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Chlamydia trachomatis infection causes mitotic spindle pole defects independently from its effects on centrosome amplification

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Abstract

Chlamydiae are gram negative, obligate intracellular bacteria, and *Chlamydia trachomatis* is the etiologic agent of the most commonly reported sexually transmitted disease in the United States. Chlamydiae undergo a biphasic life cycle that takes place inside a parasitophorous vacuole termed an inclusion. Chlamydial infections have been epidemiologically linked to cervical cancer in patients previously infected by human papillomavirus (HPV). The inclusion associates very closely with host cell centrosomes, and this association is dependent upon the host motor protein dynein. We have previously reported that this interaction induces supernumerary centrosomes in infected cells, leading to multipolar mitotic spindles and inhibiting accurate chromosome segregation. Our findings demonstrate that chlamydial infection causes mitotic spindle defects independently of its effect on centrosome amplification. We show that chlamydial infection increases centrosome spread and inhibits the spindle assembly checkpoint delay to disrupt centrosome clustering. These data suggest chlamydial infection exacerbates the consequences of centrosome amplification by inhibiting the cells' ability to suppress the effects of these defects on mitotic spindle organization. We hypothesize that these combined effects on mitotic spindle architecture identifies a possible mechanism for *Chlamydia* as a cofactor in cervical cancer formation.

Keywords

Chlamydia; Centrosomes; Dynein; Microtubule Spindles; Mitosis; Cell Cycle; Cyclin B1; Securin

Introduction

Chlamydia trachomatis infection is the leading bacterial cause of sexually transmitted disease (STD) in the United States, approaching four million cases annually (1). If left untreated chlamydial infections can lead to more serious sequela such as pelvic inflammatory disease, ectopic pregnancy, and tubal infertility (2–9). *Chlamydia* has also been reported as a cofactor in increased cancer rates in patients previously infected by human papillomavirus (HPV) (10–17). At least 90% of all cervical carcinomas are associated with infection by high risk HPV types 16 and 18 (18), but only a small number of women infected with HPV progress to invasive cervical cancer (ICC) (19). Therefore, the development of ICC depends on cofactors in conjunction with HPV, such as other STDs, hormones, smoking, and host genetic background and immunologic responses.

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The gain or loss of chromosomes due to mitotic defects can greatly accelerate tumor progression, and as a result, supernumerary centrosomes are seen in carcinomas of many origins (20–22). The presence of bipolar spindles at mitosis is critical for the proper segregation of chromosomes. Failure to form bipolar spindles results in chromosome segregation errors leading to chromosome instability, and ultimately aneuploidy (20). We established previously that chlamydial infection induces centrosome number defects and multipolar spindles (23). Recently, Johnson et al. confirmed our findings and demonstrated that chlamydial infection dysregulates the centrosome synthesis pathway (24). However, the presence of multiple centrosomes does not necessarily lead to mitotic spindle defects. Cells with supernumerary centrosomes have the ability to suppress multipolar spindles and undergo normal bipolar mitosis by clustering centrosomes to form only two active organizing sites (21, 25).

Clustering of centrosomes appears to require two mechanisms; organization of the microtubules by microtubule motor proteins and the spindle assembly checkpoint (SAC). The microtubule motor protein dynein along with the protein NuMA are proposed to focus microtubules allowing the cell to form bipolar spindles even in the presence of extra centrosomes (25–27). The SAC on the other hand, is a mechanism to monitor microtubule attachment to the kinetochores, delaying the onset of anaphase until the microtubules and chromosomes are properly organized (28).

In the current study we show that chlamydial infected cells are unable to suppress the multi-centrosome phenotype to form bipolar spindles, as cells with pre-existing extra centrosomes infected with *Chlamydia* are not able to sequester these centrosomes into functional bipolar spindles. This defect in centrosome clustering is not due to deregulation of microtubule motor proteins, as dynein and NuMA are still present in the spindles and functional, but is instead due to coordinate events of increasing centrosome spread during interphase, as well as overriding the SAC delay. This is likely an important factor in pathogenesis especially in co-infection with HPV.

Results

Centrosome defects and spindle defects are linked

We previously reported that chlamydial infection resulted in an increase in the number of cells with defective centrosome numbers as well as an increase of cells with multipolar spindles during mitosis. These two phenotypes, while functionally linked, are not mutually interdependent. It has recently become clear that eukaryotic cells have mechanisms to suppress the disruptive effects of extra centrosomes and can form normal bipolar mitotic spindles even when additional centrosomes are present (25). To further investigate the impact of chlamydial infection on both centrosome number defects as well as spindle pole organization defects, these phenotypes were quantitated over the time course of the chlamydial infectious cycle. The number of cells with centrosome number defects increased linearly over time before peaking at about 98% (Figure 1).

The number of mitotic cells with spindle defects followed the same trend. The rate of increase in both centrosome number defects and spindle defects was plotted against each other resulting in a line that had a slope approaching 1 (0.76 ± 0.08) (Figure 1A). The one to one linkage of these two phenotypes suggested that *Chlamydia* is inhibiting the innate centrosome clustering mechanism of mammalian cells reported by Quintyne et. al 2005 (25).

