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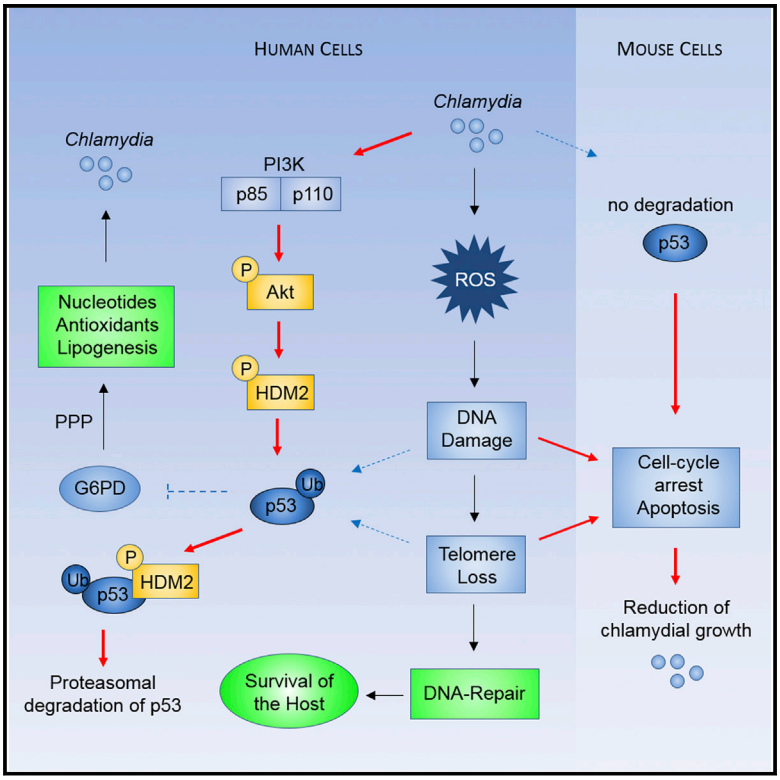
Tumor Suppressor p53 Alters Host Cell Metabolism to Limit *Chlamydia trachomatis* Infection

Graphical Abstract



Highlights

Chlamydia induces degradation of the tumor suppressor p53

Stabilization of p53 severely impairs chlamydial development

Degradation of p53 unleashes the pentose phosphate pathway for metabolic support

G6PD expression overcomes the DNA damage response and rescues chlamydial growth

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In Brief

Nutrient supply from the host cell is essential for the survival of obligate intracellular bacteria. The tumor suppressor p53 plays a crucial role in host cell metabolism. Siegl et al. now highlight the role of p53 in host cell defense during infection by *Chlamydia* and show how this pathogen counteracts p53 for survival.

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Tumor Suppressor p53 Alters Host Cell Metabolism to Limit *Chlamydia trachomatis* Infection

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SUMMARY

Obligate intracellular bacteria depend entirely on nutrients from the host cell for their reproduction. Here, we show that obligate intracellular *Chlamydia* downregulate the central tumor suppressor p53 in human cells. This reduction of p53 levels is mediated by the PI3K-Akt signaling pathway, activation of HDM2, and subsequent proteasomal degradation of p53. The stabilization of p53 in human cells severely impaired chlamydial development and caused the loss of infectious particle formation. DNA-damage-induced p53 interfered with chlamydial development through downregulation of the pentose phosphate pathway (PPP). Increased expression of the PPP key enzyme glucose-6-phosphate dehydrogenase rescued the inhibition of chlamydial growth induced by DNA damage or stabilized p53. Thus, downregulation of p53 is a key event in the chlamydial life cycle that reprograms the host cell to create a metabolic environment supportive of chlamydial growth.

INTRODUCTION

Chlamydia trachomatis is the cause of trachoma, the leading infectious blinding disease worldwide, and the predominant sexually transmitted bacterial pathogen that causes pelvic inflammatory disease and ectopic pregnancy. *Chlamydia* have a unique biphasic developmental life cycle consisting of infectious, nonreplicative elementary bodies (EBs) and noninfectious, replication-competent reticulate bodies (RBs). Sequence analysis of *Chlamydia*'s small (~1.04 Mb) genome has revealed that numerous metabolic pathways in *C. trachomatis* are incomplete or entirely missing (Stephens et al., 1998). *Chlamydia* overcome their reduced metabolic capacity by taking up nutrients such as nucleotides, amino acids, and lipids from intact and living host cells (Saka and Valdivia, 2010) during their entire developmental cycle.

As part of its intracellular lifestyle, *Chlamydia* actively interferes with host cell apoptosis (Fan et al., 1998) to protect its replication niche. These antiapoptotic strategies prevent cell death induced by death receptor activation, diverse cellular stresses (Fan et al., 1998), and double-stranded RNA, which imitates viral infection (Böhme et al., 2010). Since defective apoptosis signaling is a hallmark of cancer cells and *Chlamydia* infection has been connected to ovarian cancer (Shanmughapriya et al., 2012), it has been hypothesized that *Chlamydia*-induced inhibition of apoptosis may support the survival of damaged cells that are prone to transform into cancer cells.

Several bacteria have been shown to cause damage to host cell DNA during infection (Bergounioux et al., 2012; Elsen et al., 2013; Nougayrède et al., 2006; Toller et al., 2011; Vielfort et al., 2013). One recent report demonstrated the induction of DNA double-strand breaks (DSBs) during *Chlamydia* infection (Chumduri et al., 2013). Interestingly, despite severe DNA damage, a normal DNA damage response, including repair, is not observed in *Chlamydia*-infected cells due to inhibition of the recruitment of pATM and p53BP to damaged DNA.

Here, we investigated the role of the tumor suppressor p53 during *Chlamydia* infections and its impact on the DNA damage response. As DSBs predominantly occurred at chromosomal ends, causing telomere shortening, host cells were subjected to cell-cycle arrest or senescence. However, *Chlamydia* infection induced the degradation of p53 and repair of damaged DNA in primary human cells. Depletion of p53 in infected cells prevented repression of the pentose phosphate pathway (PPP), which is responsible for DNA damage repair, and ensured the provision of metabolites that are essential for chlamydial growth. Our data provide a link to a p53-dependent antibacterial mechanism that limits the metabolite resources required for the survival of obligate intracellular bacteria and promotes host cell death in response to infection-induced oxidative stress and genotoxicity.

RESULTS

p53 Is Downregulated in *Chlamydia*-Infected Human Cells

Chlamydia infection induces DSBs (Chumduri et al., 2013) and transient telomere shortening (Prusty et al., 2013). Upon detection of single-strand breaks, DSBs, or telomere shortening, the tumor suppressor p53 is stabilized and induces cell-cycle arrest and the DNA damage response, cellular senescence, or apoptosis (Bálint and Vousden, 2001). In the presence of a

