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Chlamydial Infection Induces Host Cytokinesis Failure at Abscission

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Abstract

Chlamydia trachomatis is an obligate intracellular bacteria and the infectious agent responsible for the sexually transmitted disease Chlamydia. Infection with *Chlamydia* can lead to serious health sequelae such as pelvic inflammatory disease and reproductive tract scarring contributing to infertility and ectopic pregnancies. Additionally, chlamydial infections have been epidemiologically linked to cervical cancer in patients with a prior human papillomavirus (HPV) infection. Chlamydial infection of cultured cells causes multinucleation, a potential pathway for chromosomal instability. Two mechanisms that are known to initiate multinucleation are cell fusion and cytokinesis failure. This study demonstrates that multinucleation of the host cell by *Chlamydia* is entirely due to cytokinesis failure. Moreover, cytokinesis failure is due in part to the chlamydial effector CPAF acting as an anaphase promoting complex mimic causing cells to exit mitosis with unaligned and unattached chromosomes. These lagging and missegregated chromosomes inhibit cytokinesis by blocking abscission, the final stage of cytokinesis.

Introduction

Chlamydia trachomatis is a major human pathogen with biovars that can cause both sexually transmitted disease (STD) and trachoma, the leading cause of infectious blindness (Hu et al. 2010). Infections caused by genital serovars of *C. trachomatis* can persist and lead to serious health sequelae such as pelvic inflammatory disease and tubal infertility in untreated women (Cates & Wasserheit 1991). *Chlamydia* has also been clinically associated with cytologic cervical atypia (Kiviat et al. 1985) and epidemiologically linked to an increase in the risk for cervical cancer (Anttila et al. 2001; Smith et al. 2002; Madeleine et al. 2007). A direct molecular mechanism responsible for this link has yet to be described.

The obligate intracellular nature of the chlamydial infectious life cycle requires *Chlamydia* to reprogram many aspects of cellular function to render the cell hospitable to growth. *Chlamydiae* have multiple systems to secrete protein effectors into the host, modulating cellular behavior to create its unique intracellular niche. *Chlamydia* expresses both a type III secretion system as well as an uncharacterized type II dependent secretion system to efficiently deliver toxins/effectors to the cell to carry out these functions (Zhong 2011; Dehoux et al. 2011). These effectors induce a variety of cytopathic effects such as centrosome amplification, degradation of host proteins and multinucleation of the host cell (Grieshaber et al. 2006; Paschen et al. 2008; Greene & Zhong 2003; Johnson et al. 2009). Multinucleated cells are common in all solid tumors and contribute to aneuploidy and chromosome instability (Weihua et al. 2011). The induction of multinucleation of the host cell during chlamydial infection is a potential contributing factor in the observed increased risk of cancer in infected patients.

Multinucleation can occur via cell fusion or cytokinesis failure, both of which have been shown to occur during viral infections. Disregulation of the cell cycle by viral infection

leads to cytokinesis failure, and the membrane fusion events of budding viruses results in cell to cell fusion of neighboring cells (Duelli & Lazebnik 2007; Liu et al. 2005). Previous studies in *Chlamydia* suggest that either of these mechanisms could contribute to multinucleation. Egress of the chlamydial inclusion has been reported to be mediated, at least in part, by budding of the inclusion from the cell suggesting that *Chlamydia* may mediate membrane fusion events (Kevin Hybiske 2007). Alternatively, we have recently determined that *Chlamydia* infection affects the cell cycle of the host cell by overriding the Spindle Assembly Checkpoint (SAC) causing infected cells to prematurely exit mitosis (Knowlton et al. 2011).

In this study we demonstrate that chlamydial induced multinucleation is entirely due to cytokinesis failure. Furthermore, we show that chlamydial induced multinucleation occurs due to failure late in cytokinesis at the abscission step. Finally, we establish that *Chlamydia* protease-like activity factor, CPAF, acts as an anaphase promoting complex by cleaving cyclin B1 and securin, allowing the chromosomes to prematurely separate. This activity drives cells through metaphase before the chromosomes are properly aligned and attached, leading to lagging chromosomes. The lagging chromosomes persist in the midbody between the newly formed daughter cells and ultimately interfere with abscission.

Results

Cytokinesis fails very late in infected cells

We previously reported that chlamydial infected cells proceed through mitosis faster than uninfected cells (Knowlton et al. 2011). To determine if this impacts cell division, we directly examined infected dividing cells using live cell imaging. HeLa cells were transfected with GFP and infected with serovar L2. These cells were imaged every 10 minutes for 24 hours. Imaging was initiated at 20 hours post infection (hpi), as this is the start of the mature stage of the chlamydial infection. Images were collected for both infected and uninfected cells for a total of 41 infected cells and 39 uninfected cells that completed mitosis during imaging. The vast majority of the infected cells became multinucleated after mitosis (80.5% infected, Fig 1 C). This was a significant increase in multinucleation over the uninfected cell population (5.1% uninfected, Fig 1 C). In addition, cells were transfected with GFP-Bicaudal D1 and imaged 24 hpi every 15 minutes for 18 hours. Bicaudal D1 was used to localize the chlamydial inclusion (Matanis et al. 2002; Moorhead et al. 2007). We observed that multinucleation often occurred many hours after the completion of mitosis and in some cases occurred after differential expression of the GFP-Bicaudal D1 protein (Fig 1A-B). The observed differential GFP-Bicaudal D1 expression between the apparent daughter cells that ultimately form a single multinucleated cell could be explained by two potential mechanisms; fusion of the closely associated cells after completion of cytokinesis or by failure late in the cytokinesis process after free diffusion is blocked by the formation of the midbody, a late cell division intermediate.

