



## Research Paper

# *Chlamydia trachomatis* Prevents Apoptosis Via Activation of PDPK1-MYC and Enhanced Mitochondrial Binding of Hexokinase II



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## ABSTRACT

The intracellular human bacterial pathogen *Chlamydia trachomatis* pursues effective strategies to protect infected cells against death-inducing stimuli. Here, we show that *Chlamydia trachomatis* infection evokes 3-phosphoinositide-dependent protein kinase-1 (PDPK1) signaling to ensure the completion of its developmental cycle, further leading to the phosphorylation and stabilization of MYC. Using biochemical approaches and imaging we demonstrate that *Chlamydia*-induced PDPK1-MYC signaling induces host hexokinase II (HKII), which becomes enriched and translocated to the mitochondria. Strikingly, preventing the HKII interaction with mitochondria using exogenous peptides triggers apoptosis of infected cells as does inhibiting either PDPK1 or MYC, which also disrupts intracellular development of *Chlamydia trachomatis*. These findings identify a previously unknown pathway activated by *Chlamydia* infection, which exhibits pro-carcinogenic features. Targeting the PDPK1-MYC-HKII-axis may provide a strategy to overcome therapeutic resistance of infection.

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## 1. Introduction

*Chlamydia trachomatis* is the most common sexually transmitted bacterial infection and the leading cause of infertility (Hammerschlag, 2004). Infection is further correlated with an increased risk of invasive cervical cancer in human papillomavirus positive women (Smith et al., 2004) and indirect evidence further suggests a role in ovarian cancer (Lin et al., 2011), but the molecular mechanism underlying this remains elusive.

*Chlamydia trachomatis* has a unique biphasic developmental cycle. Upon infection, the highly infectious elementary bodies (EBs) are sequestered within a vacuole, termed inclusion, in which they differentiate into metabolically active reticulate bodies (RBs) that divide by binary fission (Elwell et al., 2016). After 24–72 h, RBs differentiate back into EBs that are released to start a new round of infection. Like other obligate intracellular bacteria, *Chlamydia trachomatis* has a small

genome of around 1 Mb (Carlson et al., 2005), which lacks the enzymes for many metabolic pathways as well as hexokinase (Omsland et al., 2014). It compensates for this reduced metabolic capacity by importing phosphorylated sugars as well as other nutrients such as amino acids, nucleotides, and lipids from the host cell (Saka and Valdivia, 2010). It therefore depends on viable host cells to complete its life cycle and has evolved effective strategies to protect them from apoptosis during the course of infection (Sharma and Rudel, 2009).

We previously demonstrated that *Chlamydia* inhibits host cell apoptosis by activating the MDM2-p53 axis to reduce p53 levels (Gonzalez et al., 2014). This inhibition is only partial, however, suggesting that there are other pathways contributing to the apoptosis resistance. In addition, it is clear that p53 reduction not only keeps host cells alive but is also directly required for successful Chlamydial replication - by stimulating the pentose phosphate pathway (PPP), shifting cellular metabolism towards aerobic glycolysis to provide increased anabolic substrates. In this way, *Chlamydia* is capable of exploiting the functional interdependence between metabolic and cell survival pathways (Mason and Rathmell, 2011; Siegl et al., 2014). This process is seen in many cancers, where loss of tumor suppressors or activation of oncogenes results in metabolic reprogramming with enhanced nutrient uptake and glycolysis as well as resistance to apoptosis.

Several *Chlamydia trachomatis*-triggered signaling pathways are known to regulate apoptosis resistance, prominently among them the

**Abbreviations:** EB, elementary body; RB, reticulate bodies; PI3K, phosphatidylinositol 3-kinase; HKII, hexokinase II; VDAC, voltage-dependent anion channel; CTL2, *Chlamydia trachomatis* L2; p.i., post-infection; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; PARP, poly-(ADP-ribose) polymerase; MOI, multiplicity of infection.

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phosphatidylinositol 3-kinase (PI3K) signaling cascade (Olive et al., 2014; Verbeke et al., 2006); (Zhong et al., 2006). PI3K, via activation of AKT, is a major regulator of MDM2 (Ogawara et al., 2002). It also stabilizes the oncogene MYC, via activation of PDPK1 (Tan et al., 2013). MYC promotes cell growth and proliferation, acquisition of the required bioenergetic substrates (Eilers, 1999; Hirvonen et al., 1990; Ryan and Birnie, 1997) and shifting of metabolism towards aerobic glycolysis and lipid synthesis (Li and Simon, 2013). One of its transcriptional targets is hexokinase II (HKII), a key enzyme for integrating energy production and cell viability. It catalyses the first step of glycolysis (Dang et al., 2008) to produce glucose-6-phosphate, a crucial intermediate for many biosynthetic pathways – including the PPP (Wilson, 2003). Key steps in metabolic re-programming in cancer consist of upregulation of HKII by MYC and enhanced association with the mitochondrial voltage-dependent anion channel (VDAC). This association leads to increased rates of glycolysis and oxidative metabolism (Robey and Hay, 2006) and inhibition of competing apoptotic signals for binding to VDAC (Majewski et al., 2004; Mason and Rathmell, 2011; Pastorino et al., 2002).

Here, we investigated whether *Chlamydia trachomatis* modulates the MYC-HKII axis of host cells and whether this plays a role in apoptosis protection. We show that infection induces stabilization of MYC via phosphorylation at serine-62 mediated by PDPK1. This leads to enhanced binding of HKII to mitochondria and inhibits host cell apoptosis required for chlamydial survival. Our data provide a link between activation of PDPK1, an important component of the PI3K pathway, and MYC protein accumulation during chlamydial infections. It provides a rationale for the reprogramming of host metabolism to promote cell survival.

## 2. Materials and Methods

### 2.1. Human Samples, Antibodies, Cell Lines and Reagents

Human fallopian tube tissue samples were provided by the Department of Gynecology, Charité University Hospital, Campus Virchow Clinic, Berlin, Germany, with signed, informed patient consent and under approval by the Charité Ethics Commission (EA1/002/07). Fragments were sourced from standard surgical procedures for benign gynecological diseases and only anatomically normal pieces were utilized for subsequent experiments. Primary mesenchymal stem cells and fallopian tube organoids were prepared from these samples as described elsewhere (Abu-Lubad et al., 2014; Kessler et al., 2015).

The following antibodies were used: rabbit monoclonal antibodies against total MYC (D84C12, 5605), total HKII (C64G5, 2867) and total PDHK1 (C47H1, 3820), rabbit polyclonal antibodies against total PDPK1 (3062) and anti-P-PDPK1 (Ser-241, 3061) (all Cell Signaling); rabbit polyclonal anti-P-MYC (T58, ab28842), mouse monoclonal anti-P-MYC (S62, 33A12E10, ab78318, both from Abcam); mouse monoclonal anti-VDAC1 (B-6, sc-390996), Rho A (24C4, sc-418) and anti-MYC (9E10, sc-40 all from Santa Cruz Biotechnology Inc.); mouse monoclonal anti-*Chlamydia trachomatis* MAb species-specific KK-12 IgG2a (supplied by David Grayston, University of Washington, Seattle); rabbit polyclonal anti-*Chlamydia* genus-specific antibody (AG, 3-090, Milan Analytica), mouse monoclonal anti-*Chlamydia trachomatis* Hsp60 (ALX-804-072-R100, Enzo Life Sciences), rabbit anti-INCA antibody (Banhart et al., 2014); goat anti-chlamydia (1990-0404, AbD Serotec); rabbit monoclonal cleaved PARP (Asp214) and rabbit polyclonal anti-cleaved caspase 3 (Asp175, 9661, both from Cell Signaling). Secondary labelled antibodies for immunofluorescence and Western immunoblot analyses were purchased from Jackson ImmunoResearch Laboratories (711-165-152, 115-166-072, 111-485-144, 115-225-146), and Amersham (NA934 and NA931). BX-912 (SML1086) and clotrimazole (6019) were from Sigma Aldrich. A cell-permeable HKII VDAC binding domain peptide (N-HKII) was purchased from Merck (376816) and JQ1 from Cayman Chemical (CAS 1268524-70-4). HeLa cells (ATCC CCL-2) were

maintained in RPMI-1640 medium (52400) from Gibco-Life Technologies (52400) supplemented with 10% fetal bovine serum (FBS, S0115, Biochrom AG).

