HHS Public Access

Author manuscript

Biochem Biophys Res Commun. Author manuscript; available in PMC 2020 January 08.

Published in final edited form as:

Biochem Biophys Res Commun. 2019 January 08; 508(2): 421–429. doi:10.1016/j.bbrc.2018.11.034.

The Molecular Mechanism of Induction of Unfolded Protein Response by *Chlamydia*

Zenas George[1], **Yusuf Omosun**[1,2], **Anthony A. Azenabor**[3], **Jason Goldstein**[1], **James Partin**[1], **Kahaliah Joseph**[1], **Debra Ellerson**[1], **Qing He**[1,2], **Francis Eko**[2], **Melissa A. McDonald**[1], **Matthew Reed**[1], **Pavel Svoboda**[1], **Olga Stuchlik**[1], **Jan Pohl**[1], **Erika Lutter**[4], **Claudiu Bandea**[1], **Carolyn M. Black**[1], and **Joseph U. Igietseme**[1,2]

[1]Centers for Disease Control and Prevention (CDC), Atlanta GA USA

[2]Morehouse School of Medicine, Atlanta GA USA

[3]University of Wisconsin, Milwaukee WI USA

[4]Oklahoma State University, Stillwater Ok USA.

Abstract

The unfolded protein response (UPR) contributes to chlamydial pathogenesis, as a source of lipids and ATP during replication, and for establishing the initial anti-apoptotic state of host cell that ensures successful inclusion development. The molecular mechanism(s) of UPR induction by *Chlamydia* is unknown. *Chlamydia* use type III secretion system (T3SS) effector proteins (e.g, the Translocated Actin-Recruiting Phosphoprotein (Tarp) to stimulate host cell's cytoskeletal reorganization that facilitates invasion and inclusion development. We investigated the hypothesis that T3SS effector-mediated assembly of myosin-II complex produces activated non-muscle myosin heavy chain II (NMMHC-II), which then binds the UPR master regulator (BiP) and/or transducers to induce UPR. Our results revealed the interaction of the chlamydial effector proteins (CT228 and Tarp) with components of the myosin II complex and UPR regulator and transducer during infection. These interactions caused the activation and binding of NMMHC-II to BiP and IRE1α leading to UPR induction. In addition, specific inhibitors of myosin light chain kinase, Tarp oligomerization and myosin ATPase significantly reduced UPR activation and *Chlamydia* replication. Thus, *Chlamydia* induce UPR through T3SS effector-mediated activation of NMMHC-II components of the myosin complex to facilitate infectivity. The finding provides

Correspondence: Joseph U Igietseme, PhD, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention (CDC), 1600 Clifton Rd, Atlanta, GA 30333 USA, jigietseme@cdc.gov, Tel: +1404-639-3352; Fax: +1404-718-4011.

Ethics Statements: I declare that none of the authors has a financial interest related to this work. All of the authors consented to this submission and the results are not under consideration for publication elsewhere. All animal protocols were approved by the CDC Institutional Animal Care and Use Committee (IACUC) under Protocol # 2894IGIMOUC-A1. The CDC IACUC is guided by Title 9, Chapter I, Subchapter A--Animal Welfare (USDA Regulations).

Note: The conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry.

greater insights into chlamydial pathogenesis with the potential to identify therapeutic targets and formulations.

Keywords

Chlamydia; pathogenesis; unfolded protein response; T3SS; myosin II complex

1. Introduction

Pelvic inflammatory disease, tubal factor infertility and ectopic pregnancy are serious complications of human genital infection by the Gram-negative intracellular bacterium *Chlamydia trachomatis* (1–3). The rising infections with attendant healthcare cost is a major burden on the public healthcare system (4). A better understanding of the molecular pathogenesis of *Chlamydia* diseases will aid the design of therapeutic measures. *Chlamydia* acquire nutrients such as lipids and ATP from host cells (1, 2) and require living cells for their intracellular survival, replication and inclusion development (5). Therefore, chlamydial infection produces conditions such as extra protein expression from the microbe that perturb the protein folding and modification functions of the endoplasmic reticulum (ER), causing ER stress that can lead to cell death if no cellular adaptation is induced (6, 7). The unfolded protein response (UPR) is a cellular response to ER stress due to calcium displacement, increased protein expression and demand for protein modification by an intracellular microbial parasite, or excessive accumulation of misfolded proteins (8, 9). The induction of UPR aims at restoring cellular homeostasis by enhancing host cell survival through autophagy promotion (10); increases protein folding capacity of ER (11); activates ER-associated protein degradation to relieve the stress on ER protein-folding machinery; or, if stress is not resolved, activates apoptotic pathways (12). The three ER membrane proteins that are transducers of the UPR signaling are: inositol-requiring enzyme-1α (IRE1α), protein kinase RNA-activated (PKR)-like ER kinase (PERK), and the activating transcription factor-6α (ATF6α) (13). These UPR transducers are kept inactive by binding to the ER chaperone GRP78/BiP (the master regulator of UPR) when the ER folding capacity is operating normally. Activators of UPR either cause the ER stress through depletion of Ca^{2+} from the ER (14), or the accumulation of unfolded/misfolded, overexpressed or modified proteins in the ER, any of which dissociates the UPR transducers from BiP to activate UPR signaling (15, 16).

Indeed, recent reports have demonstrated that *Chlamydia*, like certain other obligate intracellular microbial agents such as *Brucella melitensis*, *Listeria monocytogenes* and Hepatitis C virus, activate the UPR pathways to enhance their intracellular survival and replication (15, 17–19). Mechanistically, Shiga toxigenic strains of *Escherichia coli* produce AB5 subtilase cytotoxin that binds to and inactivates BiP to activate the UPR transducers (20); Hepatitis C virus activates UPR at least in part due to the accumulation of immature core protein (Core 199) in the ER lumen (15), while *L. monocytogenes* require its cholesterol-dependent cytolysin toxin (listeriolysin O) to induce UPR (18). However, the mechanism of UPR activation by *Chlamydia* is not known. *Chlamydia* utilize various mechanisms including clathrin-mediated endocytosis for its uptake by host cells (21). Upon

host cell invasion, *Chlamydia* recruit and activate elements of the cytoskeleton such as actin, myosin complex and microtubules to enhance entry, facilitate the establishment of parasitophorous inclusion and its structural stability, replication and extrusion of EBs from host cell (22–26). To achieve this, *Chlamydia* translocate certain T3SS effector proteins e.g., Tarp and CT166 into the host cell cytoplasm (27, 28) to rapidly recruit and activate members of the host's GTPase proteins (such as Rac1 and Cdc42) at site of entry and around the vesicle (29, 30). Specifically, the Tarp protein possesses G and F actin binding domains (22), induces actin nucleation, polymerization and filament formation (31). Also, Tarp regulates the recruitment and activation of host cell kinases (e.g., the ROCK and Src family kinases) around the inclusion and acts as a scaffold for Rac1 guanine nucleotide exchange (32). The CT166 protein post-translationally modifies the GTPase itself (28) while the CT228 is involved in the recruitment of components of the myosin II complex and co-localizes with Src family of kinases around the inclusion membrane (23). These kinases inactivate the myosin light chain phosphatase (MYPT1) through phosphorylation at Threo-852, causing its release from the myosin light chain 2 (MLC2) and also the activation of the myosin heavy chain II (NMMHC-II) (33). Interestingly, activation of the NMMHC-II is required for the activation and modulation of the most conserved IRE1α arm of UPR in eukaryotes (34–36). Besides, the chlamydial inclusion membrane protein CT813 also known as InaC (37) recruits ADP-ribosylation factor 1 (ARF1) and 4 (ARF4) to effect the acetylation and detyrosination of microtubules required for interaction with the Golgi complex and production of infectious EBs (24). These findings suggest that chlamydial infection may activate the cytoskeletal network and associated molecules that are required for UPR activation and stabilization.

In this study, we investigated the hypothesis that the release of the T3SS effector proteins (e.g., Tarp and CT228) into host cells by *Chlamydia* leads to the recruitment and activation of the NMMHC-II which then binds to BiP and IRE1α, resulting in UPR activation. Our results identified NMMHC II as molecules that bind to BiP and IRE1α during *Chlamydia* infection. Specific inhibitors that block the activation and function of NMMHC II, also suppressed UPR activation and *Chlamydia* replication. Thus, *Chlamydia* induce UPR through the activation of NMMHC-II components of the myosin complex, providing greater insights into *Chlamydia* pathogenesis with potential therapeutic targets.

2. Materials and Methods

2.1. *Chlamydia* strains and Cell Cultures

Chlamydia muridarum Nigg (the agent of mouse pneumonitis, MoPn - animal specific strain), *C. trachomatis* (human specific strain) serovars L2/LGV-434 and D/UW-3 were grown in HeLa 229 cells (ATCC, Rockville, MD USA) and purified elementary bodies (EBs) were tittered as infectious forming units per millimeter (IFU/ml) using standard procedures previously described (19)(38)(39).