Multinucleation is not caused by cell fusion

The genital biovars of *Chlamydia* are reported to egress from the cell without lysis, suggesting chlamydial infection may induce cell membrane fusion events in a manner similar to budding viruses (Kevin Hybiske 2007; Todd & Caldwell 1985). We employed a differential cell staining technique to determine if cell fusion occurs during infection. Two populations of cells were labeled with different fluorescent dyes and fusion events were scored by counting cells that fluoresced in both channels. HeLa cells were intrinsically labeled with either CytoTracker green or CytoTracker red fluorescent dyes. These two cell populations were then independently infected with *Chlamydia* L2. The two cell populations were trypsinized and co-plated onto coverslips 6 hpi and allowed to incubate for a total of 36

hours. These experiments were repeated with the genital serovar G but incubated for 48 hours, due to the slower growth rate. As a control to verify our ability to detect multinucleation, two uninfected cell populations were labeled with CytoTracker red or CytoTracker green, co-plated and subjected to an electrical current to induce fusion. Yellow stained cells indicating fusion between red and green dyed cells were enumerated at the indicated time points. No fusion could be detected in either serovars L2 or G infected samples with greater than 1000 multinucleated cells counted (Fig 1D). Conversely, yellow stained cells indicating cell fusion were readily evident in the electro-fused samples. These data rule out fusion as a contributing mechanism for multinucleation.

Cytokinesis fails after ingression

Upon ruling out fusion as a mechanism for multinucleation, we sought to determine at which step cytokinesis fails in the chlamydial infected cells. Cytokinesis can be divided into multiple stages; specification of the cleavage plane and ingression of the cleavage furrow, followed by formation of the midbody and abscission (Normand & King 2010). Ingression of the cleavage furrow initiates with the formation of an actin-myosin ring during late anaphase in order to mechanically separate the daughter cells. To morphologically assess the structural organization of the contractile ring, infected cells were stained with fluorescently labeled phalloidin. The actin organization at the cleavage furrow appeared to be unperturbed by the chlamydial inclusion as there was no significant observable differences between infected and uninfected dividing cells (Fig 2A).

As an additional line of evidence that the actin cleavage furrow was not disrupted during infection, we compared infected cells to those treated with the actin depolymerizing compounds cytochalasin D or latrunculin A. Disruption of the actin contractile ring during cytokinesis results in multinucleation and immediate arrest in G1 of the cell cycle (Lohez et al. 2003). Centrosome amplification induced by chlamydial infection is dependent on progression of the cell cycle into G2 (Johnson et al. 2009). We used this observation to assess the effects of cytokinesis failure induced by *Chlamydia* on cell cycle progression. As a measure of cell cycle progression we calculated the number of centrosomes per cell. The infected cells not treated with the actin depolymerizing drugs failed cytokinesis and had a clear increase in centrosome numbers (average centrosomes per cell 2.06, Fig 2B). However, infected cells treated with either cytochalasin D or latrunculin A became multinucleated but had no increase in centrosome numbers over treated uninfected cells (average centrosome per cell 1.71, Fig. 2B). The observation that the centrosome phenotype (no amplification) induced by disruption of the actin contractile ring was dominant over the chlamydial induced phenotype (amplification) demonstrates that chlamydial induced cytokinesis failure occurs at a step after formation and function of the contractile ring.

In addition to the the lack of disruption of the actin cleavage furrow, the invaginated region of the cell did not appear to be sterically hindered by the chlamydial inclusion. To specifically address the effects of steric interference on cell division, we compared the multinucleation rate in cells infected with *Coxiella burnetii* with cells infected with *C. trachomatis* L2. *C. burnetii* is a bacterial pathogen with a eukaryotic intracellular life cycle that takes place in a vacuole termed the *Coxiella* containing vacuole (CCV) (Heinzen et al. 1999). Although the growth rate and characteristics of the intracellular niche differ between *C. trachomatis* and *Coxiella*, both organisms construct and maintain a large intracellular parasitophorous vacuole in which replication occurs (Heinzen et al. 1996). We measured the multinucleation rate of cells infected with *C. trachomatis* L2 and in agreement with previous reports, multinucleation was significant by 18 hpi (Fig 2D). The size of the inclusion at these early time points was similar to the size of the CCV at 96 hours due to *Coxiella*'s slower growth rate. Cells infected with *Coxiella* for 96 hours displayed no increase in multinucleation compared to uninfected cells (Fig 2C and D). To specifically compare the

steric effects of CCVs that were of similar size to the chlamydial inclusions, we measured the size of inclusions in chlamydial infected multinucleated cells at 18 hpi (average size of $5,588 \mu\text{m}^2 \pm 450$). We then calculated the multinucleation rate in *Coxiella* infected cells containing CCVs of comparable size (average size of $6,429 \mu\text{m}^2 \pm 350$, Fig 2C). There was no increase in multinucleation in this population of cells over uninfected cells. This demonstrates that simple steric effects exerted by the presence of the chlamydial inclusion does not account for the rate of cytokinesis failure observed.

The lack of cell cycle arrest, correct formation of the contractile ring, lack of steric interference from the chlamydial inclusion and the inhibition of diffusion prior to multinucleation all suggest that cytokinesis failure is occurring very late, likely at abscission.