### 2.1.1. Chlamydia Infections

*Chlamydia* infections were conducted using *Chlamydia trachomatis* L2 (ATCC VR-902B) at the indicated MOIs, as described previously (Al-Zeer et al., 2013). In brief, cells were infected for 2 h at 35 °C in normal RPMI growth medium containing 5% FCS, 2 mM glutamine, and 1 mM sodium pyruvate. The medium was then replaced with fresh growth medium and the cells cultured for the indicated times at 35 °C.

Fallopian tube organoids, from healthy fallopian tube epithelial tissue were generated and maintained in Matrigel under control of paracrine growth factor cocktails, as previously described (Kessler et al., 2015). For infection with CTL2, organoids were removed from Matrigel by washing in ice cold medium (Advance, Invitrogen), mechanically fragmented by passing the suspension 5 times through a 20G syringe and centrifuged at 1200 rpm. The pellet was incubated with CTL2 frozen stock to achieve MOI 5 (based on the number and size of the organoids in the starting culture) and incubated for 20 min on ice. Subsequently, organoid fragments were seeded back into fresh Matrigel and provided with standard growth medium.

### 2.1.2. Western Immunoblotting

Western immunoblotting was conducted on cell monolayers lysed directly with SDS lysis buffer (3% 2-mercaptoethanol, 20% glycerine, 0.05% bromophenol blue, 3% SDS). Cell lysates were harvested and boiled at 95 °C for 10 min. Equal amounts of protein were separated using SDS-PAGE and immunoblotting as described previously (Al-Zeer et al., 2013).

### 2.1.3. Infectivity Assay

Infectious progeny assays were conducted as follows. HeLa cells seeded in 24-well plates were infected with CTL2 as described. Infected cells were lysed at 48 h p.i. by adding Nonidet P40 (NP40) at a final concentration of 0.06% per well. Pipetting steps were performed using a robotic station (Biomek FX pipetting robot, Beckman Coulter). Lysates were diluted in infection medium and transferred to infect fresh HeLa cells at a final dilution of 1:40. Plates were incubated for 24 h at 35 °C and 5% CO<sub>2</sub> and processed for immunolabeling.

### 2.1.4. Small Interfering RNAs (siRNAs)

Small interfering RNAs (siRNAs) targeting MYC 5'-CCCAAGGTAGTTA TCCTTAAA-3', HKII 5'-ACCGTTCGCTTTCGACCAAAA-3' and 5'-CAGCATG AAATGAACTGGT-3', and firefly luciferase (5'-AACUUACCCUGAGUAC UUCGA-3') were purchased from Qiagen (Hilden, Germany). The transfection of siRNAs was performed using Hyperfect transfection reagent (Qiagen) according to the manufacturer's instructions. Cells were harvested to determine the knockdown efficiency and infectious progeny using Western blot and infectivity titration assay, respectively.

### 2.1.5. Cytosol-Mitochondria Fractionation

Cytosolic and mitochondrial fractions were prepared from HeLa cells infected with CTL2 or control cells using the Mitochondria Isolation Kit from Thermo Scientific, in accordance with the manufacturer's instructions.

### 2.1.6. Indirect Immunofluorescence

Indirect Immunofluorescence was conducted as described elsewhere (Abu-Lubad et al., 2014).

### 2.1.7. Apoptosis Assay

HeLa cells seeded in 24-well plates were infected with CTL2 as described for infectious progeny assay. Cells were treated with peptide at the indicated concentrations from 24 h p.i., recombinant human TNF $\alpha$  (BD Pharmingen, 554618) was added at the indicated concentrations

together with 10 µg/ml cycloheximide (Sigma, 065K12261) and incubated for 5 h until harvesting, immunoblotting and immunofluorescence.

### 2.1.8. Antibody Conjugation

50 µl of anti-P-PDPK1 antibody (Ser-241, 3061, Cell Signaling, containing 0.1 mg/ml BSA) were concentrated to 20 µl by ultrafiltration (Amicon Ultra-0.5 ml, Ultracel-30K). 2 µl of ATTO-550 NHS-Ester (20 mg/ml in DMSO) were added to the protein and incubated for 4 h at room temperature in the dark. The reaction was terminated by the addition of 2 µl ethanolamine (1 M) and incubated for 30 min. The reaction mixture was incubated with 50 µl (30 mg/ml) of magnetic Dynabeads Protein G (Novex, Life Technologies) for 30 min. After removal of the supernatant, the beads were washed 2 times with 1 ml of PBS, 0.1% Tween-20. Bound antibody was eluted with 50 µl 0.1 M glycine HCl, pH 3.0. The eluate was immediately neutralized by addition of 5 µl 1 M Tris HCl (pH 7.5).

### 2.1.9. Treatment With Chemicals

Cells were treated with different concentrations of BX-912, clotrimazole, N-HKII peptide and JQ1 for the indicated time points.

### 2.1.10. Assessment of Host Cell Viability

Briefly, cells were treated with different drugs for the indicated time points. Then, supernatants were collected from cells at the end of the treatment and lactate dehydrogenase colorimetric assay (Thermo Scientific) was used to evaluate toxicity exerted by different drugs according to manufacturer's instructions. In brief, extracellular release of LDH from damaged cells converts lactate into pyruvate via NAD<sup>+</sup> reduction to NADH that reduces the developer to a colored product with absorbance at 490 nm measured by a microplate reader (Molecular Devices, USA).

### 2.1.11. Statistical Analysis

Data were analyzed using non-parametric one-way ANOVA, Dunnett's multiple comparison test or Student's *t*-test.

## 3. Results

### 3.1. *Chlamydia trachomatis* Infection Increases MYC Levels in Human Cells

Immunoblotting of HeLa cell lysates revealed a significant increase in total MYC protein levels at 24 and 48 h after infection of cells with *Chlamydia trachomatis* L2 (CTL2) (Fig. 1a and b). We also visualized MYC expression in intact HeLa cells using confocal fluorescence immunohistochemistry. Non-infected cells expressed MYC at basal levels, while infected cells (identified by their large CTL2-filled inclusions) showed increased levels of MYC at 48 h post-infection (p.i.) (Fig. 1c). Quantification of fluorescence signal intensity by ImageJ verified that the levels of nuclear MYC protein were significantly increased in infected monolayers, consistent with the immunoblotting data (Fig. 1d). Notably, we also observed an increase of MYC expression in neighboring non-infected cells in the infected culture (Fig. 1c and d). Thus, acute CTL2 infection exerts paracrine effects that lead to MYC induction also in bystander cells although we cannot exclude that the inclusion-free cells were arisen from infected cells during mitosis.

Regulation of MYC stability is mediated by phosphorylation at two main sites, Thr-58 and Ser-62 (Sears, 2004), which are near the amino terminus and are highly conserved in all mammalian isoforms. In response to a growth-stimulatory signal, MYC gene expression is increased, along with protein phosphorylation on the Ser-62 residue, resulting in its stabilization (Vervoorts et al., 2006). To monitor MYC phosphorylation, we carried out immunoblotting of CTL2-infected protein samples using specific antibodies against MYC phosphorylated at Ser-62 or Thr-58. Notably, while the levels of MYC phosphorylated at Ser-62 increased, levels of MYC phosphorylated at Thr-58 decreased significantly following infection (Fig. 1a and b), suggesting that in CTL2-

infected cells, too, MYC is stabilized through phosphorylation at Ser-62. To examine the impact of CTL2 infection in non-transformed cells, human primary mesenchymal cells (Fig. 1e–f) and fallopian tube primary epithelial organoids (Fig. 1g–h) were prepared from fallopian tubes, surgically removed from patients undergoing procedures for benign gynecological disease as described previously (Abu-Lubad et al., 2014; Kessler et al., 2015). Infection led to a significant decrease in the levels of MYC phosphorylated at Thr-58 (Fig. 1e–h) at 48 h p.i., while levels of MYC phosphorylated at Ser-62 were significantly increased (Fig. 1e–h). We conclude that CTL2 infection leads to an increase in the levels of MYC in host cells by shifting the phosphorylation state towards the stabilized form of MYC phosphorylated at Ser-62.