2.2. Immunoprecipitation assays

Immunoprecipitation kit (abcam Cambridge, MA, USA) was used in pull down experiments with UPR specific antibodies. M-PER® Mammalian Protein Extraction Reagent (Thermo

Fisher Scientific, Houston TX, USA) was used in harvesting protein samples; 4–20% PROTEAN® TGX™ protein gel, nitrocellulose membrane and Clarity™ and Clarity Max™ Western ECL Blotting Substrates (Bio-Rad, Hercules, CA, USA) were used in protein separation and western blotting. Pathfinder® *Chlamydia* Culture Confirmation System (Bio-Rad, Hercules, CA, USA) was used in confirmation of *Chlamydia* inclusions. Using anti-BiP, anti-IRE1α [p-Ser724] and anti-PERK [p-Thr981] antibodies, a pull down assay was conducted according to manufacturer's procedure (Immunoprecipitation kit; Abcam, Cambridge, MA, USA). Briefly, cells infected with *Chlamydia* or non-infected control at different time points were lysed using non-denaturing lysis buffer (1 ml lysis buffer/10^7 cells). Total protein concentration was measured using Bradford reagents and 500μg of protein were pre-cleared using Protein A/G Sepharose® beads. After preclearance, proteins were incubated with appropriate antibody overnight with gentle rocking at 4°C. Antigen-Antibody complex were mixed with Protein A/G Sepharose® beads and incubated for 1 h at 4°C. Antigen-Atibody-Protein A/G Sepharose® beads complex were washed and bound proteins eluted with SDS sample buffer heated at 90°C for 5 min.

2.3. Proteomics analysis

SDS PAGE separation of immunoprecipitated proteins was conducted using 4–20% PROTEAN® TGX™ protein gel system. Protein bands of interest were excised and analyzed with nanoLC-MS/MS system as previously described (40). Briefly, each gel slice was subjected to in-gel trypsin digestion and 10% of each digest was analyzed with nanoLC-MS/MS system. The data was searched using the MASCOT search engine against a custom database containing human, mouse, and chlamydial proteins.

2.4. Peptide design and assay

A peptide spanning amino acid 618–654 of Tarp (NCBI Reference Sequence: YP_001654788.1) was designed to mimic the proline-rich, Tarp homo-oligomerization domain (22). Tarp is usually translocated into the host cell cytoplasm via the T3SS but the initial *in silico* structural analysis indicated that it was not cell permeable (41). To facilitate the translocation of the peptide into host cells, the peptide was conjugated to the C-terminus of HIV's trans-activator of transcription (TAT) peptide known to translocate conjugated cargos into mammalian cells (42). A non-functional TAT-fused peptide was also designed using a randomly scrambled peptide generated from the proline-rich amino acid 618–654 using PepControls web server tool (43). The efficacy of the peptides in blocking Tarp functions were assayed by pre-treating HeLa cells with different concentrations for 1 hour followed by infection with *C. trachomatis* L2 for 48h and enumeration of *Chlamydia* inclusions.

2.5. Western blot analysis

At appropriate time points, total protein were prepared from cells by lysing *in situ* using M-PER® Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, lL, USA) supplemented with EDTA-Free protease inhibitor cocktail according to manufacturer's instruction. Briefly, 1ml of protein extraction reagent supplemented with 1× protease inhibitor was added to 10^7 cells and incubated on ice for 5 min. Lysed cells were aspirated into 15ml tube and vortexed for 30 sec. Protein concentration was determined using

Bradford method (44). Equal amount of protein were loaded onto 4–20% PROTEAN® TGX™ protein gel (Bio-Rad, Hercules, CA, USA) and electrophoresed for 1 h, and the separated protein bands were transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were washed, blocked with either 2% casein or 5% non-fat milk or 5% BSA for 1 h, probed with primary antibody overnight at 4°C, and horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1 h. Membranes were analyzed using ChemiDoc XRS+ system (Bio-Rad) and pictures with band intensity analysis were performed using Image Lab™ software.

2.6. Myosin-II complex inhibition

In vitro studies for the effect of inhibition of myosin light chain kinase (MLCK) and Myosin-II ATPase activities on UPR pathways activation and *Chlamydia* replication were performed by treating cultures with 20μM of ML-7 hydrochloride or 50μM of (S)-(-)-Blebbistatin (Santa Cruz Biotec., Inc, Dallas, TX, USA) or equal amount of DMSO solution that was used in preparing each inhibitor in the infection medium. Cell cultures were pre-treated for 1h with respective inhibitor or DMSO before *Chlamydia* infection and the treatments continues post infection till end of experiments. The effect of myosin-II complex inhibition on UPR activation was measured using the level of IRE1α phosphorylation at Ser724. The effect of myosin light chain kinase (MLCK) and Myosin-II ATPase inhibition on *Chlamydia* replication was measured using Pathfinder® *Chlamydia* Culture Confirmation System (Bio-Rad, CA, USA), by standard procedures. ML-7 hydrochloride inhibits myosin light chain kinase while (S)-(-)-Blebbistatin inhibits Myosin-II ATPase activity. Preliminary studies showed that the concentrations used did not affect cell viability.

2.7. Immunocytochemistry

Chlamydia- infected cells were treated with inhibitors of Myosin-II complex, fixed with cold acetone (stored in −20°C freezer) for 5 minutes in −20°C freezer, washed, blocked with 10% normal goat serum (NGS) in PBS for 30 minutes at 4°C, and incubated with anti-pIRE1 [Ser724] antibody diluted in 1% NGS with rocking overnight at 4°C. Cells were then washed and incubated with secondary antibody (Rabbit IgG-Alexa Fluor 488; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1hr with rocking at room temperature. The slides were viewed face-up on microscope with mounting medium. Quantification of fluorescence was performed by scanning fluorescent-stained cells at 20X objective on a Nikon fluorescent microscope using the NIS-Elements Imaging Software version 3.20 (Nikon Instruments Inc, Melville NY, USA).

2.8. Statistical analysis

Statistical analyses were performed with SigmaPlot software. The data derived from different experiments were analyzed and compared by performing a 1- or 2-tailed *t* test, and the relationship between different experimental groupings were assessed by analysis of variance (ANOVA). Statistical significance was judged at $P < 0.05$.

3. Results

3.1. Several species and strains of Chlamydia induce UPR

We extended previous studies that the mouse agent of pneumonitis (*C. muridarum*) activated all the three arms of UPR (19), by establishing that different strains and serovars of *Chlamydia* induce UPR. Thus, the assessment of UPR induction in epithelial cells using the human *C. trachomatis* (Ct.) serovars D and L. or the murine strain *C. muridarum*, revealed that all the chlamydial strains activated the IRE1α arm of the UPR (Figure 1a). The activation of PERK arm of UPR was observed at 24hr post infection, while the activation of ATF6α arm was not detected in HeLa 229 cells used in these experiments. The activation of the IRE1α arm of the UPR pathways by all chlamydial strains and serovars reflected the fact that it is the most conserved of all the three UPR arms in eukaryotes (36). We therefore confirmed and extended previous studies with primary mouse epithelial cells that all the three arms of UPR were activated by *Chlamydia* (19). Since UPR induction may involve cytoskeletal protein assembly and interaction with the master regulator (BiP) and transducers of UPR signaling especially IRE1α, and chlamydial T3SS effector proteins bind to cytoskeletal proteins (34), we tested the hypothesis that Tarp and CT228 are involved in UPR initiation.

3.2. Tarp and CT228 are present in EBs and upregulated during C. trachomatis infection

Since UPR is a relatively early event during chlamydial infection (19), we predicted that the cytoskeleton-binding *Chlamydia* T3SS effectors proteins involved in UPR induction would be present either in the elementary bodies (EBs) and/or expressed early following host cell invasion by *Chlamydia*. Thus, the presence of Tarp and CT228 in EBs of *C. trachomatis* serovar L2 (Ct L2) and their expression pattern during infection were analyzed with western blot using anti-Tarp and anti-CT228 antibodies. The time period of analysis post-infection (48 h) covered the developmental cycle of *Chlamydia* (45). The results shown in figure 1b (A) indicate that chlamydial EBs possess preformed Tarp whose expression was detectable up to 48hr post-infection, as previously reported (22). Likewise, Figure 1b (B) shows that Ct. L2 EBs contained pre-formed CT228 that, like the Tarp protein is present during the course of infection, although only up to 24hr post infection. The results suggested that the chlamydial-encoded cytoskeleton-binding and activating effectors proteins (Tarp and CT228) are available during infection and may be involved in the initiation of UPR.