Infected dividing cells make midbodies

Following formation of the actin-myosin contractile ring, cleavage furrow formation and ingression, the spindle microtubules are compacted to form an intracellular bridge containing densely packed microtubules termed the midbody (Caballe & Martin-Serrano 2011). Abscission occurs after resolution of the membrane within the midbody to form the final independent daughter cells (Caballe & Martin-Serrano 2011). To visualize the progression of cytokinesis, chlamydial infected cells were stained with anti- β -tubulin allowing us to quantify the effect of infection on the formation of the midbody, the last step before completion of cytokinesis. In uninfected HeLa cells, $74.2 \pm 4.6\%$ of post-metaphase mitotic cells possessed fully formed midbodies. This number increased to $92.2 \pm 0.6\%$ in the 36 hour chlamydial infected cell population (Fig 3A and B). UV treatment of uninfected cells was used as a control as UV treatment causes failure in cytokinesis by inducing DNA cross-bridging and forcing cells to abort cytokinesis at the abscission stage (Norden et al. 2006). We found that midbodies were present in $83.0 \pm 1.2\%$ of post-metaphase cells post UV exposure (Fig 3B) indicating that cells that fail cytokinesis due to DNA bridges successfully form midbodies. We also assessed midbody formation in cells that failed cytokinesis due to failure in the function of the actin contractile ring using cytochalasin D and latrunculin. These cells never progressed to midbody formation (Data not shown). The proper formation of midbodies during infection further indicates that chlamydial infected cells successfully proceeded through the actin-myosin contractile ring stage into this late stage of cytokinesis. The observation that chlamydial infection increased the number of cells with intact midbodies also suggested that there is a delay in the resolution of the midbody and that infected cells fail in cytokinesis at this stage. This is further supported by the observed differential expression of GFP observed in infected dividing cells because diffusion was limited by the compact midbody structure (Fig 1.).

Lagging chromosomes are increased in frequency in the midbody of infected cells and correlate with multinucleation

Previous studies from our lab demonstrated that infected cells prematurely exit mitosis (Knowlton et al. 2011). A cell that exits mitosis before metaphase is complete can acquire chromosome segregation defects. These defects are commonly associated with cytokinesis failure as chromosome segregation errors can lead to chromosome non-disjunction and lagging chromosomes that bridge the midbody (King 2008). We therefore examined dividing cells for the presence of DNA between the forming daughter cells. Cells infected with serovar L2 for 32 hours and uninfected cells were stained for DNA, microtubules and *Chlamydia* (Fig 4A). Mitotic cells in these populations were quantified for the presence of DNA in the midbody between the two daughter cells. The infected cell population had a significant increase in the number of cells containing DNA in the midbody compared to uninfected (26.3% and 6.7% respectively, Fig 4B). Due to chromosome decondensation

during mitotic exit limiting detection, the number of cells with DNA in the midbody may be higher (Steigemann et al. 2009). In order to confirm that DNA in the midbody resulted in cytokinesis failure, we performed live cell imaging (Fig 4C). Uninfected cells succeed in mitosis 94.8% of the time, correlating with the low level of multinucleation observed in HeLa cells (Fig 4D). Surprisingly, we observed that uninfected cells displayed some level of DNA disorganization after metaphase. In this population 41.0% of successful cytokinesis events had evidence of DNA disorganization. The number of uninfected cells that failed cytokinesis was small (5.1%), however only half of these cells that failed cytokinesis had evidence of DNA disorganization suggesting that cytokinesis failure in uninfected cells was rarely caused by the presence of DNA in the midbody. In contrast, the rate of cytokinesis failure in the infected cell population rose to 80.5% with 93.9% of these demonstrating lagging DNA between daughter cells (Fig 4D). These data further suggest that the increase in lagging chromosomes and chromosome non-disjunction directly contributes to chlamydial induced cytokinesis failure.

Chlamydial infected cells do not arrest in metaphase

The spindle assembly checkpoint (SAC) regulates the timing of mitotic exit. This checkpoint blocks the progression at the metaphase to anaphase transition until chromosomes are properly aligned and have obtained bipolar attachment (McLean et al. 2011). This helps guarantee that the chromosomes are properly segregated to the poles and allows the chromosomes to be cleared from the cleavage furrow. As chlamydial infection increased the incidence of chromosomes found in the midbody between mitotic cells, we asked whether infection affects the SAC. To determine if *Chlamydia* infection is affecting the SAC, we triggered mitotic arrest with the drug monastrol. Monastrol specifically inhibits kinesin Eg5 resulting in the formation of monopolar spindles (Kapoor & Mitchison 2001). The disruption of bipolar spindle triggers the SAC, resulting in mitotic arrest in metaphase (Kapoor & Mitchison 2001). HeLa cells were infected with *C. trachomatis* serovar L2 and treated with monastrol for 24 hours. To assay for induction of the SAC, the mitotic index was measured by counting the percentage of cells in mitosis. Mitotic cells were stained with Hoechst to visualize DNA. The uninfected population had a mitotic index of $6.6 \pm 0.3\%$ which decreased to $4.4 \pm 0.1\%$ after infection (Fig 5A). Monastrol treatment led to an increase to $27.0 \pm 1.1\%$ and again when infected with *Chlamydia* this decreased to $21.9 \pm 0.6\%$. These decreases showed statistical significance ($p < 0.003$ and $p < 0.01$, respectively). HeLa cells are a transformed cancer cell line and likely have changes in cell cycle checkpoint control. Thus, we also looked at the effects of chlamydial infection on the SAC in a primary cell line. The mitotic index of primary human foreskin fibroblasts was $2.9 \pm 0.4\%$ which dropped to $0.3 \pm 0.1\%$ when infected with *C. trachomatis* L2 (Fig 5B). Treatment of the fibroblasts with monastrol resulted in an increase of the mitotic index to $21.4 \pm 0.8\%$ indicating mitotic arrest. Monastrol treated infected primary fibroblasts showed a decrease in the mitotic index. The index dropped to $1.9 \pm 0.1\%$ suggesting that these cells no longer arrested in mitosis. Although fibroblasts are not a typical target cell of a natural chlamydial infection cell cycle control in all non transformed cells is regulated by the same highly conserved checkpoint proteins. These data together demonstrate that infected cells do not arrest as efficiently during mitosis, even when the SAC is triggered.