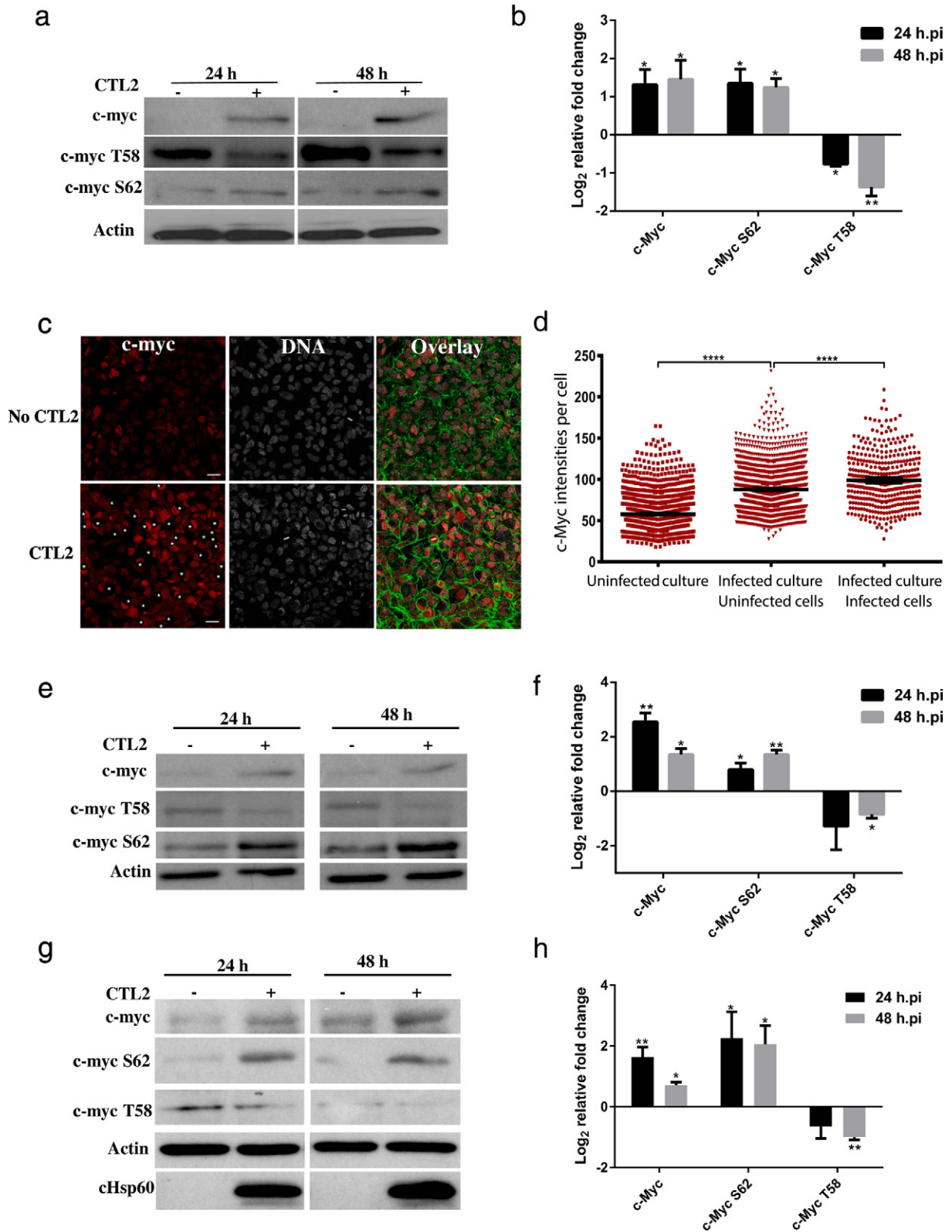
### 3.2. PDPK1 is Phosphorylated and Recruited to *Chlamydia trachomatis* Inclusions

PDPK1 has recently been linked to PLK1-dependent MYC stabilization via phosphorylation at Ser-62 (Tan et al., 2013). To monitor PDPK1 activation and phosphorylation, we carried out immunoblotting of CTL2-infected HeLa cells using anti-phosphorylated PDPK1 (Ser-241) and total-PDPK1 antibodies. We observed a significant increase in phosphorylated PDPK1 Ser-241 at 24 and 48 h p.i. compared to non-infected cells (Fig. 2a and b). These results prompted us to investigate the cellular localization of PDPK1 phosphorylated at Ser-241 in 48 h CTL2-infected HeLa cells, using the same antibodies. PDPK1 distribution in infected cells was similar to control, in the form of vesicular staining throughout the cytoplasm with minor accumulation at the chlamydial inclusions (arrowheads, Supplementary Fig. S1a). No increase in expression levels was observed, as examined using immunoblotting (Fig. 2a and b). Similarly, PLK1 exhibited vesicular staining throughout the cytoplasm of infected cells (arrowheads, Supplementary Fig. S1b and c), in congruence with what has been described by Sun et al. (2011), while phosphorylated PLK1 exhibited nuclear as well as some vesicular staining throughout the cytoplasm upon infection, with minor accumulation at the inclusions (Supplementary Fig. S1b and c). By contrast, phosphorylated PDPK1 Ser-241 was induced and recruited to the chlamydial inclusion, suggesting that this might be an essential step for chlamydial development (Fig. 2c). Recruitment was specific to phosphorylated PDPK1, because the cytosolic protein RhoA was not recruited to the chlamydial inclusion at the respective time points (Supplementary Fig. S2a). Further, phosphorylated PDPK1 recruitment was restricted to mid- and late-stages of chlamydial development, as no significant recruitment to the inclusion was observed at early times of infection (Supplementary Fig. 2b). Levels of MYC phosphorylated at Ser-62 were also not increased at early infection times (Supplementary Fig. S2c) and cycloheximide treatment did not inhibit the increase of MYC phosphorylated at Ser-62 at late stages of infection (Supplementary Fig. S2d). Recruitment of phosphorylated PDPK1 to the inclusion was also observed in human primary MSCs (Fig. 2d) and human fallopian tube organoids infected with CTL2 for 48 h (Fig. 2e). Overall, our data indicate that PDPK1 activation functions in CTL2 infection-induced signaling.

### 3.3. PDPK1 Activation is Required for the Phosphorylation of MYC at Ser-62 and Chlamydial Growth

To dissect the link between CTL2-induced MYC phosphorylation at Ser-62 and PDPK1 phosphorylation, we used the established pharmacological inhibitor BX912, which blocks phosphorylation of MYC at Ser-62 (Tan et al., 2013) by inhibiting PLK1. Cells were infected with CTL2 for 48 h, treated with BX912 (10 µM) from 12 h p.i., and subjected to immunoblotting. BX912 on its own had no apparent toxic effect on the host, as measured by LDH assay (Supplementary Fig. S3a), but markedly reduced the degree to which phosphorylated PDPK1 Ser-241 was induced after chlamydial infection. In line with this, the levels of MYC phosphorylated at Ser-62 were also reduced, whereas levels of total PDPK1 remained unchanged (Fig. 3a). Further, phosphorylated PDPK1, MYC,