The linked increase in spindle pole defects with increased centrosome numbers was specific to chlamydial infected cells. The over-expression of Plk4 (polo-like kinase 4) induces supernumerary centrosomes. Transfection of GFP-Plk4 in HeLa cells led to a dramatic

increase in average centrosome numbers but did not lead to an increase in spindle defects demonstrating that HeLa cells can actively suppress the effects of extra centrosomes (Figure 1C and D). These data suggest that chlamydial infection is causing spindle pole defects in addition to increasing centrosome numbers.

Chlamydial infection causes spindle defects independently of centrosome number defects

To verify that chlamydial infection causes spindle defects independently from inducing centrosome number defects we infected the murine neuroblastoma cell line N1E-115. This cell line has a unique phenotype in that cells possess a large number of extra centrosomes with every cell having more than two centrosomes (25). However, these cells have predominantly normal bipolar spindles demonstrating an inherent ability to suppress the effects of extra centrosomes on spindle organization. We hypothesized that infection of this cell type would allow us to separate the effects of chlamydial infection on centrosome number defects from effects on spindle organization. Neuroblastomas were infected for 36 hours with *C. trachomatis* serovar L2. This time point was chosen as it corresponds with a dramatic increase in both spindle pole defects and centrosome number defects, but is well before cell lysis which occurs after 48 hour infection with serovar L2. Infected neuroblastoma cells had elevated centrosome numbers when compared to uninfected cells, 11.8 ± 0.5 and 9.0 ± 0.5 respectively (Figure 2A). However, all the cells in both infected and uninfected populations had greater than two centrosomes. To investigate the effect of chlamydial infection on spindle pole architecture, cells were stained for microtubules and the number of metaphase cells with improperly organized mitotic spindles was quantitated. The uninfected neuroblastoma cells were able to suppress the effects of extra centrosomes as only $33.5 \pm 2.9\%$ had multipolar spindles (Figure 2B). However, the spindle poles of infected cells were greatly disrupted with $71.7 \pm 4.1\%$ displaying a multipolar spindle phenotype at 36 hours post infection (Figure 2B). This dramatic increase in defective spindles cannot be explained by the modest increase in centrosomes per cell as the percentage of cells with intrinsic centrosome number defects (100%) did not change. This suggested that *Chlamydia* not only induces centrosome number defects but also interferes with the cells' innate ability to suppress the effects of extra centrosomes.

Genital serovars also cause spindle pole defects

The ability of a chlamydial infection to disrupt mitotic spindle organization may be an important virulence factor for *Chlamydia*'s epidemiological link to cervical cancer. We asked whether the more common STD causing serovars caused the same clustering defect. Neuroblastoma cells were infected with serovars G and L2, and with the mouse pathogen *C. muridarum*. *C. muridarum* is used as a model for human reproductive tract diseases. Infection with all three chlamydial strains resulted in increased spindle pole defects at similar levels to that of serovar L2 (Figure 2).

Centrosome function

The primary function of the centrosomes is to act as the minus end anchoring and nucleating site for microtubules. There are multiple mechanisms used by cells to suppress the effects of extra centrosomes, clustering and selective suppression of centrosome activity (29). To ask whether chlamydial infection affected the ability of the centrosome to nucleate microtubules, neuroblastoma cells were transfected with GFP-EB1. EB1 is a plus end microtubule tracking protein and labels the growing ends of the microtubules. The centrosomes of EB1 transfected N1E-115 cells all showed robust microtubule minus end anchoring and nucleation, as demonstrated by bright EB1 staining co-localizing with all centrosomes (Figure 3A). This staining pattern was unchanged in chlamydial infected cells; every centrosome had equivalent amounts of EB1 fluorescence, demonstrating that all the

centrosomes in both infected and uninfected cells were functional. However, the centrosomes of the infected cells appeared to be more disorganized than in uninfected cells.

To further examine the affect of chlamydial infection on centrosome function a nocodazole washout was performed on uninfected and infected neuroblastoma cells to test the ability of centrosomes to nucleate microtubules following depolymerization. After nocodazole treatment and subsequent recovery, the centrosomes in uninfected and infected cells were compared at 0, 5, 10, 15, 20, and 25 minutes. Representative images from 0 minutes, and 25 minutes post-washout confirm that centrosomes from both uninfected and infected cells are equally able to reestablish microtubule nucleation. At 25 minutes post-washout there is bright β -tubulin staining co-localizing with all centrosomes (Figure 3B). To this end we also performed nocodazole washouts in HeLa cells and HeLas transfected with Plk4 resulting in similar staining patterns as with the neuroblastoma cells (Figure S1 and S2).

Chlamydial infection increases the spread of extra centrosomes during interphase

The physical grouping of centrosomes is a necessary step for normal bipolar mitosis. We hypothesized that chlamydial infection may affect the physical grouping of centrosomes and that this may be a contributing mechanism to chlamydial induction of multipolar spindles. To answer this question we measured the geometric spread of centrosomes in infected and uninfected interphase neuroblastoma cells. The geometric spread is a measure of the area taken up by a bounding circle that encompasses the cells' centrosomes (Figure 4). The centrosomes in the uninfected neuroblastoma cells were clustered in an average area of 34.4 μm^2 (Figure 4, center box) with the middle 50% clustered between 1.5 and 24 μm^2 (Figure 4, box) and a fairly tight standard deviation of 69 (Figure 4, whiskers). This changed dramatically upon infection with *C. trachomatis* L2. The average area occupied by the geometric spread of the centrosomes was 122 μm^2 (Figure 4, center box), with the middle 50% spread between 10 and 164.5 μm^2 (Figure 4, box). The standard deviation was also much greater suggesting much more variability in the localization of the centrosomes (Figure 4, whiskers). This result suggests that chlamydial infection interferes with the cells' ability to localize centrosomes together in a small area; perhaps making it more difficult to physically group the centrosomes into only two active spindle poles.

