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RESEARCH ARTICLE

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Infection of HeLa cells with *Chlamydia trachomatis* inhibits protein synthesis and causes multiple changes to host cell pathways

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Abstract

The obligate intracellular bacterium *Chlamydia trachomatis* replicates in a cytosolic vacuole in human epithelial cells. Infection of human cells with *C. trachomatis* causes substantial changes to many host cell-signalling pathways, but the molecular basis of such influence is not well understood. Studies of gene transcription of the infected cell have shown altered transcription of many host cell genes, indicating a transcriptional response of the host cell to the infection. We here describe that infection of HeLa cells with *C. trachomatis* as well as infection of murine cells with *Chlamydia muridarum* substantially inhibits protein synthesis of the infected host cell. This inhibition was accompanied by changes to the ribosomal profile of the infected cell indicative of a block of translation initiation, most likely as part of a stress response. The Chlamydia protease-like activity factor (CPAF) also reduced protein synthesis in uninfected cells, although CPAF-deficient *C. trachomatis* showed no defect in this respect. Analysis of polysomal mRNA as a proxy of actively transcribed mRNA identified a number of biological processes differentially affected by chlamydial infection. Mapping of differentially regulated genes onto a protein interaction network identified nodes of up- and down-regulated networks during chlamydial infection. Proteomic analysis of protein synthesis further suggested translational regulation of host cell functions by chlamydial infection. These results demonstrate reprogramming of the host cell during chlamydial infection through the alteration of protein synthesis.

KEYWORDS

Chlamydia trachomatis, infection, microarray, proteomics, stress response, translation

1 | INTRODUCTION

Chlamydia trachomatis is an obligate intracellular bacterium that replicates in a vacuole (the inclusion) in the cytosol of human epithelial cells. The epidemiologically most important biovars of *C. trachomatis* are frequent causes of sexually transmitted disease throughout the world (estimated incidence of 300 million cases per year; Newman et al., 2015) and of blinding trachoma, a chronic, scarring infection of the eye (Bourne et al., 2017).

During its development in the inclusion, *C. trachomatis* has to acquire numerous nutrients from the host cell, has to escape the cell's defence reactions, and has eventually to organise its release from the infected cell (Bastidas, Elwell, Engel, & Valdivia, 2013). During infection, numerous processes of the host cell are altered, presumably

reflecting the bacterial efforts to manipulate the host cell, together with the host cell reaction. One outcome of this interplay is a substantial change in host cell transcription during chlamydial infection, indicating a response of the host cell to the infection (Hess et al., 2003; Humphrys et al., 2013). However, protein expression in human cells is also subject to numerous posttranscriptional regulatory mechanisms, such as regulation of translation and of protein degradation (Sonenberg & Hinnebusch, 2009). Indeed, altered degradation of host cell proteins has been demonstrated in cells infected with *C. trachomatis* (Olive et al., 2014), and a number of other bacteria have been found to target host cell translation (Lemaitre & Girardin, 2013). During chlamydial infection, a number of instances are known where the levels of individual host proteins are unexplained by transcriptional regulation. Thus, expression of the Bcl-2-family member Bim is reduced despite unchanged mRNA-levels (Fischer et al., 2004), and the transcription factor p53 has been found to be down-regulated, accompanied by an increase in its proteasomal turnover during chlamydial infection (Siegl, Prusty, Karunakaran, Wischhusen, & Rudel, 2014).

Translation is achieved through the hugely complex ribosome RNA-protein machineries. Translational changes in mammalian cells have been reported during infection with *Legionella pneumophila* and with *Shigella* or *Salmonella* bacteria, as well as through the action of bacterial toxins (Lemaitre & Girardin, 2013). *C. trachomatis* infection affects a large number of signalling pathways in the infected cell, much of which is not understood (Bastidas et al., 2013). We therefore in this study set out to test the hypothesis that infection of human epithelial cells with *C. trachomatis* alters protein translation in infected host cells. We found a pronounced reduction of protein synthesis, changes in ribosomal profiles, and a number of predicted effects on numerous signal transduction pathways.

2 | RESULTS

2.1 | *Chlamydia trachomatis* infection reduces host cell protein synthesis

To test for global protein synthesis, we measured the metabolic incorporation of the chemically modified methionine analogue L-azidohomoalanine (AHA), followed by fluorescent detection through Click-iT-chemistry. HeLa human cervical epithelial cells were infected with *C. trachomatis* L2 strain, and newly synthesised proteins were labelled. Fluorescence microscopy revealed strong incorporation of the amino acid into the bacteria with a signal also in the host cell. Bacterial signal incorporation was blocked by doxycycline (Figure 1a), an inhibitor of bacterial protein synthesis, or by rifampicin, an inhibitor of bacterial RNA synthesis (not shown). Quantification by flow cytometry showed enhanced protein synthesis in infected cells (Figure 1b). However, when doxycycline was added prior to addition of the AHA-label to block bacterial protein synthesis, a substantial reduction of protein synthesis was noted (Figure 1b), indicating reduced protein synthesis by the infected host cell. The inhibitory effect of the infection was approximately equivalent to 100–200 ng ml^{-1} of the inhibitor of eukaryotic protein synthesis, cycloheximide (Figure S1). We also used a second approach to confirm the effect of chlamydial infection on host cell protein synthesis (surface sensing of translation; Schmidt, Clavarino, Ceppi, & Pierre, 2009). This method also detected a reduction of protein synthesis in HeLa cells infected with *C. trachomatis* (Figure S1b). An inhibitory effect on host cell protein synthesis was seen from about 20 hr post-infection, and inhibition was maintained up to 48 hr post-infection (Figure S2a).

RNA-synthesis of *C. trachomatis*, as measured by incorporation of uridine and sensitive to the bacterial RNA-polymerase inhibitor rifampicin, was also detectable in infected HeLa cells (Figure S3). Despite the known changes in mRNA induced by chlamydial infection, no difference between uninfected cells and cells infected and treated with rifampicin was observed. This suggests that the synthesis of ribosomal RNA, the largest share of RNA in a human cell, is not substantially affected by the infection, and changes to mRNA may not be detectable by this method.

We also tested protein synthesis in cells infected with a *C. trachomatis* serovar E strain. In these experiments, infected cells showed reduced total protein synthesis already in the absence of bacterial protein synthesis inhibition, and the effect of the addition of doxycycline was weaker than observed for HeLa cells infected with *C. trachomatis* L2 (Figure 1c). We further infected mouse oviduct epithelial cells (Johnson, 2004) with the species *Chlamydia muridarum*, which is commonly used experimentally to infect mice. We again observed a reduction of protein synthesis (even though less pronounced) upon infection with *Chlamydia* (Figure 1d), and a similar effect was seen in HeLa cells infected with *C. muridarum* (Figure S2b). Microscopy showed pronounced uptake of the AHA-label into the inclusion of infected HeLa cells (Figure S2c). The reduction of de novo protein synthesis during chlamydial infection therefore appears to be a feature of several species and strains in both humans and mice.

2.2 | *Chlamydia trachomatis* infection causes alteration to the ribosomal profile of host cells

The reduction in de novo protein synthesis suggested the possibility of altered translation during chlamydial infection. To test for this possibility, we analysed the ribosomal profile of infected cells by separation of ribosomes over a sucrose gradient (Chasse, Boulben, Costache, Cormier, & Morales, 2017). This technique permits the identification of the relative abundance of ribosomal subunits (40S, 60S), monosomes (80S), and polysomes. As shown in Figure 2a, there was a substantial increase of the 80S (monosome) peak in ribosomes of *C. trachomatis*-infected cells, associated with a relative reduction in polysome abundance (although an absolute reduction of polysome abundance is not certain). When expressed as the ratio of polysomes to monosomes, an approximately twofold reduction was found (Figure 2b). Because polysomes represent the actively translating fraction of ribosomes, this change in ribosomal profile indicates a reduced translation in human cells infected with *C. trachomatis*.

