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Authors

Wang, Kevin

Muñoz, Karissa J

Tan, Ming

et al.

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# *Chlamydia* and HPV induce centrosome amplification in the host cell through additive mechanisms

**Kevin Wang**[a], **Karissa J. Muñoz**[b], **Ming Tan**[a,c], **Christine Sütterlin**[b]

[a]Department of Microbiology and Molecular Genetics, University of California, Irvine, California, USA

[b]Department of Developmental and Cell Biology, University of California, Irvine, California, USA

[c]Department of Medicine, University of California, Irvine, California, USA.

## Summary

Based on epidemiology studies, *Chlamydia trachomatis* has been proposed as a co-factor for human papillomavirus (HPV) in the development of cervical cancer. These two intracellular pathogens have been independently reported to induce the production of extra centrosomes, or centrosome amplification, which is a hallmark of cancer cells. We developed a cell culture model to systematically measure the individual and combined effects of *Chlamydia* and HPV on the centrosome in the same host cell. We found that *C. trachomatis* caused centrosome amplification in a greater proportion of cells than HPV and that the effects of the two pathogens on the centrosome were additive. Furthermore, centrosome amplification induced by *Chlamydia*, but not by HPV, strongly correlated with multinucleation and required progression through mitosis. Our results suggest that *C. trachomatis* and HPV induce centrosome amplification through different mechanisms with the chlamydial effect being largely due to a failure in cytokinesis that also results in multinucleation. Our findings provide support for *C. trachomatis* as a co-factor for HPV in carcinogenesis and offer mechanistic insights into how two infectious agents may cooperate to promote cancer.

### Keywords

host-pathogen interaction; co-infection; sexually-transmitted infection; multinucleation; cell cycle progression; cervical cancer

## Introduction

Each year, cervical cancer causes 300,000 deaths in the world, making it the fourth most common cancer in women (Arbyn et al. 2018). Human papillomavirus (HPV) is its main etiologic agent, with 90% of cervical carcinomas linked to “high-risk” HPV types, such as HPV16 and 18 (Muñoz et al. 2003). However, not all women

Address correspondence to: Christine Sütterlin, PhD, Department of Developmental and Cell Biology, University of California, Irvine, California, USA, Phone: (949) 824-7140, Fax: (949) 824-4709, suetterc@uci.edu.

Conflict of interest:
None

infected with HPV develop cervical cancer, which suggests that additional factors are involved in carcinogenesis. A number of co-factors, including smoking, long-term use of oral contraceptives, and *Chlamydia trachomatis* infection, have been proposed (Fonseca-Moutinho 2011; International Collaboration of Epidemiological Studies of Cervical Cancer et al. 2007; Silva, Cerqueira, and Medeiros 2014). The evidence for *C. trachomatis* as co-factor is based on sero-epidemiology studies showing that women with cervical cancer were more likely to have had a prior *Chlamydia* infection (Smith et al. 2002; Zhu et al. 2016).

HPV contributes to oncogenesis through multiple mechanisms. It causes aberrant proliferation of host cells, which supports viral DNA replication (Münger et al. 2004). It also promotes genomic instability in an infected host cell by inactivating cell cycle checkpoints, dysregulating host DNA repair pathways, and inducing centrosome abnormalities (Thomas and Laimins 1998; Banerjee et al. 2011; Spardy et al. 2009; Duensing et al. 2000). As a consequence, HPV-infected cells accumulate cellular mutations while undergoing enhanced proliferation, which together lead to malignant transformation.

The centrosome, an organelle with a key role in microtubule organization, is dysregulated in many cancer cells (Salisbury et al. 1999; Chan 2011). Normal diploid cells have a single centrosome, which duplicates in parallel to DNA in S-phase of the cell cycle. Cancer cells often contain extra, or supernumerary, centrosomes, which is a phenomenon called centrosome amplification (Lingle et al. 1998; Pihan et al. 1998). Such supernumerary centrosomes contribute to carcinogenesis by leading to chromosome missegregation, genomic instability, and enhanced cell invasiveness (Ganem, Godinho, and Pellman 2009; Godinho et al. 2014). Centrosome amplification is caused by at least three distinct mechanisms, which include cell-cell fusion, cytokinesis defects, and dysregulation of the centrosome duplication machinery (Godinho and Pellman 2014).

