaction Buffer Kit were purchased from Invitrogen and fixed with 4% PFA. The bacteria were stained with cHsp60 antibody and subjected to microscopy or further analysed by FACS (Accuri).

**Copy number of Chlamydia genomes in axenic culture**
Chlamydia were grown in HeLa229 for 48 h. The cells were lysed and the EBs were purified by renografin gradient separation as explained above. The bacteria were pooled by centrifugation and re-suspended in axenic medium without Gln. The re-suspended Chlamydia were split into two aliquots and Gln was added into one of them and incubated at 35°C for 24 h. The bacteria were further pelleted and DNA was isolated using DNAzol reagent (Thermo Fisher Scientific).

Quantitative PCR was used to enumerate Chlamydia genome copy number. The following primers were used for amplifying the C. trachomatis lytA gene that was cloned into the vector: forward primer 5′-TCTAAAGCGTCTGGTGAAAGCT-3′ and reverse primer 5′-GAAATAGCGTAGTAATAATACCCG-3′. Data were analysed by using the Step One Plus software package (Applied Biosystems). GraphPad Prism 7 was used to generate the graph.

Cell culture and transfection

HeLa229 were used for propagating bacteria and for basic experiments. Epithelial cells isolated from human fimbriae (Fimb cells), U2OS (ATCC®HTB-96™), HCT116 (ATCC®CRL-247™) and HUVECs (ATCC®CRL-1730™) were also used in the study. HUVECs were used in the high throughput RNA sequencing. All cell lines were tested negative for mycoplasma contamination via PCR. HeLa229 and human Fimb cells were grown in RPMI1640 + GlutaMAX™ (Gibco™ 72400-054) with 10% heat inactivated FCS (Sigma-Aldrich F7524). U2OS and HCT116 cells were cultured in DMEM (Sigma-Aldrich D6429) with 10% heat inactivated FCS. HUVECs were cultured in Medium 200 (Gibco™ M200500) containing 1x LSGS (Gibco™ S00310). For glutamine deprivation experiments, the cells were first seeded in RPMI1640 + GlutaMAX™ or DMEM, high glucose (Sigma-Aldrich D6429). The following day the medium was changed to the basic formulation of DMEM (Sigma-Aldrich D5030) supplemented with 5% dialyzed FCS (Sigma-Aldrich F0392), 1 or 4.5 g/l D-glucose (for HeLa229/ U2OS or human Fimb respectively) and varying concentrations of L-glutamine (according to experimental setup). All cell lines obtained from ATCC were authenticated by the company.

Cells were transfected with plasmid DNA at a confluency of 60% with Polyethylenimine (PEI) or X-treme GENE™ HP DNA transfection reagent (Roche) and OptiMEM transfection medium (Gibco) in 5% FCS medium. After 5 h, transfection medium was replaced by fresh RPMI
supplemented with 5% FCS medium. The plasmids used in the study are described in the Extended data file. siRNA against SLC1A5 (sc-60210) and c-Myc (sc-29226) was obtained from Santa cruz Biotech.