We observed an additional peak in the profile (between 40S and 60S) in preparations from infected cells. We were unable to resolve the nature of this peak. On SDS-PAGE, no protein bands could be

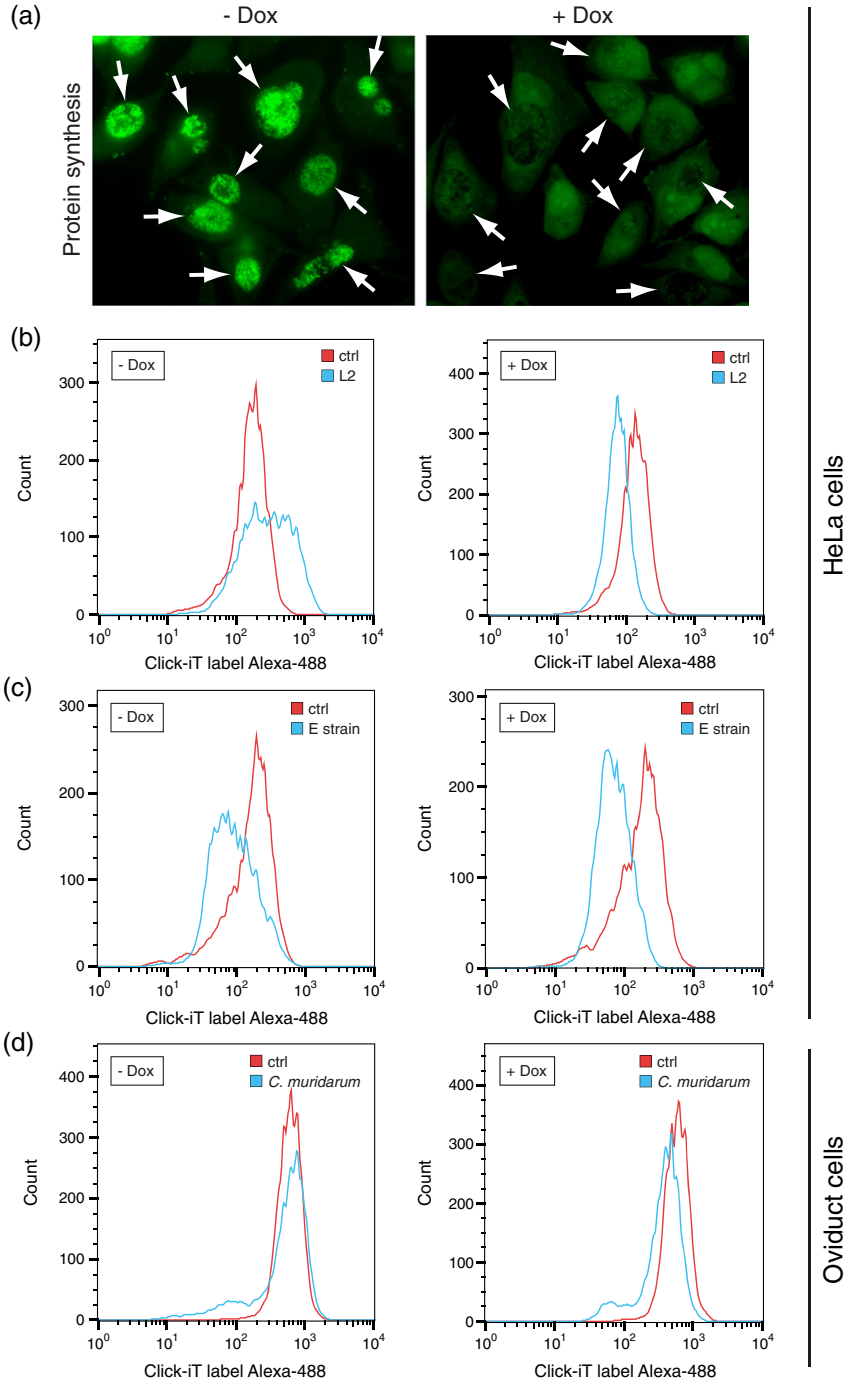

FIGURE 1 Infection with different strains and species of Chlamydia reduces protein synthesis of the host cell. (a) HeLa cells were infected with *Chlamydia trachomatis* for 28 hr. Cells were labelled with AHA, which is incorporated in newly synthesised proteins. Where indicated, doxycycline (2.25 μM) was added prior to labelling for 2 hr in total. After fixation, the *Click-iT* reaction was performed in the well for 30 min at RT. Cells were analysed by epifluorescence microscopy. Chlamydial inclusions are indicated by arrows (MOI = 1). (b, c) Uninfected (ctrl, red line) HeLa cells or HeLa cells infected with *C. trachomatis* (serovar L2 (b) or E (c), blue lines) for 28 hr (b) or 45 hr (c) were labelled with AHA (MOI = 3). Where indicated, doxycycline (2.25 μM) was added 1 hr prior to labelling for 2 hr in total. After harvesting and fixation, cells were labelled using the *Click-iT* reaction and analysed by flow cytometry. Data are representative of at least three independent experiments. (b) Six experiments were performed; both increase of AHA-incorporation (p = 0.000287) in the absence and decrease in the presence of doxycycline (p = 0.004472) were significant (paired t-test). (c) Four experiments were performed; a statistical trend was observed for the increase of AHA-incorporation (p = 0.121467) in the absence and a significant decrease in the presence of doxycycline (p = 0.025112; paired t-test); differences in the mean fluorescence intensity measured. (d) Uninfected (ctrl, red line) or infected (*Chlamydia muridarum*, 19 hr, MOI = 3, blue line) C57epi.1 mouse oviduct cells were labelled with AHA. Where indicated, doxycycline (2.25 μM) was added 1 hr prior labelling for 2 hr in total. After harvesting and fixation, the *Click-iT* reaction was performed, and cells were analysed by flow cytometry. Data are representative of at least three independent experiments. Four experiments were performed. The decrease in AHA-incorporation was statistically significant (p = 0.04631, paired t-test of mean fluorescence values). For all conditions used in this study, we have tested whether antibiotic (mostly doxycycline, also rifampicin) altered protein synthesis in uninfected cells. We have never observed such an effect (not shown)

identified that corresponded to this peak. When we isolated *C. trachomatis*, lysed the bacteria the same way, and subjected a similar amount of bacteria to sucrose gradient centrifugation, no such peak was seen.

This ribosomal profile is characteristic of a block at the initiation stage of translation (Coudert, Adjibade, & Mazroui, 2014). A similar profile was seen when cells were treated with a number of known stressors such as brefeldin A, tunicamycin, or serum starvation, whereas inhibition of ribosomal elongation by cycloheximide as expected caused a decrease in the 80S peak (Figure S4a).

Inhibition of protein synthesis in conditions of various forms of stress, such as viral infection, endoplasmic reticulum (ER) stress, or starvation, is mediated by the phosphorylation of the eukaryotic initiation factor 2 alpha (eIF2α). Phosphorylation of eIF2α was also observed during infection with *C. trachomatis* (Figure 2c), similar to the stress response to other stimuli (Figure S4b). We also observed increased phosphorylation of a second initiation factor, eIF4B (Figure 2c), which was not seen during the other forms of stress investigated (Figure S4b). eIF4B is an RNA-binding protein that facilitates recruitment of mRNA to ribosomes and is involved in translation of