**Fig. 1.** MYC is upregulated and stabilized at 24–48 h p.i. with CTL2. a) Western blot showing levels of total MYC protein as well as MYC phosphorylated at Ser-62 or Thr-58 at 24 and 48 h p.i. in CTL2-infected (MOI 1) whole cell lysates.  $\beta$ -actin served as loading control. b) Band densities from a) quantified and normalized to corresponding band densities of the  $\beta$ -actin loading control. Alterations in expression levels compared to non-infected controls are represented as mean fold change  $\pm$  SEM, \* $p < 0.05$ , \*\* $p < 0.01$ ,  $t$ -test,  $n = 3$ . c) Confocal microscopy images of HeLa cells infected with CTL2 (MOI 0.5) at 44 h p.i. Total MYC and nuclei were labelled with anti-MYC antibody and DAPI. Scale bar: 30  $\mu$ m. d) Quantification of MYC intensities revealed a significant increase in MYC signal in cells infected with CTL2 compared to non-infected cells, irrespective of whether they harbored chlamydial inclusions.  $n = 14$  images. Mean pixel intensities of MYC per nuclei were quantified from confocal images with ImageJ. Black bars indicate the means  $\pm$  SD, \*\*\*\* $p < 0.0001$ ,  $t$ -test,  $n \geq 403$ . e–h) Western blotting analysis from human primary fallopian mesenchymal stem cells (e) and human epithelial primary cell organoids (g) from fallopian tube, showing upregulation of total MYC protein expression as well as MYC phosphorylated at Ser-62 between 24 and 48 h p.i. in CTL2 (MOI 1) infected whole cell lysates.  $\beta$ -actin served as loading control. (f and h) The band densities were quantified and normalized to the  $\beta$ -actin loading control. Alterations in expression levels compared to non-infected controls are represented as fold change  $\pm$  SEM, \* $p < 0.05$ , \*\* $p < 0.01$ ,  $t$ -test,  $n = 3$ .

and MYC phosphorylated at Ser-62 signals from DMSO-treated and BX912-treated infected monolayers were analyzed and quantified using ImageJ. Consistent with the immunoblotting data, BX912 inhibitor significantly reduced the expression of MYC and MYC phosphorylated at Ser-62 (Fig. 3b). Then, infected samples treated with BX912 were prepared for immunofluorescence microscopy using antibodies against phosphorylated PDPK1 Ser-241. Confocal analysis confirmed that BX912 added at 12 h p.i. was sufficient to reduce the induction of phosphorylated PDPK1 Ser-241 expression levels in response to infection with CTL2 (Fig. 3c).

The induction of phosphorylated PDPK1 upon CTL2 infection prompted us to investigate the consequences of BX912 treatment on the formation of infectious CTL2 progeny. Cells were infected with CTL2 for 12 h to establish infection and then treated with 10  $\mu$ M BX912 for a further 36 h. The cells were lysed and lysates used to infect a second population of HeLa cells for 24 h. Quantifying the number of resulting inclusions showed that there was a dramatic decrease in infectious progeny after treatment with the inhibitor, suggesting that the pathogen was unable to complete its normal developmental cycle (Fig. 3d). Therefore, we conclude that a functional PDPK1 signaling pathway is necessary for proper bacterial development.

#### 3.4. Hexokinase II is the Major Target of the PDPK1-MYC Axis, Which is Crucial for Chlamydial Growth

As MYC is known to induce HKII and we found that stabilization of MYC upon infection depends on the PDPK1 signaling pathway (Fig. 3a), we sought to determine expression levels of HKII in infected cells treated with PDPK1 inhibitors. Cells were infected with CTL2 for 12 h then treated with 10  $\mu$ M BX912 from 12 h p.i. for a further 36 h before Western immunoblotting. Infection induced the expression of HKII, which was inhibited by treatment with BX912 (Fig. 4a). Further, HKII signals from DMSO-treated and BX912-treated infected monolayers were analyzed and quantified using ImageJ. Consistent with the immunoblotting data, BX912 inhibitor significantly reduced the induction of HKII upon CTL2 infection (Fig. 4b), suggesting that it depends on the PDPK1 cascade. A continuous uptake of glucose and the production of glucose-6-phosphate is known to be essential for the survival and growth of *Chlamydia trachomatis* in host cells (Engstrom et al., 2015). Therefore, we investigated the effect of MYC inhibition on the formation of infectious CTL2 progeny using the small molecule inhibitor JQ1 (Bandopadhyay et al., 2014). As HeLa cells overexpress MYC due to viral insertion, JQ1 on its own had no apparent toxic effect on host cells, as measured by LDH assay (Fig. S3a). Cells were infected with CTL2 for 2 h, then treated with increasing concentrations of JQ1 for another period of 44 h before using the cell lysates to infect a second population of HeLa cells for 24 h. JQ1 treatment significantly abrogated the production of bacterial progeny (Fig. 4c). Similarly, JQ1 treatment significantly inhibited CTL2 replication in primary cells treated with JQ1 (Supplementary Fig. S3b). Confocal microscopy further confirmed that JQ1 treatment abrogated the expression of MYC in infected and non-infected compared to untreated cells (Supplementary Fig. 3b). Treatment with JQ1 (20  $\mu$ M) 2 h p.i. with CTL2 for additional 44 h also blocked the increase in HKII levels observed in untreated infected HeLa cells by Western blotting (Fig. 4d), suggesting that the induction of HKII by *Chlamydia* is MYC dependent. Quantification of MYC and HKII signals from DMSO-treated and JQ1-treated infected monolayers using ImageJ revealed that JQ1 inhibitor significantly reduced the induction of MYC