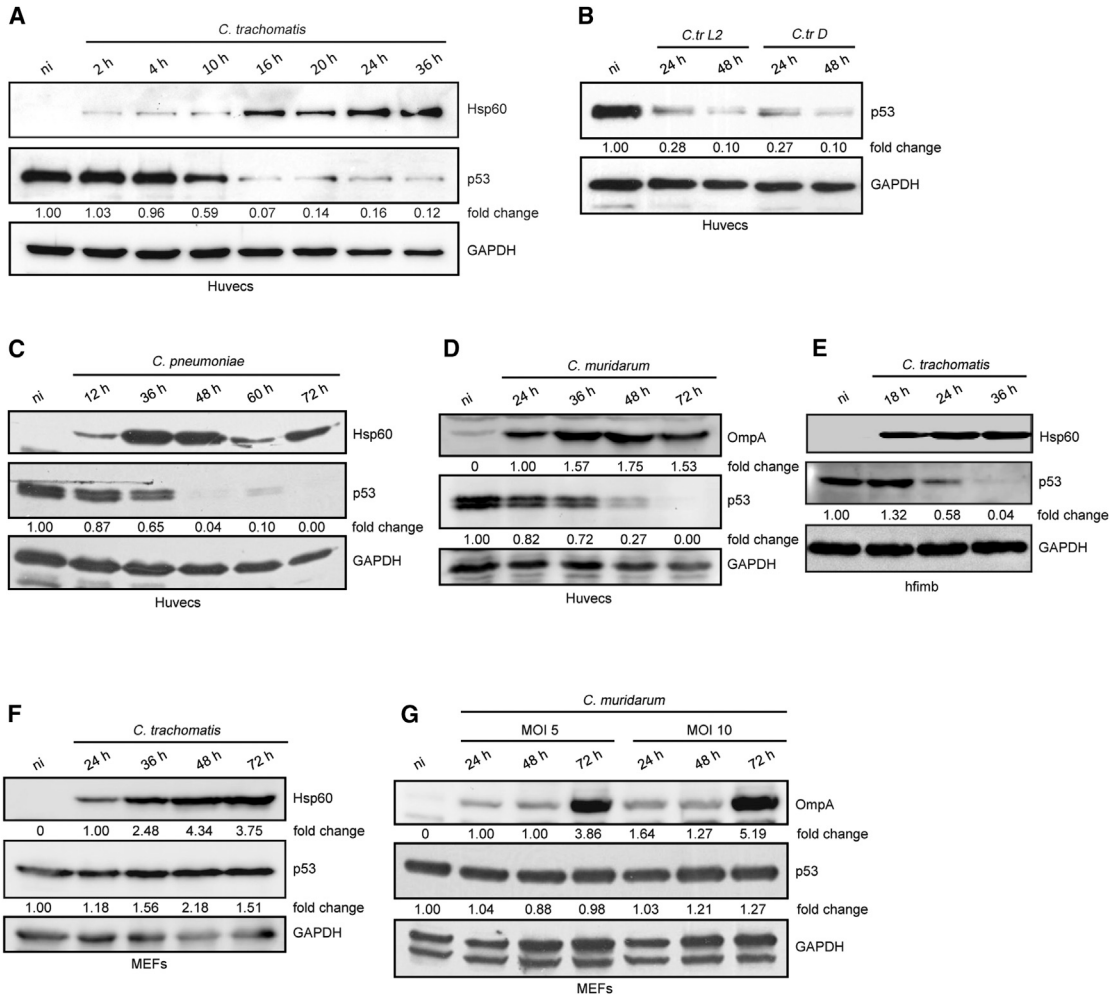


Figure 1. p53 Is Downregulated in *Chlamydia*-Infected Human Cells

(A) HUVECs were infected with *C. trachomatis* for different time periods, and p53 and chlamydial Hsp60 were detected by immunoblotting. GAPDH was used as a loading control. Fold-change values of p53 were derived by normalization to GAPDH and are indicated below the blots.

(B and C) HUVECs were infected with *C. trachomatis* serotype L2 and serotype D (B) and *C. pneumoniae* (C).

(D) HUVECs were infected with *C. muridarum* for different time periods and p53 was detected by immunoblotting.

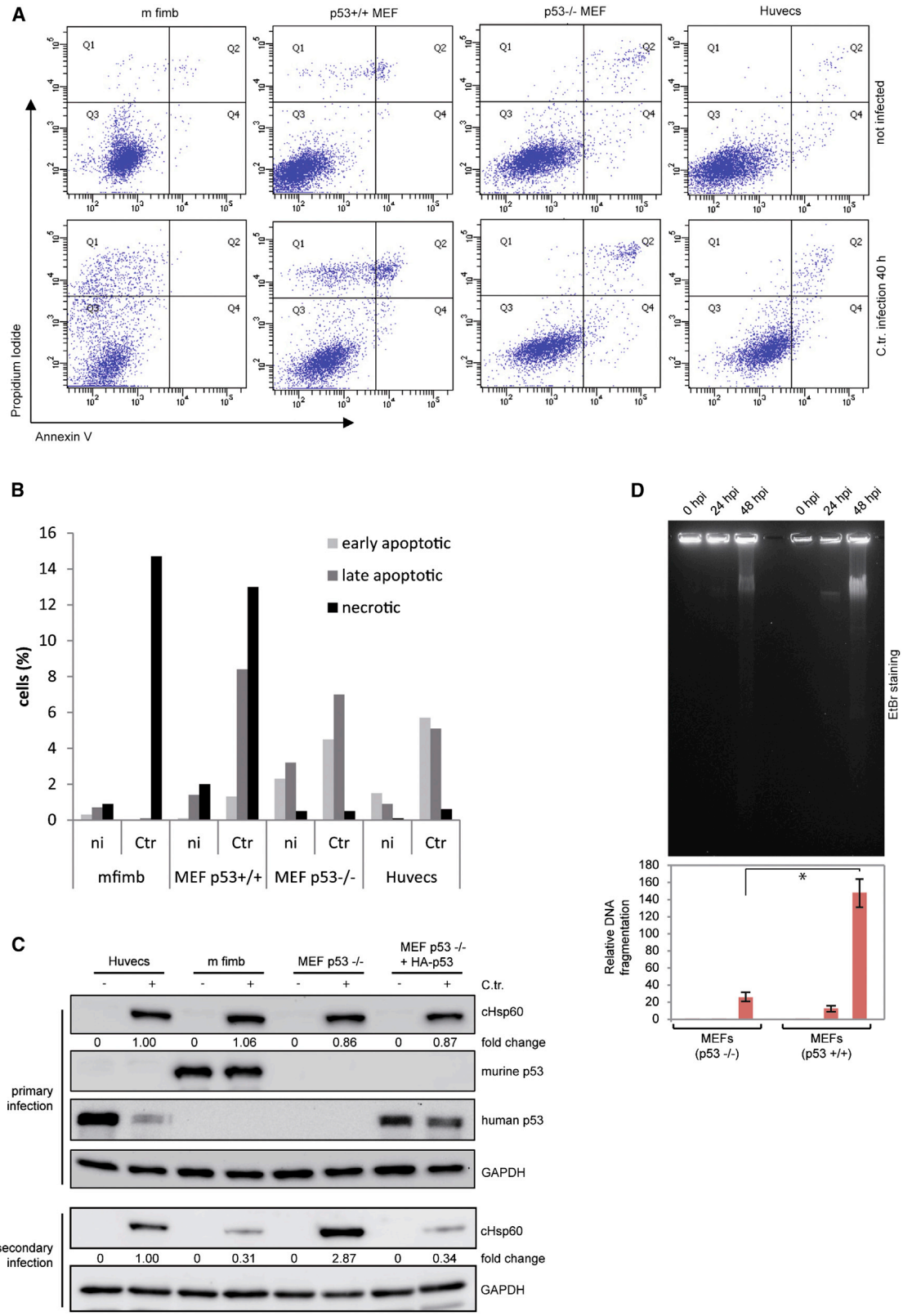
(E) Epithelial cells of human fallopian tube fimbriae (hfimb) were infected with *C. trachomatis* for different time periods and p53 was detected by immunoblotting.

(F) p53 is not downregulated in MEFs. MEFs were infected with *C. trachomatis* for different time periods and processed as described above.

(G) MEFs were infected with *C. muridarum* for different time periods and p53 was detected by immunoblotting.

Chlamydia infection, even high doses of the DNA-damage-inducing drug etoposide could not induce apoptosis (Figure S1A). Etoposide-induced apoptosis is p53 dependent and results in outer mitochondrial membrane permeabilization (Green and Kroemer, 2009). Since *Chlamydia* was clearly able to prevent this, we suspected manipulation of p53-induced signaling pathways.