We previously demonstrated that cyclin B1 and securin were degraded during chlamydial infection (Knowlton et al. 2011). Both of these proteins are important regulatory components of the SAC, which controls mitotic exit. (Kim & Yu 2011; Pines 2011; van Leuken et al. 2008) The secreted chlamydial protease CPAF has been reported to degrade cyclin B1 (Paschen et al. 2008; Balsara et al. 2006). We reasoned that CPAF may be acting as an anaphase promoting complex mimic, causing premature exit from mitosis. The anaphase promoting complex/cyclosome (APC/C) is a ubiquitin ligase and regulates the

SAC by targeting key molecules for degradation by the proteasome. (Baker et al. 2007; van Leuken et al. 2008) To test the hypothesis that CPAF is the chlamydial factor acting as an APC/C we purified a crude cytosolic fraction from chlamydial infected cells and tested the ability of this fraction to degrade cyclin B1 and securin. This fraction contains the active CPAF molecule that is secreted from *Chlamydia* (Huang et al. 2008). We analyzed the degradation of both cyclin B1 and securin by western blot analysis. Lysates from chlamydial infected cells but not uninfected cells resulted in degradation of both cyclin B1 and securin purified from mitotic uninfected cells (Fig 6A). We tested whether this degradation was due to CPAF proteolytic activity by using a specific CPAF inhibitory peptide. This peptide is derived from a self inhibitory fragment of the CPAF zymogen that blocks access to the substrate binding pocket as described by Huang et al (Huang et al. 2008). This inhibitory peptide was demonstrated to have high potency against CPAF and is very specific as it does not affect the proteasome or CPAF from the closely related bacteria *C. caviae* (Jorgensen et al. 2011). Western blotting revealed that only infected lysates could actively degrade cyclin B1 and securin. This degradation could be inhibited by the CPAF inhibitory peptide, but a scrambled peptide did not block degradation. Furthermore, treatment of infected lysates with a protease inhibitor cocktail did not inhibit degradation (Fig 6B), confirming that host proteases were not responsible for the degradation of cyclin B1 and securin. To test whether CPAF mediated degradation of cyclin B1 resulted in its loss of function we assayed for the activity of cyclin dependent kinase 1 (Cdk1). Cdk1 is active when bound to cyclin B1 and becomes phosphorylated on T161. We assessed the activity state of Cdk1 in infected mitotic cells using an antibody specific for this species. Western blotting revealed a drastic reduction in levels of phosphorylated active Cdk1 in chlamydial infected cells, supporting the hypothesis that degradation of cyclin B1 results in inactivation of the SAC (Fig 6C).

Cyclin B1-Cdk1 must accumulate and become active in order for the cell to enter mitosis (Gavet & Pines 2010; Marangos & Carroll 2008). To determine if degradation of cyclin B1 by CPAF alters the host cell's ability to enter mitosis we performed a mitotic index at various points after a thymidine release. Both uninfected and infected cells began to enter mitosis at 10 hour post release, but infected cells did not undergo a robust entry into mitosis (Fig 7A). Instead, infected cells had a larger variability in the time it takes them to enter mitosis as was shown by the shallower slope on the graph (Fig 7A). Uninfected cells had a peak of mitotic entry at about 12 hours after release from the block. In comparison, the chlamydial infected cells still had not reached a peak of mitotic entry by 13 hours after block release. This delay tracks with cyclin B1 levels in these two populations. The uninfected cell population showed a larger and faster increase in cyclin B1 levels after release from the block then did the infected cells as could be seen by the western blot and was confirmed by quantification (Fig 7B,C). Additionally, we examined the length of S/G2 in uninfected and infected cells using the FUCCI cell cycle sensor (Sakaue-Sawano et al. 2008). HeLa cells were transduced with Premo FUCCI Cell Cycle Sensor and then infected with *Chlamydia*. The length of S/G2 was calculated by measuring the time from the degradation of Cdt1-RFP (degraded at the beginning of S phase) to nuclear membrane breakdown. We determined that in uninfected cells S/G2 was 507.3 ± 78.4 minutes, while infected cells had a longer S/G2 that lasted 774.6 ± 68.7 minutes (Fig 7D). Together these data demonstrate that the length of S/G2 is altered in infected cells and that this delay in entering mitosis correlates with a slower accumulation of cyclin B1.