Dynein localization and function

Dynein localization to the spindle poles is reported to be necessary for focusing and clustering extra centrosomes in order to form normal bipolar mitotic spindles (25, 30). We previously demonstrated that chlamydial infection causes re-localization of centrosomes from their normal cellular position and that the microtubule motor protein dynein was necessary for this effect (23). We also showed that dynein is recruited and maintained on the chlamydial inclusion (31). For these reasons we hypothesized that dynein sequestration by the chlamydial inclusion was a likely mechanism for the decrease in centrosome clustering we observed. To measure this effect, we quantified the fluorescent signal of dynein staining in the spindle poles as a percentage of the total dynein fluorescence in the entire cell including the chlamydial inclusion. If dynein was sequestered by the chlamydial inclusion we would expect that the percentage of the dynein fluorescence signal would be reduced in the spindles. However, just the opposite was observed, in uninfected neuroblastomas 18.4% of the total dynein fluorescence signal was contained in the spindle poles. The percentage of dynein fluorescence signal in chlamydial infected cells rose to about 32.7% (Figure 5A and B). This finding is likely the result of an increase in the number of spindles in mitotic cells due to a chlamydial infection. Thus, sequestration of dynein by the inclusion is not likely to be involved in the induction of mitotic spindle defects. Western blot analysis demonstrated that total amount of dynein in the cell was unchanged (Figure 5C).

We next asked whether chlamydial infection affected dynein function in infected cells. Dynein is the major minus directed motor protein complex in the cell and is responsible for the transport of a wide variety of cargo. We tested the effect of chlamydial infection on two cellular processes dependent on dynein trafficking, retrograde trafficking and trafficking to the lysosomes. Cells were infected with *C. trachomatis* L2 for 32 hours and the delivery of fluorescently labeled Cholera toxin B subunit (CtxB) was measured. CtxB is delivered to the Golgi apparatus after binding to the lipid GM1 on the plasma membrane in a dynein dependent manner. Fluorescently labeled transferrin (Tf) was used to measure dynein dependent trafficking from the plasma membrane to the lysosome. Intensity plots demonstrate that in both chlamydial infected and uninfected HeLa cells, Tf and CtxB are efficiently delivered from the plasma membrane to the perinuclear region by 30 minutes post infection (Figure 5D). Normalizing the percent fluorescent signal delivered over time demonstrated that there was no delay as the rate of delivery was not affected by the chlamydial infection.

Chlamydial nascent inclusions also require dynein function for delivery to the MTOC of the host cell. To determine if chlamydial infection impacted nascent inclusion trafficking we infected cells with *C. trachomatis* serovar G for 48 hours followed by a 4 hour infection with *C. trachomatis* serovar L2. Antibodies specific for serovar L2 were used to differentiate between the established serovar G chlamydial infection and the trafficking of the early nascent L2 inclusions. In both uninfected and serovar G infected cells, the L2 inclusions were delivered efficiently to the MTOC of the cells with > 95% of the L2 signal delivered to the perinuclear region (Figure 5E).

Chlamydial infection does not alter the recruitment of NuMA to the mitotic spindle

NuMA is a nuclear protein that localizes to the mitotic spindle during mitosis. In the spindle, NuMA is complexed with dynein and is required for spindle architecture. Decreased amounts of NuMA in mitotic spindles inhibits centrosome clustering and leads to multipolar spindles in many cancers (25, 26, 32). We therefore, asked if chlamydial infection caused defects in NuMA localization that could potentially be a mechanism for spindle defects. To measure NuMA localization neuroblastoma cells were transfected with a plasmid expressing GFP tagged NuMA, and the percentage of NuMA recruited to the spindles in relation to total GFP signal in the cell was calculated. In uninfected cells 26.7% of the GFP signal was present in the spindles and after infection with *C. trachomatis* L2 at 36 hours post infection this increased slightly to about 28.7% of the GFP signal present in the mitotic spindles (Figure 6). This is consistent with the results from the dynein recruitment experiment and is likely due to chlamydial infection increasing the number of mitotic spindles.

Taken together these experiments demonstrate that chlamydial infection does not cause multipolar spindles by inducing gross defects in the microtubule motors involved in centrosome focusing. However, chlamydial infection does lead to a disorganization of the centrosomes, likely by interfering with the local microtubule organization near the chlamydial inclusion.