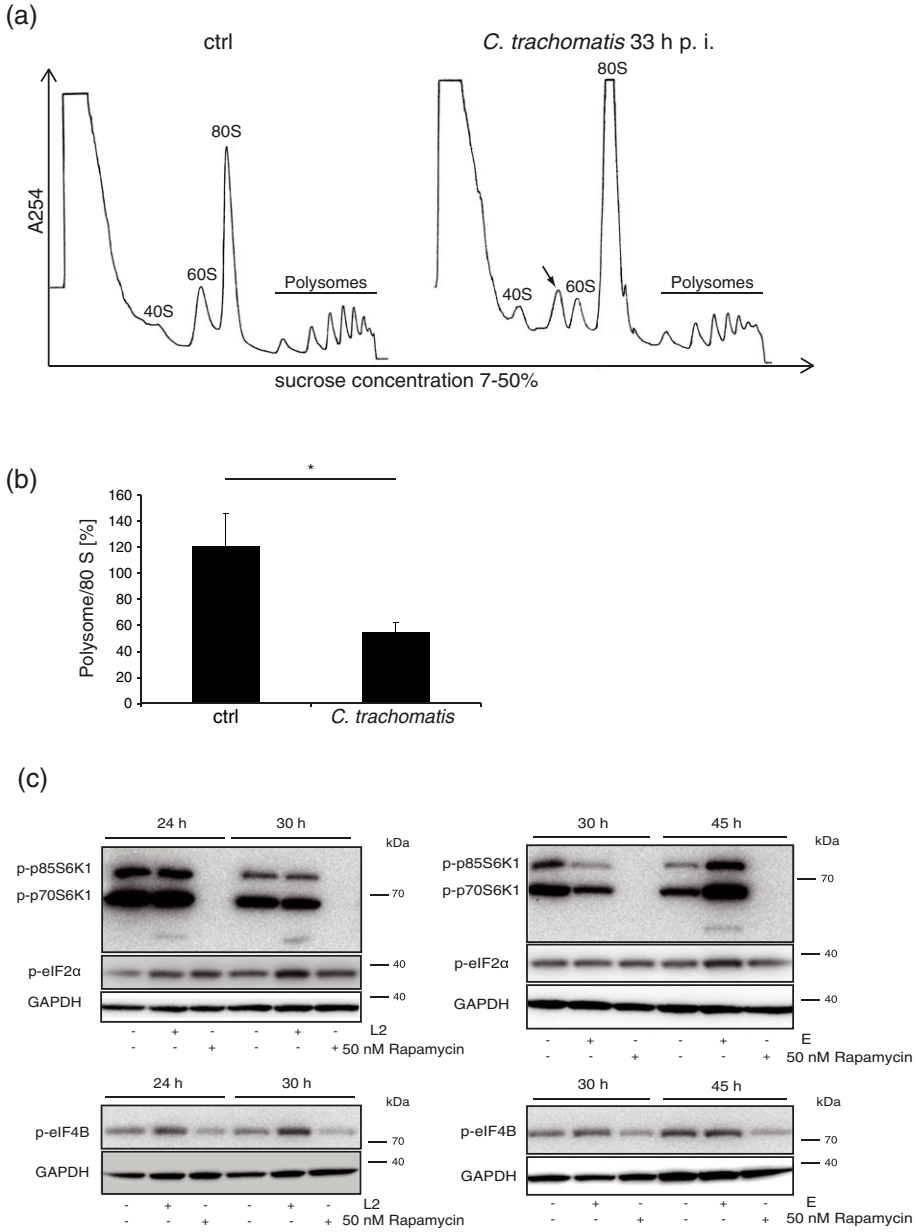

FIGURE 2 Reduced protein synthesis is associated with changes in ribosomal profile and altered phosphorylation of eukaryotic initiation factors. (a) Ribosomal profiles of uninfected (left) or *Chlamydia trachomatis* L2-infected cells (33 hr, MOI = 1, right) were obtained by loading equal protein amounts of lysate on top of a linear 7–50% sucrose gradient (11 ml). Ribosomes were separated by centrifugation. Profiles were recorded by monitoring RNA absorbance at A254 with a UV detector. The arrow indicates an additional peak in infected cells. One representative profile of more than three independent experiments is shown. (b) Relative abundance of 80S monosomes versus polysomes of untreated or *C. trachomatis*-infected cells for 30–33 hr (MOI = 1) is shown. Areas under the curve were measured using ImageJ 1.51. Means and SD of three independent experiments are shown. Statistical significance was analysed using two-tailed paired *t*-test ($^{*}p < 0.05$). (c) Uninfected HeLa cells or HeLa cells infected with *C. trachomatis* (MOI = 3) for the indicated times were lysed in 8 M urea. Proteins were detected by western blotting. GAPDH served as a loading control. Data are representative of at least three independent experiments. (c) Phosphorylation of S6 kinase and of initiation factors during chlamydial infection. HeLa cells were infected with *C. trachomatis* L2 or E strains (MOI = 3). Two hours before lysis, rapamycin (50 nM) was added where indicated. Cells were lysed directly in 8 M urea (supplemented with phosSTOP) at the times post-infection indicated. Lysates were analysed by western blotting for phosphor-S6 kinase and the phosphorylated forms of the initiation factors eIF2α and eIF4B. The mTOR-inhibitor rapamycin reduced S6-phosphorylation as expected

numerous mRNAs (Gingras, Raught, & Sonenberg, 1999; Methot, Pause, Hershey, & Sonenberg, 1994). Enhanced phosphorylation of eIF4B is not a common event during cell stress but has also been observed during infection with the Kaposi sarcoma-associated herpesvirus (Kuang, Fu, Liang, Myoung, & Zhu, 2011). Enhanced eIF2α phosphorylation was also observed during infection of HeLa cells with the *C. trachomatis* E strain although it appeared later in the infection (Figure 2c). eIF4B phosphorylation was only minimally enhanced during infection with the E strain (Figure 2c), despite the strain's activity in reducing protein synthesis (see Figure 1c), suggesting that this event may be specific to the L2 strain and perhaps not obligatorily linked to altered protein synthesis.

One possibility how *Chlamydia* may affect translation is the consumption of host cell nutrients, perhaps most obviously amino acids. Amino acid starvation can affect translation through inactivation of mTOR (Jewell & Guan, 2013); a prominent effect of amino acid starvation (when amino acids are removed from the culture media) is the dephosphorylation of S6 kinase (Hara et al., 1998). Surprisingly, we found no decrease in S6-kinase phorphorylation during infection with the L2 strain, and an early but not late decrease during infection with the E strain (Figure 2c). This argues against the idea that translation alteration through amino acid starvation and mTOR-inactivation is the reason for the effects observed here, at least for the L2 strain.

The mammalian stress response is known to lead to the formation of stress granules, consecutive to the phosphorylation of eIF2α, which sequester mRNAs and may have additional signalling functions (reviewed in Kedersha, Ivanov, & Anderson, 2013). However, targeting the core stress granule protein TIAR by RNAi had no effect on the observed reduction in protein synthesis during *Ctr*-infection (data not shown). *C. trachomatis* infection therefore appears to cause a distinctive pattern of stress response that is different from the other stimuli tested.

We next tested for a potential role of the chlamydial protease CPAF for the observed reduction in protein synthesis. CPAF is produced by the bacteria at approximately the same time as the reduction in protein synthesis occurs (around midcycle of *C. trachomatis* development; Zhong, Fan, Ji, Dong, & Huang, 2001). CPAF is secreted from the chlamydial inclusion into the cytosol (Zhong et al., 2001). Although the degradation of host cell proteins by CPAF is more strongly observed during extraction of infected cells with detergent (Chen, Johnson, Lee, Sutterlin, & Tan, 2012), some host cell proteins have been found to be degraded in intact cells during chlamydial growth (Snavely et al., 2014). Because a cytosolic protease is likely to cause stress to a human cell, we tested whether CPAF played a role in host cell protein synthesis.

We first tested whether isolated cytosolic CPAF-expression in the absence of infection would reproduce the phenotype observed in infected cells. Indeed, when expression of active CPAF was induced in human 293T cells using an established system (Paschen et al., 2008), a reduction in protein synthesis but not in RNA-synthesis (or only minimally) was seen (Figure 3a,b). CPAF therefore appears to have the principal capacity to reduce protein synthesis in human cells. Ribosomal fractionation of CPAF-expressing cells also showed an increase in the 80S peak similar to the effect of chlamydial infection (Figure S4c).