Discussion

Multinucleation has been reported during chlamydial infections of cultured cells previously by Greene et al. and Sun et al. (Greene & Zhong 2003; Sun et al. 2011). In this study we demonstrated that multinucleation during chlamydial infection is entirely caused by failure in cytokinesis and, in many cases, this failure occurs hours after apparent separation of the

two daughter cells. By careful examination of the stages of cytokinesis in infected cells, we were able to determine that infected cells progressed through actin-myosin contractile ring formation and formed the typical condensed microtubule midbody structure that precedes the final stages of cell division, abscission. It is during this final stage that cytokinesis fails and multinucleation occurs. Abscission is irreversible for cytokinesis and is therefore regulated by a variety of pathways. By observing live infected cells by fluorescent microscopy we demonstrated that the population of cells failing cytokinesis were those that had DNA segregation errors caused by chlamydial infection. The presence of DNA in the midbody of dividing cells is a trigger to the cell to abort cytokinesis (Steigemann & Gerlich 2009; Norden et al. 2006). Our evidence supports the hypothesis that induction of DNA segregation errors during cytokinesis is a significant factor leading to cytokinesis failure in chlamydial infected cells. We demonstrated in previous studies that chlamydial infection shortens mitosis of the host cell, causing a disorganized metaphase to anaphase transition (Knowlton et al. 2011). In this study we were able to connect the observed high rate of multinucleation to the induction of disorganized mitosis. During chlamydial infection both cyclin B1 and securin are degraded reducing the time dividing cells spend in metaphase (Knowlton et al. 2011). Here, we extended this observation demonstrating that the chlamydial toxin CPAF acts as an anaphase promoting factor mimic, directly degrading cyclin B1 and securin resulting in the inactivation of Cdk1. This activity is likely responsible for overriding the SAC allowing cells to proceed to anaphase with disorganized chromosomes, resulting in DNA segregation errors. A recent study from the Harrison lab suggested a role for steric effects of the chlamydial inclusion leading to asymmetric cleavage furrow formation (Sun et al. 2011). In our studies we focused on events midway through the infectious cycle, before the chlamydial inclusion became the majority of the cell volume. We directly compared the effects of *Coxiella* vacuoles of equal or greater size than those formed in chlamydial infected cells. In cells with large *Coxiella* vacuoles there was no increase in multinucleation, demonstrating that the presence of a large inclusion body is insufficient to cause multinucleation. It is possible that when the inclusion increases in size that steric effects also contribute to cytokinesis failure. Our results indicate that early exit from metaphase, caused by CPAF activity, is capable of causing lagging chromosomes that inhibit cytokinesis. However it is possible other chlamydial factors also contribute to cytokinesis failure. The inc proteins CT223 and to a lesser extent CT224 and CT225 cause cytokinesis failure when ectopically expressed in mammalian cells (Alzhanov et al. 2009). The mechanism by which they contribute to this failure is not known and ectopic expression is not always a reliable assay to assess the function of bacterial proteins.

Cyclin B1 and activated Cdk1 are required for cells to enter mitosis leading to the question of how degradation of cyclin B1 during the chlamydial infection effects entry into mitosis. In this study we show that infected cells have a delay in mitotic entry after S phase arrest and spend more time in S/G2 than uninfected cells. Cyclin B1 is synthesized in the cytosol but is shuttled into the nucleus. In the nucleus cyclin B1 and its partner active Cdk1 reach a threshold level triggering mitosis. We speculate that during chlamydial infection cyclin B1 levels are reduced as CPAF targets it for destruction in the cytosol, however, after cyclin B1 enters the nucleus it is protected from the activity of CPAF. This would explain the delay in mitotic entry. After NMBD cyclin B1 again becomes exposed to CPAF and is degraded pushing cells out of mitosis prematurely.

Over all, our data support a model where the combined cytopathic effects of chlamydial infection subsequently lead to multinucleation. In this model chlamydial infection leads to centrosome amplification and de-clustering of centrosomes (Knowlton et al. 2011; Grieshaber et al. 2006; Johnson et al. 2009). Additionally, CPAF degrades cyclin B1 and securin during mitosis leading to deactivation of Cdk1 and early exit from metaphase. Because of centrosome and spindle defects, the DNA is never properly aligned during

metaphase leading to segregation errors during cytokinesis. These events ultimately lead to DNA bridging between daughter cells causing abscission failure (Fig. 8). By inducing aneuploidy through lagging chromosomes and cytokinesis failure, infection with *Chlamydia* may contribute to cellular transformation and cervical cancer.

The target of a naturally occurring chlamydial infection is the epithelial cell layer. These cells are highly differentiated and are reported to have a low population of replicating cells (Ramos et al. 2002). We speculate that the infection of replicating cells during a natural infection is a rare occurrence and therefore the induction of multinucleation is uncommon. However, HPV infection can drive cells out of quiescence and cause cells to reenter the cell cycle (Korzeniewski et al. 2011; McLaughlin-Drubin & Münger 2009). We theorize that this population of cells would be more sensitive to cell cycle effects caused by chlamydial infection during a co-infection occurrence.

Materials and Methods

Organisms and Cell Culture

Chlamydia trachomatis serovar L2 (LGV 434) and G (G/UW-57) were grown in HeLa 229 cells, and EBs were purified by Renografin density gradient centrifugation as previously described (Caldwell et al., 1981). *Coxiella burnetii* (Nine mile strain in phase II) was obtained from Dr. Robert Heinzen. All cell lines were obtained from the American Type Culture Collection. HeLa 229 cells were grown in RPMI-1640 (Cellgro) supplemented with 10% fetal bovine serum (FBS) and 10 µg/ml gentamicin. BJ Human Foreskin Fibroblasts were grown in DMEM (Cellgro) supplemented with 10% FBS and 10 µg/ml gentamicin.