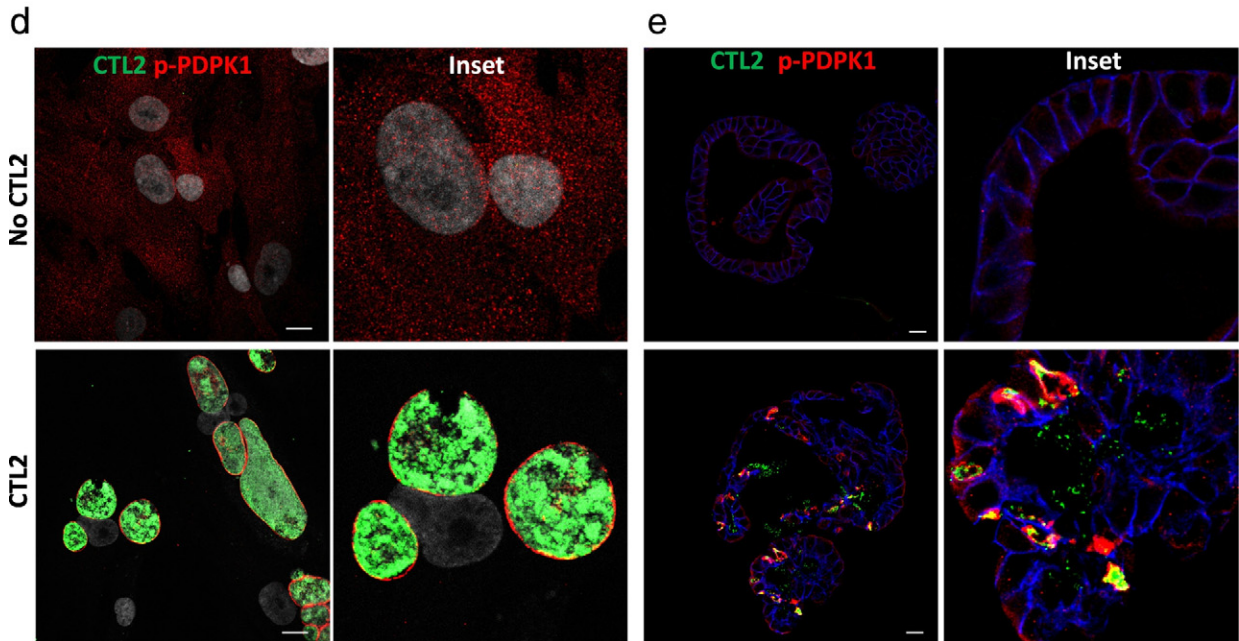
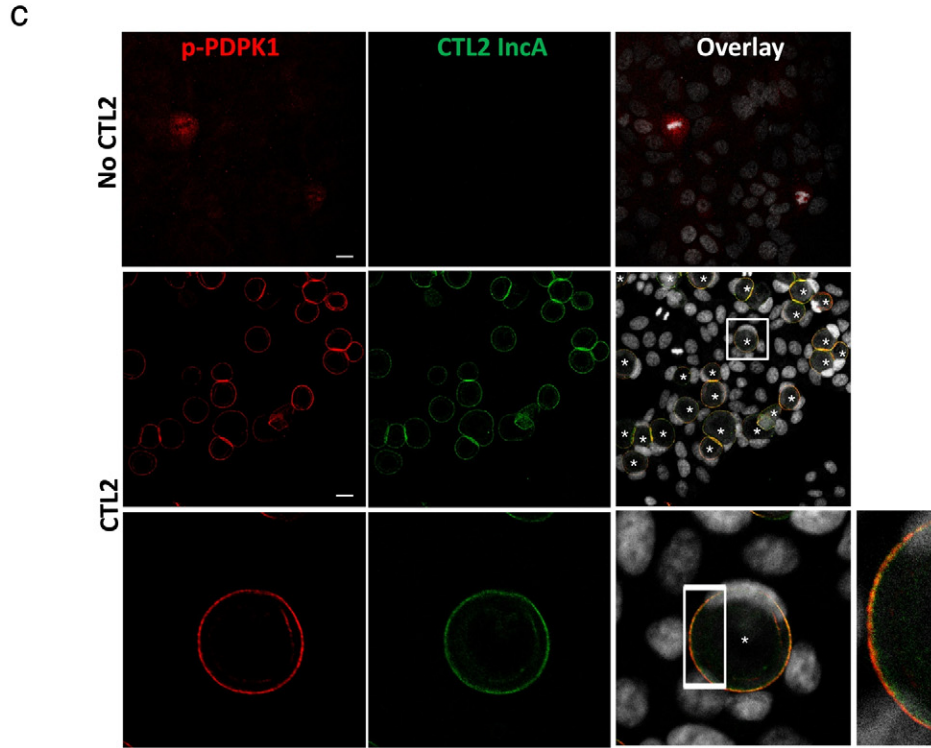
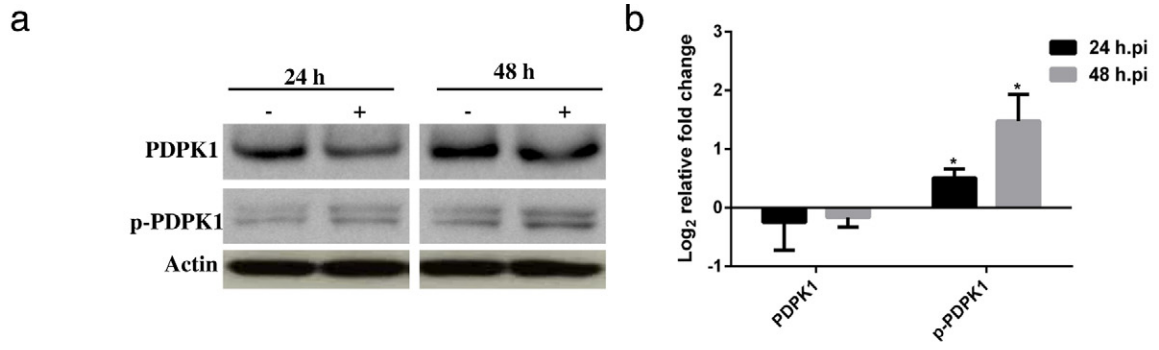
and HKII upon infection (Fig. 4e). Similar results were obtained with human primary MSCs and human fallopian tube organoids, respectively, infected with CTL2 for 48 h (Supplementary Fig. S3d and e). In line with these findings, the expression of PDHK1 in infected cells was upregulated in a MYC-dependent manner (Fig. 4d). The induction of HKII expression after infection was confirmed to be MYC-dependent using specific RNA interference oligonucleotides against HKII and MYC (Fig. 4f). Knockdown of HKII or MYC using RNAi abrogated bacterial replication (Fig. 4g), indicating that MYC-induced HKII expression in infected cells is essential for bacterial development.

#### 3.5. Chlamydia trachomatis Infection Enhances HKII-Mitochondria Interaction to Block Apoptosis

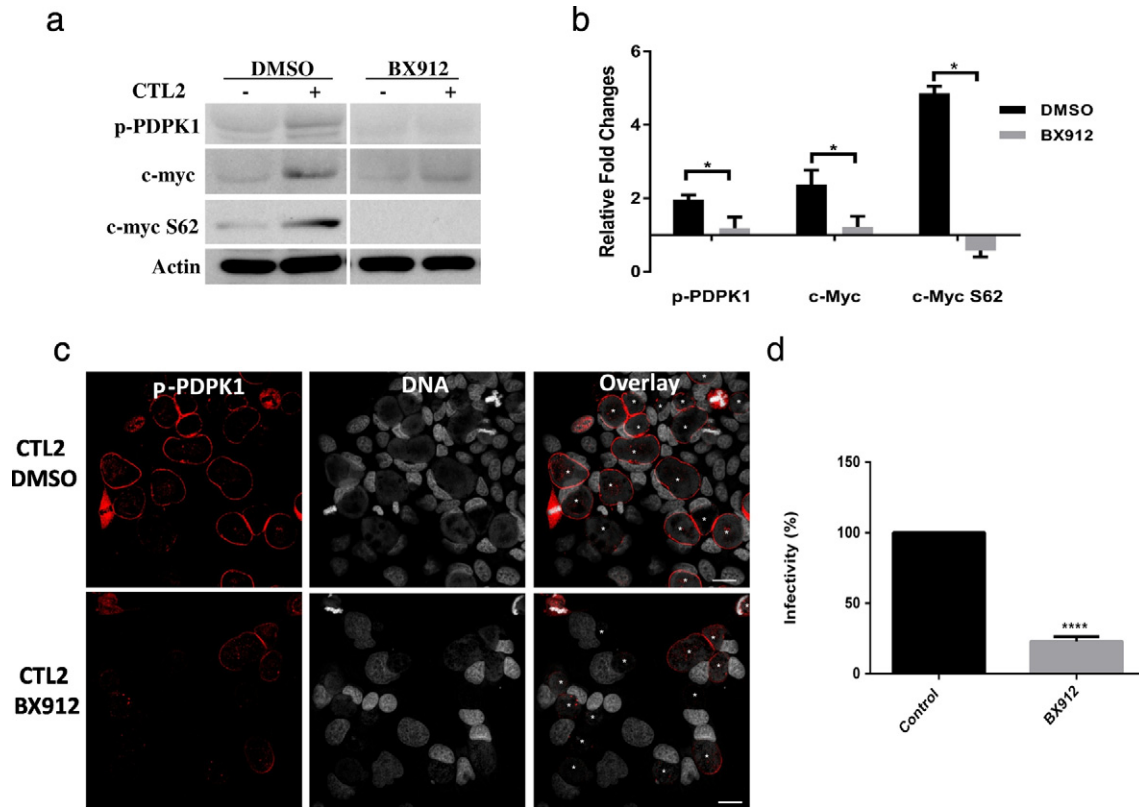
Cell survival relies on the prevention of apoptosis largely mediated by pathways related to PI3K signaling (Franke et al., 2003; Kandel and Hay, 1999), coupled to metabolism and the requirement of glucose availability (Danielsen et al., 2015; Vander Heiden et al., 2001). As HKII inhibits apoptosis by binding mitochondrial VDAC (Vyssokikh and Brdiczka, 2003), we examined its localization in HeLa cells during CTL2 infection by immunoblotting of cytosolic and mitochondrial fractions, using VDAC as a mitochondrial marker. In infected cells, HKII was significantly enriched and localized at mitochondria different from non-infected cells (Fig. 5a and b). To examine the role of the HKII-mitochondria association for chlamydial infection we used clotrimazole, a drug that efficiently dissociates HKII from mitochondria in several cell models (Pastorino et al., 2002). Clotrimazole had no apparent toxic effect on host cells, as measured by LDH assay, Supplementary Fig. S3a. However, a 24 h clotrimazole treatment initiated 24 h p.i. abrogated chlamydial replication in a concentration-dependent manner, as determined by progeny assay (Fig. 5c). A competitive peptide of HKII (N-HKII) that corresponds to the N-terminal hydrophobic domain has recently been shown to effectively displace HKII from mitochondria (Majewski et al., 2004). Treatment of MSCs with N-HKII during 24 h CTL2 infection significantly inhibited bacterial replication, as determined by progeny assay (Supplementary Fig. S4a). Yet, N-HKII peptide treatment had no toxic effect on host cells according to the LDH assay (Supplementary Fig. S3a).