High risk HPV induces centrosome amplification through its oncoproteins E6 and E7. Co-expression of E6 and E7 is sufficient to increase centrosome number in normal human keratinocytes, which leads to multipolar spindles and ultimately to genomic instability (Duensing et al. 2000). Additionally, mice expressing E6 and E7 have cervical and skin lesions containing cells with multiple centrosomes (Schaeffer et al. 2004). E7 expression has been proposed to promote centrosome amplification by altering the centrosome duplication machinery, whereas the mechanism for E6-induced centrosome amplification is less understood (Duensing et al. 2006; Duensing and Münger 2002).

*C. trachomatis* also induces centrosome amplification (Grieshaber et al. 2006; Johnson et al. 2009). Tissue culture cells infected with this obligate intracellular bacterium formed extra centrosomes in interphase and multipolar spindles in mitosis (Grieshaber et al. 2006). Intriguingly, these phenotypes persisted after the cells were cured of the infection. *C. trachomatis* has been proposed to induce centrosome abnormalities by dysregulating the centrosome duplication machinery and by causing cytokinesis defects in host cells (Johnson et al. 2009; Alzhanov et al. 2009). An important caveat is that prior mechanistic investigations were mostly done in *C. trachomatis*-infected HeLa cells, which contain HPV-18 DNA (Schwarz et al. 1985). Thus, these studies measured the combined effects

of *Chlamydia* and HPV on the centrosome, but not the individual contribution of *C. trachomatis*.

In the present study, we investigated the respective roles of HPV and *Chlamydia* in causing centrosome abnormalities. To accomplish this goal, we developed a cell culture system that allowed us to determine the individual and combined effects of these two sexually transmitted pathogens on the centrosome in the same host cell. Our results provide biologic plausibility for a role of *Chlamydia* as a co-factor for HPV in the development of cervical cancer.

## Experimental procedures

### Antibodies used in this study

Primary antibodies: anti-Centrin (Millipore, 04–1624), anti-γ-tubulin (Abcam, ab11321), anti-α-tubulin (Sigma, T5168), anti-Cep164 (Santa Cruz, sc-240226). Secondary antibodies for immunofluorescence microscopy: Donkey anti-Rabbit IgG Alexa Fluor 488 (Invitrogen, A21206), Donkey anti-Mouse IgG Alexa Fluor 555 (Invitrogen, A31570), Goat anti-Rat IgG Alexa Fluor 564 (Invitrogen, A11081), Donkey anti-Goat IgG Alexa Fluor 488 (Invitrogen, A21202).

### Cell culture and *Chlamydia* infection

The parental hTERT-RPE-1 cell line was obtained from ATCC and cultured at 37°C and 5.0% $CO_2$ in DMEM (Gibco, 11995–065) supplemented with 10% FBS (Atlanta Biologicals, S11550). A549 cells stably expressing HPV16 E6/E7 were a generous gift from Dr. Ashok Aiyar (LSU New Orleans) and were grown in DMEM supplemented with 10% FBS.

*Chlamydia* infection was done by infecting near-confluent cell monolayers with *C. trachomatis* serovar L2 (ATCC) at an MOI of 3 in SPG (200 mM sucrose, 20 mM sodium phosphate and 5 mM glutamate; pH 7.2) followed by centrifugation at 700×*g* for 1 hour at room temperature. As control, cells were mock infected with SPG alone. After centrifugation, the inoculum was removed and replaced with DMEM containing 10% FBS. The same infection conditions were used for RPE-1 and A549 cells.

### Generation of RPE-1 cell lines expressing HPV16 E6/E7 oncoproteins

The pLXSN-HPV16 E6/E7 retroviral vector was obtained from Addgene (Plasmid #52394). From this vector, the pLXSN-Empty control vector was generated using Gibson assembly with forward primer 5′-TCCTCTAGAGTCCTGTAATCCTACCATGGCTGATCCTGCAG-3′ and reverse primer 5′-GATTACAGGACTCTAGAGGATC-3′. The pLXSN retroviral vectors and helper plasmid were co-transfected in 293T cells with calcium phosphate (293T cells and helper plasmid were generous gifts from Dr. Aimee Edinger, UC Irvine). Viral particles were collected 48 hours post transfection and used to infect RPE-1 cell monolayers with 10ug/mL polybrene (Sigma). Colonies were pooled after 10 days of selection with 600ug/mL G418 (Fisher, BP-918). HPV16 E6/E7 expressions were confirmed via

RT-PCR. Forward primer 5′-GCAAGCAACAGTTACTGCG-3′ and reverse primer 5′-GGTTTCTCTACGTGTTCTTG-3′ were used to detect HPV16 E6 expression and primer pair 5′-CAGCTCAGAGGAGGAGGATG-3′ and 5′-GCCCATTAACAGGTCTTCCA-3′ were used to detect HPV16 E7 expression.