Infections

Cells were incubated with *C. trachomatis* EBs at an MOI of approximately 3 in Hank's balanced salt solution (HBSS) (Gibco) for 45 minutes at room temperature while rocking. The inoculum was removed and replaced with fresh RPMI-1640 or DMEM containing 10% FBS and 10 µg/ml gentamicin. For *C. burnetii* infections, the same procedure was used except the incubation was for 4 hours and no gentamicin was used.

Immunohistochemistry

Cells for fluorescent microscopy were grown on 12-mm number 1.5 borosilicate glass coverslips coated with Poly-L-lysine (Sigma). For antibody staining, the coverslips were fixed in ice cold methanol for 10 minutes and incubated with the primary antibody described for each experiment. The antibodies used for these experiments were: mouse monoclonal anti-β-tubulin (Sigma), mouse monoclonal anti-γ-tubulin (Sigma) and AlexaFluor 488 phalloidin (Invitrogen). *Chlamydia trachomatis* and *Coxiella burnetii* were stained with platelet poor human serum (Sigma). To visualize the primary antibodies, AlexaFluor 488 conjugated secondary antibodies against mouse IgG were used (Molecular Probes/Invitrogen). The far-red fluorescent DNA dye DRAQ5 (Biostatus) or Hoechst was used to visualize nuclei. Images were acquired using a spinning disk confocal system connected to a Leica DMIRB microscope with a 63x oil-immersion objective, equipped with a Photometrics cascade-cooled EMCCD camera, under the control of the Open Source software package µManager (<http://www.micro-manager.org/>). Images were processed using the image analysis software ImageJ (<http://rsb.info.nih.gov/ij/>). Images displayed for figures are maximal intensity projections of the 3d data sets, adjusted for brightness and contrast.

Transfections and plasmids

HeLa cells were seeded on 25-mm number 1.5 glass coverslips in 6-well plates and grown to 70% confluency. Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. A stable cell line of RFP-H2B HeLa cells were selected with 5 μ g/ml blasticidin (Fisher) and expression from the GFP vector was allowed to proceed for 48 hours to permit time for expression. Human H2B was cloned from pBOS-H2BGFP (BD Biosciences) into a RFP-destination vector using the Gateway Cloning System (Invitrogen) according to the manufacturer's instruction. Similarly, Bicaudal D1 was cloned from pOTB7-Bicaudal (ATCC) into a GFP-destination vector using the Gateway Cloning System.

Live Cell Imaging

Cells were transferred to high glucose DMEM media with 25 mM HEPES. The coverslips were overlaid with mineral oil and the chamber was supplemented with CO₂. Images were acquired as previously described.

Cell fusion assay

HeLa cells were pre-stained with CytoTracker Red CMTPX or Green CMFDA (Molecular Probes) using recommended protocol. Both population of cells were infected with *Chlamydia trachomatis* at an MOI of 3. The two populations of cells were seeded together on 25-mm number 1.5 glass coverslips in 6-well plates 6 hpi. Cells were fixed with cold methanol at 30 hpi and stained with Draq5. Alternatively, cells were pre-stained and seeded without infection. The cells were then electroporated at 175V, 0 Ω , 975 μ F. Cells were imaged and orange multinucleated cells were counted as positive for cell fusion.

Inclusion measurement

HeLa cells were imaged using confocal microscopy. mageJ was used to measure the area of the inclusion or CCV. We calculated and compared the 2 dimensional areas occupied by the CCV and inclusion to simplify quantification. In both the *Coxiella* CCV and chlamydial inclusion the Z component was comparable and did not significantly change over time. The flat cell shape highly constrained the shape of the vacuoles in the Z dimension and significant growth only occurs in the X and Y dimensions.

Contractile ring and Midbody formation

In order to disrupt the formation of the actin-myosin ring, cells were treated with either 10 μ M cytochalasin D (Sigma) or 250 nM latrunculin A (Sigma) for the duration of the infection. Cells infected with *C. trachomatis* were immediately treated with an actin inhibitor. Cells were fixed with methanol and stained as described.

Cell-free degradation assay

Mitotic cell proteins were obtained through a mitotic shake-off of HeLa cells in Phosphate Buffered Saline (PBS). Cells were pelleted and resuspended in 0.5 mL of NP-40 Buffer (1% NP-40, 150 mM NaCl, 0.5% Triton X-100 in 50 mM Tris pH 8) Protease inhibitor cocktail (Sigma) was added. Extraction was carried out on ice for 20 minutes and then centrifuged to pellet the debris. Supernatant was collected and kept at -80°C until use. Chlamydial proteins were collected through harvesting the cytosolic fraction of cells infected with *C. trachomatis* serovar L2. Cells were scraped and centrifuged to form a pellet. Cells were resuspended in a douncing buffer (10mM KCl, 1.5mM MgCl₂, 1mM EDTA, 1mM DTT, 250mM sucrose in 20mM Hepes-KOH pH 7.5). Cells were dounced and the supernatants were harvested with a series of centrifugations with the final centrifugation at 100,000g. Protease inhibitor cocktail (Roche) or CPAF peptide inhibitor (GenScript) was also added. For degradation assay,

mitotic host proteins and chlamydial cytosolic proteins were mixed and incubated for 1 hour at 37°C.

Mitotic Index Assays

The mitotic index was calculated by determining the ratio of mitotic cells to the total number of cells present. For the infected populations only infected cells were counted. A minimum of 1500 cells were counted over 20-30 fields and the procedure was repeated 3 times.