**Metabolic profiling**

For this study HUVECs were seeded in triplicates, either uninfected or infected with *C. trachomatis* serovar L2 for 36 hours. After the respective time medium was collected, snap frozen in liquid nitrogen, and the cells were washed with ice cold 154 mM ammonium acetate (Sigma) and snap frozen in liquid nitrogen. The cells were harvested after adding 480 µl cold MeOH/H$_2$O (80/20, v/v) (Merck) to each sample containing Lamivudine (Sigma) standard (10 µM). The cell suspension was collected by centrifugation and transferred to an activated (by elution of 1 ml CH$_3$CN (Merck)) and equilibrated (by elution of 1 ml MeOH/H$_2$O (80/20, v/v)) RP18 SPE-column (Phenomenex). The eluate was collected and evaporated in a SpeedVac concentrator. The residue was dissolved in 50 µL of 5mM NH$_4$OAc in CH$_3$CN/H$_2$O (25/75). Each sample was diluted 1:2 (cells) or 1:5 (medium) in CH$_3$CN. 5 µl of sample was applied to HILIC column (Acclaim Mixed-Mode HILIC- 1, 3 μm, 2.1 * 150 mm). Metabolites were separated at 30°C by LC using a DIONEX Ultimate 3000 UPLC system (Solvent A: 5 mM NH$_4$OAc in CH$_3$CN/H$_2$O (5/95), Solvent B: 5 mM NH$_4$OAc in CH$_3$CN/H$_2$O (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 alternating positive and negative mode. Peak determination and semi-quantitation were performed using TraceFinder™ Software. For determination of protein content for the data normalization, BCA assay (Thermo Fisher Scientific) was performed. The pellet of the cell samples was dried, resuspended in 0.2 M sodium hydroxide (Roth), boiled for 20 min at 95°C and absorbance was measured at 550 nm. Prism GraphPad was used for statistical analysis.

**Western blotting and antibodies**

Lysates for Western blot analysis were prepared by directly lysing cells in SDS sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 20% glycerol and 5% β-mercaptoethanol) in ice. Western blot analysis was performed as described 42. Briefly protein samples were separated in the 6-12%
SDS-PAGE (Peqlab) and transferred to a PVDF membrane (Roche) in a semidry electroblotter (Thermo Fisher Scientific). The membrane was further blocked in tris buffer saline containing 0.05% Tween20 and 5% bovine serum albumin or dry milk powder. The primary antibody against c-Myc (Y69: ab-32072), pc-Myc Thr58 (ab-28842) and pc-Myc Ser62 (ab-51156) SLC1A5 (ab-84903) glutaminase (ab-156876) was purchased from Abcam. The T-ERK (cs-9180), pERK (cs-9106), T-AKT (cs-9272), pAKT Ser473 (cs-9271), were obtained from Cell Signaling. Chlamydial HSP60 (sc-57840) and anti-ubiquitin (sc-8017) antibody was purchased from Santa Cruz Bioscience and β Actin antibody from Sigma (A5441). Proteins were detected with secondary antibodies coupled with HPR (Santa Cruz Bioscience) using ECL system (Pierce) and Intas Chem HR 16-3200 reader. Quantification of blots was done by FIJI (ImageJ) software.

**Immunoprecipitation**

Uninfected and *Chlamydia*-infected (MOI 1) HeLa229 cells were lysed using denaturing buffer (RIPA lysis buffer: 50mM Tris-HCl pH 7.5, 150mM NaCl, 1% Triton-X100, 1% NP-40, 0.1% SDS, 10% glycerol containing Complete protease inhibitor cocktail (Roche) and MG-132, proteasome inhibitor) to prevent co-precipitation of interacting partners of c-Myc. Lysates from 7 × 10⁶ cells were prepared as described before and incubated with 3 µg anti-c-Myc antibody for 1 h at 4°C followed by incubation with protein G magnetic beads (Dynabeads, Thermo Fisher Scientific) for 2 h at 4°C. The samples were washed several times and eluted by addition of 2x SDS-sample buffer and heating to 94°C. Samples were separated with SDS-PAGE and visualized by immunoblotting after probing against anti ubiquitin antibody.

**Nuclear-cytoplasmic isolation**

HeLa229 cells were plated in 150 mm dishes and either left uninfected or infected with *Chlamydia* (MOI 1) for the mentioned period of time. The cells were washed with ice cold PBS. The cells were scraped into a falcon. The cells were centrifuged and resuspended in buffer containing 10 mM Hepes-KOH pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.05% NP-40, protease inhibitors and incubated for 20 min in ice. The cells were then homogenized with a dounce homogenizer (10 strokes). The cells were centrifuged at 4,000 RPM for 5 min at 4°C,
the supernatant containing cytoplasmic proteins was collected and lysed with 2x SDS-sample buffer. The pellet was resuspended in buffer containing 20 mM Hepes-KOH pH 7.9, 400 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 15% glycerol, 0.5 mM DTT, protease inhibitors and incubated for another 20 min and vortexed. Further the sample was centrifuged at 14000 RPM for 10 min at 4°C, the pellet containing the nuclear extract was lysed in 2x SDS-sample buffer and analysed using Western blot.