### Immunofluorescence microscopy

Cells, grown and infected on glass coverslips, were fixed in 100% ice-cold methanol for 10 minutes. Cells were permeabilized and incubated in blocking buffer (2% FBS, 0.1% Triton) for 30 minutes at room temperature. *C. trachomatis* and host cell DNA was stained with NucBlue (Invitrogen, P36985). Centrosomes were detected with antibodies to Centrin to observe centrioles, Cep164 to mark mother centrioles and γ-tubulin to observe pericentriolar material. Mitotic spindles and microtubule regrowth were visualized with anti-α-tubulin antibody. Coverslips were mounted with ProLong Glass Antifade containing NucBlue (Invitrogen, P36985). Immunofluorescence microscopy images were acquired on a Zeiss Axiovert 200M microscope.

### Microtubule regrowth assay

Cells grown on coverslips in growth medium supplemented with 25mM HEPES, were first incubated on ice for 40 minutes to depolymerize microtubules and then shifted to room temperature for 4 minutes to allow microtubule regrowth. Cells were rinsed for 40 seconds with microtubule buffer (60mM PIPES, 25mM HEPES, 10mM EGTA, 2mM $MgCl_2$, 0.25nM Nocodazole, 0.25nM Paclitaxel, pH 6.9) and fixed with ice-cold methanol for 7 minutes prior to immunofluorescence microscopy analysis with antibodies to α-tubulin and γ-tubulin.

### Pharmacological inhibition of cell cycle progression and centrosome duplication

Mock or *Chlamydia*-infected RPE-1 cells were arrested in S-phase by incubating them with 2mM thymidine (ACROS Organics, 226740050) in standard growth medium for 24 hours, starting at 12 hpi. Cells were arrested in G2 by first incubating them with 2mM thymidine for 18 hours, followed by release from the thymidine block for 6 hours and incubation with 10μM RO-3306 (TOCRIS, cat # 4181) in standard medium for 12 hours. Both the S-phase and the G2 arrest experiments were analyzed at 36 hpi using immunofluorescence microscopy.

To inhibit centrosome duplication, *Chlamydia*, HPV and HPV+*Chlamydia* cells were incubated with 125nM of the Plk4 inhibitor centrinone (MedChemExpress HY-18682) in standard growth medium starting at 1 hpi. The experiment was evaluated by immunofluorescence microscopy at 36 hpi.

### EdU labeling

Cells undergoing S-phase were identified using the Click-iT EdU Cell Proliferation kit (Invitrogen, C10337). Control or HPV cells were grown on coverslips and infected with *Chlamydia* at an MOI of 3. At 36 hpi, cells were incubated with 10μM EdU for 30 minutes and fixed with 4% PFA. EdU labeled cells were detected following the manufacturer's protocol.

### Statistical analyses

For each experiment, 3 independent biological replicates were performed, and the results are presented as mean ± SD. Data were analyzed by unpaired, two-tailed t-tests with Welch's correction on Graph Pad PRISM software version 8.

## Results

### An experimental system for studying the effects of *Chlamydia* and HPV on the centrosome

We developed a cell culture model that allowed us to separate the effects of HPV and *Chlamydia trachomatis* (referred to as *Chlamydia* hereafter) on the centrosome in the same human cell (Fig. 1A). We used retinal pigment epithelial cells (RPE-1) as the host cell because these diploid epithelial cells are neither cancerous nor transformed by HPV, unlike HeLa or A2EN cells that are commonly utilized for *Chlamydia* infection (Buckner et al. 2016). To mimic the effects of HPV on the centrosome, we generated an RPE-1 cell line that stably expresses the viral oncoproteins HPV16 E6 and E7 (referred to as "HPV cells") (Fig. S1). Prior studies showed that ectopic co-expression of the HPV oncoproteins E6 and E7 was sufficient to induce centrosome amplification (Duensing et al. 2000; Duensing and Münger 2002). Ectopic E6/E7 expression is proposed to mimic the effect of HPV on the centrosome, with the advantage that this approach does not require a stratified epithelium typically used in an HPV infection model (Bienkowska-Haba et al. 2018). However, because we are not performing actual HPV infections, we cannot exclude the possibility that other HPV factors may contribute to the phenotypes measured in this study. RPE-1 cells transduced with an empty vector lacking these viral oncogenes served as a negative control ("control cells").