To determine whether the observed HKII-mitochondria association contributes to apoptosis resistance of CTL2-infected cells, HeLa cells were treated with N-HKII, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and cycloheximide for 5 h, starting at 24 h p.i. Cell lysates were then examined by immunoblotting using antibodies directed against cleaved caspase-3 and cleaved poly-(ADP-ribose) polymerase (PARP). Whilst CTL2 infection alone conferred a significant resistance to TNF- $\alpha$ -induced apoptosis, inhibition of the HKII-mitochondria association led to a complete reversal of this phenotype (Fig. 5d). Similar results were obtained in infected primary cells treated with N-HKII (10  $\mu$ M), TNF- $\alpha$ , (50 ng/ml) and cycloheximide (10  $\mu$ g/ml) from 24 h p.i. for 8 h (Supplementary Fig. S4b). Finally, we used a specific antibody against cleaved caspase-3 as an alternative readout for apoptosis (Fig. 5e). HeLa cells infected for 24 h p.i. were treated with 20  $\mu$ M N-HKII, 50 ng/ml TNF- $\alpha$  and 10  $\mu$ g/ml cycloheximide for 5 h. Although CTL2 infection alone conferred resistance to TNF- $\alpha$ -induced apoptosis, inhibiting the HKII-mitochondria interaction with N-HKII peptide was sufficient to reverse this phenotype (Fig. 5e and f) and induce apoptosis in over 90% of cells. Collectively, our data show that the CTL2-induced apoptosis resistance in host cells depends on the association of HKII with mitochondria.

**Fig. 2.** CTL2 induces PDPK1 phosphorylation downstream of PI3K. a) Western blot showing an increase of PDPK1 phosphorylated at Ser-241 compared to total PDPK1 at 24 and 48 h p.i. in CTL2-infected (MOI 1) whole cell lysates.  $\beta$ -actin served as loading control. b) The band densities from a) were quantified and normalized to corresponding band densities of the  $\beta$ -actin loading control. Alterations in expression levels compared to non-infected controls are represented as mean fold change  $\pm$  SEM, \* $p < 0.05$ ,  $t$ -test,  $n = 3$ . c) CTL2 infection increases levels of PDPK1 phosphorylated at Ser-241, which is recruited as a rim-like structure at the inclusions in HeLa cells. PDPK1 phosphorylated at Ser-241, IncA and nuclei were labelled with Cy3-conjugated phosphorylated PDPK1 and IncA antibodies and DAPI. Scale bar: 30  $\mu$ m. Asterisks are CTL2 inclusions. d) Monolayers of fallopian tube mesenchymal stem cells, infected with CTL2 for 48 h p.i. (MOI 0.5) were labelled with antibodies against *Chlamydia trachomatis*, PDPK1 phosphorylated at Ser-241 and DAPI. Results were similar to c). Scale bar: 30  $\mu$ m. e) Human primary fallopian tube epithelial organoids, infected with CTL2 for 48 h p.i. were labelled with antibodies against *Chlamydia trachomatis*, PDPK1 phosphorylated at Ser-241 and E-Cadherin. Results were similar to c). Scale bar: 30  $\mu$ m.







**Fig. 3.** PDK1 activation precedes MYC stabilization and is essential for the formation of infectious progeny. a) Western blot showing reduced levels of phosphorylated PDPK1, total MYC and MYC phosphorylated at Ser-62 in whole cell lysates prepared from cells infected with CTL2 for 48 h (MOI 1) and treated with the inhibitor BX912 (10  $\mu$ M) 12 h p.i. for 36 h.  $\beta$ -actin served as loading control. b) The band densities from a) were quantified and normalized to corresponding band densities of the  $\beta$ -actin loading control. Alterations in expression levels compared to non-infected controls are represented as mean fold change  $\pm$  SEM, \* $p < 0.05$ ,  $t$ -test,  $n = 3$ . c) Treatment with BX912 (10  $\mu$ M) 12 h p.i. for 36 h reduces levels of phosphorylated PDPK1 in HeLa cells infected with CTL2 (MOI 0.5). Phosphorylated PDPK1 and nuclei were labelled with anti-phosphorylated PDPK1 antibody and DAPI. Scale bar: 30  $\mu$ m. d) HeLa cells were infected with CTL2 (MOI 0.5), treated with BX912 (10  $\mu$ M) from 12 h p.i. and the generation of infectious EBs determined by infectivity assay at 48 h. p.i. Results are depicted as mean  $\pm$  SD normalized to controls of three independent experiments;  $p < 0.0001$ ,  $t$ -test.