Chlamydial infection inhibits the function of the spindle assembly checkpoint

Clustering additional centrosomes to form bipolar spindles requires two mechanisms. The first is the dynein/NuMA focusing pathway which clusters extra centrosomes and nucleates the microtubules into spindles, and the second is to delay mitosis to allow time for the microtubules and centrosomes to properly organize (28). To determine if chlamydial infection is affecting the timing of mitosis we measured the mitotic index and determined the stages of mitosis in infected and uninfected HeLa cells. The mitotic index is determined by calculating the ratio of cells in mitosis, as determined by the phosphorylation of Histone

H3 to total interphase cells, and provides a measure of the time it takes to complete mitosis. Uninfected cells had a mitotic index of about 8.1% while infected cells the index dropped to 3.8%. To demonstrate that this drop was caused by a decrease in the time cells took to complete mitosis we measured the frequency of cells in S phase by measuring DNA synthesis rates. The proportion of cells in S phase did not significantly change after infection with *Chlamydia* as the percentage of EdU positive cells was the same between uninfected and infected cells, 61.9 ± 4.8 vs 60.6 ± 5.2 , respectively (Figure 7A). To determine the stage of mitosis chlamydial infection was affecting we categorized the mitotic cells. In the uninfected cell population $7 \pm 1\%$ were in prophase, $30 \pm 1\%$ in prometaphase, $37 \pm 1\%$ in metaphase, $12 \pm 1\%$ in anaphase, and $14 \pm 1\%$ in telophase. However, in the L2 infected population there was a significant shift from metaphase to prometaphase while the other stages did not change significantly (Figure 7B). In the infected population metaphase dropped to $21 \pm 2\%$ and prometaphase increased to $54 \pm 3\%$. This shift was significantly different from the uninfected population with a p-value less than 0.001. The difference between prometaphase and metaphase is the organization of the chromosomes at the metaphase plate. The shift to prometaphase in the context of a lower mitotic index suggests that cells are entering anaphase before the chromosomes are completely aligned.

To verify that chlamydial infected cells were exiting mitosis early we measured the length of mitosis using the fluorescence ubiquitination cell cycle indicator system (FUCCI) (33). HeLa cells were transduced with modified baculovirus encoding the two fluorescent proteins that respond to changes in the cell cycle. Multiple cells were imaged for 18 hours using live cell microscopy. Example cells are shown (Figure 8A). The timing of mitosis was plotted by normalizing the start of mitosis to the first frame before nuclear membrane breakdown (NMBD) and the green fluorescent intensity (GFP-geminin) was normalized to the fluorescent signal in the nucleus at this time point. Relative signal intensity was plotted as a function of time (Figure 8B). Complete degradation of GFP-geminin (loss of green fluorescence) was used as the marker for mitotic exit. The graph in figure 8B represents the average time and intensity for four infected and 5 uninfected cells. The infected cells spent an average of 29 minutes less time in mitosis than the uninfected cells (53 ± 4 minutes versus 82 ± 8 minutes respectively) (Figure 8C).

Taken together these experiments demonstrate the chlamydial infected cells progress normally through the cell cycle but exit metaphase before the chromosomes are properly aligned. Delay of the onset of anaphase until all the chromosomes are aligned during metaphase is mediated by the spindle assembly checkpoint (SAC). Eukaryotic cells use the SAC as a safety mechanism to ensure the fidelity of chromosome segregation (34). The SAC is switched off by the activity of the anaphase promoting complex (APC). The APC ubiquitylates securin and cyclin B1, thereby activating the protease separase and inactivating the cyclin-dependent kinase-1 (Cdk1). Separase then cleaves cohesin complexes that hold the sister chromatids together and initiates sister-chromatid separation. Cdk1 inactivation leads to the dephosphorylation of Cdk1 substrates by protein phosphatases, and by that means enables exit from mitosis (35). Previous studies have indicated that chlamydial infection can lead to degradation of cyclin B1 (36, 37). We hypothesized that the observed early onset of anaphase in chlamydial infected cells was due to degradation of cyclin B1 and securin. To test whether cyclin B1 and securin were degraded during a chlamydial infection, mitotic cells were harvested using a mitotic shake off and the levels of cyclin B1 and securin were measured by western blot and immunoprecipitation respectively. Securin levels were measured using immunoprecipitation as it was present in much lower quantities than cyclin B1. In the chlamydial infected mitotic cells nearly all the cyclin B1 was cleaved as a lower molecular weight band appeared and the expected 48 KDa band disappeared (Figure 9A). The securin immunoprecipitation experiments demonstrated that securin was also degraded in the chlamydial infected cells. GAPDH was used as a loading control (Figure 9B).

Cyclin B1 is cleaved by a chlamydial protease (CPAF) secreted into the cytosol of infected cells (36, 37). To determine if securin was similarly cleaved by a secreted protease we purified the cytosolic fraction from infected cells using the method reported for CPAF purification (38). The cytosolic fraction was incubated with mitotic host proteins and a western blot was performed to analyze degradation. Cyclin B1 and securin were both cleaved after incubation with infected cytosolic lysate and compared to keratin 8 as a control (Figure 9C). In the immunoprecipitation experiments we observed complete disappearance of the securin signal while in the cell free degradation assay we observed cleavage of the protein. We believe this is because we precipitate with and C-terminally targeted antibody and attempted detection with a N-terminally target antibody.

Discussion

In previous studies we showed that the chlamydial inclusion acquires the dynein motor protein and initiates migration from the cell periphery to the microtubule organizing center (MTOC) early after entry (31). The interaction between dynein and the chlamydial inclusion continues throughout the chlamydial life cycle (23). This association results in centrosome number defects and centrosome mislocalization (23). Recently, Johnson et al. confirmed centrosome amplification and demonstrated that chlamydial infection disregulates centrosome duplication (24). We have also demonstrated infected cells display an increased frequency of multipolar mitotic spindles (23). Multipolar spindles are found in many cancers and lead to an increase in chromosome instability that can be an important step in cellular transformation. The mechanisms that regulate spindle bipolarity and organization are not completely understood. However, centrosome number defects play a necessary role in this process. Supernumerary centrosomes provide extra nucleation sites for the formation of aberrant spindles. Extra centrosomes do not always lead to multipolar spindles as eukaryotic cells have multiple mechanisms to suppress the effects of too many centrosomes.