We first monitored p53 stabilization in primary human umbilical vein endothelial cells (HUVECs) after *C. trachomatis* infection. Astonishingly, p53 levels decreased around 10–16 hr postinfection (Figure 1A). Heat inactivation of *Chlamydia* prior to infection prevented the reduction of p53 levels (Figure S1B), demonstrating that live bacteria, and not the sensing of pathogen-associated molecular patterns such as bacterial lipopolysaccharides, are required for p53 downregulation. *Chlamydia* infection also reduced p53 in HeLa cells, which, in contrast to most tumor cells, express wild-type p53 (Figure S1C). Downregulation of p53 is a general consequence of *Chlamydia* infection, since it is also observed in cells infected with a different *C. trachomatis* serovar (Figure 1B), the human pathogenic species *C. pneumoniae* (Figure 1C), and the mouse-specific strain *C. muridarum* (Figure 1D). To confirm that *C. trachomatis* induced p53 downregulation in naturally infected tissues, we prepared epithelial cells from biopsies of human fimbriae of the fallopian tube (hfimb) and monitored p53 levels upon chlamydial infection. p53 was strongly reduced in these primary cells (Figure 1E). Concomitantly with reduced p53 protein levels, DNA binding of p53, which is indicative of its transcriptional activity, decreased during infection in a time-dependent manner (Figure S1D). Additionally, we infected murine embryonic fibroblasts (MEFs) with



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C. trachomatis and *C. muridarum* to test whether downregulation of p53 during *Chlamydia* infection was species specific or a general feature that was independent of the host. Interestingly, p53 levels remained unchanged in MEFs infected with *C. trachomatis* (Figure 1F) or *C. muridarum* (Figure 1G), demonstrating that the fate of p53 differs fundamentally between infected human and mouse cells.

Chlamydia Infection Induces p53-Dependent Cytotoxicity in Mouse Cells

To examine a possible outcome of the differential regulation of p53 in primary human and mouse cells, we analyzed the viability of HUVECs, MEFs ($p53^{+/+}$ or $p53^{-/-}$), and (as a second mouse cell line in addition to MEFs) epithelial cells of murine fimbriae (mfimb) during *C. trachomatis* infection (Figures 2A and 2B). We observed an increased number of necrotic MEFs and mfimb compared with HUVECs after *C. trachomatis* infection. In contrast to this, almost no necrotic cells were found in $p53^{-/-}$ MEFs. To verify that increased cytotoxicity during *C. trachomatis* infection of murine cells results in reduced bacterial infection, we infected mfimb, $p53^{-/-}$ MEFs, and $p53^{-/-}$ MEFs transfected with hemagglutinin (HA)-tagged p53, as well as HUVECs, with *C. trachomatis* for 2 days, and used lysates of these cells for reinfection (Figure 2C). Under these conditions, only *Chlamydia* that passed the developmental cycle will reinfect and allow progeny quantification (hereafter referred to as infectivity assay). Interestingly, chlamydial progeny formation was increased in $p53^{-/-}$ MEFs compared with chlamydial infectivity in mfimb and $p53^{-/-}$ MEFs transfected with HA-p53 (Figure 2C). In addition to the infectivity assay, one-step growth curves were generated to characterize the number of inclusion-forming units and the infectivity rate of *C. trachomatis* and *C. muridarum* in HUVECs, $p53^{+/+}$ MEFs, and $p53^{-/-}$ MEFs. Again, fewer chlamydial progeny were recovered from *C. trachomatis*- and *C. muridarum*-infected $p53^{+/+}$ MEFs compared with $p53^{-/-}$ MEFs (Figure S1E). Based on these results, we hypothesized that p53 plays an important role during *Chlamydia* infection and is responsible for restricted infection in mouse cells.

Since increased infection-induced cytotoxicity was clearly linked to p53 in mouse cells, we next investigated DNA damage caused by *Chlamydia* infection in more detail. We developed an assay, which we call the neutral DNA break (NDB) assay (see Supplemental Experimental Procedures), that allows one to differentiate between bacterial and host cell DNA and thus is suitable for characterizing the type of DNA damage that has occurred. Since chromosomal and high-molecular-weight chlamydial DNA does not migrate into the gel, only extrachromosomal and broken DNA becomes separated. Interestingly, we detected in all cells a single prominent DNA fragment that migrated into the gel and whose intensity increased in etoposide-treated or *Chlamydia*-infected cells (Figures S2A and S2B), suggesting that this band represents a major DNA-damage-associated cleaved product of the human genome. Southern hybridization identified this band as telomeric DNA, which is also present in minute amounts as extrachromosomal telomeric DNA in most normal cells (Wang et al., 2004). Interestingly, the telomeric DNA break became prominent only after 24 hr postinfection (hpi) in HeLa cells (Figure S2A) and even later (36 hpi), but visibly stronger, in MEFs (Figure S2B). Telomere restriction fragment (TRF) analysis validated the results of the NDB assay in both cell types (Figures S2C and S2D). We observed complete but transient loss of telomeres in HeLa cells infected with *C. trachomatis* (Figure S2C, left panel) or *C. pneumoniae* (Figure S2C, right panel). Telomeres were lost in MEFs upon *C. trachomatis* infection and were not repaired at all (Figure S2D, left panel). A similar effect was observed upon *C. muridarum* infection of MEFs (Figure S2D, right panel), indicating that the lack of telomere repair depends on the cell type and not on the chlamydial strain. Intriguingly, we found decreased DNA damage in $p53^{-/-}$ MEFs during *Chlamydia* infection in comparison with the $p53^{+/+}$ MEFs (Figures 2D and S2E), suggesting that p53 not only functions as a downstream effector of DNA damage but also inhibits repair of damaged DNA. Increased p53-mediated cytotoxicity may also enhance DNA degradation in infected cells. Thus, we concluded that the presence of active p53, as well as severe and prolonged shortening of telomeres, might be the cause of increased cytotoxicity in *Chlamydia*-infected MEFs, suggesting that p53 downregulation plays an important role in ensuring efficient infection of the host cell.

Downregulation of p53 in Infected Cells Depends on PI3 Kinase and HDM2 Activation

C. trachomatis infection activates the PI3K pathway to stabilize the antiapoptotic Mcl-1 and cIAP-2 proteins (Rajalingam et al., 2008). However, activation of this pathway also induces enhanced degradation of p53 by targeting it for proteasomal-mediated degradation. To examine the impact of PI3K on p53 downregulation in primary HUVECs, we analyzed PI3K activity during infection by monitoring phosphorylation of the PI3K substrate Akt at serine 473 (Ser473). In line with previous studies (Rajalingam et al., 2008; Verbeke et al., 2006), *C. trachomatis* infection resulted in increased phosphorylation and activation of Akt in a multiplicity of infection (moi)- and time-dependent manner in primary cells (Figures 3A and 3B). Furthermore,

Figure 2. *Chlamydia* Infection Induces p53-Dependent Cytotoxicity in Mouse Cells