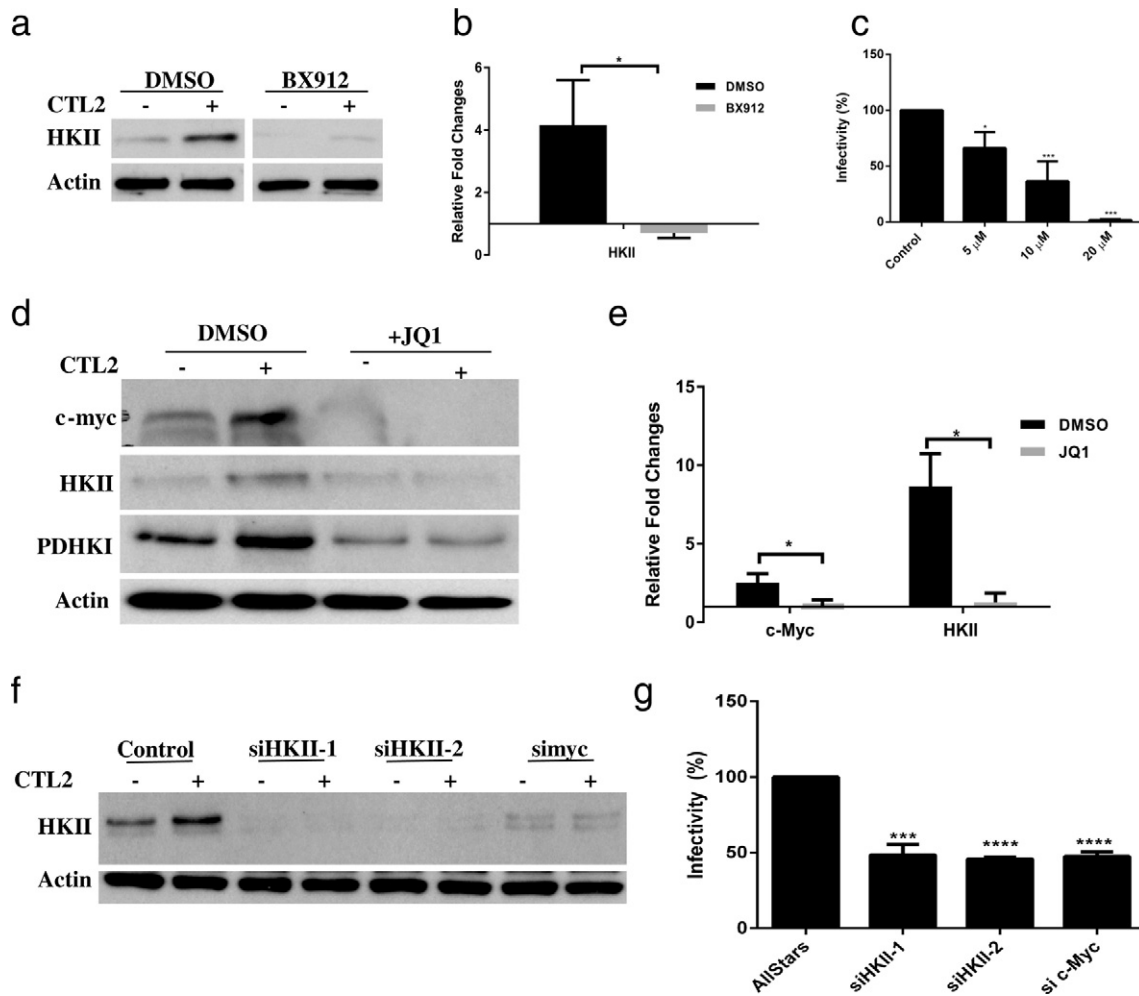
#### 4. Discussion

Here we demonstrate a functional link between PDPK1 and HKII. We show that PDPK1-MYC signaling is activated in cells infected with CTL2 and that this activation is essential for increased expression of HKII and its mitochondrial association. Interestingly, forced dissociation of HKII from mitochondria rendered cells sensitive to apoptosis, and blocking of the pathway at the level of PDPK1, MYC, or HKII led to impaired bacterial growth. Evidently, in order to replicate productively, CTL2 not only has to keep host cells alive but also needs to reprogram their metabolism to secure sufficient nutrients for its growth. In healthy tissues, induction of aerobic glycolysis and inactivation of cell cycle checkpoints occur in rapidly proliferating cells and depend on growth factor signaling. However, a limited supply of glycolytic substrates or insufficient generation of ATP to meet the cell's energy requirements will trigger apoptosis (Izzyumov et al., 2004; Okoshi et al., 2008). Under the condition of cellular transformation the oncogenic kinase PDPK1 has been found to be overexpressed and hyperactivated (Fyffe and Falasca, 2013). Our analyses uncover a startlingly similar phenotype of CTL2-infected cells in that infection activated two intrinsically oncogenic proteins, PDPK1 and MYC.

The PI3K pathway plays a central role in regulating cell survival (Kennedy et al., 1997; Khwaja et al., 1997) and is considered to be the main signaling cascade associated with the ability of *Chlamydia* to inhibit apoptosis induction by various stimuli, including staurosporine, TNF- $\alpha$ , or etoposide (Verbeke et al., 2006). *Chlamydia trachomatis*-infected cells also exhibit increased levels of HIF-1 $\alpha$  and subsequently Mcl-1, which blocks apoptosis at the mitochondrial level (Sharma et al., 2011). Here we show that an important signaling component of the

PI3K pathway, PDPK1, promotes apoptosis resistance via MYC stabilization through phosphorylation at Ser-62. PDPK1 is a master regulator with multiple roles in metabolism, proliferation and cell survival (Raimondi and Falasca, 2011). MYC stabilization in turn leads to upregulation of HKII. The expression level and location of HKII are crucial for a proper balance of metabolism and cell survival. The mitochondria-HKII association favorably influences mitochondrial energetics and cell survival by preventing pro-apoptotic Bak and Bax oligomerization and binding at the level of the mitochondrion (Robey and Hay, 2006). It is also critical for preventing cytochrome *c* release and apoptosis, which is regulated by the PI3K cascade (Kennedy et al., 1999). Recently, it has been shown that *Chlamydia* preserves mitochondrial integrity through upregulation of miR-30c, which downregulates p53 to ensure its normal development inside the host (Chowdhury et al., 2017). Our previous observation that restoring p53 levels in infected cells with Nutlin3a treatment was unable to fully re-sensitize cells to apoptotic stimuli (Gonzalez et al., 2014) strongly suggests that additional mechanisms are employed by the pathogen to suppress apoptosis. In support of this, we found that chemically removing HKII from mitochondria had a severe impact on pathogen replication and fully re-sensitized infected cells to TNF- $\alpha$  induced apoptosis.

This observation also has broader implications. Activation of PDPK1-MYC-HKII signaling during CTL2 infection is likely to play a direct role in metabolic reprogramming. MYC regulates several other genes involved in glucose metabolism, in particular glucose transporter 1 (Osthus et al., 2000). HKII plays a critical role in maintaining the gradient required for glucose entry into the cell by catalyzing the first step in glucose metabolism to generate glucose-6-phosphate (Dang et al., 2008). This critical metabolite can serve as



**Fig. 4.** MYC-HKII axis is crucial for bacterial development. a) Western blotting analysis showing that in HeLa cells infected with CTL2 (MOI 1) and treated with BX912 (10  $\mu$ M) for 36 h, induction of total HKII protein expression is reduced compared to control cells. Samples were collected at 48 h p.i.,  $\beta$ -actin served as loading control. b) The band densities from a) were quantified and normalized to corresponding band densities of the  $\beta$ -actin loading control. Alterations in expression levels compared to non-infected controls are represented as mean fold change  $\pm$  SEM, \* $p$  < 0.05,  $t$ -test,  $n$  = 3. c) HeLa cells were infected with CTL2 (MOI 0.5) treated with different doses of JQ1 from 10 h p.i. and the generation of infectious EBs determined via infectivity assay. Results are depicted as mean percentage  $\pm$  SD normalized to controls of three independent experiments. \* $p$  < 0.05 \*\*\* $p$  < 0.001, one-way ANOVA, Dunnett's multiple comparison test. d) Western blot showing that in HeLa cells infected with CTL2 (MOI 1) HKII and MYC protein levels are significantly increased by 48 h p.i. compared to control cells. This upregulation was significantly inhibited by JQ1 (20  $\mu$ M for 44 h).  $\beta$ -actin served as loading control. e) The band densities from d) were quantified and normalized to corresponding band densities of the  $\beta$ -actin loading control. Alterations in expression levels compared to non-infected controls are represented as mean fold change  $\pm$  SEM, \* $p$  < 0.05,  $t$ -test,  $n$  = 3. f) Western blot showing reduced induction of total MYC and HKII protein expression in HeLa cells transfected with siRNA specific for MYC or HKII, compared to luciferase transfected CTL2-infected (MOI 1) whole cell lysates. Samples were collected 48 h p.i.,  $\beta$ -actin served as loading control. g) HeLa cells transfected as shown in f) infected with CTL2 (MOI 1) were examined for generation of infectious EBs by infectivity assay. Results are depicted as mean  $\pm$  SD normalized to controls of three independent experiments; \*\*\* $p$  < 0.001, \*\*\*\* $p$  < 0.0001, one-way ANOVA, Dunnett's multiple comparison test.

a biosynthetic precursor or for lactate production by aerobic glycolysis, as well as for nucleotide biosynthesis by PPP. Enhanced glucose uptake and lactate production has indeed been observed in *Chlamydia trachomatis*-infected cells (Ojcius et al., 1998). Further, enhanced expression of glucose-6-phosphate dehydrogenase, the key enzyme of the PPP, has been shown to be a central element in the chlamydial life cycle that reprograms the host cell metabolism in support of bacterial development (Gehre et al., 2016; Siegl et al., 2014). In congruence, our results show that increased mitochondrial HKII is required for CTL2 to complete its life-cycle.