In this study we demonstrated that chlamydial infection causes spindle defects during mitosis independently of its affect on centrosome amplification. Proper spindle formation in the presence of extra centrosomes requires at least two major cellular functions, centrosome clustering and a delay in mitosis for the clustering-focusing pathway to successfully form only two spindle poles. The centrosome clustering and spindle focusing pathway requires localization of both dynein and the NuMA protein in the mitotic spindles (25, 26). We were surprised to find that chlamydial infection did not measurably affect the recruitment of either of these proteins to the mitotic spindle or did infection affect diverse dynein functions within the cell. We initially hypothesized, that like the mechanism in many cancer cells, sequestration of dynein by the chlamydial inclusion would lead to a loss of dynein and NuMA in the spindles and would be a likely mechanism for the disruption of centrosome clustering (25, 26). Instead it appears that infection with *Chlamydia* induces spindle defects by disrupting centrosome organization during interphase and inhibiting mitotic arrest.

Chlamydial infection of cells (cell line N1E-115) with inherent centrosome number defects resulted in an increase in the spread of the supernumerary centrosomes. This result is likely mediated by the same mechanism that leads to displacement of the centrosomes from their normal juxta-nuclear position in cells with normal centrosome numbers (23). We have previously demonstrated that the centrosomes interact directly with the chlamydial inclusion and that this interaction is mediated by dynein (23). We believe that the increase in the spread of supernumerary centrosomes caused by chlamydial infection is the result of a direct cis-interaction between the chlamydial inclusion and centrosomes mediated by dynein. This spread is likely due to a direct interaction with the chlamydial inclusion prohibiting normal centrosome movement.

Chlamydial infection significantly decreased the mitotic index of infected cells but didn't change the rate of S phase in the cell cycle. Other labs have also reported that chlamydial infection does not significantly change the length of the cell cycle (39–41). Infection also lead to a shift in the mitotic cells of the infected population from metaphase to prometaphase. These data suggest that infected cells are spending less time in mitosis and prematurely transitioning from metaphase to anaphase, indicating that infection inhibits the function of the SAC. The SAC acts to arrest mitosis, giving the spindles and the chromosomes time to properly attach and align at the metaphase plate. It is triggered by unattached kinetochores and lack of tension between sister chromatids (34). The SAC arrests mitosis by negatively regulating cdc20 activation of the APC (anaphase promoting complex) which mediates polyubiquitylation of cyclin B1 and securin (34). It is the inhibition of the degradation of these two proteins that keeps the cell from progressing to anaphase (34). We show here that chlamydial infection actively overrides cell cycle arrest by causing the degradation of the two targets of the APC, cyclin B1 and securin. We do not yet know what chlamydial factor(s) are involved in the cleavage of securin but it is likely that the chlamydial effector CPAF is involved as it has been demonstrated to target cyclin B1 (36, 37). Other factors may also contribute to chlamydial regulation of the cell cycle. For example the type III secretion effector CT847 is reported to interact with the cellular protein GCIP and is proposed to drive chlamydial infected cells through the G1/S cell cycle checkpoint (42).

Taken together these data in combination with the data from Johnson et al. support the idea that chlamydial infection actively promotes spindle defects and chromosome instability by affecting at least three independent cellular pathways, dysregulation of centrosome duplication (Johnson et. al.), capture of centrosomes through dynein resulting in mislocalization of centrosomes (this study) and lastly, overriding the SAC driving infected cells through mitosis in the presence of spindle defects and unaligned chromosomes.

The role that dysregulation of the SAC plays in the chlamydial infectious cycle is not clear, as most of the cells infected by a natural chlamydial infection are terminally differentiated. However, one class of cells that would be impacted by the inhibition of cell cycle checkpoints leading to increased spindle defects are cells co-infected with HPV. We hypothesize that the combined effects of centrosome number defects, increases in centrosome spread, and inhibition of the SAC and other cell cycle checkpoints may contribute to cervical cancer in patients previously infected with HPV.

Materials and Methods

Organisms and Cell Culture

Chlamydia trachomatis serovar L2 (LGV 434) were grown in HeLa 229 cells, and EBs were purified by Renografin density gradient centrifugation as previously described (43). All cell lines were obtained from the American Type Culture Collection. Neuroblastoma (N1E-115) cells (CRL-2263) were grown in RPMI-1640 (Lonza) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 10 µg/ml gentamicin (Gibco). HeLa 229 cells (CCL-2.1) were grown in RPMI-1640 supplemented with 10% FBS and 10 µg/ml gentamicin.