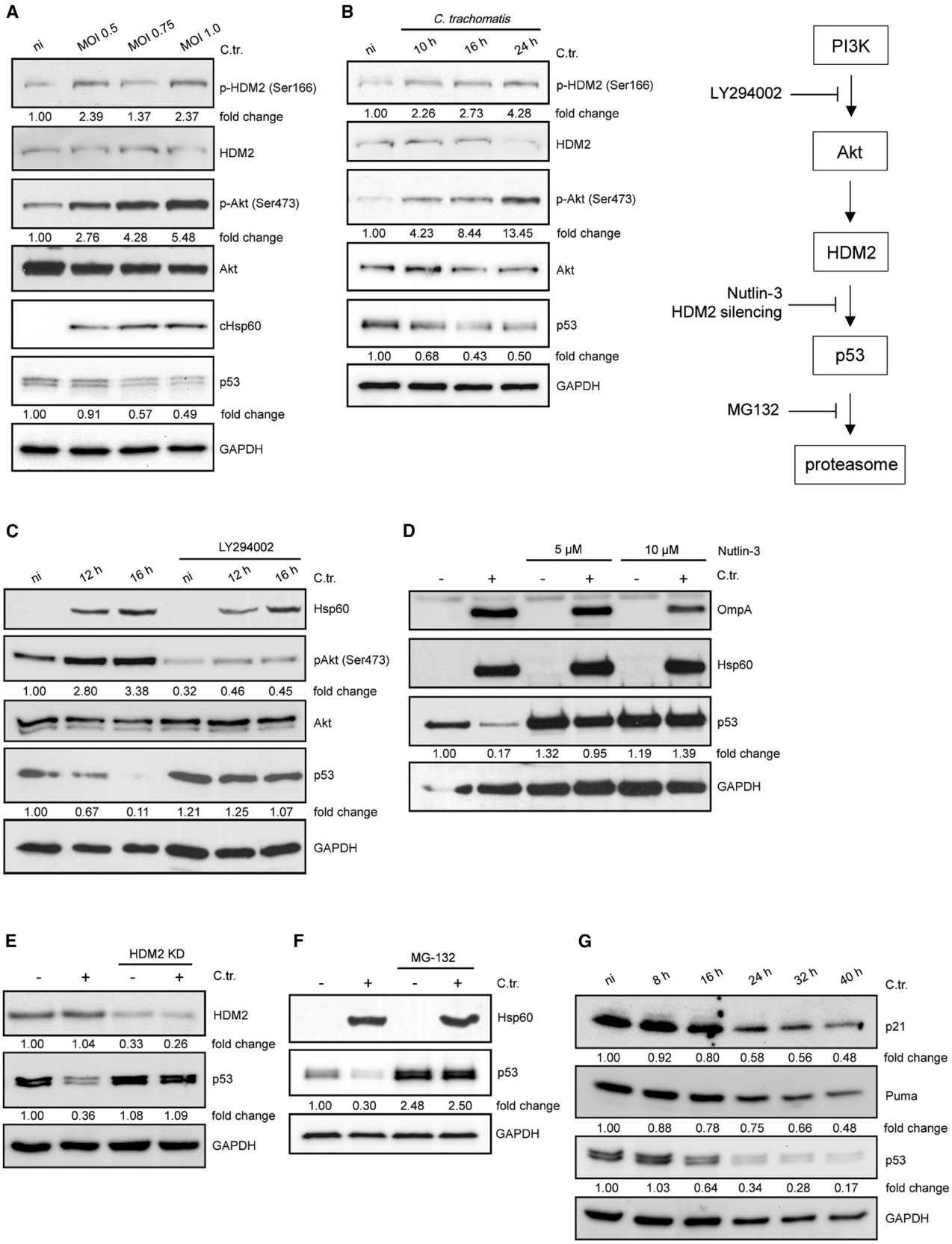
(A) *C. trachomatis* infection induces cytotoxicity in epithelial cells of murine fimbriae (mfimb) and wild-type MEFs, but not in MEFs deficient of p53. HUVECs, mfimb, $p53^{+/+}$, and $p53^{-/-}$ MEFs were infected with *C. trachomatis* for 40 hr. Annexin-V/propidium iodide staining was followed by flow-cytometric analysis.

(B) Early apoptotic cells after 40 hr of *Chlamydia* infection are displayed in Q4, late apoptotic cells are shown in Q2, and necrotic cells are shown in Q1.

(C) Cytotoxicity in MEFs reduces chlamydial infectivity. Epithelial cells of mfimb, $p53^{-/-}$ MEFs, and $p53^{-/-}$ MEFs transfected with HA-tagged p53, as well as HUVECs, were infected with *C. trachomatis* (C.tr.) for 48 hr and chlamydial development was analyzed by an infectivity assay.

(D) An NDB assay was carried out using $p53^{+/+}$ and $p53^{-/-}$ MEFs infected with *C. trachomatis* for different time periods. Gels were stained with ethidium bromide (EtBr). Fragmented DNA and DNA within the wells were quantified and used to determine the relative fragmented DNA level, presented as bar diagrams. Mean ± SEM of three independent experiments is shown; *$p < 0.05$.

See also Figures S1 and S2.



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the PI3K inhibitor LY294002 rescued p53 protein levels (Figure 3C), indicating a crucial role for the PI3K pathway in infection-induced p53 degradation. In contrast, infection of MEFs with *C. trachomatis* only slightly induced activation of PI3 kinase (Figure S3A), which explains the constant levels of p53 in infected mouse cells.

HDM2 (the human form of murine double minute 2 [MDM2]) is the main regulator of p53 protein levels and mediates p53 degradation via its E3 ligase activity, which catalyzes the ubiquitination of p53. Akt directly activates HDM2 by phosphorylation of Ser166 and Ser186 (Ogawara et al., 2002), and in agreement with this, we found increased phosphorylated HDM2 in infected cells (Figures 3A and 3B). HDM2 binds to p53 in the nucleus, mediates its export to the cytoplasm, and catalyzes the formation of ubiquitin chains on p53. To block the interaction between HDM2 and p53, we used Nutlin-3, a *cis*-imidazoline that specifically binds to a pocket in HDM2 and thereby inhibits its interaction with the N terminus of p53 (van Leeuwen et al., 2011). Application of Nutlin-3 at 10 μM resulted in a strong stabilization of p53 protein levels that could not be overcome by *Chlamydia* infection (Figure 3D). To rule out any off-target effects of Nutlin-3 and to confirm HDM2 as a target of *Chlamydia*-induced p53 degradation, we silenced HDM2 expression using small hairpin RNAs (shRNAs). Depletion of HDM2 expression interfered with p53 downregulation in infected cells as well (Figure 3E). Furthermore, infection-induced p53 downregulation was prevented by treating the cells with the proteasomal inhibitor MG-132 (Figure 3F). We confirmed the inhibition of p53 function by analyzing two important transcriptional targets of p53: the cell-cycle arrest protein p21 and the proapoptotic protein Puma. As expected, the protein levels of both p53 targets decreased during *Chlamydia* infection (Figure 3G). In addition, the RNA levels of Puma decreased after *C. trachomatis* infection (Figure S3B), consistent with a functional downregulation of p53.

Stabilization of p53 Interferes with Chlamydial Development

The results obtained so far strongly indicated that the downregulation of p53 during chlamydial infection was promoted by the pathogen rather than an antibacterial response of the host cell to limit replication of *Chlamydia.* In line with this notion, HeLa cells with stable silencing of p53 expression (HeLa-p53-KD) using shRNAs were infected with the same efficiency as the wild-type cells (HeLa-pLVTHM) (Figure S3C). Furthermore, *Chlamydia* propagated in both HeLa-pLVTHM- and HeLa-p53-KD-generated infectious EBs with the same efficiency (Figures S3D and S3E), suggesting a benefit of p53 downregulation for the pathogen rather than the host.