**Immunofluorescence analysis**

The immunostaining was performed as described earlier. Briefly, HeLa229 and HUVEC cells were grown on cover slips and infected with the indicated *C. trachomatis* strain at an MOI 1 for indicated time points. The cells were washed with PBS, fixed with 4% PFA/Sucrose and permeabilized with 0.2% Triton-X-100/PBS for 30 min. Samples were blocked with 2% FCS/PBS for 1 h. All primary antibodies were incubated for 1 h at room temperature. Primary antibodies were used in the following dilutions in 2% FCS/PBS: anti-Hsp60 (1:500), c-Myc (1:200). Samples were washed three times and incubated with a Cy2-/Cy3-/Cy5-conjugated secondary antibody for 1 h in the dark. The cells were mounted on microscopic slide using Mowiol. Slides were air dried for at least 24 h and examined using Leica DM2500 fluorescence microscope, the images were analysed using LAS AF and Image J software.

**Inhibitor studies**

Hela229 cells were grown in the RPMI1640 +GlutaMAX™ or in the basic DMEM medium supplemented with 5% dialysed FCS and 1 g/l of D-Glucose and 4 mM L-glutamine. The cells were treated with 30 μM MAPK inhibitor UO126 (Cell Signaling Technology), 30 μM PI3K inhibitor LY297004 (Cell Signaling Technology), the cell-permeable thiazolidinone c-Myc inhibitor 10058-F4 (Sigma-Aldrich), 5 μM glutaminase inhibitor BPTES (Sigma-Aldrich) or the 5 mM SLC1A5 inhibitor GPNA (Sigma-Aldrich) for the respective period of time. In general, cells were infected with *Chlamydia* at an MOI 1 for different time points. The vehicle used for each inhibitor was used in the appropriate concentration. The cells were lysed and analysed using Western blotting.

**RNA Sequencing and NGS Data Analysis**
Differential gene expression from infected host cells: Total RNA was isolated from uninfected or infected cells (12, 24 and 36 h post infection) and was used for RNA sequencing (RNA seq). Libraries for RNA Seq were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® with 12 PCR cycles for amplification and sequenced on Illumina NextSeq500 platform. FASTQ generation was carried out using CASAVA and the quality check was performed using FastQC. Reads from FASTQ files were aligned to hg19 genome using Bowtie2 and differential gene regulation calculation was carried out based on edgeR algorithm. The gene expression changes between infected and uninfected cells were normalized to library size were used for carrying out Gene Set Enrichment Analysis (GSEA) using weighted parameters on C2, C5 and Hallmark gene sets downloaded from MSigDB database. In this analysis, the nominal p-value is calculated using an empirical phenotype-based permutation test procedure. The permutation-based false-discovery rate (FDR) Q value is generated by correcting for gene set size and multiple hypothesis testing. Heat map for Fig 6a was generated using heat map package in R with default settings.

Differential gene expression of *Chlamydia* in axenic culture: Renografin purified *Chlamydia* were cultured either in DMEM media (without Glucose/G6P without Gln) or in DMEM with only G6P or with DMEM with G6P and Gln for 12 h at 35°C. The bacteria were pelleted by centrifugation at 12,000 g for 10 min at 4°C. Total RNA was isolated from the bacteria and was used for RNA sequencing (RNA-seq). Libraries for RNA-seq were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® with 12 PCR cycles for amplification and sequenced on Illumina NextSeq500 platform. FASTQ generation was carried out using GALAXY platform (DKFZ, Heidelberg) and the quality check was performed using FastQC. Reads from FASTQ files were aligned to the *C. trachomatis* D serovar 434/Bu genome using alignment with HISAT2. Differential gene regulation calculation was carried out based on feature Counts algorithm. The differential gene expression heat map was created with Graph pad 7.