3.3. Myosin IIa[p- Ser1943], Myosin IIb and CT228 are co-immunoprecipitated from Chlamydia-infected cells using antibodies against the regulators and transducers of UPR

One of the mechanisms of UPR activation involves the binding of either unfolded/misfolded, post-translationally modified or UPR-inducing proteins to BiP, and the propagation of the UPR signaling pathways through the transducers (20) or IRE1 (46, 47). To investigate *Chlamydia*:host protein interaction during UPR, and the possible involvement of Tarp or CT228, we used anti-BiP and anti-IRE1α[p-Ser724] antibodies to pull down and identify proteins that may be bound to these UPR regulators and transducers during *Chlamydia* infection. The immunoprecipitated proteins were identified using nanoLC-MS/MS system with MASCOT search engine against a custom database containing human, mouse, and *Chlamydia* proteins as previously described (40). Table 1 shows that Myosin-IIa

(NP_002464.1), Myosin-IIb (NP_005955.3), Myosin-IIc (NP_079005.3), and Heat shock protein (HSP) 90-beta isoform a (NP_031381.2) known to play a role in UPR were immunoprecipitated by antibodies against UPR regulator and transducers. The identities of the nanoLC-MS/MS system-identified UPR related proteins were further confirmed using western blot analysis. As shown in figure 2, Myosin IIa[p-Ser1943], Myosin IIb and CT228 were confirmed to be present in the samples immunoprecipitated using anti-BiP and anti-IRE1α[p-Ser724] antibodies. The Western blot analysis also revealed that the binding of BiP/IRE1 to cytoskeletal proteins was more specific for Myosin IIa[p- Ser1943], Myosin IIb and CT228 at early time points up to 9hr post infection than later times after infection. Thus, our results have demonstrated the binding of the heavy chain components of myosin II complex and *Chlamydia* effector protein CT228 to UPR regulator and transducer, and the interactions may lead to UPR induction.

3.4. Inhibition of myosin II activation during Chlamydia infection significantly reduce UPR induction

It has been established that *Chlamydia* uses the T3SS effector proteins Tarp to rapidly recruit and activate members of the host's GTPase proteins (such as Rac1) and Rac1 regulates the recruitment and activation of host cell kinases (32). These kinases inactivate the myosin light chain phosphatase subunit 1 (MYPT1), causing its release from the myosin light chain 2 (MLC2) and also the activation of the NMMHC II (33) required for the induction and modulation of the IRE1α arm of UPR (34, 35). To confirm that activation of myosin heavy chain during *Chlamydia* infection causes UPR induction, we used inhibitor studies to examine the effect of blocking the activation of myosin II complex on UPR induction. To inhibit the activation of the myosin II complex, two important enzymatic activities associated with the activated complex were targeted. These activities include the kinase activity of myosin light chain kinase and the ATPase activity of the myosin heavy chain component. The myosin light chain kinase phosphorylates myosin light chain 2 at Ser19 and Thr18 which allows for activation of the ATPase activity of the heavy chain components of the myosin II complex (48, 49). To block the activity of the myosin light chain kinase, the potent and specific non-competitive inhibitor ML7-hydrochloride was used, while (s)-(-)-Blebbistatin was used for the inhibition of the ATPase activity (50). The activation of the IRE1α arm of UPR was used as a measure of the effect of blocking the activation of myosin II complex on UPR activation because it is the most conserved in eukaryotes (36) and was activated by all strains and serovars of *Chlamydia* tested in this study. As shown in figure 3a, the inhibition of the myosin light chain kinase and the ATPase activities resulted in the reduction of UPR activation as measured by the level of IRE1α phosphorylation. However, the effect was more pronounced with the kinase inhibitor. Predictably, the process involved the dimerization of IRE1α that activates its kinase activity (46) and high oligomer formation that activate the regulated, site-specific endoribonuclease activity for generation of the mRNA for the XBP1 transcription factor (51). The results indicated that the inhibition of the activation or function of the myosin II complex prevented chlamydial-induced UPR.

3.5. Inhibition of myosin II complex activation significantly reduced chlamydial inclusion development

We previously demonstrated that *Chlamydia* relies on UPR activation for its inclusion development and replication (19). Since results shown in figure 3a indicated that the activation of myosin II complex is involved in UPR activation, we extended the study to determine the effect of inhibiting the activation of the myosin II complex on *Chlamydia* replication and inclusion development. As shown in figure 3b, inhibiting the activation of myosin II complex resulted in a significant reduction in *Chlamydia* replication similar to what was observed when IRE1α activity was inhibited (19). Specifically, treatment of infected cells with the myosin light chain kinase inhibitor (ML-7 hydrochloride) produced a significant reduction in number and size of inclusions (P= 0.0001), representing 79% reduction in *Chlamydia* replication. Treatment of infected cells with the myosin ATPase inhibitor ((s)-(-)-Blebbistatin) also produced a significant reduction in number of inclusions (P =0.003), representing 56% reduction in *Chlamydia* replication. The results indicated that the chlamydial activation of the myosin II complex possibly via the T3SS effectors leads to UPR regulator/transducer binding and activation of UPR.

3.6. Role of Tarp in UPR induction and chlamydial replication

We previously demonstrated that inhibition of UPR reduced chlamydia replication as measured by inclusion development (19). We hypothesized that the inhibition of T3SS effector function in cytoskeletal activation would result in UPR inhibition and suppression of chlamydial inclusion development. Accordingly, when chlamydial-infected cells were treated with a TAT-fused peptide mimetic of the proline-rich, Tarp oligomerization domain that confers actin nucleation and polymerization ability (52), UPR induction (Figure 4a) and chlamydial inclusion development (Figure 4b) were also inhibited. It should be observed that the peptide inhibition was specific and TAT-fusion that enhanced the cell permeability of the peptide was necessary for the inhibitory effect of the peptide mimic, since neither the nominal peptide without TAT, nor TAT-fused scrambled peptide suppressed chlamydia replication (Figure 4b). The TAT-fused peptide mimetic approach was applied because the initial *in silico* structural analysis indicated that it was not cell permeable (41). The results indicate that the actin-binding and cytoskeletal assembly promotion function of Tarp that requires its oligomerization is a requirement for the heavy chain myosin II activation, UPR activation and promotion of chlamydial replication and inclusion development. The result also provides greater insights into the role of Tarp in chlamydial pathogenesis with the potential to identify peptide mimetics-based small molecules as therapeutic formulations.

4. Discussion

UPR plays a major role in chlamydial inclusion development and pathogenesis, since the its inhibition is deleterious to the survival and replication of *Chlamydia* (19). UPR also plays a role in the replication of other bacterial pathogens such as *Listeria* (18) and *Brucella* spp (53). However, the mechanism of UPR induction by *Chlamydia* remained unknown. The chlamydial T3SS system is essential for infection since *Chlamydia* uses T3SS effectors to recruit and modify host cell cytoskeletal components as a requirement for a productive infection (22). Interestingly, one of the mechanisms of UPR induction is the binding of

activated cytoskeletal proteins (specifically the type-II myosin) to the master regulator and transducers of UPR (34, 35). Therefore, we tested the hypothesis that the chlamydial T3SS effector proteins recruit, assemble and activate the host's myosin-II complex via the host's GTPases and kinases to produce the activated non-muscle myosin heavy chain II (NMMHC-II), which then binds BiP and/or IRE1α to induce UPR. Our results demonstrated that the chlamydial T3SS effectors Tarp and CT228 are present in the EBs with some expression immediately (within hours) following infection of the host cell, as previously reported (22). Results from immunoprecipitation and proteomics studies, using antibodies against the master regulator (BiP) and transducer (IRE1α) of UPR, and mass spectrometric analysis, confirmed that T3SS effectors were associated with the components of the cytoskeleton and UPR regulators and transducers during chlamydial infection. The binding of components of the cytoskeleton to BiP and IRE1α during infection was also established. Thus, western blot analysis using antibodies specific for myosin IIa and IIb identified the 230kDa bands as having a preference for binding to BiP and IRE1α during the early period of *Chlamydia* infection (Figure 2). The results indicated that the interaction of chlamydial T3SS effectors and host's cytoskeletal proteins with UPR regulators and transducers during *Chlamydia* infection lead to UPR induction.

The myosin II complex of the cytoskeletal network is a hexameric molecule composed of two heavy chains, two essential light chains and two regulatory light chains also called myosin light chain 2 (MLC2) (54). The difference between the isoforms of myosin II is the heavy chain components that may be homodimeric IIa, IIb or IIc, which determines their specific roles (55). The MLC2 component regulates the motor activity of the heavy chain, and the phosphorylated form of MLC2 is required for the myosin II complex activation. However, the myosin II complex is kept inactive through the interaction of MLC2 with the myosin phosphatase target subunit-1 (MYPT1) component of the neighboring Myosin phosphatase (MP) complex (56). MLC2-MYPT1 interaction causes the constitutive dephosphorylation of MLC2, which enhances its binding to the heavy chain component of the myosin II complex, thus keeping the complex inactive (33). The activation of the myosin II complex involves the phosphorylation of MLC2, requiring the phosphorylation of MYPT1 in a Rac1 dependent manner that dissociates MLC2 from both MYPT1 and the heavy chain, thereby activating the latter (57). The myosin II complex is activated during chlamydial infection in a Rac1-dependent manner (23), apparently initiated by the T3SS effector proteins Tarp and CT228 released into host cell. These effectors recruit/activate host GTPases (e.g., Rac1) that activate host kinases (e.g., the ROCK and Src family kinases) at the inclusion surface (23). The host kinases inactivate MYPT1 through its phosphorylation at threonine-853, while MLC2 is activated by phosphorylation at serine-19 and threonine-18 positions, resulting in its release from MYPT1 and the myosin heavy chain II, thereby activating the latter (23, 29, 58). Thus, chlamydial infection causes the activation of the myosin II complex and our results have shown that the inhibition of myosin heavy chain II reduces UPR induction, as measured by phosphorylation of IRE1α at serine-724. Also, our result showing that activated myosin IIb binds to IRE1α is corroborated by published data that Myosin IIb is required for the stabilization of IRE1α oligomer (34). In addition, these results underscored the important role of Tarp in chlamydial pathogenesis. We have shown that the actin-binding and cytoskeletal assembly promotion function of Tarp that requires its

oligomerization is a requirement for the heavy chain myosin II activation, UPR activation and promotion of chlamydial inclusion development. Thus, a TAT-fused peptide mimetic of the proline-rich, Tarp oligomerization domain that confers actin nucleation and polymerization ability (52) inhibited UPR induction and chlamydial inclusion development. This finding provides greater insights into chlamydial pathogenesis with the potential to identify therapeutic targets and peptide-based small molecules as therapeutic formulations. Thus, *Chlamydia* T3SS effector proteins such as Tarp and CT228 recruit and activate host cell's cytoskeletal molecules and this process leads to UPR induction that supports chlamydial development.