To investigate whether elevated p53 levels affect chlamydial development, we increased the protein stability of p53 in infected HUVECs using Nutlin-3 and etoposide. Indirect effects of these inhibitors on *Chlamydia* could be excluded, since preincubation of chlamydial EB with Nutlin-3 or etoposide for 30 min did not affect infection efficiency (Figure S3F), inclusion formation, or infectivity. Pretreatment of cells with the DNA-damaging agent etoposide at 50 μM prior to infection increased p53 levels (Figure S3G). HUVECs pretreated with etoposide to activate p53 and infected with *C. trachomatis* showed a decline in p53 protein levels after 18 hr of infection (Figure 4A). Surprisingly, inclusion formation was strongly impaired in these cells. Confocal and electron microscopy revealed that *Chlamydia* were able to infect cells in the presence of high levels of p53, since individual bacteria could be seen inside the cell (Figures 4B and 4C); however, the *Chlamydia* appeared to be in a persistent, nonreplicative form (Figures 4B and 4C). The same effect on *Chlamydia* inclusion formation was observed in the presence of the HDM2 inhibitor Nutlin-3, which also stabilized p53 (Figures 4D and 4E). High levels of p53 not only affected inclusion formation but also caused a loss of infectivity, as no infectious *Chlamydia* could be obtained from cells grown with stabilized p53 (Figure 4F). An effect similar to that observed with Nutlin-3 was seen after cells were treated with the PI3K inhibitor LY294002 before *Chlamydia* infection (data not shown), which also caused increased stabilization of p53 (Figure 3C). Unexpectedly, removal of Nutlin-3 at 16 hpi resulted in complete recovery of chlamydial inclusion formation within several hours (Figure 4G) and the successful completion of the chlamydial life cycle, since infectivity of *Chlamydia* was restored under these conditions (Figure 4H). In addition to the role of DNA damage in chlamydial growth inhibition, p53 might directly function as a suppressor of *Chlamydia* development. To demonstrate that etoposide affects chlamydial development via stabilization of p53, we made use of a set of isogenic HCT116 cells, which differ only in their p53 status (Bunz et al., 1998). Etoposide treatment of the parental HCT116 line, which contains wild-type p53 (HCT116 $p53^{+/+}$), strongly repressed chlamydial development, whereas the same treatment of the p53-deficient line (HCT116 $p53^{-/-}$) allowed

Figure 3. Downregulation of p53 in Infected Cells Depends on PI3 Kinase and HDM2 Activation

(A) *C. trachomatis* (C.tr.) infection induces phosphorylation of Akt and HDM2. HUVECs were infected with *C. trachomatis* at different moi values or for different time periods.

(B) p-HDM2 (Ser166), HDM2, p-Akt (Ser473), Akt, cHsp60, and p53 were detected by immunoblotting and GAPDH was used as loading control.

(C) Inhibition of PI3K blocks p53 depletion. HUVECs were treated with 10 μM of the PI3K inhibitor LY294002 followed by infection with *C. trachomatis* (C.tr.) for different time periods. p53, pAkt, and Akt were detected by immunoblotting.

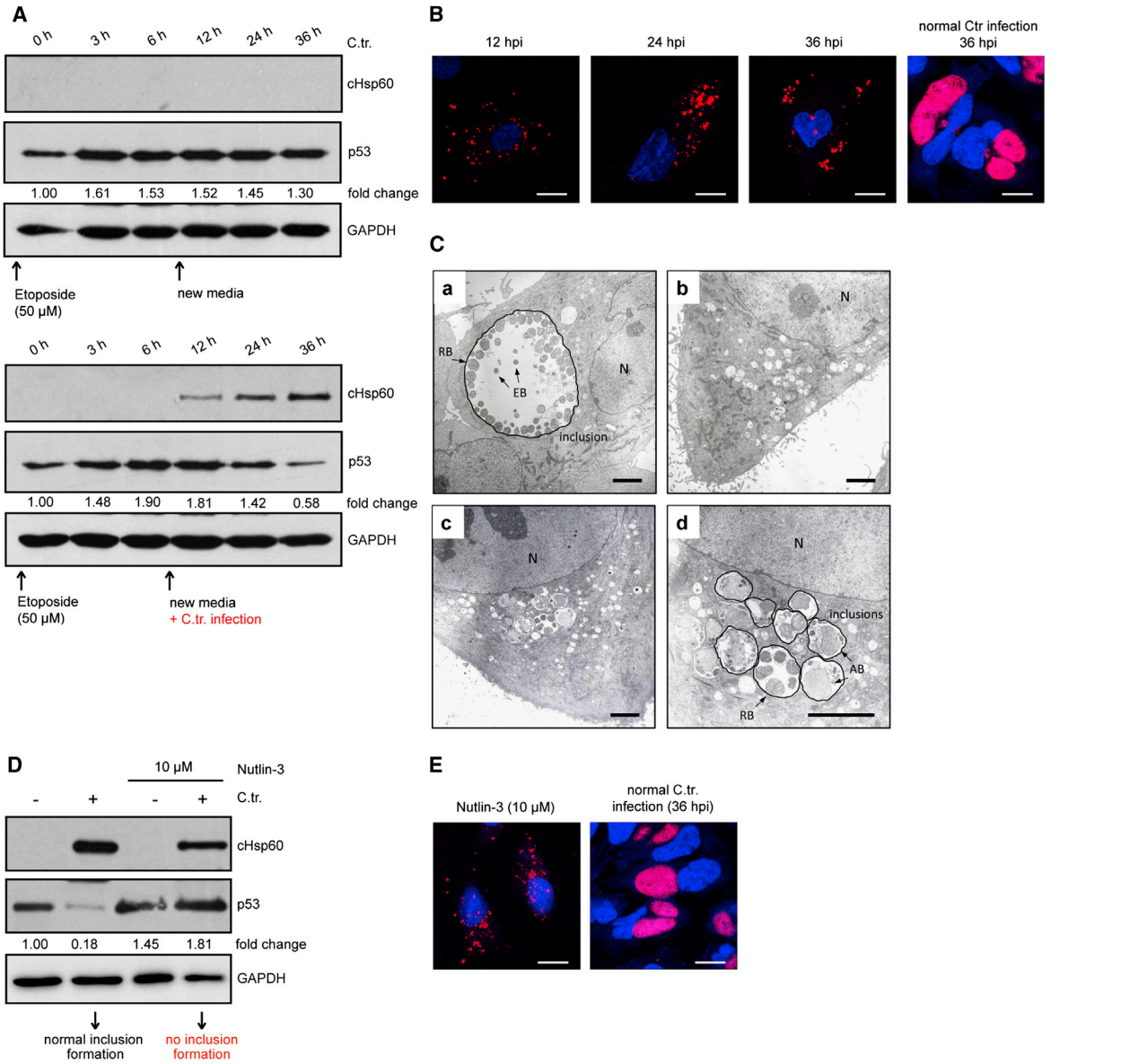
(D) Inhibition of HDM2 results in p53 stabilization. HUVECs were treated with 5 and 10 μM of the HDM2 inhibitor Nutlin-3, followed by infection with *C. trachomatis* (C.tr.) for 24 hr. p53, chlamydial Hsp60, and chlamydial OmpA were detected by immunoblotting. GAPDH was used as the loading control.

(E) Silencing of HDM2 resulted in stabilization of p53. HDM2 was silenced by lentivirus-mediated transduction of shRNA constructs in HUVECs and the knockdown efficiency was checked with an antibody against HDM2.

(F) Inhibition of the proteasome prevents p53 degradation. HUVECs were treated with 3 μM of the proteasome inhibitor MG-132, followed by infection with *C. trachomatis* (C.tr.) for 24 hr.

(G) HUVECs were infected with *C. trachomatis* (C.tr.) for different time periods. p21, Puma, and p53 were detected by immunoblotting.

See also Figure S3.



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chlamydial growth (Figure S3H). These results demonstrate a direct inhibitory effect of p53 on chlamydial development.