To test whether CPAF indeed has this effect during *C. trachomatis* infection, we tested a genomically CPAF-deficient mutant strain (Rst17), together with an isogenic but CPAF-competent mutant (Rst5). In these experiments, we observed however no difference between the chlamydial strains: Rst17 and Rst5 showed comparable levels of reduction of protein synthesis (Figure 3c). Therefore, although CPAF can reduce protein synthesis, it does not seem to be required for this effect during chlamydial infection.

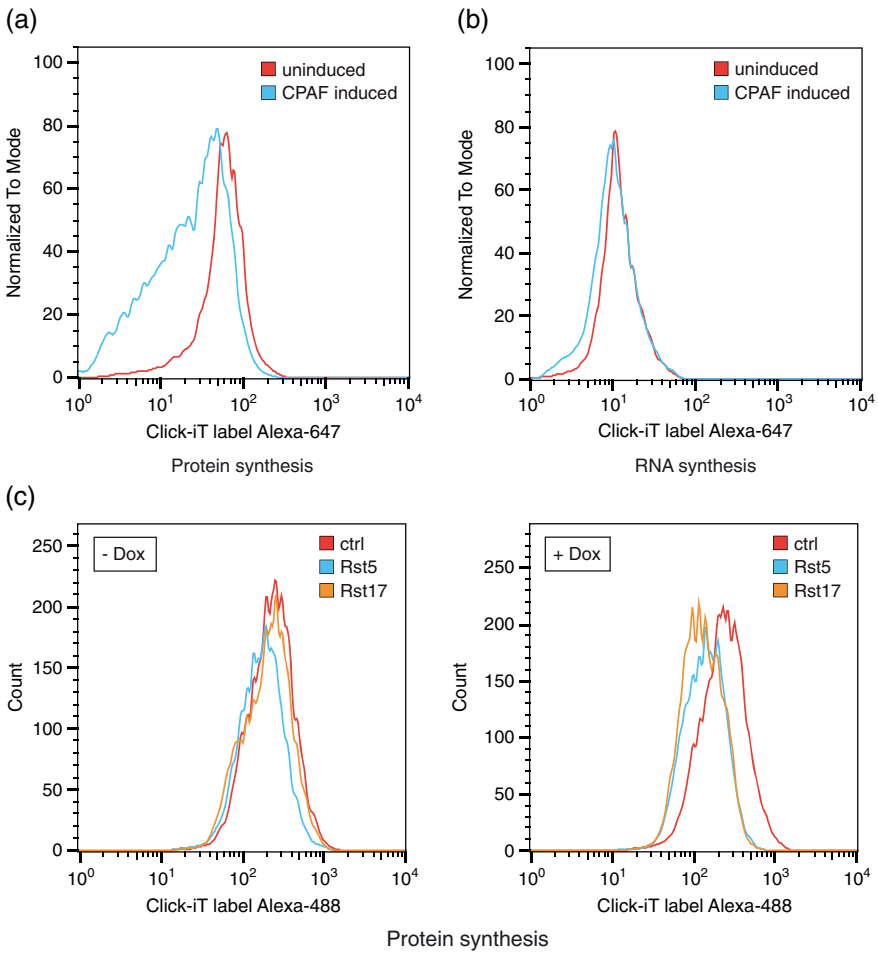

FIGURE 3 Effect of the expression of CPAF on protein and RNA synthesis. (a) Active CPAF was induced in 293 TRex-CPAF cells by adding anhydrotetracycline (5 ng ml^{-1}) and CM (1 μM) for 6 hr in total (blue line). As control, cells were cultured without inducing CPAF (red line). Three hours before harvesting, growth medium was changed to Met and Cys free medium and after 1 hr, AHA was added. Cells were harvested and fixed before the *Click-iT* reaction was performed for 3 hr at RT. Cells were analysed by flow cytometry. Data are representative of at least three independent experiments. (b) Where indicated, CPAF was induced as described in (a). One hour prior to harvesting, cells were labelled with EU (100 μM), which is incorporated in newly synthesised RNA. After harvesting and fixation, the *Click-iT* reaction was performed. Cells were analysed by flow cytometry. Data are representative of two independent experiments. (c) Uninfected (ctrl, red line) HeLa cells or HeLa cells infected with Rst5 (CPAF competent L2 strain; blue line) or Rst17 (CPAF-deficient isogenic L2 strain; orange line, both 40 hr, MOI = 3) were labelled with AHA. Where indicated, doxycycline (2.25 μM) was added for 2 hr in total. After harvesting and fixation, the *Click-iT* reaction was performed and cells were analysed by flow cytometry. Data are representative of at least three independent experiments

2.3 | Evidence for differential regulation of protein expression during *Chlamydia trachomatis* infection

Translation is a very complex process, and changes induced by *C. trachomatis* infection on protein synthesis may non-specifically affect all mRNAs or may alternatively be more specific and target some mRNAs but not others. To test for global versus more limited changes, we performed two types of further analyses. First, we analysed polysomal mRNA versus total mRNA during *C. trachomatis* infection. Because polysomal mRNA is being translated, this fraction may be expected to give a better representation of actual proteins synthesised than total mRNA. Second, we performed proteomic analyses of protein production in infected cells.

We fractionated cell lysates from uninfected and *C. trachomatis*-infected cells as above, collected polysomal fractions, and extracted mRNA from these fractions. Total mRNA was also extracted, and gene expression was compared using gene arrays (Table S5). Gene set enrichment analysis showed substantial down-regulation of a number of gene sets. Most strongly down-regulated were genes coding for components of the mitochondrial inner membrane, structural components of the ribosome, and genes involved in nucleosome and chromatin organisation. Translation elongation factors were also down-regulated (Figure 4). This suggests that the reduction in protein synthesis is linked to the down-regulation of the synthesis of the proteins driving translation. At least part of this effect on protein synthesis may therefore be the result of transcriptional regulation, amplifying the effect of reduced translation.

To deduce more biological information from this approach, we mapped differentially regulated genes onto a protein interaction network (based on the STRING database, Version 10; Szklarczyk et al., 2015). We extracted a maximally scoring subgraph that indicates the functional interaction of the most strongly differentially regulated genes (infected vs. non-infected, analysed separately for total mRNA and polysomal mRNA; Fang & Gough, 2014). We stipulated that the networks have 40 nodes and allowed genes that may not be differentially regulated but link other genes that are (Figure 5).

The size of each node correlates with the number of connections in the plot. The shaded background shows individual "modules" (highly connected networks). The colours represent the strength of the regulation (infected vs. non-infected cells). The module most strongly up-regulated in polysomal mRNA (Figure 5a) by *C. trachomatis* infection represents signalling through epidermal growth receptor (EGFR). Activation of the EGFR-signalling pathways has been noted before upon *C. trachomatis* infection, and RNAi against the EGFR reduced chlamydial development (Patel et al., 2014). Enhanced signalling through pathways downstream of EGFR has further been documented during infection with *C. trachomatis* (Buchholz & Stephens, 2007; Subbarayal et al., 2015). The up-