Antibodies and Microscopy

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 either fixed in ice cold methanol for 10 minutes, or 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, and incubated with the primary antibody described for each experiment. The antibodies used for these experiments were: mouse monoclonal anti-

tubulin (Sigma), mouse monoclonal anti- β -tubulin (Sigma), rabbit monoclonal anti- β -tubulin (Cell Signaling Technology) and mouse monoclonal anti-dynein intermediate chain 74.1 (Covance). For most experiments *Chlamydia trachomatis* was stained with human serum from male AB plasma purchased from Sigma, specifically for Figure 5E, *Chlamydia trachomatis* serovar L2 was stained with a monoclonal anti-MOMP antibody, a gift from Harlan Caldwell, Rocky Mountain Labs, NIAID, NIH. To visualize the primary antibodies appropriate AlexaFluor conjugated secondary antibodies were used; 488/568/647 against mouse, rabbit or human IgG (Molecular Probes/Invitrogen). The far-red fluorescent DNA dye DRAQ5 (Biostatus) 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 μ Manager (<http://www.micro-manager.org/>). Images were processed using the image analysis software ImageJ (<http://rsb.info.nih.gov/ij/>), or Imaris (Bitplane Scientific Solutions). Representative confocal micrographs displayed in the figures are maximal intensity projections of the 3D data sets, adjusted for brightness and contrast.

Infections

Cells were incubated with *C. trachomatis* EBs at an MOI of approximately three in Hank's balanced salt solution (HBSS) (Gibco) for 30 minutes at room temperature while rocking. The inoculum was removed and replaced with fresh RPMI-1640 containing 10% FBS and 10 μ g/ml gentamicin. Infections were allowed to proceed for 36 hours unless otherwise noted.

Transfections and Plasmids

Cells were seeded on 12 mm number 1.5 glass coverslips in 24-well plates and grown to 50% confluency. Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The GFP-NuMA plasmid construct was a kind gift from Dr. Andreas Merdes, Centre National de la Recherche Scientifique-Pierre Fabre, Toulouse, France. Transfection with GFP-Plk4 was carried out as above. Human Plk4 (ATCC) was cloned from pBlueScript into a GFP-destination vector using the Gateway Cloning System (Invitrogen) according to the manufacturer's instructions. The GFP-EB1 plasmid was a kind gift from Dr. Jennifer S. Tirnauer, University of Connecticut Health Center.

Nocodazole Washout

HeLa cells, neuroblastomas, or HeLas transfected with GFP-Plk4 were treated with 5 μ g/ml nocodazole in complete media for 30 minutes at 37°C. The cells were then washed twice with fresh media and placed at 37°C to recover. The cells were fixed at 0, 5, 10, 15, 20, and 25 minutes following the washout. The cells were subsequently stained for β -tubulin, γ -tubulin, and *Chlamydia* when necessary.

Mitotic Index, DNA Synthesis Assays, and Stages of Mitosis Analysis

The mitotic index was calculated by determining the ratio of mitotic cells to the total number of cells present in a field of view. HeLa cells were stained with anti-phospho-Histone H3 (Thermo Scientific) to easily discern mitotic cells, and for *Chlamydia* when necessary. 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 three times. The rate of DNA synthesis in HeLa cells was determined by uptake of the thymidine analogue EdU (5-ethynyl-2'-deoxyuridine), with the use of the Click-iT EdU kit (Invitrogen). For uninfected and infected populations EdU positive cells were compared with the total number of cells

present in a field of view. 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 three times. The stages of mitosis were determined by staining cells for DNA and establishing the number of cells in different stages of mitosis for multiple coverslips. A minimum of 200 cells were counted for each stage of mitosis for both uninfected and infected populations, and the procedure was repeated three times.

Western Blot Analysis

The western blot analysis for dynein localization was carried out as follows. Uninfected and infected cells were mechanically lysed in MEPS buffer and microtubules were stabilized with taxol, the samples were centrifuged at low and then high speeds. Each of the pellets and supernatant from the high-speed spin were run on SDS-PAGE and blotted for anti-dynein 74.1. The western blot procedure for *in vivo* cyclin B1 was carried out as follows. HeLa cells were infected with *C. trachomatis* L2 for 30 hours. Mitotic cells were shaken off the flask in PBS and centrifuged. Pellets were lysed with sample buffer (Thermo Scientific). A western blot was performed using mouse monoclonal anti-cyclin B1 (Cell Signaling) or mouse monoclonal anti-GAPDH (EnCor) followed by sheep anti-mouse HRP antibody (AbCam). The substrate was a chemo-luminescent reagent (Pierce).

Cell-free Degradation

Mitotic host cell proteins were collected as follows. Mitotic cells were shaken off the flask in PBS and centrifuged. Cells were lysed with NP-40 Buffer [1% NP-40, 0.5% Triton X-100, 0.15 M NaCl, 50 mM Tris pH 8.0] for 20 minutes on ice. Sample was centrifuged at 15,000 rpm to remove debris and stored at -80°C . Chlamydial proteins present in the cytoplasm were collected as follows. Cells were scraped into PBS and pelleted. Cells were resuspended in douncing buffer [10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM DTT, 250 mM sucrose, 20 mM Hepes-KOH pH 7.5] and dounced 5 times. Cells were centrifuged for 5 minutes at 1500g and the supernatant collected and further spun for 15 minutes at 100,000g. Supernatant was used as enzyme source. A western blot was performed as previously described. Rabbit monoclonal anti-cytokeratin 8 (Abcam) followed by goat anti-rabbit HRP antibody (Millipore) was used as a loading control.