We infected either control cells with *C. trachomatis* to produce "*Chlamydia* cells", or HPV cells to generate "HPV+*Chlamydia* cells". For these infections, we used *C. trachomatis* serovar L2 because this strain has been used as an experimental model to study *C. trachomatis*-induced centrosome amplification (Grieshaber et al. 2006; Johnson et al. 2009). This strain is representative of other *C. trachomatis* strains, such as the genital serovars D and G, that produce comparable levels of centrosome amplification (Grieshaber et al. 2006).

We then compared the percentage of control, HPV, *Chlamydia* and HPV+*Chlamydia* cells with amplified centrosomes. We detected centrosomes by immunofluorescence microscopy with antibodies to the centrosomal marker proteins γ-tubulin and centrin2, which stain the pericentriolar material (PCM) and centrioles, respectively. Centrosome amplification was defined as cells harboring more than 2 centrosomes (n>2 γ-tubulin dots) (Fig. 1B).

### *Chlamydia* and HPV dysregulated centrosome number in an additive manner

This systematic approach revealed different effects of these two pathogens on the centrosome. Control cells had only few supernumerary centrosomes (Prevalence of 1.9%). *Chlamydia* cells showed a higher prevalence of amplified centrosomes than HPV cells (Prevalence of 32.2% vs 21.1%) (Fig. 1C), but the highest percentage of extra centrosomes (59.5%) was seen in HPV+*Chlamydia* cells. These data demonstrated that *Chlamydia* had a greater effect on the centrosome than HPV, and that together, they caused more centrosome

amplification than either infectious agent alone (Fig. 1C). Thus, HPV and *Chlamydia* cause centrosome amplification in a host cell through additive mechanisms.

We also examined effects of HPV and *Chlamydia* on the centrosome of A549 cells, which are HPV-negative lung carcinoma cells. Similar to our results with RPE-1 cells, there was a greater prevalence of centrosome amplification in *Chlamydia* cells when compared to HPV cells. However, because E6/E7 expression did not cause a statistically significant increase in the percentage of A549 cells with amplified centrosomes, we were unable to test if the effects of HPV and *Chlamydia* on centrosome amplification are additive (Fig. S2). We conclude from these experiments that unlike for HPV, the prominent effects of *Chlamydia* in dysregulating centrosome number is independent of the cell type used as the host cell.

## *Chlamydia*- and HPV-induced centrosome amplification did not disrupt the function of the centrosome in organizing microtubules

We next tested if *Chlamydia* and HPV altered the function of the centrosome, which organizes microtubules by controlling their nucleation and anchoring. HPV, *Chlamydia* and HPV+*Chlamydia* cells all formed a radial array of interphase microtubules that was indistinguishable from that of control cells (Fig. 2A). There was also no difference in the growth kinetics of microtubules as measured in regrowth assays (Fig. 2B).

We further analyzed spindle formation to assess centrosome function in mitosis (Fig. 2C). HPV and HPV+*Chlamydia* cells showed a higher prevalence of abnormal spindles, including multipolar and pseudo-bipolar spindles, than control or *Chlamydia* cells (Figs. 2C and 2D). This finding suggests that the abnormal interphase centrosomes in HPV and HPV+*Chlamydia* cells may promote the formation of abnormal mitotic spindles. In contrast, while a high percentage of *Chlamydia* cells had supernumerary centrosomes in interphase (Fig. 1C), only few of these cells actually progressed into mitosis and formed abnormal spindles. We conclude that all cells with abnormal centrosomes were able to form mitotic spindles and that cell cycle progression to reach mitosis may be disrupted in *Chlamydia* cells with supernumerary centrosomes.

## *Chlamydia* cells with amplified centrosomes are multinucleated and defective in cell cycle progression

We observed that *Chlamydia* was more likely to cause host cell multinucleation than HPV. 32.9% of *Chlamydia* cells and 32.1% of HPV+*Chlamydia* cells were multinucleated, compared to only 3.9% of HPV cells and 1.0% of control cells (Fig. 3A). In *Chlamydia* cells, multinucleation and centrosome amplification were often present in the same cell. Consistent with this observation, we determined a $\phi$ coefficient of 0.94 between the two phenotypes, which indicates a strong correlation (Fig. 3B). In contrast, HPV cells had a low $\phi$ coefficient of 0.23. HPV+*Chlamydia* cells displayed an intermediate correlation ($\phi$ coefficient = 0.49), consistent with a mixed effect (Fig. 3B).