Our previous studies showed that *Chlamydia*-induced UPR resulted in the splicing of XBP1 mRNA due to activation of IRE1α (19). The activation of IRE1α for XBP1 splicing involves both the dimerization/autophosphorylation step and the formation of a higher-order oligomers of the dimerized-phosphorylated IRE1α (59). Cytoskeletal components such as F-actin, myosin heavy chain 1 (MYO1) and myosin heavy chain IIb could activate and stabilize IRE1 oligomers during productive UPR activation (34, 35, 59, 60). Also, the over-expression of smooth muscle myosin heavy chain II could activate PERK-eIF2α arm of UPR resulting in increased protein turnover through activation of autophagy (61). Our results suggested that myosin IIa (an isoform of myosin IIb) may also be involved in activating IRE1 possibly through stabilizations of the oligomers since they are both kept inactive by the common myosin light chain 2 that is activated during *Chlamydia* infection (23). In addition, our results show that the inhibition of the activation of myosin components of the cytoskeleton before *Chlamydia* infection using ML-7 hydrochloride (inhibitor of myosin light chain kinase) or (s)-(-)-Blebbistatin (inhibitor of myosin heavy chain ATPase) significantly reduce *Chlamydia* replication and inclusion formation (Figure 3b). This observation correlates with reduction in oligomer formation and the level of IRE1α[p-Ser724] phosphorylation. It is noteworthy that the pre-treatment of host cells with inhibitors of the myosin light chain kinase and ATPase activities before infection effectively caused a reduction in chlamydial replication in these studies, while treatment 24hr after infection did not have any effect (23). The lack of effect on *Chlamydia* replication when ATPase activity was blocked 24hr post infection is not surprising because *Chlamydia* developmental cycle would have advanced by 24hr post infection (45).

In summary, our findings highlighted the roles of cytoskeletal proteins recruited and activated during *Chlamydia* infection in UPR induction. Since the recruitment and modulation of actin, microtubules and the myosin II complex are early events during *Chlamydia* infection, these findings have identified possible targets for prevention of *Chlamydia* infection as an alternative to antibiotic treatment.

Acknowledgements:

We thank Ted Hackstadt of the Laboratory of Intracellular Parasites, NIAID/NIH, Hamilton MT USA and Travis Jewett of the University of Central Florida, Orlando, FL USA for their resource and intellectual contributions to the study.

Funding: This work was supported by the Centers for Disease Control and Prevention (CDC) and PHS grants (AI41231, GM 08248, RR03034 and 1SC1GM098197) from the NIH.

References

1. Omsland A, Sixt BS, Horn M, Hackstadt T. 2014 Chlamydial metabolism revisited: interspecies metabolic variability and developmental stage-specific physiologic activities. FEMS Microbiol Rev 38:779–801. [PubMed: 24484402]
2. Cox JV, Abdelrahman YM, Peters J, Naher N, Belland RJ. 2016 *Chlamydia trachomatis* utilizes the mammalian CLA1 lipid transporter to acquire host phosphatidylcholine essential for growth. Cellular Microbiology 18:305–318. [PubMed: 26381674]
3. Park ST, Lee SW, Kim MJ, Kang YM, Moon HM, Rhim CC. 2017 Clinical characteristics of genital chlamydia infection in pelvic inflammatory disease. BMC Womens Health 17:5. [PubMed: 28086838]
4. Anonymous 2017 Centers for Disease Control and Prevention. Sexually Transmitted Disease Surveillance 2016 Atlanta, U.S. Department of Health and Human Services.
5. Sixt BS, Bastidas RJ, Finethy R, Baxter RM, Carpenter VK, Kroemer G, Coers J, Valdivia RH. 2017 The *Chlamydia trachomatis* Inclusion Membrane Protein CpoS Counteracts STING-Mediated Cellular Surveillance and Suicide Programs. Cell Host Microbe 21:113–121. [PubMed: 28041929]
6. Schwarz DS, Blower MD. 2016 The endoplasmic reticulum: structure, function and response to cellular signaling. Cell Mol Life Sci 73:79–94. [PubMed: 26433683]
7. Samtleben S, Jaepel J, Fecher C, Andreska T, Rehberg M, Blum R. 2013 Direct imaging of ER calcium with targeted-esterase induced dye loading (TED). J Vis Exp:e50317. [PubMed: 23685703]
8. Díaz-Villanueva JF, Díaz-Molina R, García-González V.2015 Protein Folding and Mechanisms of Proteostasis. Int J Mol Sci 16:17193–230. [PubMed: 26225966]
9. Walter P, Ron D. 2011 The unfolded protein response: From stress pathway to homeostatic regulation. Science 334:1081–1086. [PubMed: 22116877]
10. Blázquez A-B, Escribano-Romero E, Merino-Ramos T, Saiz J-C, Martín-Acebes MA.2014 Stress responses in flavivirus-infected cells: Activation of unfolded protein response and autophagy. Frontiers in Microbiology 5:1–7. [PubMed: 24478763]
11. Luo B, Lee AS. 2013 The critical roles of endoplasmic reticulum chaperones and unfolded protein response in tumorigenesis and anticancer therapies. Oncogene 32:805–18. [PubMed: 22508478]
12. Ruggiano A, Foresti O, Carvalho P. 2014 Quality control: ER-associated degradation: protein quality control and beyond. J Cell Biol 204:869–79. [PubMed: 24637321]
13. Carrara M, Prischi F, Ali MM. 2013 UPR Signal Activation by Luminal Sensor Domains. Int J Mol Sci 14:6454–66. [PubMed: 23519110]
14. Lytton J, Westlin M, Hanley MR. 1991 Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. J Biol Chem 266:17067–71. [PubMed: 1832668]
15. Takahashi S, Sato N, Kikuchi J, Kakinuma H, Okawa J, Masuyama Y, Iwasa S, Irokawa H, Hwang GW, Naganuma A, Kohara M, Kuge S. 2017 Immature Core protein of hepatitis C virus induces an unfolded protein response through inhibition of ERAD-L in a yeast model system. Genes Cells 22:160–173. [PubMed: 28097745]
16. Bellucci A, Navarria L, Zaltieri M, Falarti E, Bodei S, Sigala S, Battistin L, Spillantini M, Missale C, Spano P. 2011 Induction of the unfolded protein response by α-synuclein in experimental models of Parkinson's disease. J Neurochem 116:588–605. [PubMed: 21166675]
17. Smith JA, Khan M, Magnani DD, Harms JS, Durward M, Radhakrishnan GK, Liu YP, Splitter GA. 2013 Brucella induces an unfolded protein response via TcpB that supports intracellular replication in macrophages. PLoS Pathog 9:e1003785. [PubMed: 24339776]
18. Pillich H, Loose M, Zimmer KP, Chakraborty T. 2012 Activation of the unfolded protein response by *Listeria monocytogenes*. Cell Microbiol 14:949–64. [PubMed: 22321539]
19. George Z, Omosun Y, Azenabor AA, Partin J, Joseph K, Ellerson D, He Q, Eko F, Bandea C, Svoboda P, Pohl J, Black CM, Igietseme JU. 2017 The Roles of Unfolded Protein Response Pathways in *Chlamydia* Pathogenesis. J Infect Dis 215:456–465. [PubMed: 27932618]
20. Paton AW, Beddoe T, Thorpe CM, Whisstock JC, Wilce MC, Rossjohn J, Talbot UM, Paton JC. 2006 AB5 subtilase cytotoxin inactivates the endoplasmic reticulum chaperone BiP. Nature 443:548–52. [PubMed: 17024087]