The SAC was induced with the kinesin-5 inhibitor monastrol (Sigma) and the mitotic index was again calculated. Cells were treated with monastrol at a final concentration of 100 μ M for a total of 36 hours. The cells were methanol fixed and stained with anti- β -tubulin to visualize monopolar spindle formation, DRAQ5, and anti-L2 antibodies where appropriate.

Western Blot

Proteins from the cell-free degradation assay were separated by SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The primary antibodies were: mouse monoclonal anti-cyclin B1, rabbit polyclonal anti-cdc2, rabbit polyclonal anti-cdc2 (Thr161), (Cell Signaling Technology), rabbit polyclonal anti-PTTG-1 (Invitrogen), mouse monoclonal anti-GAPDH (EnCor). Proteins were visualized with either goat anti-mouse peroxidase conjugated secondary antibodies (Thermo) or goat anti-rabbit peroxidase conjugated secondary antibodies (Sigma) and SuperSignal West Dura Extended Duration Substrate (Thermo). The blot was imaged using a CoolSnap CCD camera and quantified using ImageJ (<http://rsb.info.nih.gov/ij/>)

Double Thymidine Block

HeLa cells were grown in 2mM thymidine in RPMI for 18 hours. Cells were then switched to fresh RPMI for 8 hours followed by infection with *Chlamydia* as previously described. Immediately following the infection the cells were put back in 2 mM thymidine in RPMI. After 12 hours, the cells were released with fresh media and samples were collected at the times indicated.

Fluorescence ubiquitination cell cycle indicator

HeLa cells were transduced with PromoTM FUCCI Cell Cycle Sensor (Invitrogen) according to the manufacturer's instructions. When necessary, the cells were infected with *Chlamydia* after the transduction. The cells were then imaged live for 18 hours, collecting frames every 10 minutes. For infected cells, this time period captures 18-36 hours post infection. This experiment was repeated 3 times each for both uninfected and infected cell populations. Data sets were exported to an OMERO imaging data base (<http://www.openmicroscopy.org/site/products/omero>). Omero's quantification tools were used for the live cell intensity analysis.

Statistical Analyses

Numerical data are presented as the mean $\pm$ SEM, and were analyzed by the unpaired t-test using GraphPad Prism4 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Multinucleation of host cells is due to cytokinesis failure and not cell fusion

[A-B] HeLa cells were transfected with GFP-Bicaudal D1 and then infected *C. trachomatis* L2 for 24 hours. The cells were then imaged every 15 minutes for 18 hours. [A]. Images are time points from live cell imaging. Time 0 is started at telophase of the cell. Stars mark nuclei while the arrow points to the inclusion. [B] GFP signal was graphed with an intensity plot to allow visual comparison of differing intensities between forming daughter cells. [C] Live cell imaging was performed on *Chlamydia* infected HeLa cells. Beginning at 20 hpi cells were imaged every 10 minutes for 24 hours. Uninfected mitotic cells resulted in multinucleation in 5.1% of the cells, while infected mitotic cells became multinucleated 80.5%. $n=39$ and $n=41$, respectively. [D] Two different populations of HeLa cells were stained with Cytotracker Red or Green. Cells were co-plated and either electroporated or infected with *C. trachomatis* L2 or G. Electroporation caused fusion as shown by the yellow multinucleated cell. Neither serovars of *C. trachomatis* induced fusion. 1000 multinucleated cells were counted and all multinucleated cells were due to cytokinesis failure.

Figure 2. *Chlamydia* does not sterically hinder cytokinesis

[A] HeLa cells were infected for 30 hours then fixed and imaged using immunofluorescence. Cells were stained with either phalloidin (green), Draq 5 for DNA (blue) and human serum for *Chlamydia* (red). The actin ring was able to properly form in *Chlamydia* infected cells. Arrows point to the actin ring. [B] HeLa cells were treated with cytochalasin D or latrunculin A and the centrosome to nuclei ratio was evaluated using anti- γ -tubulin and Draq5. Uninfected cells and infected cells treated with cytochalasin D did not show a significant difference in centrosome to nuclei ratio, having a ratio of 1.7 ± 0.1 and 1.7 ± 0.1 respectively. Similarly, when cells were treated with latrunculin A, uninfected cells had a centrosome to nuclei ratio of 1.6 ± 0.1 , while infected cells had a ratio of 1.7 ± 0.1 . Untreated infected cells showed a significant increase with an average of 2.1 ± 0.1 centrosomes per cell $p = 0.001$. $N = 3$ experiments with at least 150 nuclei per experiment. [C] Cells were infected with *Chlamydia* for 18 hours or *Coxiella* for 96 hours, then fixed. Cells were stained with anti- β -tubulin (green), human serum (red), and Draq5 (blue). The arrow indicates the location of the CCV. [D] Cells were infected with *Chlamydia* or *Coxiella* for the indicated amounts of time and percent multinucleation of the population of infected cells were counted. Uninfected HeLa had a multinucleation rate of $2.9 \pm 0.3\%$ while infection increased multinucleation with time. The infected rates were $8.02 \pm 0.81\%$, $8.26 \pm 0.50\%$, $13.2 \pm 1.3\%$, $17.2 \pm 0.5\%$ for 18, 20, 22, 24 hpi respectively. All chlamydial time points investigated demonstrated a significance of $p < 0.005$. *Coxiella* had a multinucleation rate of $3.4 \pm 0.2\%$. $N = 3$ experiments with at least 600 cells per experiment.