Real Time PCR

RNA was isolated from uninfected and *Chlamydia*-infected HeLa229 cells using RNA easy kit (Qiagen, Germany). RNA was reverse transcribed using a Revert Aid First Strand synthesis Kit
(Fermentas) according to the manufacturer’s instructions and diluted 1:10 with RNase free water. qPCR was performed as previously described. Briefly, qPCR reactions were prepared with Quanta SYBR (Quanta Bio) and PCR was performed on a Step One Plus device (Applied Biosystems). Data was analysed using ΔCt method, Step One Plus software package (Applied Biosystems) and Excel (Microsoft). Endogenous control was GAPDH. Primers were designed by q Primer Depot. The details of the primer are listed in the table in Extended data file.

**Transcervical mouse infections and determination of bacterial burden**

ASCT2/SLC1A5 KO mice generated in a C57BL/6 background were obtained from the Australian National University (ANU). All animal experiments were performed in accordance with protocols approved by animal care and experimentation of German Animal Protection Law approved under the Animal (Scientific Procedures) Act 1986 (project license 55.2-2532-2-762). The mice used for experiment were between 10-14 weeks old. Five days before transcervical infection, mice were treated subcutaneously with 2.5 mg of DepoProvera (medroxy-progesterone acetate). The mice were transcervically infected with either 1 × 10^7 infection-forming units (IFU) of *C. trachomatis* or 1 × 10^4 IFU of *C. muridarum* using a non-surgical embryo transfer device (ParaTechs Corp.). The mice were euthanized 7 days post-infection and the uterine horns were taken for further analysis. The uterine horns were homogenized in SPG buffer and DNA was isolated using DNeasy blood and tissue kit (Qiagen). Quantitative PCR was used to enumerate *Chlamydia* and host genome copy number. The following primers were used for amplifying the *C. trachomatis lytA* gene that was cloned into the vector: fwd, 5′-TCTAAAGCGTCTGGTGAAAGCT-3′ and rev, 5′-GAAATAGCGTAGTAATAATACCCG-3′. Normalization of bacterial genome to that of the host was performed using mouse synectin primers: fwd, 5′-ACTAATGTCAAGGAGCTGTACG-3′ and rev, 5′-CCTCCGACTTGAAACCTTCC-3′. Quantitative PCR with reverse transcription (RT–PCR) was performed as described below. Data were analysed using Step One Plus software package (Applied Biosystems) and expressed as the ratio of chlamydial genome to host genome (*lytA/synectin*). GraphPad Prism 7 was used to generate a scatter column chart.
and perform statistical analysis. One-way analysis of variance (ANOVA) with Newman–Keuls multiple-comparison tests was performed with the significance level set to less than 0.01. Statistical analysis was performed to decide the sample size used in mouse infection by the Institute of Mathematics, University of Würzburg under the allowance A2 55.5-2531.01-49/12. All mouse experiments were carried out with 9 or 10 female mice per treatment group. Mice in each experiment were age-matched and cage mates were randomly distributed into different treatment groups to avoid cage effects.