Glycolysis Is Not a Major p53 Target that Is Essential for Chlamydial Development

p53 is a major suppressor of tumor growth (Vousden and Prives, 2009) and affects multiple pathways, such as the cell cycle, DNA repair, and apoptosis (Levine and Oren, 2009). In more than half of all human cancers, p53 is either mutated or downregulated, emphasizing its role as a major tumor suppressor. However, a cell-intrinsic antibacterial role of p53 has not been established so far. Since etoposide treatment failed to induce efficient apoptosis in infected cells (Figure S1A), we reasoned that the antichlamydial effect of p53 might not depend on its function as an inducer of cell death. Recently, an important role for p53 in the regulation of metabolic pathways, including glycolysis, was demonstrated (Madan et al., 2011). As obligate intracellular bacteria, *Chlamydia* depend on the uptake of glucose from the host cells (Omsland et al., 2012), and p53 has been shown to downregulate glucose transport and glycolysis (Schwartzenberg-Bar-Yoseph et al., 2004). We observed upregulation of glucose transporter 1 (Glut1) after *Chlamydia* infection and downregulation after etoposide treatment (Figure S3I). However, we did not observe decreased expression of Glut4 after etoposide treatment (Figure S3I). p53 negatively regulates glycolysis through activation of TIGAR (a direct inhibitor of cellular fructose-2,6-bisphosphate levels) (Bensaad et al., 2006). Indeed, we found that TIGAR was downregulated in *Chlamydia*-infected cells (Figure S3J) and slightly stabilized after etoposide treatment (Figure S3J), suggesting that TIGAR downregulation depends on p53 degradation. However, supplementation of the growth medium with cell-permeable sodium pyruvate, one of the major end products of glycolysis, to overcome the TIGAR-mediated block in glycolysis did not rescue chlamydial growth (Figure S3K). It is still possible that intermediates of glycolysis (e.g., amino acids) might be essential for chlamydial growth in vivo. However, under cell culture conditions, amino acids are supplied in the culture medium, suggesting that other p53-dependent metabolic pathways are important for *Chlamydia* growth under these conditions.

Glucose-6-P-Dehydrogenase Is the p53 Target that Is Critical for Chlamydial Development

The PPP is another glycolytic pathway that is downregulated by stabilized p53. One of the major p53 targets in the PPP is glucose-6-P-dehydrogenase (G6PD) (Jiang et al., 2011), the first and rate-limiting enzyme of the PPP that is involved in NADPH production. We selectively inhibited the PPP by treating HUVECs with 6-aminonicotinamide (6-AN), a well-established inhibitor of the $NADP^+$-dependent 6-phosphogluconate dehydrogenase, and observed a dose-dependent strong reduction of chlamydial growth (Figure 5A). p53 was previously shown to inhibit G6PD activity by binding directly to G6PD and preventing active dimer formation (Jiang et al., 2011). To further characterize the nature of the p53-mediated antichlamydial activity, we used $p53^{-/-}$ H1299 cells and H1299 cells stably expressing tumor-associated p53 mutants, which are mostly missense mutations that affect p53 function and fail to inhibit G6PD activity (Jiang et al., 2011). We transiently transfected H1299 cells with p53 and observed a strong reduction of chlamydial infectivity and impaired inclusion formation (Figures 5B and 5C), further supporting a direct role for p53 in the suppression of *Chlamydia* growth. Infection of $p53^{-/-}$ H1299 cells or H1299 cells expressing high levels of mutant p53 R175H and p53 R273H (Petitjean et al., 2007) allowed partial chlamydial growth recovery (Figure S4A). These results indicated that impaired chlamydial growth was possibly caused by p53-mediated inhibition of G6PD and subsequent inhibition of the PPP. Indeed, G6PD activity was strongly reduced after overexpression of p53, but not of the mutants p53 R175H and p53 R273H, in noninfected and infected cells (Figure 5D). Interestingly, G6PD activity upon etoposide treatment was rescued in noninfected and infected $p53^{-/-}$ H1299, p53 R175H, and p53 R273H H1299 cells (Figure 5D), demonstrating that p53 is a main inhibitor of G6PD activity. As a consequence, we expected that overexpression of G6PD would overcome the inhibitory activity of stabilized p53 and rescue chlamydial development. In agreement, overexpression of G6PD rescued chlamydial development in etoposide- and Nutlin-3-treated cells in a dose-dependent manner (Figures 5E and S4B), strengthening the hypothesis that p53-mediated inhibition of G6PD is the crucial point for the antichlamydial activity of p53. However, rescue of

Figure 4. Stabilization of p53 Adversely Affects *C. trachomatis* Inclusion Formation and Infectivity

(A) HUVECs were treated with etoposide (50 μM) for 6 hr to induce p53 stabilization. Cells were washed with fresh medium to remove the inhibitor and subsequently infected with *C. trachomatis* (C.tr.) at an moi of 1 for 30 hr or left uninfected. Cells were lysed at the indicated time points, and p53 and Hsp60 were detected by immunoblotting. Fold-change values were derived by normalization to GAPDH.

(B) Upregulation of p53 results in impaired chlamydial inclusion formation. Cells were treated and infected as described in (A), fixed and stained with an antibody against chlamydial Hsp60 (cHsp60) and Draq5 to visualize nuclei, and analyzed by immunofluorescence analysis. Scale bar, 20 μm.

(C) Transmission electron microscopy was performed to investigate the ultrastructure of single dispersed inclusions. HUVECs were infected with *C. trachomatis* (a), treated with 50 μM etoposide (b), or treated with etoposide for 6 hr followed by *C. trachomatis* infection (c and d) for 24 hr. Panel (d) is a magnification of (c). Bars represent 2 μm at 3,000× (a–c) and 7,000× (d) magnification. RB, reticulate body; EB, elementary body; AB, aberrant body.