21. Hybiske K, Stephens RS. 2007 Mechanisms of *Chlamydia trachomatis* entry into nonphagocytic cells. Infect Immun 75:3925–34. [PubMed: 17502389]
22. Parrett CJ, Lenoci RV, Nguyen B, Russell L, Jewett TJ. 2016 Targeted Disruption of Chlamydia trachomatis Invasion by in Trans Expression of Dominant Negative Tarp Effectors. Front Cell Infect Microbiol 6:84. [PubMed: 27602332]
23. Lutter EI, Barger AC, Nair V, Hackstadt T. 2013 *Chlamydia trachomatis* inclusion membrane protein CT228 recruits elements of the myosin phosphatase pathway to regulate release mechanisms. Cell Rep 3:1921–31. [PubMed: 23727243]
24. Wesolowski J, Weber MM, Nawrotek A, Dooley CA, Calderon M, St Croix CM, Hackstadt T, Cherfils J, Paumet F. 2017 Chlamydia Hijacks ARF GTPases To Coordinate Microtubule Posttranslational Modifications and Golgi Complex Positioning. MBio 8.
25. Wesolowski J, Paumet F. 2017 Taking control: reorganization of the host cytoskeleton by *Chlamydia*. F1000Res 6:2058. [PubMed: 29225789]
26. Kumar Y, Valdivia RH. 2008 Reorganization of the host cytoskeleton by the intracellular pathogen Chlamydia trachomatis. Commun Integr Biol 1:175–7. [PubMed: 19704885]
27. Clifton DR, Fields KA, Grieshaber SS, Dooley CA, Fischer ER, Mead DJ, Carabeo RA, Hackstadt T. 2004 A chlamydial type III translocated protein is tyrosine-phosphorylated at the site of entry and associated with recruitment of actin. Proc Natl Acad Sci U S A 101:10166–71. [PubMed: 15199184]
28. Bothe M, Dutow P, Pich A, Genth H, Klos A. 2015 DXD motif-dependent and -independent effects of the Chlamydia trachomatis cytotoxin CT166. Toxins (Basel) 7:621–37. [PubMed: 25690695]
29. Carabeo RA, Grieshaber SS, Hasenkrug A, Dooley C, Hackstadt T. 2004 Requirement for the Rac GTPase in *Chlamydia trachomatis* invasion of non-phagocytic cells. Traffic 5:418–25. [PubMed: 15117316]
30. Subtil A, Wyplosz B, Balañá ME, Dautry-Varsat A 2004 Analysis of Chlamydia caviae entry sites and involvement of Cdc42 and Rac activity. J Cell Sci 117:3923–33. [PubMed: 15265988]
31. Jiwani S, Ohr RJ, Fischer ER, Hackstadt T, Alvarado S, Romero A, Jewett TJ. 2012 *Chlamydia trachomatis* Tarp cooperates with the Arp2/3 complex to increase the rate of actin polymerization. Biochem Biophys Res Commun 420:816–21. [PubMed: 22465117]
32. Lane BJ, Mutchler C, Al Khodor S, Grieshaber SS, Carabeo RA. 2008 Chlamydial entry involves TARP binding of guanine nucleotide exchange factors. PLoS Pathog 4:e1000014. [PubMed: 18383626]
33. Chen CP, Chen X, Qiao YN, Wang P, He WQ, Zhang CH, Zhao W, Gao YQ, Chen C, Tao T, Sun J, Wang Y, Gao N, Kamm KE, Stull JT, Zhu MS. 2015 In vivo roles for myosin phosphatase targeting subunit-1 phosphorylation sites T694 and T852 in bladder smooth muscle contraction. J Physiol 593:681–700. [PubMed: 25433069]
34. He Y, Beatty A, Han X, Ji Y, Ma X, Adelstein RS, Yates JR, Kemphues K, Qi L. 2012 Nonmuscle myosin IIB links cytoskeleton to IRE1α signaling during ER stress. Dev Cell 23:1141–52. [PubMed: 23237951]
35. Kwartler CS, Chen J, Thakur D, Li S, Baskin K, Wang S, Wang ZV, Walker L, Hill JA, Epstein HF, Taegtmeyer H, Milewicz DM. 2014 Overexpression of smooth muscle myosin heavy chain leads to activation of the unfolded protein response and autophagic turnover of thick filament-associated proteins in vascular smooth muscle cells. J Biol Chem 289:14075–88. [PubMed: 24711452]
36. Zhang L, Zhang C, Wang A. 2016 Divergence and Conservation of the Major UPR Branch IRE1-bZIP Signaling Pathway across Eukaryotes. Sci Rep 6:27362. [PubMed: 27256815]
37. Kokes M, Dunn JD, Granek JA, Nguyen BD, Barker JR, Valdivia RH, Bastidas RJ. 2015 Integrating chemical mutagenesis and whole-genome sequencing as a platform for forward and reverse genetic analysis of *Chlamydia*. Cell Host Microbe 17:716–25. [PubMed: 25920978]
38. Igietseme JU, He Q, Joseph K, Eko FO, Lyn D, Ananaba G, Campbell A, Bandea C, Black CM. 2009 Role of T lymphocytes in the pathogenesis of *Chlamydia* disease. J Infect Dis 200:926–34. [PubMed: 19656067]
39. Rajaram K, Giebel AM, Toh E, Hu S, Newman JH, Morrison SG, Kari L, Morrison RP, Nelson DE. 2015 Mutational Analysis of the *Chlamydia muridarum* Plasticity Zone. Infect Immun 83:2870–81. [PubMed: 25939505]

40. Igietseme JU, Omosun Y, Stuchlik O, Reed MS, Partin J, He Q, Joseph K, Ellerson D, Bollweg B, George Z, Eko FO, Bandea C, Liu H, Yang G, Shieh WJ, Pohl J, Karem K, Black CM. 2015 Role of Epithelial-Mesenchyme Transition in Chlamydia Pathogenesis. PLoS One 10:e0145198. [PubMed: 26681200]
41. Gautam A, Chaudhary K, Kumar R, Sharma A, Kapoor P, Tyagi A, Raghava GP, consortium Osdd. 2013 In silico approaches for designing highly effective cell penetrating peptides. J Transl Med 11:74. [PubMed: 23517638]
42. Kristensen M, Birch D, Mørck Nielsen H. 2016 Applications and Challenges for Use of Cell-Penetrating Peptides as Delivery Vectors for Peptide and Protein Cargos. Int J Mol Sci 17.
43. Holton TA, Pollastri G, Shields DC, Mooney C. 2013 CPPpred: prediction of cell penetrating peptides. Bioinformatics 29:3094–6. [PubMed: 24064418]
44. Noble JE, Bailey MJ. 2009 Quantitation of protein. Methods Enzymol 463:73–95. [PubMed: 19892168]
45. Elwell C, Mirrashidi K, Engel J. 2016 Chlamydia cell biology and pathogenesis. Nat Rev Microbiol 14:385–400. [PubMed: 27108705]
46. Gardner BM, Pincus D, Gotthardt K, Gallagher CM, Walter P. 2013 Endoplasmic reticulum stress sensing in the unfolded protein response. Cold Spring Harb Perspect Biol 5:a013169. [PubMed: 23388626]
47. Gardner BM, Walter P. 2011 Unfolded proteins are Ire1-activating ligands that directly induce the unfolded protein response. Science 333:1891–4. [PubMed: 21852455]
48. Kassianidou E, Hughes JH, Kumar S. 2017 Activation of ROCK and MLCK tunes regional stress fiber formation and mechanics via preferential myosin light chain phosphorylation. Mol Biol Cell 28:3832–3843. [PubMed: 29046396]
49. Mizutani T, Haga H, Koyama Y, Takahashi M, Kawabata K. 2006 Diphosphorylation of the myosin regulatory light chain enhances the tension acting on stress fibers in fibroblasts. J Cell Physiol 209:726–31. [PubMed: 16924661]
50. Kovács M, Tóth J, Hetényi C, Málnási-Csizmadia A, Sellers JR 2004 Mechanism of blebbistatin inhibition of myosin II. J Biol Chem 279:35557–63. [PubMed: 15205456]
51. Credle JJ, Finer-Moore JS, Papa FR, Stroud RM, Walter P. 2005 On the mechanism of sensing unfolded protein in the endoplasmic reticulum. Proc Natl Acad Sci U S A 102:18773–84. [PubMed: 16365312]
52. Jewett TJ, Fischer ER, Mead DJ, Hackstadt T. 2006 Chlamydial TARP is a bacterial nucleator of actin. Proc Natl Acad Sci U S A 103:15599–604. [PubMed: 17028176]
53. Qin QM, Pei J, Ancona V, Shaw BD, Ficht TA, de Figueiredo P. 2008 RNAi screen of endoplasmic reticulum-associated host factors reveals a role for IRE1alpha in supporting *Brucella* replication. PLoS Pathog 4:e1000110. [PubMed: 18654626]
54. Dulyaninova NG, Bresnick AR. 2013 The heavy chain has its day: regulation of myosin-II assembly. Bioarchitecture 3:77–85. [PubMed: 24002531]
55. Liu Z, Ho CH, Grinnell F. 2014 The different roles of myosin IIA and myosin IIB in contraction of 3D collagen matrices by human fibroblasts. Exp Cell Res 326:295–306. [PubMed: 24768700]
56. Qiao YN, He WQ, Chen CP, Zhang CH, Zhao W, Wang P, Zhang L, Wu YZ, Yang X, Peng YJ, Gao JM, Kamm KE, Stull JT, Zhu MS. 2014 Myosin phosphatase target subunit 1 (MYPT1) regulates the contraction and relaxation of vascular smooth muscle and maintains blood pressure. J Biol Chem 289:22512–23. [PubMed: 24951589]
57. Shibata K, Sakai H, Huang Q, Kamata H, Chiba Y, Misawa M, Ikebe R, Ikebe M. 2015 Rac1 regulates myosin II phosphorylation through regulation of myosin light chain phosphatase. J Cell Physiol 230:1352–64. [PubMed: 25502873]
58. MacKay CE, Shaifta Y, Snetkov VV, Francois AA, Ward JPT, Knock GA. 2017 ROS-dependent activation of RhoA/Rho-kinase in pulmonary artery: Role of Src-family kinases and ARHGEF1. Free Radic Biol Med 110:316–331. [PubMed: 28673614]
59. Tam AB, Koong AC, Niwa M. 2014 Ire1 has distinct catalytic mechanisms for XBP1/HAC1 splicing and RIDD. Cell Rep 9:850–8. [PubMed: 25437541]