**Generation of mouse organoids**

Generation of mouse organoids was adapted from 44. Mice were sacrificed and fallopian tube tissue was prepared and processed within 2 h. Briefly, tissue samples were washed with DPBS (Gibco) and placed into a sterile Petri dish (Corning) where they were cut into small pieces. Then, on the top of the minced tissue, a glass slide (VWR) was placed and strongly pressed down to obtain smaller pieces. The cells were washed with DPBS, placed into a 15 ml falcon tube and centrifuged at 1,000 g for 10 minutes. The supernatant was removed, the pellet was resuspended in Matrigel (Corning) and plated in 50 µl drop in wells in a 24-well plate. The plate was carefully transferred to 37°C incubator to allow the Matrigel to get solidified for 20 min following the addition 500 µl/well of pre-warmed media (DMEM advanced (Sigma), Wnt (25%), R-Spondin (25%), Noggin (10%), B27 (2%; Thermo Scientific), Nicotinamide (1 mM; Sigma), human EGF (50 ng/ml; Thermo Scientific), FGF (100 ng/ml; Thermo Scientific), TGF-β inhibitor (0.5 mM; Tocris), Rock inhibitor (10 mM; Abmole Bioscience).

**Splitting organoids**: Approximately in 7 days later, the drop was carefully resuspended in cold DMEM medium and centrifuged at 1000 g at 4°C for 5 min. The supernatant was discarded and 50 µl Matrigel was added and further processed as explained above.

**Infectivity assay in organoids**: For infection with *C. trachomatis* mature organoids were released from a confluent Matrigel drop by resuspending it with ice-cold DPBS (Gibco). The suspension was collected in a low-binding Eppendorf tube and 2 µl of a frozen stock of *C. trachomatis* L2 expressing GFP was added (5 x 10⁵ IFU). The suspension was mixed and placed on ice for 30 min following centrifugation. 50 µl of Matrigel was added to each tube and seeded
into a 24-well plate (Corning) with following 20-minute incubation at 37°C to allow the Matrigel drop solidify. 6 days post infection the organoids were fixed with 4% PFA and used for immunostaining. In addition, infected organoids were lysed with glass beads and different dilutions were used to infect freshly plated HeLa cells to analyse the infectivity of the progeny.

Statistical analysis

In all experiments, a minimum of three technical replicates was used and the n number refers to the number of independent experiments performed. The data are presented as box plots with the mean and s.e.m. Statistical analyses were performed with the Prism 7.2 package (GraphPad Software). ExactTest () function as a part of edgeR module in R 3.3.4 was used to carry out pairwise comparison and calculate p-values. False discovery rates were calculated as q-values using Benjamini-Höchberg algorithm implemented in edgeR module.

Author contribution

KR and TR designed the experiments. The experiments were performed by KR, NV and TW. Next generation sequencing on Illumina platform was performed by AB, EW and resulting RNA-Seq data was analysed by RS and KR. Samples for Mass Spectrometry were prepared by KR and SJ. NV, CH, MS and WE performed the metabolic flux analysis of axenic culture and data interpretation. SJ, WS and AS performed metabolic flux analysis in host cell culture. FD provided plasmids and cell lines. Click reagents were synthesized by JF and JS. KR and TR wrote the manuscript.

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Data availability

Microarray data have been deposited in the Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo/) of the National Center for Biotechnology Information and can be accessed with the GEO accession numbers GSE147538 and GSE147539. Any other data that support the findings of this study are available from the corresponding author upon request.

Code availability

Code described here has been integrated into the Galaxy codebase and released under the Academic Free License (AFL) v. 3.0 (https://github.com/galaxyproject/galaxy). A free public instance of Galaxy can be accessed at https://usegalaxy.org. For the transcriptomics analysis on sequencing data deposited at gene expression omnibus (GSE147538), open access code implemented in bowtie, samtools, edgeR and GSEA tool and packages was utilized.