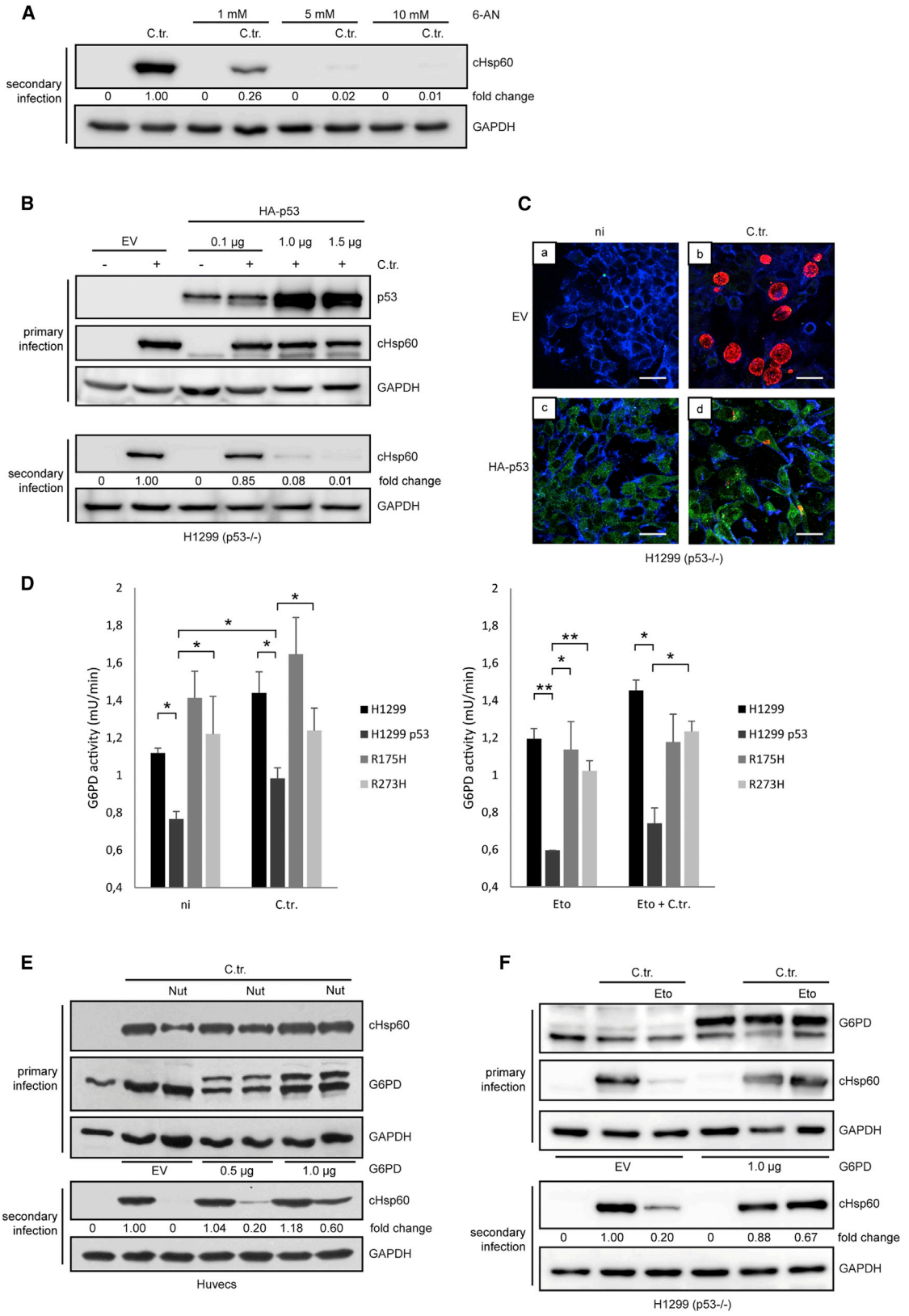
(D) HUVECs were treated with 10 μM of Nutlin-3 and infected with *C. trachomatis* (C.tr.) for 24 hr. p53 and chlamydial Hsp60 were detected by immunoblotting and fold-change values were determined by normalization to GAPDH.

(E) To show impaired inclusion formation after addition of Nutlin-3, cells infected with *C. trachomatis* (C.tr.) for 24 hr were treated as described in (B). Scale bar, 20 μm.

(F) High levels of p53 result in loss of chlamydial infectivity. HeLa229 cells infected and treated as described in (A) were lysed at 48 hpi and the lysate was transferred to fresh cells to monitor chlamydial infectivity. Infectivity was determined by quantifying Hsp60 levels normalized to GAPDH.

(G) Immunofluorescence analysis of restored chlamydial inclusion formation after removal of Nutlin-3. HUVECs were treated with 10 μM Nutlin-3 and infected with *C. trachomatis* (C.tr.) for 16 hr. The medium was replaced and cells were fixed at the indicated time points after removal of Nutlin-3. Scale bar, 20 μm.

(H) Nutlin-3-induced loss of infectivity can be rescued by removing Nutlin-3. HUVECs were treated with 10 μM of Nutlin-3 and infected with *C. trachomatis* (C.tr.) for 48 hr. In one set of cells, Nutlin-3 containing media was replaced by fresh medium at 16 hpi (lane 4). Cells of the primary infection were lysed and supernatants were used to determine chlamydial infectivity by secondary infection of fresh cells.



(legend on next page)

Chlamydia after etoposide and Nutlin-3 treatment was further enhanced after G6PD overexpression in a p53 null background (Figures 5F and S4C), demonstrating that p53-independent effects have to be considered. Since G6PD has been shown to have a protective role during oxidative DNA damage (Pandolfi et al., 1995), we verified the level of etoposide-mediated DNA damage in the presence of transient expression of G6PD (Figure S4D) in $p53^{-/-}$ cells. We observed considerably less DNA fragmentation in the presence of G6PD, underlining the protective potential of the PPP against oxidative stress and DNA damage. In line with this, chlamydial growth was rescued in $p53^{+/+}$ MEFs after G6PD overexpression in the presence of Nutlin-3. Chlamydial recovery was further improved after overexpression of G6PD in $p53^{-/-}$ MEFs (Figure S4E).

To further investigate the impact of oxidative stress on the p53-dependent effects during infection, we measured the levels of reactive oxygen species (ROS) in $p53^{+/+}$ and $p53^{-/-}$ cells. ROS levels increased after Nutlin-3 treatment of $p53^{+/+}$ cells, but not after etoposide treatment or p53 overexpression (Figure S5A). In line with these results, antioxidants quenching ROS failed to rescue chlamydial progeny formation (Figure S5B).

Our results show that p53 degradation serves an important function during *Chlamydia* infection to remove the inhibitory block on G6PD and highlight the importance of the PPP itself, not only for DNA repair but also for providing the necessary metabolites for optimal chlamydial growth.

DISCUSSION

Obligate intracellular pathogenic bacteria represent a group of bacteria that live in a special conflict situation because they entirely depend on metabolites of the host but at the same time harm the cell. In the case of *Chlamydia*, the dramatically reduced genome size and the resulting limited coding capacity forces the bacteria to receive key metabolites, such as amino acids and nucleotides, from the host cell. Rapid replication to more than 1,000 bacteria per cell is certainly one of the most harmful and stressful insults to the host cell during infection, but, intriguingly, is not the only one. Like many other bacteria, *Chlamydia* induces the formation of ROS early in infection (Boncompain et al., 2010). However, in contrast to other bacteria, increased ROS production at early infection time points and strong ROS scavenging at later infection time points are essential for the normal development of *Chlamydia* (Boncompain et al., 2010). The dependence on ROS generation that causes severe genotoxicity (Chumduri et al., 2013) is a paradoxical situation for an obligate intracellular bacterium that undergoes a developmental cycle and therefore has to protect its replication niche until noninfectious RBs retransform into infectious EBs. Here, we show that sustained p53 downregulation is the key event that prevents apoptosis and thus allows the repair of cytotoxic DNA damage and derepression of the PPP, which is central to host nucleotide biosynthesis during chlamydial infection.

Previous studies have indicated that many different kinds of bacterial pathogens, including *Chlamydia*, can induce severe DNA damage, particularly DSBs (Bergounioux et al., 2012; Chumduri et al., 2013; Toller et al., 2011; Vielfort et al., 2013). DSBs cause some of the most severe insults to DNA because they lead to a block in the cell cycle and eventually cell death if not repaired immediately (Nelson and Kastan, 1994). We observed that *Chlamydia* infection causes mainly severe shortening of telomeres (Prusty et al., 2013). Excessive shortened telomeres trigger the p53/pRb pathways that induce apoptosis or senescence to limit the lifespan of the damaged cell (Saretzki et al., 1999). Interestingly, shortened telomeres were repaired in infected human cells, but not in mouse cells, pointing to a fundamental difference in balancing infection-induced DNA damage. This was in line with the observation that *Chlamydia* infectivity was severely affected in p53 wild-type compared with $p53^{-/-}$ mouse cells, implying that p53 plays a central role in counteracting chlamydial development.

Downregulation of the tumor suppressor p53 by *Chlamydia* depended on activation of the PI3 kinase/HDM2 pathway, which controls p53 stability via proteasomal degradation. Interestingly, PI3 kinase was not significantly activated in mouse cells, suggesting that the activation of this pathway is decisive for the fate of p53 and the development of *Chlamydia*. This is in line with previous reports demonstrating that inhibitors of the PI3 kinase prevent normal chlamydial development (Rajalingam et al., 2008). However, we found that not only was downregulation of p53 crucial for *Chlamydia* to overcome the DNA-damage-driven cytotoxic response, but stabilization of p53 by etoposide or Nutlin-3 treatment at early time points of infection dramatically affected the development and progeny formation of *Chlamydia*.