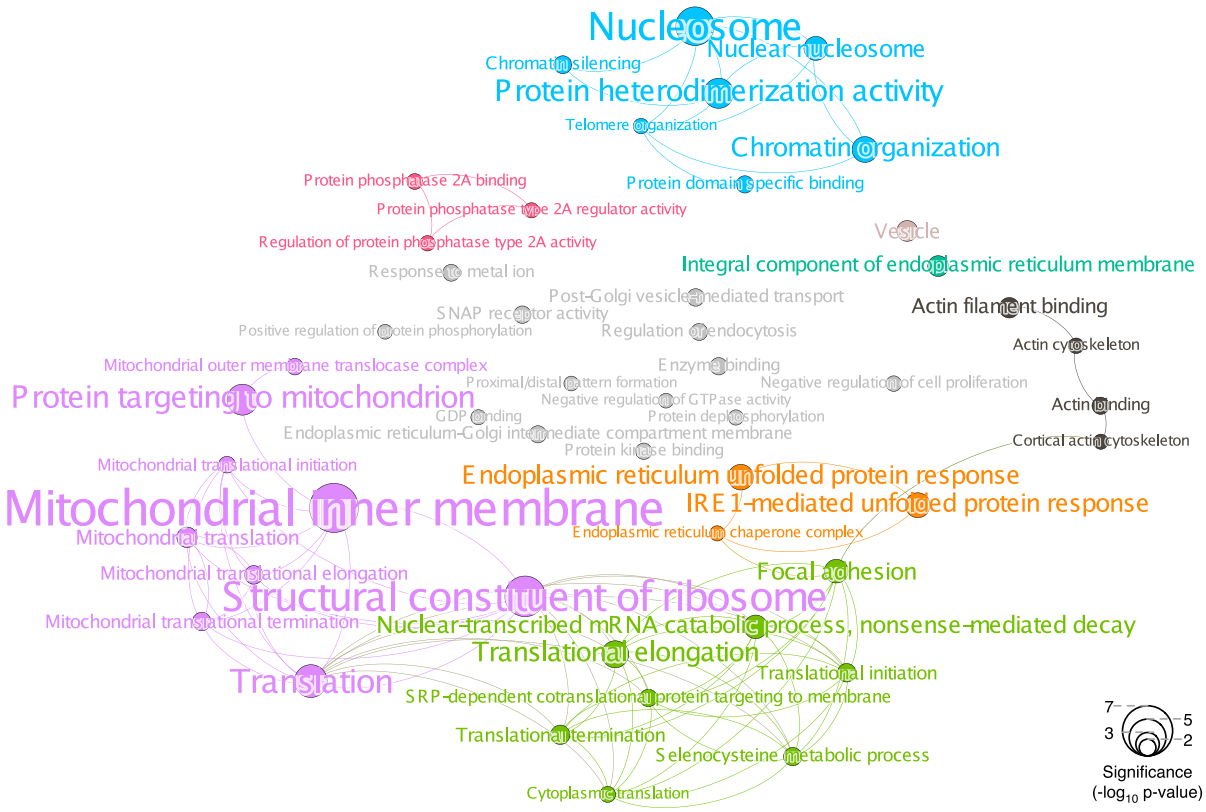

FIGURE 4 Gene set enrichment analysis. The network depicts Gene Ontology (GO) terms significantly down-regulated in the polysomes after infection with *Chlamydia trachomatis* (33 hr) compared with control (p value < 0.01). Network nodes correspond to GO terms with diameter and label size denoting the regulation significance calculated using a Gene Set Enrichment analysis. The largest and smallest nodes correspond to $p = 10^{-7}$ and $p = 0.01$, respectively. Nodes are connected by an edge if they share at least 30% of their genes. Nodes are coloured according to their cluster membership (Blondel, Guillaume, Lambiotte, & Lefebvre, 2008), and the network has been laid out with the Force Atlas algorithm as implemented in Gephi (v. 0.92)

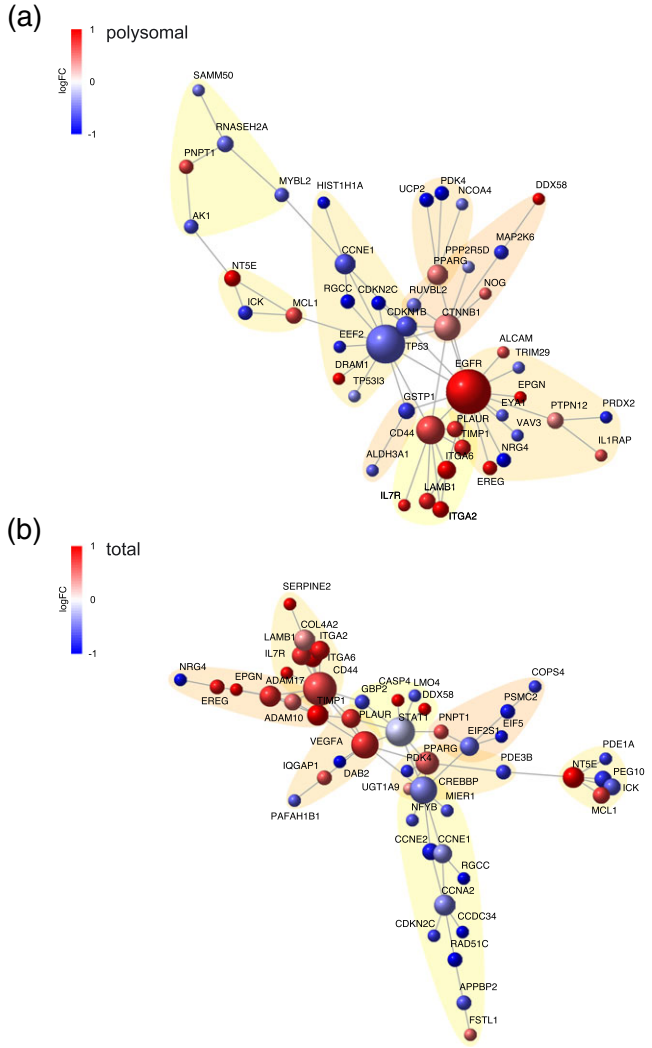

FIGURE 5 Subgraphs of genes differentially expressed in *Chlamydia trachomatis* infected and uninfected HeLa cells. The nodes and edges correspond to genes mapped to their protein product with the edges indicating known interactions between them. Nodes colour and size are proportional to the fold change between the two conditions (infected with *C. trachomatis* and non-infected) and the node degree, respectively. The shaded backdrops denote network modules that have been calculated via a spin-glass model and simulated annealing as implemented in the igraph R package (Csardi, 2006). (a) polysomal RNA, (b) total RNA isolated simultaneously

regulation of this signalling network through enhanced protein synthesis, as reflected by the specifically enhanced mRNA-levels in the polysomal fraction, may support such signalling enhancement.

The most strongly down-regulated module was the transcription factor p53 and connected genes. Although it has been suggested to be the result of post-translational loss of p53, a reduction of p53-signalling has been described independently before (Gonzalez et al., 2014; Siegl et al., 2014). Our data suggest that reduced p53-signalling is at least partly the result of reduced protein synthesis.

This analysis indicated a very different pattern depending on whether polysomal mRNA or total mRNA was analysed (Figure 5a,b). This suggests that the analysis of total mRNA might be of limited value when predicting the protein levels because the actively transcribed mRNA (polysomal fraction) appeared quite different from the total mRNA. For both pools of mRNA, both up- and down-regulated genes were identified, which, however, showed little overlap. The Venn diagram (Figure S6) depicts the differentially regulated genes between the different conditions and RNA fractions. One finding was a substantially higher number of genes whose expression was reduced by *C. trachomatis* infection in the polysomal fraction (443 genes) than in the total mRNA fraction (259 genes). Table S7 shows the individual genes that were up- or down-regulated specifically in polysomes (but not on the level of total mRNA) or that were up-regulated in both polysomal and total RNA.

In the second approach, we measured protein synthesis directly by metabolic labelling of infected and uninfected cells using "light" and "heavy" labelled lysine and arginine (Ong et al., 2002). Total cell lysates were subjected to mass spectrometry, and differentially synthesised proteins were recorded. Eight hundred twenty proteins were consistently identified in five separate proteomic experiments, and 692 were quantified in at least 70% of all samples. As predicted from the AHA-incorporation experiments above, the de novo protein synthesis was found to be distinguishably reduced in *C. trachomatis*-infected host cells (Figure 6a,b). Protein set enrichment analysis identified a number of biological processes and structures that were down-regulated in samples from infected cells (Table S8). A number of these protein/gene sets had also been identified in the analysis of polysomal mRNA-samples (Figure 4, above). The protein data were mostly not in the bracket normally considered statistically significant. This may have been a function of lower sensitivity of this method, because low-abundance proteins are not easily found in total cell extracts. However, main biological terms were found to be down-regulated both by polysomal mRNA and by proteome analysis (highlighted in Table S8), suggesting that the translational changes in infected cells indeed cause substantial changes to protein synthesis during infection of human cells with *C. trachomatis*.