60. Ishiwata-Kimata Y, Yamamoto YH, Takizawa K, Kohno K, Kimata Y. 2013 F-actin and a type-II myosin are required for efficient clustering of the ER stress sensor Ire1. Cell Struct Funct 38:135–43. [PubMed: 23666407]

61. van Vliet AR, Giordano F, Gerlo S, Segura I, Van Eygen S, Molenberghs G, Rocha S, Houcine A, Derua R, Verfaillie T, Vangindertael J, De Keersmaecker H, Waelkens E, Tavernier J, Hofkens J, Annaert W, Carmeliet P, Samali A, Mizuno H, Agostinis P. 2017 The ER Stress Sensor PERK Coordinates ER-Plasma Membrane Contact Site Formation through Interaction with Filamin-A and F-Actin Remodeling. Mol Cell 65:885–899.e6. [PubMed: 28238652]

Highlights

- *Chlamydia* induces UPR for ATP, nutrients and to protect host cell from apoptosis
- Chlamydial T3SS effectors activate non-muscle myosin heavy chain II (NMMHC-II)
- Activated NMMHC-II binds UPR master regulator (BiP) or transducers to induce UPR
- Inhibition of UPR activation prevents *Chlamydia* replication and inclusion formation

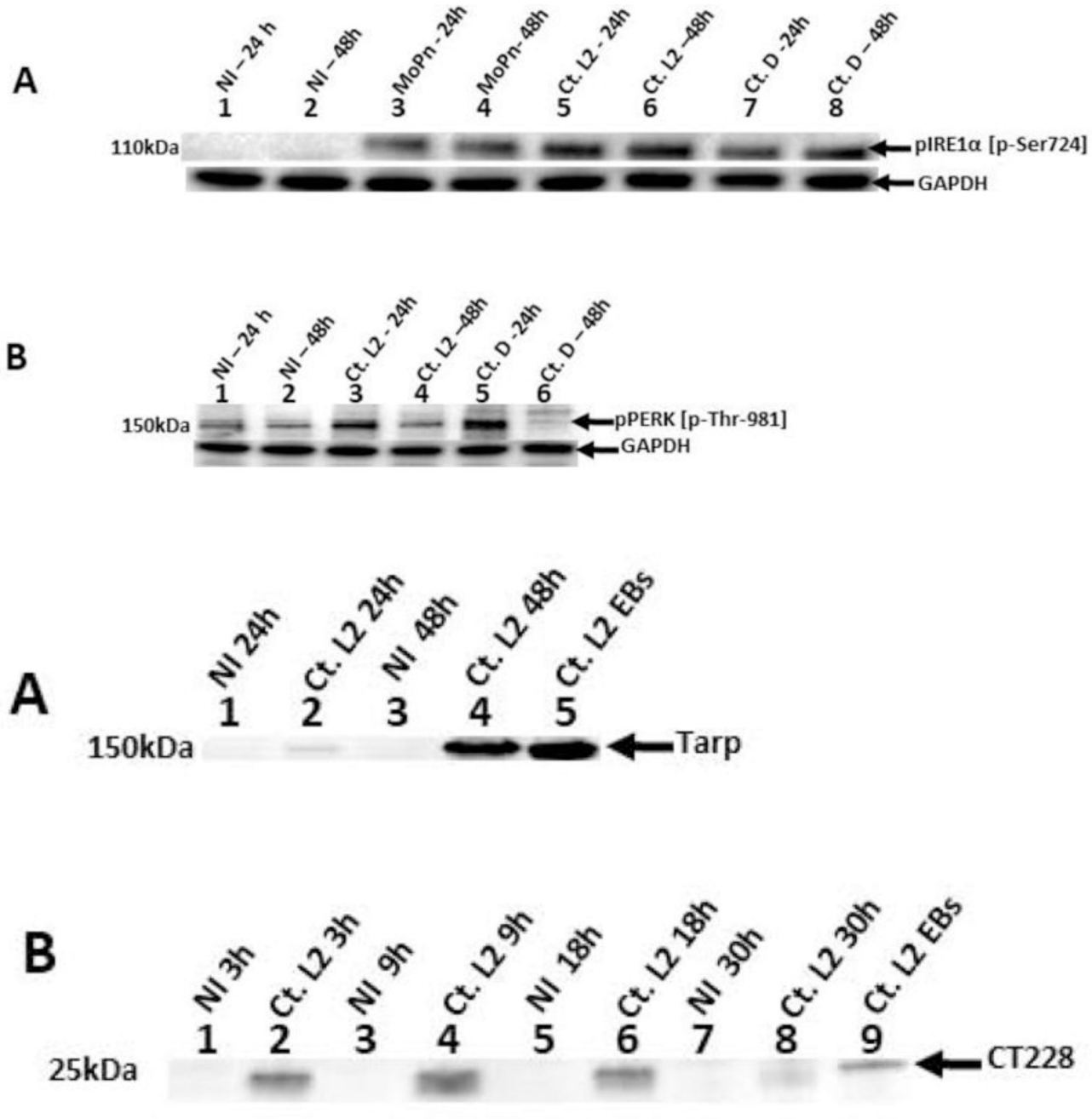

Figure 1. Different strains and serovars of *Chlamydia* activate IRE1α and PERK arms of UPR and chlamydial elementary bodies (EBs) contain preformed Tarp and CT228 proteins whose expression are upregulated during infection.

Figure Ia. 20μg of total protein from HeLa 229 cells were infected with strains and serovars of Chlamydia at MOI = 1 and analyzed by western blotting. Equal amount of total protein from non-infected HeLa cells were included as negative controls. **(A)** Analysis to determine the level of pIRE1α[p-Ser724] phosphorylation after 24 and 48hr infection with different Chlamydia strains and serovars (top bands) and GAPDH protein loading control (lower bands). Lane 1: 24hr non-infected cells; Lane 2: 48hr non-infected cells; Lane 3: 24hr MoPn infected cells; Lane 4: 48hr MoPn infected cells; Lane 5: 24hr Ct. L2 infected cells; Lane 6:

48hr Ct. L2 infected cells; Lane 7: 24hr Ct. D infected cells; Lane 8: 48hr Ct. D infected cells. **(B)** Analysis to determine the level of pPERK[p-Thr980] phosphorylation after 24 and 48hr infection with different C. trachomatis serovars L2 and D (top bands) and GAPDH protein loading control (lower bands). Lane 1: 24hr non-infected cells; Lane 2: 48hr non-infected cells; Lane 3: 24hr Ct. L2-infected cells; Lane 4: 48hr Ct. L2-infected cells; Lane 5: 24hr Ct. D-infected cells; Lane 6: 48hr Ct. D-infected cells. Figure 1b. **(A)** An image of western blot analysis to determine the presence of Tarp in Ct. L2 EBs and its expression level at 24 and 48hr post-infection. Lane 1: 24hr non-infected HeLa cells; Lane 2: 24hr Ct. L2-infected cells; Lane 3: 48hr non-infected cells; Lane 4: 48hr Ct. L2-infected cells; Lane 5: Ct. L2 total EB proteins. **(B)** A representative image of western blot analysis to determine the presence of CT228 in Ct. L2 EBs and its expression level at 3, 9, 18 and 30hr post-infection. Lane 1: 3hr non-infected HeLa cells; Lane 2: 3hr Ct. L2-infected cells; Lane 3: 9hr non-infected cells; Lane 4: 9hr Ct. L2-infected cells; Lane 5: 18hr non-infected cells; Lane 6: 18hr Ct. L2-infected cells; Lane 7: 30hr non-infected cells; Lane 8: 30hr Ct. L2-infected cells; Lane 9: Ct. L2 total EB proteins. Experiments were repeated 3 times with essentially the same results.