Declaration of interests

There is no conflict of interest
**Figure legends**

**Fig 1:** Cell wall synthesis of *Chlamydia* grown in axenic medium is induced by glutamine.

a. Central metabolic pathways of the chlamydial and host TCA cycle. b. Diagram showing the setup of the experiment. c. The relative amounts of the indicated metabolites compared to time point 0 were determined in the supernatant of axenic cultures with *Chlamydia* at different time points by GC/MS using internal standards (see Methods). The stability of Gln is shown as the green line where the supernatant with only Gln was analysed at 0 and 24 h. Number of independent experiments to confirm uptake: Gln: n=6, Glu: n=5, KG: n=4. d. $^{13}$C-enrichments and isotopologue distributions of selected metabolites in *Chlamydia* cultured in axenic medium with [U-$^{13}$C$_5$] Gln for 24 h. n=3. e. Purified EBs (Input) were incubated in axenic medium with only G6P or G6P together with Gln or Glu. After 24 h, the bacteria were pelleted and analysed by EM. Representative images from three independent experiments are shown. Scale bar = 2 µm. f. Purified EBs were incubated with G6P or G6P and Gln. Both samples were treated with DA-DA for 12 h. Click chemistry was used to detect DA-DA in peptidoglycan (green). Bacteria were stained for cHsp60 (red). The samples were analysed using Structured Illumination Microscopy. Representative images from two independent experiments. The insets show magnifications of the peptidoglycan signal (lower panel). Scale bar = 0.5 µm. g. FACS analysis of the bacteria from (f). The fluorescence is presented as bar plot overlaid with data points. Data were averaged (± s.e.m.) from three independent experiments (n=3). Statistical significance was calculated using an unpaired t test (two tailed) (t=5.138, Df=4, p value =0.0068). ** Indicates p value <0.01. h. EBs were incubated in axenic medium containing only G6P or G6P together with Gln. After 12 h the bacteria were pelleted and total RNA was isolated for RNA-seq (see Methods). The heat map shows expression levels of known early genes. i. Genome copy number of *Chlamydia* was determined at the indicated times. Shown is the mean from three independent experiments (± s.e.m.) n=3. Statistical significance was calculated using two-way ANOVA with Dunnett’s multiple comparison test (Df=24). * p value=0.034; ** p value =0.002; ns, non-significant.
**Fig. 2:** Chlamydial infection triggers altered glutamine metabolism in infected cells.

HUVECs were either left uninfected or infected with *Chlamydia* at an MOI of 1 for 36 h. Cells or medium samples were extracted and metabolites were analysed by LC-MS (see Methods). **a.** Depletion of amino acids from the culture medium from HUVECs with and without infection (only significant results are shown). Data were averaged (± s.e.m.) from three independent experiments. n=3. **b/c.** Content of Gln and Glu in control and infected cells. Data from three independent experiments are depicted as bar plot overlaid with data points with the mean (± s.e.m.). Paired two tailed t-test was used to calculate statistical significance (b) t=10.75, Df=2, p value=0.0085; (c) t=1,664, Df=2) p value= 0.2379. **p value <0.01, ns non-significant.** Error bar was defined as mean with SEM. **d.** Control or *Chlamydia* infected HUVECs were cultured in the presence of [U-13C5] Gln for 36 h. 13C-enrichments and isotopologue distributions of selected metabolites were determined by LC-MS. Data present the mean of three independent samples. **e.** Diagram outlining the synthesis of the D-aminopimelic acid (DAP) in *Chlamydia* from host or chlamydial derived metabolites. **f.** Mass isotopologue distribution of DAP extracted from infected host cells using acid hydrolysis. Data present the mean of three independent samples.