Hierarchical clustering of proteins with altered synthesis identified a number of candidates that were up- or down-regulated in terms of protein synthesis during chlamydial infection. Figure 6c shows the most significantly regulated proteins of this analysis and Table S9 compares those with the microarray data.

3 | DISCUSSION

In this study, we report reduced protein synthesis in HeLa cells infected with *C. trachomatis*. This reduction was associated with a change in the ribosomal profile. The infection caused a substantial change in abundance of mRNAs in the polysomal fractions isolated from infected cells, as well as detectable differences in protein synthesis. The data suggest that a stress response to the infection causes a reduction in translation initiation, which has a specific effect on the synthesis of genes of various biological areas, affecting structure and signalling of the host cell. Although the chlamydial protease CPAF also reduced protein synthesis when expressed in human cells in the absence of infection, it was not required for a reduction of protein synthesis by the infection.

Stress granules do not seem to play a role in infected cells. We also tested for a potential role of ribophagy by blocking autophagy

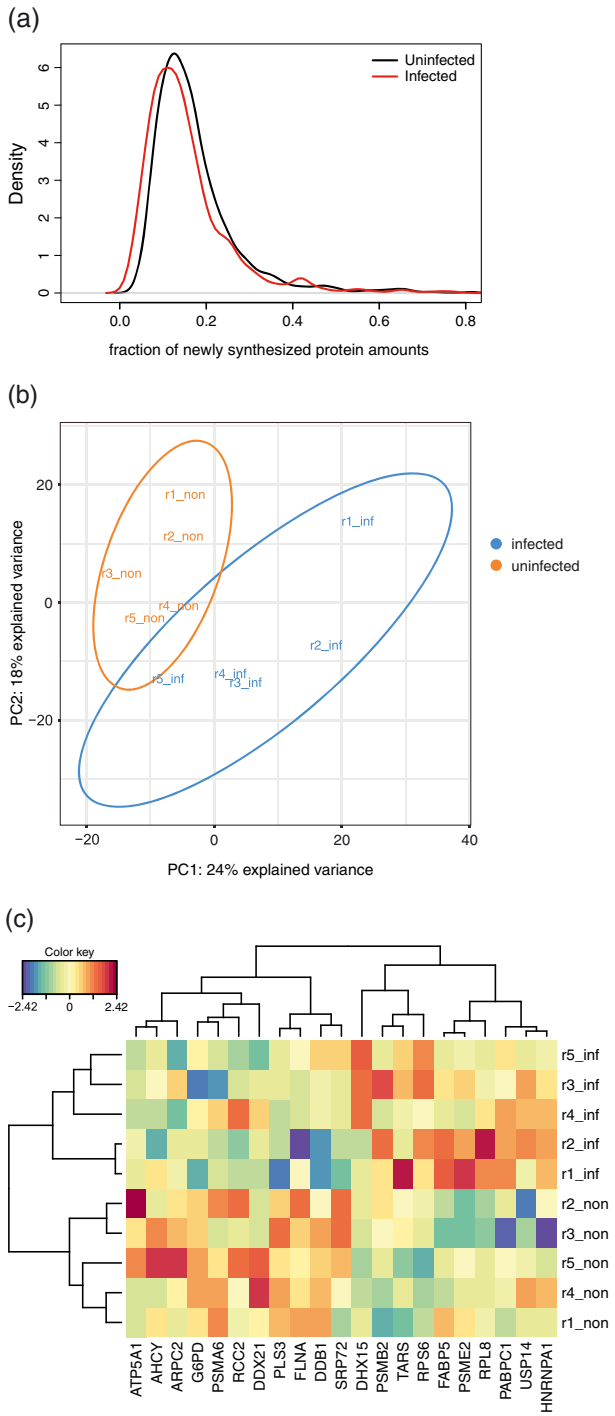

FIGURE 6 Protein synthesis in uninfected and *Chlamydia trachomatis*-infected HeLa cells. (a) Proteomic probing of protein synthesis according to (Boisvert et al., 2012). The density plot shows the fraction of newly synthesised protein amounts based on the abundance of identified individual peptides. Data are averaged from five replicate experiments (30–33 hr of infection), only considering human proteins. (b) Supervised partial least squares discriminant analysis, showing partial separation of the protein synthesis rates for infected and non-infected cells; further emphasising that *Chlamydia* infection has a mild but notable impact on the global protein synthesis of the host cell (PC: principal component). (c) Hierarchical clustering of significantly differentially synthesised proteins (quantified in each condition, limma-moderated p value < 0.05) and conditions by *Euclidean* distance. Infected and non-infected cells cluster into two according groups. The colour code indicates $\log_2$ H/L ratios of protein intensities

pathways. However, HeLa cells deficient in LC-III and fibroblasts deficient in ATG5 showed, when infected by *C. trachomatis*, protein synthesis reduction similar to wild type cells (data not shown). Phosphorylation of eIF2α occurred in a way comparable with other situations of stress and indicates a cellular stress response. Phosphorylation of eIF4B, as observed during chlamydial infection (although consistently only with the L2 but not the E strain), is a less frequent feature of cellular stress, and we did not detect it in cells exposed to other forms of stress such as serum starvation. This phosphorylation has notably been found during infection with the Kaposi sarcoma-associated herpesvirus (Kuang et al., 2011). eIF4B can bind both mRNA and the 40S ribosomal subunit (Altmann, Wittmer, Methot, Sonenberg, & Trachsel, 1995). It is a target of p70 S6 kinase of the PI3K-mTOR-signalling pathway, and the phosphorylation of eIF4B by either S6K or by protein kinase B stimulates protein synthesis (Dennis, Jefferson, & Kimball, 2012). This suggests that the phosphorylation of eIF4B during chlamydial infection is more likely a cellular attempt to up-regulate translation, in response to other signals.

One possibility how stress is caused is the depletion of amino acids through the bacterial growth. When amino acids are removed from the culture medium, S6 kinase is rapidly dephosphorylated (Hara et al., 1998), presumably through the regulation of mTOR, although this is not very well understood (Jewell & Guan, 2013). However, there was no consistent reduction in S6-phosphorylation during chlamydial infection, unlike the findings reported for *Salmonella*-infection HeLa cells where amino acid depletion correlated with the dephosphorylation of S6 kinase (Tattoli et al., 2012). This argues against amino acid starvation as the driving force of the observed alteration of protein synthesis.

Although (at least in yeast) 80S monosomes are transcriptionally active (Heyer & Moore, 2016), most translation occurs on polysomes (Warner & Knopf, 2002). Total mRNA levels may therefore not always be good predictors of actual protein synthesis (Sonenberg & Hinnebusch, 2009), and this appears to be the case during chlamydial infection. Analysis of polysomal mRNA, followed by gene ontology analysis, identified a number of biological processes whose activity is predicted to be down-regulated. A number of these biological processes were also found down-regulated by proteomic analysis of infected cells, providing validation of the results and confirming that this polysomal analysis indeed reflected protein synthesis, at least in some processes.

One regulated process was translation itself: Genes involved in translation were less highly expressed in infected cells. This may act as a feedback mechanism, following a stress-induced reduction of ribosomal initiation. Mitochondrial proteins appear to be affected on two levels, both by down-regulation of the import machinery and by direct reduction of the synthesis of proteins of the inner mitochondrial membrane. Structural alterations to mitochondria during *C. trachomatis* infection have been described (Chowdhury et al., 2017). Such alterations may be linked to reduced synthesis of these protein groups. Smaller alterations were noted in biological systems such as ER-physiology and cytoskeleton. The ER is targeted by chlamydial infection, causing close apposition of the ER-membrane to the inclusion membrane as well as the recruitment of ER-proteins to the contact sites with the inclusion (Derre, 2015). Chlamydial proteins are further

known that target the ER and appear to connect it to the chlamydial inclusion (Stanhope, Flora, Bayne, & Derre, 2017). The actin cytoskeleton is also known to respond to chlamydial infection (Wesolowski & Paumet, 2017). Changes in protein synthesis may be one basis of such changes to these cellular structures.