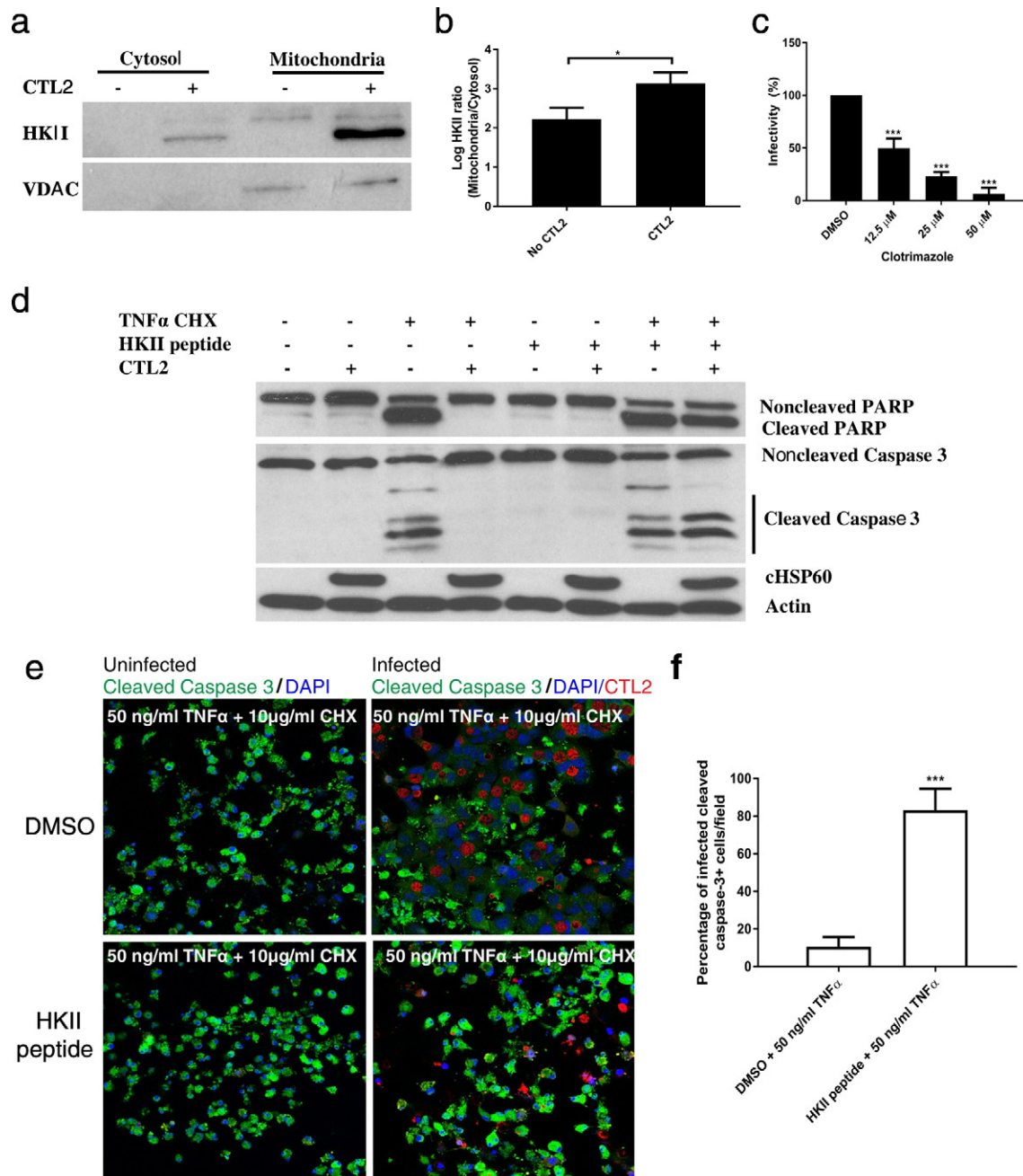
In addition, Akt, another kinase required for apoptosis inhibition during *Chlamydia trachomatis* infection (Verbeke et al., 2006), converges onto MYC signaling during metabolic reprogramming, by enhancing the activity of both HKII and glucose transporters (Robey and Hay, 2006; Wieman et al., 2007). This suggests that activation of PDK1 during *Chlamydia trachomatis* infection acts as a master switch modulating Akt (Verbeke et al., 2006), p53 (Gonzalez et al., 2014;

Siegl et al., 2014), and HKII, to ensure host cells are able to meet the bacteria's metabolic requirements without triggering apoptosis.

The fact that *Chlamydia trachomatis* infection leads to degradation of the master tumor suppressor p53 is particularly poignant in the context of MYC stabilization, as under normal conditions balance of these two pathways is crucial for preventing tumorigenesis, and p53 normally represses MYC transcription (Sachdeva et al., 2009). They constitute two of the most significant tumor related genes in the human genome, and are increasingly linked to effects on host metabolism and cell survival. Inhibition of p53 is known to enhance the Warburg metabolism, characteristic of rapidly proliferating and cancer cells – directing metabolic pathways to aerobic glycolysis and the PPP – both of which are beneficial also for *Chlamydia trachomatis* growth (Eisenreich et al., 2013; Siegl et al., 2014).

Several therapeutic inhibitors have been developed to counteract the fateful role of deregulated MYC activation in human cancers (Stine et al., 2015). Use of the small molecule bromodomain inhibitor JQ1,





**Fig. 5.** CTL2 enhances HKII-mitochondria association to induce anti-apoptotic effect. a) Western blot of mitochondrial and cytosolic fractions from infected cells and controls showing that in infected cells with CTL2 (MOI 1) HKII protein levels are markedly increased by 48 h p.i. and translocated into the mitochondria compared to control cells. VDAC served as loading control for the mitochondrial fraction. b) HKII expression levels (cytosolic and mitochondria fractions) were quantified from infected and non-infected cells. Log HKII expression (mitochondria/cytosolic) from infected and non-infected controls are represented as mean fold change  $\pm$  SEM, \* $p$  < 0.05,  $t$ -test,  $n$  = 3. c) HeLa cells were infected with CTL2 (MOI 1) treated with different doses of clotrimazole from 24 h p.i. and the generation of infectious EBs determined via infectivity assay at 48 h p.i. Results are depicted as mean percentage  $\pm$  SD normalized to controls of three independent experiments; \*\*\* $p$  < 0.001, one-way ANOVA, Dunnett's multiple comparison test. d) The extent of apoptosis induced by TNF- $\alpha$  treatment was analyzed by western blotting using antibodies to cleaved PARP and caspase 3 in HeLa cells infected with CTL2 (MOI 1). Infected cells showed greatly reduced levels of cleaved PARP and caspase 3. Treatment with a peptide inhibitor of HKII (20  $\mu$ M) for 5 h fully sensitized cells to the levels seen in non-infected control cells.  $\beta$ -actin served as loading control. e) HeLa cells infected with CTL2 (MOI 1) and immunolabelled for cleaved caspase-3 (green) showed markedly fewer positive cells upon TNF- $\alpha$  treatment. HKII peptide inhibitor treatment (20  $\mu$ M) for 5 h largely restored the number of cleaved caspase-3-positive cells. CTL2 inclusions and nuclei were labelled with anti-*Chlamydia trachomatis* antibody (red) and Hoechst (blue). f) Quantitative analysis of the number of cleaved caspase-3-positive nuclei per image (12 images) in infected HeLa cells treated with HKII peptide inhibitor during apoptosis induction with TNF- $\alpha$  and cycloheximide, shows that HKII peptide inhibitor significantly increases the number of apoptotic nuclei in infected cell populations. Results are depicted as mean  $\pm$  SD; \*\*\* $p$  < 0.001,  $t$ -test,  $n$  = 3.

which decreases MYC expression, has been shown to be effective in inhibiting cancer cell viability (Jung et al., 2015). In our setting, genetic and pharmacological means to inhibit MYC and PDPK1 also block chlamydial replication, suggesting that infected cells may be susceptible to therapies targeting the PDPK1-MYC axis via inhibition of the major

metabolic regulator HKII. The finding that CTL2 activates the PDPK1-MYC-HKII axis in host cells increases our understanding of how CTL2 survives in its intracellular niche despite its reduced genome size and how it prevents the host cell from outstripping its nutrient supply as infection progresses.

## Conflicts of Interest

The authors declare no conflict of interest.

## Author Contributions

M.A.Z and A.X conducted Fig. 1a, c and d. M.A.Z conducted the immunofluorescence for Fig. 1b. M.A.L contributed to the western immunoblotting for Fig. 1c. M.K prepared the samples for Fig. 1d. M.A.Z conducted Figs. 2, 3, 4, and 5 and Supplementary Figs S1 and S3. M.A.Z and A.X conducted Supplementary Fig. S2. M.A.Z wrote the manuscript. T.F.M. supervised the project and the manuscript preparation.

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## Appendix A. Supplementary Data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2017.08.005>.

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