**Fig. 3:** Signalling pathways involved in the stabilization of c-Myc in infected cells. **a.** Plot of gene set enrichment analysis (GSEA) performed with RNA-seq data of the gene set “Positive regulation of c-myc targets”. Gene expression profiles were generated as described in the Methods section and a selected gene set is shown. Vertical black bars indicate the position of genes in the “Positive regulation of c-myc targets” gene set and the enrichment score is shown as a green line. This analysis demonstrates that genes induced upon infection are known as target genes of c-Myc (NES: normalized enrichment score). Three independent experiments were performed. n=3. See the methods section for statistical analysis. **b.** HeLa229 cells were infected with *Chlamydia* for different time points and the samples were analysed using Western blotting for phosphorylated c-Myc (pc-Myc serine 62), pc-Myc (threonine 58), cHsp60 and ß Actin. **c.** c-Myc was immunoprecipitated from lysates of HeLa229 cells infected with *Chlamydia* for different time points. Ubiquitin was detected in the precipitate by immunoblotting. The input
from the same experiment was probed against c-Myc, cHsp60 and β Actin. **d.** HeLa229 cells were either left uninfected or infected with *Chlamydia* for 24 or 36 hpi. The cells were treated with the translation inhibitor cycloheximide (CHX) for 10, 30 or 60 min. The cells were then lysed and analysed by Western blotting for c-Myc, cHsp60 and β Actin. For quantification, values were normalized for each condition to the 0 time point of CHX-treated sample. **e.** Diagram showing the signalling pathway leading to c-Myc stabilization. **f/g.** HeLa229 cells were treated with the MAPK inhibitor, (U0126) (f) or the PI3K inhibitor (LY294002) (g) for 4 h and infected with *Chlamydia* for 24 h. The cells were lysed and analysed by Western blotting. n=3. **h.** HeLa229 cells were infected with *Chlamydia* for different time points. The cells were harvested for Western Blot analysis. n=3. **i.** HeLa229 cells were either left uninfected or infected with *Chlamydia* and treated with the PI3K inhibitor. The cells were lysed and analysed by Western. In all Western blots, detection of cHsp60 served as an infection and of β Actin as a loading control. The immunoblots shown in this figure are representative of at least three independent experiments.

**Fig. 4: c-Myc is downstream of PI3K/MAPK pathway and critical for chlamydial growth.**

**a/b.** U2OS cells harboring an inducible c-Myc gene under the control of a Tet-inducible promoter were induced with AHT for 12 h and either treated with MAPK inhibitor U0126 (a), or the PI3K inhibitor LY294002. n=3. (b) or left untreated as indicated. The cells were further infected with *Chlamydia* and analysed by Western blotting for bacterial infection. n=3. **c.** The cells from the experiment shown in (a) and (b) were fixed and immunostained to detect chlamydial inclusions (green) and actin (phalloidin, red). Images are representative for three independent experiments. UI, uninfected control; U0: treated with U0126; LY, treated with LY294002; AHT, induced. n=3. Scale bar = 15 µm. **d.** HeLa229 cells were treated with the chemical c-Myc inhibitor 10058-F4. The cells were infected with *Chlamydia* for 24 or 36 hpi and then analysed for *Chlamydia* infection by Western blotting (cHsp60). n=3. **e.** HCT116 cells engineered to express a shRNA to silence c-Myc expression under the control of an inducible Tet<sup>on</sup> promoter were treated with AHT (1 µg/ml) for 24 h and then infected with *Chlamydia* for another 24 h. The cells were lysed and analysed for *Chlamydia* infection by probing for cHsp60. n=3. **f.** An
unrelated control siRNA or a siRNA pool directed against c-Myc was used to knock down c-Myc in HeLa cells. After 48 h of transfection, the cells were infected with *Chlamydia* for 24 h. The cells were harvested and analysed by Western blotting for chlamydial infection. The immunoblots shown in the figures are representative of at least three independent experiments.

**Fig. 5: Glutamine is a limiting metabolite for chlamydial intracellular growth.**

a. Cells isolated from human fimbriae (Human Fimb) were grown in basic formulation of DMEM containing 1 g/l D-glucose and different concentrations of glutamine. The cells were infected with *Chlamydia* (Ct) for 24 h, lysed and analysed via Western blotting to detect chlamydial Hsp60, c-Myc and β Actin. n=3.

b. HeLa229 cells were grown in basic formulation of DMEM containing 1 g/l D-glucose. The cells were treated with or without glutamine (2 mM) and infected with *Chlamydia* for different time points. The cells were lysed and analysed for chlamydial growth using Western blotting to detect chlamydial Hsp60, c-Myc and β Actin. n=3.