CPAF is a prominent chlamydial protease. CPAF can cleave many host cell substrates, although most of these cleavage events appear to be only detectable when infected cells are lysed and CPAF is not experimentally inhibited during the lysis process, suggesting that these cleavage events are lysis artefacts (Chen et al., 2012). Nevertheless, CPAF-dependent cleavage events have been demonstrated (Snavely et al., 2014), and biological processes have been attributed to the genomic presence of CPAF in *C. trachomatis* (Brown et al., 2014; Yang et al., 2015). The effect of active CPAF on protein synthesis of human cells we observed in this study may therefore play a role during infection of human cells with *C. trachomatis*, even though reduction of protein synthesis was still seen in cells infected with a CPAF-deficient strain of *C. trachomatis*.

The separate analysis of polysomal versus total mRNA levels showed intriguing differences. Regulatory modules were identified that were clearly visible in polysomal mRNA analysis but absent from the same analysis when performed on total mRNA. The two main signalling nodes identified, namely, EGFR-signalling as up-regulated and p53-signalling as down-regulated, are consistent with earlier biological reports.

Besides protein synthesis, enhanced host cell protein degradation has also been observed during chlamydial infection, although a mechanism is not known (Olive et al., 2014). The protein levels thus appear to be regulated on many levels during chlamydial infection, and the focus on only one change may be misleading when a particular gene is investigated.

Our results suggest the necessity to investigate several levels of protein expression when the levels of a particular host gene are interrogated during chlamydial infection. The data identify a new layer of regulation in which *Chlamydia* affects the function of the host cell. It seems likely that the reduction of protein synthesis is a cellular response to the stress generated by the infection. *Chlamydia*, on the other hand, must have learned to handle the changes to protein synthesis.

4 | EXPERIMENTAL PROCEDURES

4.1 | Cell culture

Human and mouse cell lines were cultured at 37°C/5% CO_2. HeLa cells and 293-TRex-3xGyrB-CPAF cells (Paschen et al., 2008) were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich and PAA Laboratories). For the 293-TRex-3xGyrB-CPAF cells, 5-μg ml^{-1} blasticidin (Invivogen, #ant-bl-05) and 350-μg ml^{-1} zeocin (Invivogen, #ant-zn-1) were added. To induce and activate CPAF, 5-ng ml^{-1} anhydrotetracycline (IBA Life Sciences) and 1-μM coumermycin (Sigma) were added (Paschen et al., 2008). Mouse oviduct cells (C57epi.1; Johnson, 2004) were a kind gift from Dr Raymond Johnson and were maintained in epithelial growth medium (1:1 DMEM and Nutrient Mixture F12-Ham [Thermo Fisher Scientific, #11320033], with 10% FCS, 1% MEM non-essential amino acids solution [Thermo Fisher Scientific, #11140050], 1% sodium pyruvate [Thermo Fisher Scientific, #11360-039], 5-μg ml^{-1} bovine insulin solution [Sigma, #I4011], and 12.5-ng ml^{-1} keratinocyte growth factor [Sigma #K1757]).

4.2 | Infection with *Chlamydia trachomatis*

C. trachomatis biovar LGV L2/434/Bu was obtained from the American Type Culture Collection. The isogenic strains Rst5 (CPAF-competent) and Rst17 (CPAF-deficient) were a generous gift from Dr Raphael Valdivia, Duke University. The strains were amplified in HeLa cells and stored in sucrose-phosphate-glutamic acid (SPG) medium (0.2-M sucrose, 8.6-mM Na_2HPO_4, 3.8-mM KH_2PO_4, and 5-mM glutamic acid, pH 7.4) at −80°C. Host cells were seeded the day before infection. *C. trachomatis* was added directly to the 80% confluent cells.

The *C. trachomatis* serovar E DK-20, isolated in 1977 by the Institute of Ophthalmology, London, UK (Treharne, Darougar, & Jones, 1977), was kindly provided by Dr J.H. Hegemann (Düsseldorf, Germany). The *C. muridarum* strain 03DC39 (MoPn) was purchased from the Federal Research Institute for Animal Health (FLI, Jena, Germany). Infection of host cells with these two bacterial strains was done by centrifugation onto the respective host cells at 2,700 × *g* for 1 hr.

4.3 | Click-iT labelling of nascent proteins or RNA

For labelling newly synthesised proteins, we made use of the "click chemistry" using the Click-iT cell reaction buffer kit (Thermo Fisher Scientific, #C10269). About 2–3 hr before harvesting, cells were incubated in medium deficient in methionine (oviduct cells in RPMI, Thermo Fisher Scientific, #A1451701) or both methionine and cysteine (HeLa cells in DMEM, Thermo Fisher Scientific, #21013024). Doxycycline (2.25 μM, Sigma, #44577) or rifampicin (60 μM, Sigma, #R3501) was added where indicated. One to 2 hr before harvesting, 25–50 μM of AHA (Thermo Fisher Scientific, #C10102) was added. After harvesting and fixation with 4% paraformaldehyde (Morphisto, #11762), cells were permeabilised with 0.2% Triton X-100 and washed with 3% bovine serum albumin. Incorporated AHA was labelled with Alexa Fluor 488 or 467 alkyne (Thermo Fisher Scientific, #10267 or #10278) and analysed by microscopy (Keyence BZ-9000) or flow cytometry (FACS Calibur, Becton Dickinson). For measuring RNA synthesis, 100 μM of 5-ethynyl uridine (Thermo Fisher Scientific, #E10345) was added for 1 hr and was "clicked" to Alexa Fluor 488 azide (Thermo Fisher Scientific, #A10266) according to the manufacturers' protocol (Thermo Fisher Scientific, #C10329).

4.4 | Detection of host cell translation by surface sensing of translation assay

For monitoring and quantification of global eukaryotic protein synthesis in mammalian cells, pulse-chase labelling with puromycin followed by immunofluorescence detection of puromycin-labelled cell surface

proteins was performed using the method described in (Schmidt et al., 2009). Around 70 min before harvest, cells were pulsed with puromycin (10 μg ml^{-1}), followed by two washes with complete medium and a chase in puromycin-free medium for 60 min. Cells were harvested with Versene (Gibco), washed twice with PBS/0,1% BSA, and stained for 30 min on ice using an AlexaFluor647-conjugated anti-puromycin antibody (Merck/Millipore, clone 12d10, MABE343-AF647, 1:50). After two washes in PBS/0,1%BSA, cells were fixed with 4% paraformaldehyde for 30 min on ice and analysed by flow cytometry (FACS Calibur, BD Biosciences).

4.5 | RNA gel electrophoresis

Total RNA was isolated from uninfected HeLa cells or cells infected with *C. trachomatis* for 30 hr by using the High Pure RNA Isolation Kit (Roche, #11828665001). RNA was run on a 0.8% agarose gel and visualised with ethidium bromide.

4.6 | Stress induction

Stress was induced in HeLa cells via treatment with tunicamycin (5 μg ml^{-1}, Sigma, #T7765), BD GolgiPlug™ Protein Transport Inhibitor containing brefeldin A (1 μl ml^{-1}, BD Biosciences, #555029), or by incubation in medium without FCS, Met, and Cys. Protein synthesis in HeLa cells was inhibited by cycloheximide (100 or 200 ng ml^{-1}, Sigma, #01810) for 2.5 hr.