Figure 3. Chlamydial infected cells form midbodies

[A-B] HeLa cells were infected for 30 hours then fixed and imaged using immunofluorescence. Cells were stained with anti- β -tubulin (green), human serum (red), and Draq 5 (blue). Arrows indicate location of the midbody. [B] All cells that were post-metaphase in mitosis were evaluated for midbody formation. Midbodies were present in $74.2 \pm 4.6\%$ of uninfected cells exiting mitosis, while this percentage rose to $92.2 \pm 0.6\%$ in cells infected with *Chlamydia*. A midbody was formed in $83.0 \pm 1.2\%$ of cells exiting mitosis that were exposed to UV light. Infected cells demonstrated a significant increase in midbody formation with a $p=0.017$. $N=3$ experiments with at least 150 cells per experiment.

Figure 4. *Chlamydia* induces lagging chromosomes in infected cells

[A] HeLa cells were infected for 30 hours then fixed and imaged. Conversely, uninfected cells were exposed to UV light for 3 minutes, allowed to grow for 24 hours and then fixed. Cells were stained with anti- β -tubulin (green), human serum (red), and Draq5 (blue) then examined in late telophase. Arrows indicate DNA bridging. [B] Uninfected cells had DNA in the midbody $6.7 \pm 1.5\%$ of the time, while infected cells had DNA in the midbody $26.3 \pm 1.2\%$ of the time. When cells were exposed to UV light there was DNA present in the midbody $19.00 \pm 1.5\%$ of the time. $N=3$ experiments with at least 200 cells per experiment. A student t-test was performed with $p < 0.02$. [C] Stable RFP-H2B HeLa cells were transfected with GFP and infected with L2 for 20 hours. Cells were then imaged every 10

minutes for 24 hours. Panels show metaphase at time 0, followed by anaphase second and finally telophase of uninfected and infected cells. [D] Cells were followed through mitosis with live cell imaging as shown in C. Cells were scored based on if they failed or succeeded in mitosis along with if DNA missegregation events were present. In uninfected cells, 94.9% of cells had successful mitosis, while only 19.5% of infected cells were successful. N=39 and N=41 cells, respectively.

Figure 5. Chlamydia infection causes the premature exit of mitosis

HeLa cells were fixed 30 hpi and stained with anti- β -tubulin (green), human serum (red), and Draq5 (blue). Mitotic cells were compared to the total number of cells after infection and/or monastrol treatment. N= 3 experiments with at least 1500 cells per experiment. [A] Uninfected HeLa cells had a mitotic index of $6.6 \pm 0.3\%$ which decreased to $4.4 \pm 0.1\%$ when infected, with a significance of $p=0.003$. When treated with monastrol $27.0 \pm 1.1\%$ of uninfected HeLa cells arrest in mitosis, but only $21.9 \pm 0.6\%$ of infected cells, $p<0.01$. [B] Fibroblasts had a mitotic index of $2.9 \pm 0.4\%$ and $0.3 \pm 0.1\%$ for uninfected and infected cells, respectively, with a significance of $p<0.002$. Monastrol treated uninfected fibroblasts had a mitotic index of $21.4 \pm 0.8\%$, while infected fibroblasts had a mitotic index of $1.9 \pm 0.03\%$, with $p<0.0001$.

Figure 6. CPAF degrades cyclin B1 and securin

[A] A cell-free degradation was performed and analyzed by western blot. Cyclin B1 and securin were degraded by infected cell lysate. Degradation could be blocked with a small peptide inhibitor of CPAF. [B] Same experiment as A, but infected cell lysate was treated with a protease inhibitor cocktail. [C] Mitotic shake-offs were performed to harvest proteins from uninfected and infected cells. Western blots show that the level of Cdk1 is unchanged due to infection, but the active T161 form of Cdk1 is not present in infected cells.

Figure 7. Host cell entry into mitosis

[A-B] A double thymidine block and infection was performed on HeLa cells. Cells were released and samples were collected at time points indicated. [A] Cells were fixed and stained for anti- β -tubulin (green), human serum (red), and Draq5 (blue) and a mitotic index was performed. In uninfected cells, a significant increase in mitotic cells begins at 10 hours post release, with the peak of mitosis occurring 12 hours after release from thymidine. Infected cells begin entering mitosis at 10 hours as well, but are not tightly synchronized. [B] Western blot analysis was performed on uninfected and infected cell lysates. Cyclin B1 levels were evaluated for cells entering mitosis. [C] The cyclin B1 western blot was analyzed for the mean pixel intensity of the bands and graphed. [D] HeLa cells were transduced using the FUCCI system and infected with *Chlamydia*. Starting 18 hpi, the cells were imaged every 10 minutes for 18 hours. The duration of S/G2 was calculated from the time of Cdt1-RFP degradation to mitotic entry (nuclear membrane breakdown). Uninfected cells were in S/G2 for 507 ± 78.4 minutes while infected required 774.6 ± 68.7 minutes with N=11, 13 respectively. The duration was significantly different with $p < 0.02$.

Figure 8. Model of chlamydial induced multinucleation

Chlamydial inclusions (red) secrete CPAF into the cytoplasm of the host cell (yellow). CPAF mimics the APC causing the cell to exit metaphase before microtubules (black) attach to the kinetochores of the chromosomes (blue). Furthermore, premature exit from metaphase does not allow supernumerary centrosomes (green) to cluster. This results in lagging chromosomes that become trapped in the midbody of the host cell. The host cell aborts abscission via the No Cut Pathway in order to protect the DNA. Lagging chromosomes can result in micronuclei.