Figure 5. p53 Downregulation and G6PD-Mediated DNA Repair Activity Are Critical for Chlamydial Development

(A) HUVECs were treated for 1 hr with 6-aminonicotinamide (6-AN) at different concentrations (1, 5, and 10 mM), followed by *C. trachomatis* (C.tr.) infection. Chlamydial development was monitored by an infectivity assay and quantification of cHsp60.

(B) Overexpression of p53 is sufficient to inhibit chlamydial development and inclusion formation. HA-tagged wild-type p53 construct was transiently transfected into $p53^{-/-}$ H1299 cells and 24 hr later the cells were infected with *C. trachomatis* (C.tr.). Chlamydial development was monitored in primary and secondary infections by quantification of cHsp60.

(C) Overexpression of wild-type p53 prevents inclusion formation. Transfection of empty vector (EV) had no adverse effect on chlamydial development (b; C.tr.). Transfection of HA-p53 did not affect cell viability (c) but prevented inclusion formation (d). Scale bar, 40 μm.

(D) G6PD activity after *C. trachomatis* infection and etoposide treatment. H1299, H1299 transfected with HA-tagged p53, p53 R175H, and R273H H1299 cells were infected with *C. trachomatis* (C.tr.) or pretreated with etoposide (50 μM) for 6 hr, followed by *Chlamydia* infection. Samples were processed and G6PD activity was measured. The graph shows mean values ± SEM of two experiments performed in triplicate. *$p < 0.05$, **$p < 0.01$.

(E) Chlamydial infectivity can be rescued despite p53 stabilization by overexpression of G6PD. HUVECs were transfected with an Myc-DDK-tagged G6PD construct for 24 hr, treated with etoposide for 6 hr, and infected with *C. trachomatis* (C.tr.) for 48 hr.

(F) G6PD overexpression rescues chlamydial infectivity in $p53^{-/-}$ H1299 cells. H1299 cells were transfected with Myc-DDK-tagged G6PD, treated with etoposide, infected with *C. trachomatis* (C.tr.), and subsequently analyzed for chlamydial infectivity.

See also Figures S4 and S5.

These results show that stabilized p53 can function to limit the growth of intracellular bacteria.

In recent years, p53 has also been linked to regulation of metabolism (Vousden and Ryan, 2009). In fast-replicating cancer cells, metabolism is shifted toward aerobic glycolysis to support mass and energy production, which is known as the Warburg effect (Warburg, 1956). As p53 is among the most important inhibitors of glycolysis in tumor cells, its inhibition enhances the Warburg effect. As for tumor cells, directing metabolic pathways to aerobic glycolysis and the PPP would also be beneficial for chlamydial growth since they entirely depend on the uptake of amino acids and nucleotides from the host (Eisenreich et al., 2013). The importance of an intact PPP for the biosynthesis of NADPH and other nucleotides is also underlined by the strong induction of nonreplicative chlamydial persistence upon silencing of G6PD (Prusty et al., 2012). G6PD deficiency is associated with an increased sensitivity for apoptosis induction by oxidative stress (Efferth et al., 1995) or ionizing radiation (Tuttle et al., 2000), suggesting that G6PD is involved in the response to DSBs. In $p53^{-/-}$ cells, ectopic expression of p53 potently inhibited G6PD expression and thus the PPP. Because this pathway has antioxidant properties and provides nucleotide precursors, it protects against DNA damage and supports DNA repair. Thus, *Chlamydia* infections, which inevitably induce the formation of ROS and DNA damage, are more efficient when the p53-mediated restriction of the PPP is avoided. Since G6PD overexpression is sufficient to overcome the p53-dependent and -independent inhibition of chlamydial development, we conclude that enhanced activity of the PPP as a consequence of p53 downregulation is the main benefit for *Chlamydia*.

Downregulation of p53 by bacterial infection has been previously demonstrated for the free-living bacteria *Shigella* (Bergounioux et al., 2012), *Helicobacter* (Wei et al., 2010), and *Neisseria* (Vielfort et al., 2013). Infection by the latter two bacteria induces DNA DSBs that would unavoidably activate p53 and eventually cause cell death. Recently, Chumduri et al. (2013) demonstrated that *C. trachomatis* also induces DNA DSBs at late time points during infection. Therefore, it is likely that downregulation of p53 is a general mechanism that enables bacterial pathogens to avoid the genotoxic response evoked by some bacterial infections. In contrast to free-living bacteria, however, obligate intracellular bacteria depend on the metabolites that they take up from the host cell. Therefore, we suggest that the downregulation of p53 during *Chlamydia* infection primarily serves to unleash the PPP shunt. Based on the data presented here, we propose that the tumor suppressor p53 plays an important role in inhibiting the growth and development of obligate intracellular *Chlamydia* by limiting metabolite flow through the PPP.

EXPERIMENTAL PROCEDURES

Infectivity Assay

C. trachomatis-infected cells (48 hpi) and uninfected control cells were washed once with PBS and lysed manually by glass beads. A second set of cells, seeded the day before, were infected by one-hundredth of the cell lysate containing infectious EBs, incubated for 24 hr at 35°C, and lysed for immunoblot analysis or fixed with 4% paraformaldehyde for immunostaining. After fixation, cells were permeabilized with PBS/0.2% Triton X-100 for 15 min at room temperature (RT) and blocked with PBS/10% fetal bovine serum for 1 hr at RT. After incubation with primary antibody against chlamydial Hsp60 (cHsp60) in PBS/2% fetal bovine serum at RT for 1 hr, cells were stained with Cy3-labeled secondary antibody for 1 hr in the dark. Cell nuclei were stained with DAPI together with the secondary antibody. Coverslips were washed and mounted with Mowiol (Carl Roth) on glass slides. The number of inclusions was determined by counting ten random fields using an epifluorescence microscope (Leica) at 40× magnification. For confocal laser scanning microscopy, samples were analyzed using a Leica TCS SPE. Infectivity was also determined by quantifying chlamydial Hsp60 (cHsp60) by immunoblotting. The hfimb cells were obtained and used according to the guidelines of the local ethics committee.

Cell Transfections

Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For overexpression of G6PD, cells were seeded into a 12-well plate and transfected with pCMV6-Myc-DDK-G6PD or empty vector control. p53 overexpression was achieved by transfecting pCDNA3-HA-p53 (Marin et al., 2000). One day after transfection, cells were treated with etoposide (50 μM) for 6 hr or Nutlin-3 (10 μM) for 1 hr and infected with *C. trachomatis* for 40 hr, followed by an infectivity assay.

Electron Microscopy

Cells were grown on glass coverslips, fixed for 45 min with 2.5% glutaraldehyde (50 mM sodium cacodylate [pH 7.2]; 50 mM KCl; 2.5 mM $MgCl_2$) at RT, incubated for 2 hr at 4°C with 2% OsO_4 buffered with 50 mM sodium cacodylate (pH 7.2), washed with dH_2O, and incubated overnight at 4°C with 0.5% uranyl acetate (in dH_2O). 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 analyzed on a Zeiss EM10 microscope (Zeiss).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.004.

AUTHOR CONTRIBUTIONS

C.S. performed all experiments except for DNA-damage assays, NDB assays, and TRF analysis. B.K.P. performed DNA damage assays, NDB assays, and TRF analysis. K.K. performed experiments with human fimbriae cells and part of the p53 overexpression experiments. J.W. provided endothelial cells of human and murine fimbriae and critically read the manuscript. T.R. supervised the research and wrote the manuscript.

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