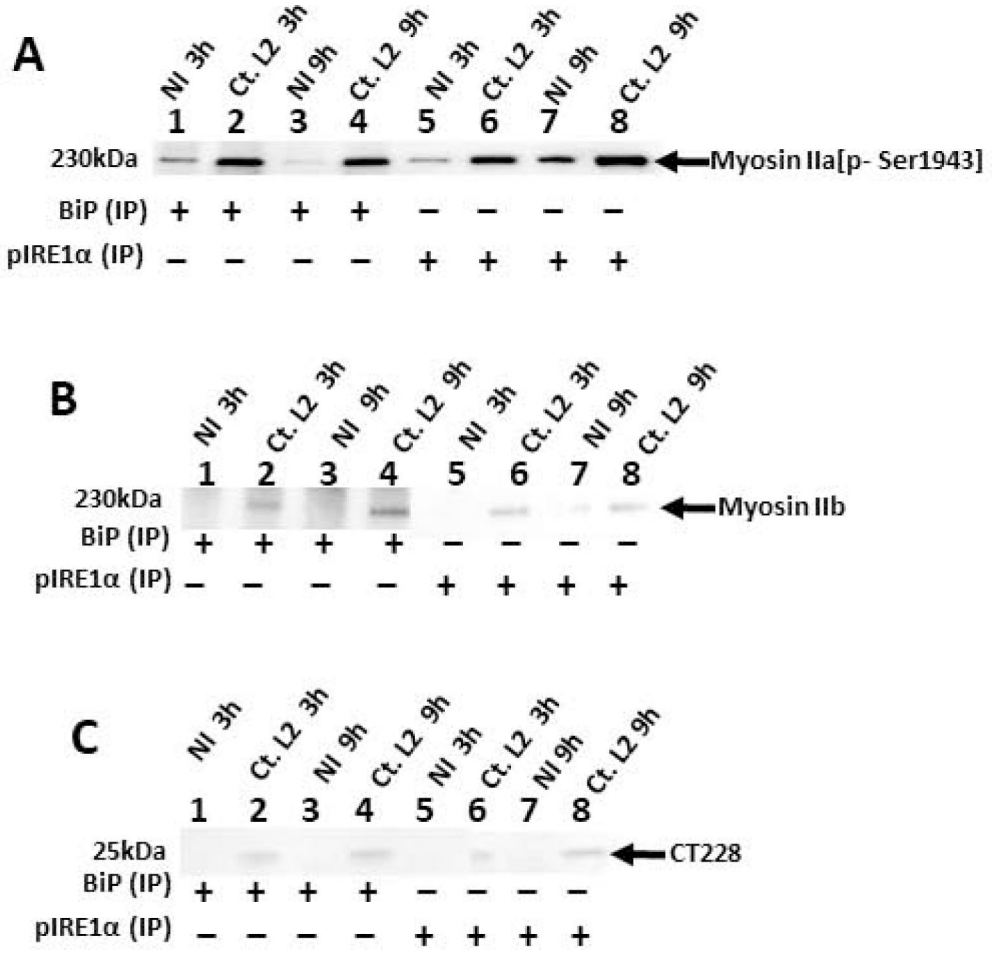

Figure 2. CT228 is associated with the activated myosin complex and UPR regulator and transducers during chlamydial infection.

(A) An image of western blot membrane probed with anti-Myosin IIa[p- Ser1943] antibody to identify the presence of Myosin IIa[p- Ser1943] in anti-BiP/anti-IRE1α[p-Ser724]-immunoprecipitated samples of Ct. L2-infected and non-infected cell lysates. Lane 1: 3hr non-infected cells immunoprecipitated with anti-BiP antibody; Lane 2: 3hr Ct. L2 infected cells immunoprecipitated with anti-BiP antibody; Lane 3: 9hr non-infected cells immunoprecipitated with anti-BiP antibody; Lane 4: 9hr Ct. L2 infected cells immunoprecipitated with anti-BiP antibody; Lane 5: 3hr non-infected cells immunoprecipitated with anti-IRE1α[p-Ser724] antibody; Lane 6: 3hr Ct. L2-infected cells immunoprecipitated with anti-IRE1α[p-Ser724] antibody; Lane 7: 9hr non-infected HeLa cells immunoprecipitated with anti-IRE1α[p-Ser724] antibody; Lane 8: 9hr Ct. L2 infected cells immunoprecipitated with anti-IRE1α[p-Ser724] antibody; **(B)** An image of western blot membrane probed with anti-Myosin IIb antibody to assess the presence of Myosin IIb in anti-BiP/anti-IRE1α[p-Ser724]-immunoprecipitated samples of Ct. L2 infected and non-infected cells after 3 and 9hr post infection. Lane 1: 3hr non-infected cells immunoprecipitated with anti-BiP antibody; Lane 2: 3hr Ct. L2 infected cells immunoprecipitated with anti-BiP antibody; Lane 3: 9hr non-infected cells immunoprecipitated with anti-BiP antibody; Lane 4: 9hr Ct. L2 infected cells

immunoprecipitated with anti-BiP antibody; Lane 5: 3hr non-infected cells immunoprecipitated with anti-IRE1α[p-Ser724] antibody; Lane 6: 3hr Ct. L2 infected cells immunoprecipitated with anti-IRE1α[p-Ser724] antibody; Lane 7: 9hr non-infected cells immunoprecipitated with anti-IRE1α[p-Ser724] antibody; Lane 8: 9hr Ct. L2 infected cells sample immunoprecipitated with anti-IRE1α[p-Ser724] antibody. **(C)** An image of western blot membrane probed with anti-CT228 antibody to assess the presence of CT228 in anti-BiP/anti-IRE1α[p-Ser724]-immunoprecipitated samples of Ct. L2-infected and non-infected cells after 3 and 9hr p.i. Lane 1: 3hr non-infected cells immunoprecipitated with anti-BiP antibody; Lane 2: 3hr Ct. L2 infected cells immunoprecipitated with anti-BiP antibody; Lane 3: 9hr non-infected cells immunoprecipitated with anti-BiP antibody; Lane 4: 9hr Ct. L2 infected cells immunoprecipitated with anti-BiP antibody; Lane 5: 3hr non-infected cells immunoprecipitated with anti-IRE1α[p-Ser724] antibody; Lane 6: 3hr Ct. L2 infected cells immunoprecipitated with anti-IRE1α[p-Ser724] antibody; Lane 7: 9hr non-infected cells immunoprecipitated with anti-IRE1α[p-Ser724] antibody; Lane 8: 9hr Ct. L2 infected cells immunoprecipitated with anti-IRE1α[p-Ser724] antibody. Experiments were repeated at least 3 times and the same results were obtained.

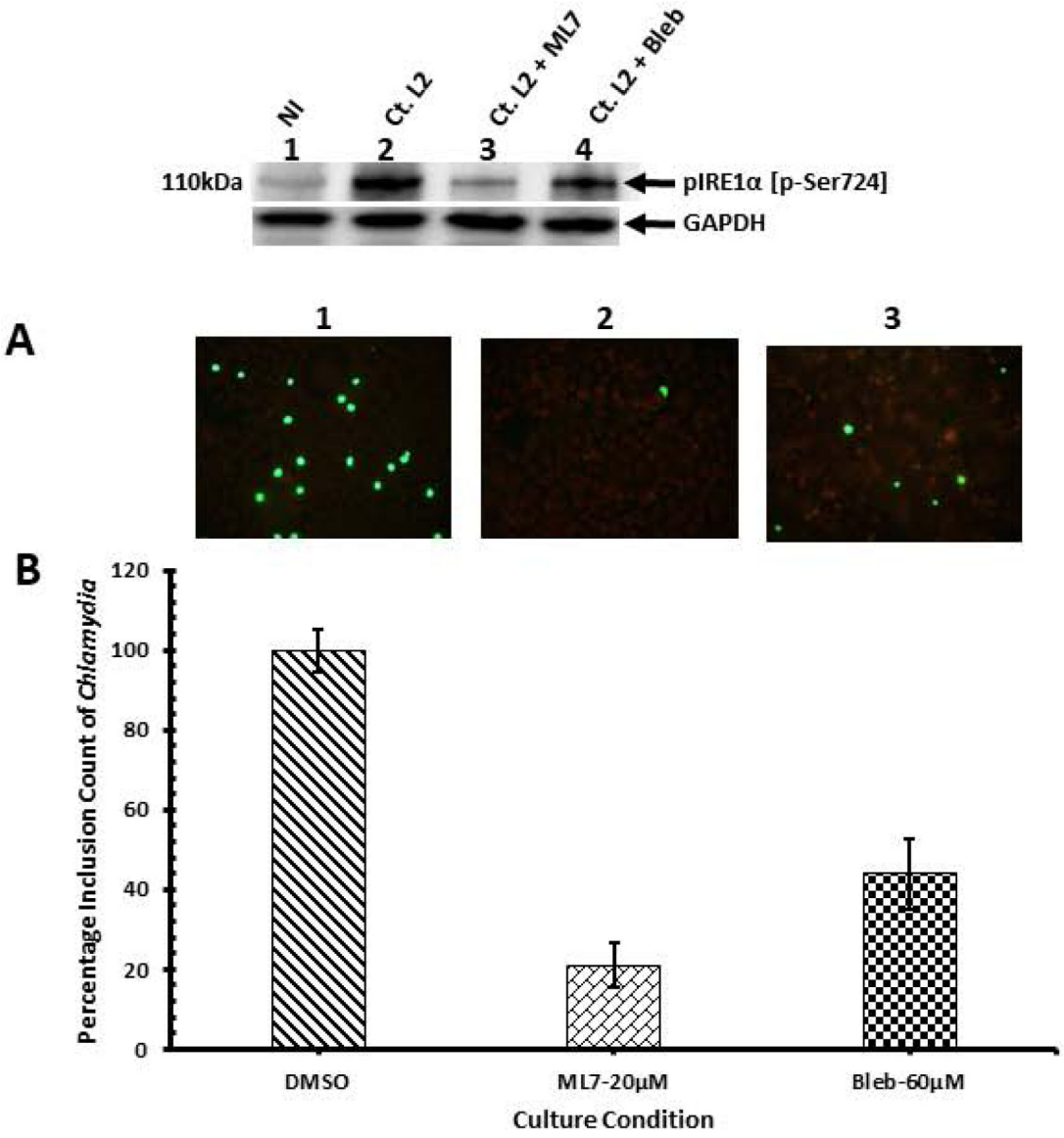

Figure 3: Inhibition of myosin light chain kinase or ATPase activity of the myosin heavy chain significantly blocks the activation of IRE1α arm of UPR whereas the inhibition of myosin light chain kinase and ATPase activities result in a significant reduction in *Chlamydia* replication.