Immunoprecipitation

HeLa cells were lysed using RIPA buffer [20 mM Tris pH 7.5, 10% glycerol, 1% triton X-100, 1% deoxycholic acid, 0.1% SDS, 2.5 mM EDTA and protease inhibitor cocktail (Sigma)] and sonicated. Cellular debris was pelleted and the supernatant was added to Protein A Agarose (Pierce) along with mouse monoclonal anti-securin (AbCam). After a four hour room temperature incubation, the complexes were pelleted and washed [25 mM Tris pH 7.5, 150 mM NaCl, 0.1% Triton X-100]. Protein was eluted with 0.2 M glycine (pH 2.0) and neutralized with 1M Tris. Western blot analysis was performed with rabbit anti-PTTG-1 (Invitrogen) followed by goat anti-rabbit HRP (Sigma).

Calculation of dynein and NuMA in mitotic spindles

Three dimensional image stacks were taken and a three dimensional region of interest was drawn around the mitotic spindles and separately around the entire cell. The ratio of fluorescence intensity was calculated by dividing the fluorescence intensity in the spindle region of interest by the total intensity of the cell. $N > 25$, and each experiment was repeated three times.

Calculation of centrosome spread

Images used for centrosome spread calculations were taken as three dimensional image stacks and maximum intensity plots were used to calculate the two dimensional spread. Because the cells are spread out on the coverslip the z dimension was very small in relation to both x and y and therefore not used in the calculation of geometric spread. The 2D spread was calculated using the ImageJ plug-in 'Hull and Circle'. A region of interest was drawn around the centrosomes, which were identified by automatic thresholding of the fluorescence intensity and the area of the bounding circle was calculated from the minimal fitted polygon.

FUCCI (Fluorescence Ubiquitination Cell Cycle Indicator)

HeLa cells were transduced with Premo™ 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 24–30 hours post infection. This experiment was repeated 3 times each for both uninfected and infected 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 plots.

Statistical Analyses

Numerical data are presented as the mean $\pm$ SEM, unless otherwise noted, and were analyzed by the unpaired Student's t-test using GraphPad Prism4 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Centrosome abnormalities correlate with spindle dysfunction

(A) HeLa cells were infected with *Chlamydia trachomatis* serovar L2, and fixed at 0, 24, 32, and 48 hours post-infection. Cells were stained with anti- γ -tubulin for centrosomes (green), anti- β -tubulin for spindle poles (red), and with human serum for *Chlamydia* (blue). The percentage of infected cells with greater than two centrosomes and greater than two spindle poles were counted for each time point (mock-infected cells were counted for the 0 hr time point). A linear regression model generated a positive slope of 0.76 ± 0.1 , and $r^2=0.97$. (B) Representative confocal micrograph, displaying an infected multipolar cell, and an uninfected bipolar cell. (C) HeLa cells were transfected with GFP-Plk4. Centrosomes (arrows) were stained with anti- γ -tubulin (green), centrosomes from transfected populations were easily identified as GFP positive. The uninfected cells were co-stained for DNA (blue), the infected cells were co-stained for *Chlamydia* (red). The GFP-Plk4 transfected cells were co-stained for microtubules (red) and DNA (blue). The cells were counted for both uninfected and infected populations resulting in 2.2 ± 0.1 average centrosomes per cell, and increasing to 3.2 ± 0.3 average centrosomes/cell 36 hours post-infection. The Plk4 transfected cells were counted for GFP positive centrosomes and this population contained 3.7 ± 0.2 average centrosomes/cell, $n > 150$ for each population. (D) Spindle poles (arrows) were stained with anti- β -tubulin and quantitated by counting the percentage of cells with more than two spindle poles in the uninfected, infected, and GFP-Plk4 transfected populations resulting in 5.5 ± 0.8 , 48.3 ± 3.4 , and 5.0 ± 4.0 percent multipolar spindles, respectively. The percentage of cells with multipolar spindles is significantly higher in infected cells compared to Plk4 positive cells, $p=0.0012$ (Student's t-test) $n > 100$ for each population. The representative confocal micrographs are co-stained as above, with the exception of the infected cell, in which *Chlamydia* is also stained with the DNA dye DRAQ5. Scale bars, 5 μ m.

Figure 2. Chlamydial infection induces slight centrosome amplification, but significant spindle defects in neuroblastomas

(A) Uninfected neuroblastomas were stained with anti- γ -tubulin for centrosomes (green), and DNA (blue). Neuroblastomas were infected with serovars L2, G, and *Chlamydia muridarum* (MoPn) and then stained for centrosomes (green), and *Chlamydia* (red). The cells were imaged, and the centrosomes (arrows) were then counted for each population, $n > 150$. The uninfected cells averaged 9 ± 0.5 centrosomes/cell, and the infected cells averaged 11.8 ± 0.5 , 12.5 ± 0.7 , and 14.5 ± 1.2 centrosomes/cell, respectively. (B) For spindle pole quantification uninfected neuroblastomas were stained with anti- β -tubulin for spindle poles (green), and DRAQ5 (blue). Infected neuroblastomas were stained for spindles (green) and for *Chlamydia* (red). Mitotic cells were counted for each population (arrows), $n > 100$. The infected population is 71.7 ± 4 , 72.3 ± 2.4 , and 72.1 ± 3.4 percent multipolar, respectively, compared to 33.5 ± 2.9 percent multipolar in uninfected controls. $*p < 0.0015$ (Student's t-test). Scale bars, $5\mu\text{m}$.