As multinucleation can cause cells to arrest in the G1 phase of the cell cycle (Ganem et al. 2009; Hart, Adams, and Draviam 2021), we compared cell cycle progression in mono- and multinucleated *Chlamydia* and HPV+*Chlamydia* cells. Mononucleated *Chlamydia* and HPV+*Chlamydia* cells, as well as multi-nucleated HPV+*Chlamydi*a, incorporated EdU, a

marker for S-phase entry, to similar extents (Fig. S3). In contrast, the percentage of EdU-positive multinucleated *Chlamydia* cells was reduced (Fig. S3), suggesting that these cells become arrested in a pre-S-phase stage of the cell cycle, likely in G1.

## *Chlamydia*-induced centrosome amplification and multinucleation result from a cytokinesis defect

The strong correlation between centrosome amplification and multinucleation in *Chlamydia* cells is indicative of a cytokinesis defect in the host cell, a reported consequence of *Chlamydia* infection (Alzhanov et al. 2009; Sun et al. 2016). To test this hypothesis, we counted the number of Cep164-positive foci in HPV, *Chlamydia*, and HPV+*Chlamydia* cells, focusing on cells with supernumerary centrosomes (Fig. S4). In a normal mitotic cell, the two mature Cep164-positive centrioles are passed on to the two daughter cell after cytokinesis (Schmidt et al. 2012). The presence of two mature centrioles in the same cell is therefore a strong indicator of a cytokinesis defect. Greater than 60% of *Chlamydia* cells had two Cep164-positive foci, whereas HPV cells contained predominantly a single Cep164 focus. HPV+*Chlamydia* cells showed an intermediate phenotype (Fig. S4).

As a complementary approach, we prevented *Chlamydia*, HPV and HPV+*Chlamydia* cells from reaching cytokinesis by blocking cell cycle progression either in S-phase or G2 and then measuring the prevalence of amplified centrosomes and multinucleation in each cell population. We induced a cell cycle arrest in S-phase by treating cells with thymidine. We also arrested cells in G2 by first synchronizing cells in S-phase, with thymidine treatment and washout, and then incubating them with the G2 inhibitor RO-3306 (Ma and Poon 2017). Each of these cell cycle manipulations decreased the prevalence of multinucleation as well as centrosome amplification in *Chlamydia* cells, but not in HPV cells (Figs. 4A and 4B). Once again, HPV+*Chlamydia* cells showed an intermediate phenotype. Thus, multinucleation and centrosome amplification are closely linked in *Chlamydia* cells, with both phenotypes depending on progression through the cell cycle. Together, these results indicate that centrosome amplification in *Chlamydia*, but not in HPV cells, may be the consequence of a cytokinesis defect. They also provide further support that these pathogens contribute to centrosome abnormalities through different mechanisms in the same host cell.

Our experimental set-up allowed us to examine an alternative model in which centrosome amplification in *Chlamydia* cells was proposed to cause multinucleation (Brown et al. 2014). To test this order of events, we prevented centrosome duplication through the use of the Plk4 inhibitor centrinone, which has been shown to block new centriole assembly in RPE-1 cells (Wong et al. 2015). Centrinone treatment significantly reduced centrosome amplification in HPV, *Chlamydia* and HPV+*Chlamydia* cells (Fig. 5A), which is consistent with published results on Plk4 inhibition (Johnson et al. 2009; Korzeniewski, Treat, and Duensing 2011). However, centrinone treatment did not prevent multinucleation in *Chlamydia* cells (Fig. 5B), suggesting that centrosome amplification is not necessary for *Chlamydia*-induced multinucleation.

## Discussion

To investigate how *C. trachomatis* could contribute to HPV-mediated carcinogenesis, we measured the respective effects of these intracellular pathogens on the centrosome in the same host cell. We found that *Chlamydia* induced more cells to have amplified centrosomes than HPV and that these pathogens together caused an even higher percentage of cells with supernumerary centrosomes. These additive effects, together with our mechanistic analyses, suggest that *Chlamydia* and HPV induce centrosome amplification through different mechanisms. This study thus provides evidence that *C. trachomatis,* as a co-factor for HPV, may contribute to the development of cervical cancer by enhancing centrosome defects.