c/d. The cells from (b) were lysed and used to infect freshly plated HeLa cells. After 24 h the cells were either fixed and immunostained for chlamydial inclusions (green) and Actin (phalloidin, red), or used for Western blot analysis as shown in panel (d). The scale bar is indicated in the figure. n=3. Scale bar = 10 µm.

e. HeLa cells were infected with the EB/RB reporter strain for 24 h and grown in basic formulation of DMEM containing 1 g/l D-glucose either without (-Gln) or with 2 mM Gln (+Gln). In one set of experiments, Gln-free medium was replaced after 24 h for medium containing 2 mM Gln (lowest panel). The cells were fixed *Chlamydia* (Red/Green) and Actin (phalloidin, grey) was detected. Loss of the green fluorescence is indicative for RBs. n=3. Scale bar = 10 µm.

f/g. HCT116 were grown in basic formulation of DMEM containing 1 g/l D-glucose, with or without L-glutamine. HCT116 expressing 4-hydroxytamoxifen (OHT)-inducible Myc-ER or the vector control were treated overnight with OHT (100 nM) (f) to activate Myc-ER or ethanol (1 µl) as control (g). The cells were then infected with *Chlamydia* at an MOI of 1 for 24 h and analysed for bacterial load by Western blotting. n=3. The immunoblots shown in the figures are representative of at least three independent experiments.
**Fig. 6: Chlamydia metabolically reprograms the host cell.**

a. HUVEC cells infected with *Chlamydia* were subjected to RNA-seq. Shown is the hierarchically clustered heat map depicting gene regulation (normalized log2FC) of gene set “Amino acid transport across the plasma membrane” which is a part of Reactome gene sets in C2 Molecular Signature Database. Three independent experiments were performed. n=3.

b. Diagram of Gln uptake pathways and the targets of the inhibitors.

c. HeLa229 cells were infected with *Chlamydia* and then subjected to Western blot analysis.

d. Control siRNA or an siRNA pool against SLC1A5 were transfected and 48 h later infected with *Chlamydia* for 24 h as indicated. The cells were lysed and further analysed by Western blot.

e. HeLa229 cells expressing a shRNA to silence GLS1 expression under the control of a Tet<sup>on</sup> promoter were induced with AHT for 7 days to deplete GLS1 and then subjected to Western blot analysis.

f. HeLa229 cells were treated with the SLC1A5 specific inhibitor GPNA or (g) the glutaminase inhibitor BPTES for 4 h and infected with *Chlamydia*. The cells were lysed and analysed by Western blotting.

h. HeLa229 cells were transfected with siRNA against c-Myc and 48 hrs later analysed by Western blotting.

i. HCT116 Tet<sup>on</sup> sh-c-Myc cells were either left untreated or treated with AHT for 24 h followed by overexpression of SLC1A5. The cells were then infected with *Chlamydia* for another 24 h and analysed by Western blotting.

j/k. SLC1A5<sup>+/+</sup> or SLC1A5<sup>−/−</sup> mice were infected with *C. trachomatis* (n=10) or *C. muridarum* (n=9) (k). The mice were sacrificed seven days post infection and the copy number of *Chlamydia* was calculated using qPCR. The data are presented as box and whisker plot overlaid with data points with the mean (± s.e.m.). Unpaired t test (two tailed) was used to calculate the significance (j) (t=3.714, df=16) and (k) (t=3.712, df=10). ** Indicates p value <0.01. n=9.

l. Organoids were derived from SLC1A5<sup>+/+</sup> or SLC1A5<sup>−/−</sup> mice and infected with *Chlamydia* (Ct) (see Methods). The infected organoids were fixed and used for immunostaining to detect *Chlamydia* (green), nuclei (blue) and actin (phalloidin, violet). Scale bar = 30 µm. The immunoblots shown in the figures are representative of at least three independent experiments.
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