Figure 3. Centrosome function is not inhibited by chlamydial infection

(A) Uninfected and infected neuroblastomas were transfected with GFP-EB1 (green), a plus-end microtubule tip-tracking protein. The cells were subsequently stained for centrosomes (red), and *Chlamydia* (blue) as necessary. The cells were imaged by confocal microscopy to evaluate the function of the centrosomes after infection. (B) Neuroblastomas were incubated with nocodazole and allowed to recover. The cells were fixed and stained at sequential time points during recovery for centrosomes (green), microtubules (red), and *Chlamydia* (blue) as necessary. The panels at time zero exhibit confocal images of cells at the time of washout. The panels at 25 minutes display cells that have been allowed to recover for 25 minutes after washout. Scale bars, 5 μ m.

Figure 4. Chlamydial infection affects centrosome positioning in interphase cells

(**A**) Both uninfected and infected neuroblastomas were stained for centrosomes (green), microtubules (red), and DNA (blue). The chlamydial inclusion is demarcated with an arrow. The area within the bounding circle translates to the geometric spread of the centrosomes (inset). (**B**) The box and whisker plot demonstrates the uninfected population have centrosomes that are clustered within an average area of $34.4\mu\text{m}^2$ (small box) ± 69.1 (standard deviation, whiskers), and the middle 50% of uninfected cells have centrosomes clustered within an area of 1.5 to $24\mu\text{m}^2$ (large box). The infected cells contain centrosomes that are spread over a larger distance with a mean average area of $121.9\mu\text{m}^2 \pm 149.8$, with the middle 50% spread between 10 and $164.5\mu\text{m}^2$, $n < 76$ for each population. Scale bars, $5\mu\text{m}$.

Figure 5. Dynein localization and function is unaffected by chlamydial infection

(A) ImageJ was used to measure the relative fluorescence intensity of uninfected and infected cells stained with anti-dynein IC (mAb 74.1). Figure A demonstrates how the images are processed. Scale bars, 5 μ m. (B) The relative dynein percentages in the spindles of the infected and uninfected populations was measured, $n > 50$, and the experiment was repeated three times. The uninfected cells contain 18.4 ± 1 percent dynein in the spindles and the infected cells contain 32.7 ± 2 percent dynein, $*p < 0.0001$ (Student's t-test). (C) This was confirmed by western blot. (D) Dynein trafficking function was determined by measuring the delivery of Alexa-transferrin (Tf) or Alexa-Cholera toxin B subunit (CtxB) to the peri-nuclear region of the cell in uninfected and infected cells. The fluorescence intensity of Tf and CtxB was determined at 0, 15, and 30 minutes post incubation with the endocytic markers. There was no significant difference in the delivery of Tf or CtxB in uninfected or infected cells. (E) Furthermore, dynein-dependent delivery of *Chlamydia* serovar L2 was not affected by previous infection of cells with serovar G.

Figure 6. Chlamydial inhibition of centrosome clustering occurs independently of NuMA
(A) Neuroblastomas were transfected with the construct GFP-NuMA. Mitotic, GFP-NuMA-positive cells were measured for NuMA association with the spindles using ImageJ. The spindles were stained with β -tubulin (red), and *Chlamydia* (blue). Scale bars, 5 μ m. (B) There was no significant difference in the relative amount of NuMA associated with the spindles in uninfected or infected populations, 26.7 $\pm$ 2 and 28.7 $\pm$ 2 percent, respectively.

Figure 7. Examination of the cell cycle demonstrates *Chlamydia* overrides the SAC

(A) The mitotic index of infected cells significantly decreases from 8.1 ± 0.4 to 3.8 ± 0.6 percent mitotic uninfected cells, $p = 0.0044$. The rates of DNA synthesis in HeLas measured by incorporation of EdU, remained the same in uninfected and infected cells (62 ± 4.8 vs 60.5 ± 5.2 percent cells positive for EdU). (B) Analysis of uninfected and infected (32hrs) HeLa cells during the different stages of mitosis revealed that cells undergo a shift from metaphase (36.6 ± 1.3 percent cells in metaphase) to prometaphase (54.0 ± 3.2 percent cells in prometaphase) during infection, $p < 0.001$ (Student's t-test).

Figure 8. *Chlamydia* induces early mitotic exit as shown by FUCCI analysis

HeLa cells were transduced with FUCCI and imaged live by confocal microscopy. Time spent in mitosis was determined by assessing the time from nuclear membrane breakdown (NMBD) to complete degradation of GFP. (A) Each panel contains a representative image from uninfected and infected cells in G2 (0 minutes), the start of mitosis (NMBD, 10 minutes), and mitotic exit (variable time). Scale bars, 5 μ m. (B) The relative fluorescence intensity is normalized to time zero and the plot displays the decrease in fluorescence intensity from NMBD to mitotic exit (arrows). Each point represents the average normalized fluorescence intensity for 4 infected cells and 5 uninfected cells. (C) The average time spent in mitosis is quantified in the graph. The time for uninfected cells is 53 $\pm$ 3 minutes, and 82 $\pm$ 8 minutes for infected cells, $p = 0.018$.

Figure 9. Chlamydial infection leads to the degradation of cyclin B1 and securin

Uninfected and infected lysates of HeLa cells were examined for the SAC proteins cyclin B1 and securin. (A) Western blot analysis of uninfected and infected lysates for cyclin B1 displayed cleavage of cyclin B1. (B) Securin was analyzed by IP followed by western blot analysis which also demonstrated degradation in infected lysates. (C) A cell-free degradation assay was performed. Cyclin B1 and securin cleavage occurs in the presence of cytosolic chlamydial proteins.