Figure 3a: Total proteins were prepared from Ct. L2 infected and non-infected HeLa cells 48hr p.i. were analyzed by western blotting and probed with an anti-IRE1α[p-Ser724] antibody. Lane 1: non-infected cells; Lane 2: Ct. L2-infected cells; Lane 3: Ct. L2-infected cells treated with 20μM of ML7-hydrochloride (ML7); Lane 4: Ct. L2-infected cells treated with 50μM of (s)-(-)-Blebbistatin (Bleb). Figure 3b: **(A 1−3)** Microscope image of fluorescent-stained Ct. L2 inclusions (green dots) in HeLa cell monolayer treated with: DMSO as a control for the effect of solvent used in reconstituting inhibitors **(A1);** 20μM of ML7-hydrochloride **(A2);** 50μM of (s)-(-)-Blebbistatin **(A3)**. **(B)** A bar graph presentation of the number of *Chlamydia* inclusions counted in the three different conditions of DMSO control **(▧)**, 20μM ML7-hydrochloride **(▨)** and 50μM (s)-(-)-Blebbistatin **(▩)**. Treatment with 20μM ML7-hydrochloride shows a highly statistical significant reduction in number

and size of inclusions with a two-tailed *P*-value 0.0001. Treatment with 50μM (s)-(-)-Blebbistatin shows a highly statistical significant reduction in number of inclusions with a two-tailed *P*-value 0.003. Results are from three independent experiments.

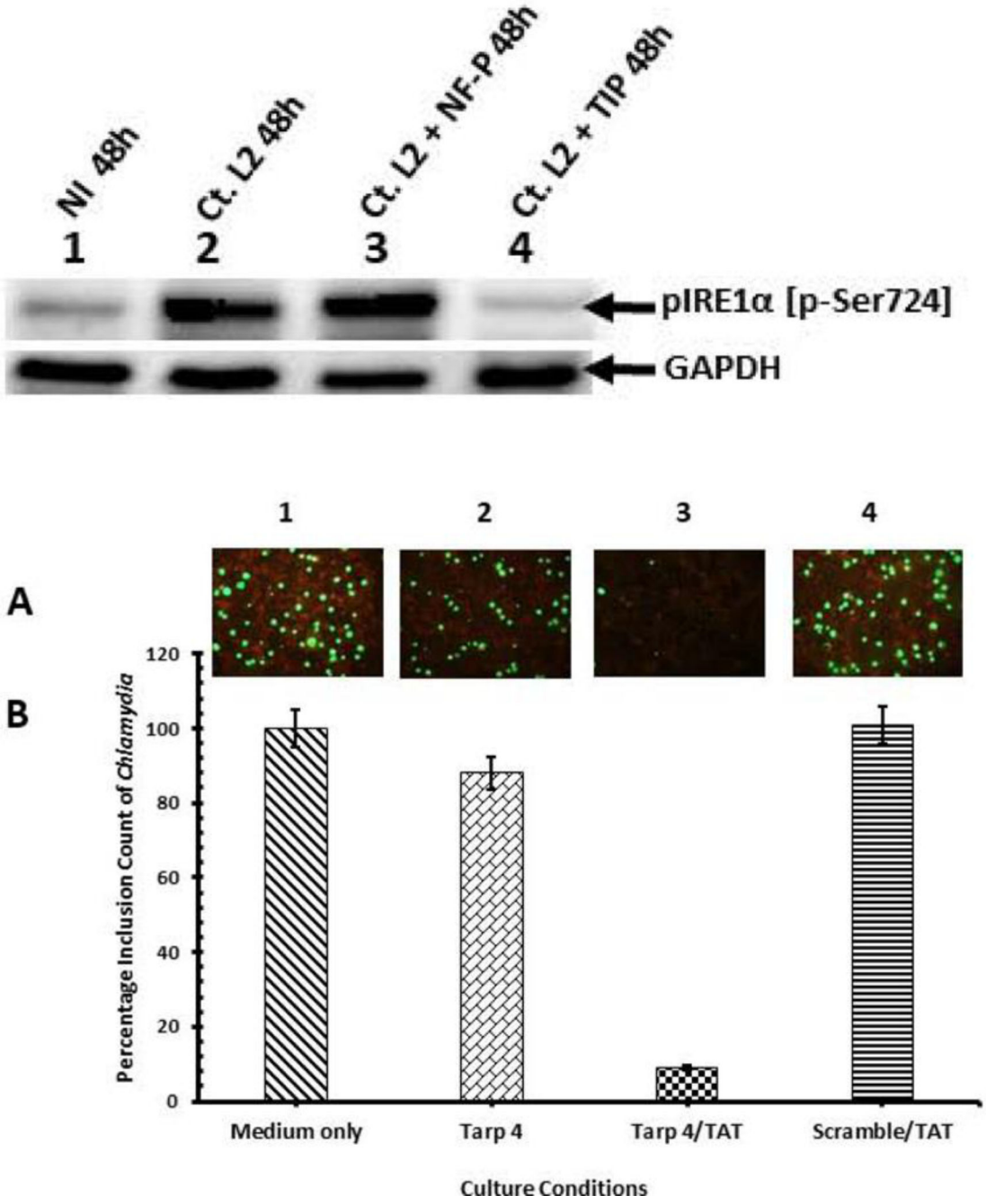

Figure 4: Inhibition of Tarp oligomerization using cell permeable Tarp peptide mimetic blocked the activation of IRE1α arm of UPR and significantly reduced *Chlamydia* replication.

Figure 4a: Total proteins were prepared from Ct. L2 infected and non-infected HeLa cells 48hr p.i. and analyzed by western blotting using an anti-IRE1α[p-Ser724] antibody. Lane 1: non-infected cells; Lane 2: Ct. L2-infected cells; Lane 3: Ct. L2-infected cells treated with 20μM of non-functional peptide; Lane 4: Ct. L2-infected cells treated with 50μM of Tarp inhibiting peptide. Figure 4b: **(A 1–4)** Microscope image fluorescent-stained Ct. L2 inclusions (green dots) in HeLa cells treated with: culture medium (Iscove) as a control for

the effect of solvent used in reconstituting inhibitory peptides **(A1)**; 100μM of non-cell permeable Tarp mimetic peptide (Tarp4) **(A2)**; 50μM of cell permeable Tarp inhibiting fusion peptide (Tarp4/TAT) **(A3)**; 100μM of non-functional (scrambled) peptide cell permeable fusion peptide (Scrambled/TAT) **(A4)**. **(B)** A bar graph presentation of the *Chlamydia* inclusions counted in the four different conditions of culture medium only as control **(▧)**, 100μM of non-cell permeable Tarp peptide (Tarp4) **(▨)**, 50μM of cell permeable Tarp inhibiting fusion peptide (Tarp4/TAT) **(▩)** and 100μM of non-functional peptide cell permeable fusion peptide (Scrambled/TAT) **(▤)**. Treatment with 50μM Tarp4/TAT peptide shows a highly statistical significant reduction in number and size of inclusions with a two-tailed *P*-value 0.0001. Two-tailed *t* test analysis for Tarp4 and Scrambled/TAT peptides gave insignificant *P*-values of 0.5 and 0.3 respectively. Results were derived from three independent experiments.

Table 1.

Select UPR-related Proteins Immunoprecipitated with Anti-BiP and Anti-IRE1α Antibodies and Identified by NanoLC-MS/MS system cum MASCOT search engine.

Antibody Used in Immunoprecipitation	Host Proteins Identified	*Chlamydia* Proteins Identified
Anti-BiP		Actin (CQB88832.1)
	Myosin-IIa (NP_002464.1);	
	Myosin-IIb (NP_005955.3;	CT228 (AFU24176.1)
	Myosin-IIc (NP_079005.3);	
	HSP 90-beta isoform-a (NP_031381.2)	
Anti-IRE1α[p-Ser724]	Myosin-IIa (NP_002464.1);	CT228 (AFU24176.1)
	Myosin-IIb (NP_005955.3;	
	Myosin-IIc (NP_079005.3);	
	HSP 90-beta isoform-a (NP_031381.2)	