4.7 | Polysome profiling

HeLa cells were infected with *C. trachomatis* or treated with stress inducers as described above. Shortly before harvesting, 50 μg ml^{-1} of cycloheximide was added for 5 min. Cells were lysed in lysis buffer containing 10-mM Tris–HCl (pH 7.5), 30-mM KCl, 10-mM $MgCl_2$, 1-mM DTT, 0.5% NP-40, 100-μg ml^{-1} CHX, and 200 U ml^{-1} of RNAsin (Promega, #N2511) for 15 min on ice and disrupted by passage through a G26 syringe. Lysates were centrifuged at 4°C for 30 min. Supernatants adjusted for protein content were layered on 7–45% sucrose density gradients (10-mM Tris pH 7.5, 60-mM KCl, 10-mM $MgCl_2$, 1-mM DTT, 7% or 50% sucrose, 10-μg ml^{-1} CHX) and centrifuged at 260,000 × *g* for 2 hr at 4°C in a Beckman SW41Ti rotor. Fractions were collected by upward displacement through a Bio-Rad EM-1 UV monitor for continuous measurement of the absorbance at 254 nm.

4.8 | Western blot

For RIPA extracts, cells were washed with PBS, trypsinized, and pelleted (3,800 × *g*, 5 min, 4°C). After a washing step with PBS, cells were resuspended in RIPA buffer (Sigma, #R0278) supplemented with protease inhibitor mix (Complete, Roche, #04693132001) and Benzonase (Merck, #70746) and incubated for 30 min on ice. For urea extracts, cells were incubated for 10 min with 8-M urea (J. T. Baker, #0345), supplemented with 325-U ml^{-1} Benzonase and 1× PhosSTOP (Roche, #4906845001), harvested by scraping, and pelleted. Where indicated, rapamycin was added at a concentration of 50 nM 2 hr before harvest. Following SDS-PAGE, proteins were blotted onto nitrocellulose membranes. Antibodies used were specific for phospho-eIF4B (Cell Signaling, #3591), phospho-eIF2α (Cell Signaling, #9721S), phospho-p70S6K1 (Cell Signaling, #9234), and GAPDH (Millipore, #MAB374).

4.9 | Microarray analysis

Polysomal RNA (from polysomal fractions isolated as above) as well as total RNA was isolated using the High Pure RNA Isolation Kit (Roche, #11828665001) and precipitated. Glycogen (300 μg ml^{-1}) was added as well as 1/10 the volume ammonium acetate (750 mM, Sigma, #A2706) and 2.5 volumes of 100% ethanol. After incubation for 20 min at −20°C, samples were washed with 75% ethanol, dried, and dissolved in water. Samples with an RNA integrity number of greater than 8.5 were further processed with the Nugen Ovation Pico WTA V2 kit (NuGEN, #3302) according to the manufacturer's protocol. The amplified cDNA was fragmented and labelled using the Affymetrix WT Terminal Labeling and Controls Kit. Labelled fragments were hybridised to GeneChip Human Gene ST 2.0 arrays for 16 hr at 45°C with 60 rpm in an Affymetrix Hybridization Oven 645. After washing and staining, the arrays were scanned with the Affymetrix GeneChip Scanner 3000 7G. CEL-files were produced from the raw data with Affymetrix GeneChip Command Console Software Version 4.0. Raw data were normalised via the Single Channel Array Normalization algorithm (Piccolo et al., 2012) mapping the probes to Entrez gene IDs via the custom chip definition file from the BrainArray resource in version 20 (Dai et al., 2005). The gene expression data are available at the Gene Expression Omnibus database under accession ID GSE114556. The reviewers can access the data using the secure token "qzklukaatbmtjab."

4.10 | Differential gene expression and pathway analysis

To find differentially enriched in polysomal transcripts during *C. trachomatis* infection, we compared the differential transcript abundance in polysomal versus total RNA during infection relative with the same transcript difference under control conditions. A positive value denotes RNA that is polysomally enriched during infection, a negative value denotes polysomal depletion during infection. Differential expression analysis was calculated using the R/Bioconductor package limma (Ritchie et al., 2015). Functional annotation of the results from limma was calculated based on the fold expression values via gene set enrichment (Luo, Friedman, Shedden, Hankenson, & Woolf, 2009) using the Gene Ontology as gene sets as provided with the org. Hs.eg.db genome-wide annotation package for humans (Bioconductor, version 3.6).

4.11 | Subgraph construction from differential gene regulation

We constructed a protein interaction network from the STRING database in version 10 (Szklarczyk et al., 2015), using all interactions that

have an experimental score >0.4. Deleting self-loops as well as the four most connected nodes resulted in a network comprising 16,486 nodes and 387,786 edges. From this, we extracted a maximally scoring subgraph of 40 nodes that is fully connected and optimised to maximise the number of most differentially regulated genes (Fang & Gough, 2014).

4.12 | Venn diagram

Differentially regulated genes between the different conditions (infection vs. control) and RNA fractions (total or polysomal RNA) were calculated using robust regression from the R/Bioconductor limma package (Ritchie et al., 2015; False Discovery Rate [FDR] corrected p value < 0.05 and log Fold Change > +/−0.5 for up- or down-regulated genes).

4.13 | Metabolic labelling and proteome comparison

For stable isotopic labelling by amino acids in cell culture (SILAC), HeLa cells were labelled with either L-arginine (Arg0) and L-lysine (Lys0; "light" amino acids) or with $^{13}C_6$ L-arginine (Arg6) and $^{2}H_4$ L-lysine (Lys4; "medium" amino acids) in DMEM supplemented with 10% dialysed FCS and glutamine (Silantes, #282946423) for at least 2 weeks. Then, cells from both conditions were either infected with *C. trachomatis* for 28–34 hr or not. Two to 6 hr before harvesting, medium of "medium" labelled cells was changed to DMEM supplemented with $^{13}C_6{}^{15}N_4$ L-arginine (Arg10) and $^{13}C_6{}^{15}N_2$ L-lysine (Lys8; "heavy" amino acids; Silantes, #282986444) to enable detection of newly synthesised proteins (ratio "heavy"/"light"). Cells were lysed on ice for 30 min in 4-M guanidine hydrochloride dissolved in 100-mM HEPES pH 7.4 containing protease inhibitor and Benzonase. After boiling and sonification, differently labelled samples were mixed equally, precipitated with acetone, reduced with 10-mM DTT for 30 min at 75°C, and alkylated using 25-mM iodoacetamide for 1 hr in the dark. Protein mixtures were separated by SDS-PAGE (Novex 4–20% Tris-Glycine Mini Gel), gel lanes were cut into six equal slices and in-gel digested using trypsin (Promega, #V5111; Shevchenko, Tomas, Havlis, Olsen, & Mann, 2006). Resulting peptide mixtures were processed on STAGE tips as described (Rappsilber, Mann, & Ishihama, 2007).

Liquid chromatography–tandem mass spectrometry measurements using a Q-Exactive+ were performed as described (Biniossek et al., 2016). MS data were analysed by MaxQuant (Cox & Mann, 2008) version 1.5.28 with the Uniprot human database downloaded on November 2015, counting 20,193 reviewed entries. Initial search tolerance was 20 ppm, main search tolerance was 6 ppm for precursors, and 20 ppm for fragment ions. Fixed modification was cysteine carbamidomethylation without variable modifications. Tryptic cleavage specificity with up to one missed cleavage was used. A minimum of 1 peptide was required for protein identification. The false discovery rate was 0.01 for peptide and protein identifications. For quantitative comparison, we used a labelling scheme based on multiplicity 3, corresponding to the SILAC scheme. The average ratios of heavy and light intensities of all quantifiable peptides in five independent experiments were calculated. Protein ratios were $\log_2$ transformed but not further normalised.

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AUTHOR CONTRIBUTIONS

MO, TT, NN, LVH, SB, JV, and SK designed, performed experiments, and analysed results. MB and HB analysed microarray data and constructed gene expression and gene regulation maps. LK analysed proteomics data, and OS supervised and analysed results of proteomics experiments. MLB conducted the mass spectrometry experiments. GH designed and supervised the study and wrote the paper.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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