*Chlamydia* has been reported to cause centrosome amplification (Grieshaber et al. 2006; Johnson et al. 2009). These prior cell culture studies have predominantly used transformed cell lines that have an HPV background, such as HeLa cells (Grieshaber et al. 2006; Johnson et al. 2009; Knowlton et al. 2013). Although *Chlamydia* was found to also induce centrosome amplification in HPV-negative cell lines, such as NIH3T3 or HFF, these studies did not separate or compare the effects on the centrosome caused by either *Chlamydia*, HPV, or both pathogens together (Grieshaber et al. 2006; Johnson et al. 2009; Knowlton et al. 2013). Furthermore, comparing centrosome amplification between different *Chlamydia*-infected HPV-negative and positive cell lines can be difficult. We avoided these issues by developing a cell culture model that uses the same cellular background to measure the respective effects of *Chlamydia* and HPV on the centrosome.

HPV has been shown to induce centrosome amplification through its oncoproteins E6 and E7. E7 is proposed to be the primary driver of centrosome amplification in an HPV infection. This conclusion is based on data showing that E7 dysregulates the centrosome duplication machinery in a Cdk2-dependent manner (Duensing et al. 2006) and that transient E7 expression is sufficient to cause centrosome amplification (Duensing et al. 2001; Duensing and Münger 2002). In contrast, E6 has been proposed to play a lesser role in centrosome amplification by disabling the p53-dependent checkpoint (Duensing et al. 2001; Duensing and Münger 2002). The loss of this checkpoint could lead to a cytokinesis defect (Bunz et al. 1998; Duensing et al. 2001), in which the nucleus and the two centrosomes duplicate normally, but the cell fails to divide, resulting in a multinucleated cell with extra centrosomes (Cosenza and Krämer 2016).

This present study compared the mechanisms through which *Chlamydia* and HPV produce supernumerary centrosomes. We propose that *Chlamydia*-induced centrosome amplification is the result of a cytokinesis defect. This idea is supported by the observation that centrosome amplification in *Chlamydia* cells strongly correlated with host cell multinucleation and required progression through mitosis. Additionally, most *Chlamydia* cells with supernumerary centrosomes had two Cep164-positive foci. In contrast, centrosome amplification in HPV cells did not correlate with multinucleation and was independent of cell cycle progression. Furthermore, most HPV cells with amplified centrosomes only contained one mature Cep164-positive mother centriole.

Together, these data suggest that HPV and *Chlamydia* induce centrosome defects through different mechanisms in the same host cell. While HPV primarily dysregulate the centrosome duplication machinery through E7 (Duensing et al. 2001; Duensing and Münger 2002), *Chlamydia* appears to cause centrosome dysregulation by disrupting cytokinesis. Consistent with this model, blocking cell cycle progression in HPV+*Chlamydia* cells partially reduced the prevalence of amplified centrosomes because it eliminated the contribution of *Chlamydia*, but not HPV, to this phenotype. Overall, our results suggest that these two pathogens activate two distinct pathways to induce centrosome dysregulation, although the respective contribution of each pathway to centrosome amplification appears to be cell type specific (Fig. S2).

*Chlamydia* is proposed to block cytokinesis through multiple mechanisms, including the physical presence of the inclusion and expression of the chlamydial proteins, CT223 (IPAM) or the protease CPAF (Greene 2001, Alzanov, 2009, Sun 2011, Brown 2014). The latter two studies both described evidence for a link between multinucleation and centrosome amplification, but Brown and colleagues suggested that multinucleation is the consequence of CPAF-induced centrosome amplification in *Chlamydia*-infected cells (Brown et al., 2014). Our data, however, suggests that the cytokinesis defect is upstream of the other two phenotypes because centrinone treatment blocked centrosome amplification in *Chlamydia* cells, but did not prevent multinucleation. Currently, it is not clear if centrosome amplification and multinucleation are functionally linked or if they are two independent consequences of the cytokinesis defect.

The presence of *Chlamydia* and HPV in the same cells produced a high prevalence of abnormal spindles but did not to affect the function of the centrosome in organizing microtubules in interphase or mitosis. As supernumerary centrosomes can lead to spindle defects, we propose that *Chlamydia* and HPV together produce abnormal spindles by altering centrosome number rather than function. These abnormal spindles may lead to chromosome mis-segregation and aneuploidy (Zhou et al. 1998), thereby providing a mechanism by which *Chlamydia* may contribute to HPV-induced carcinogenesis.

Our study provides evidence for *Chlamydia*-induced cell cycle dysregulation in the host cell. We observed that fewer multinucleated *Chlamydia* cells progressed through the cell cycle than either mononucleated *Chlamydia* or multinucleated HPV+*Chlamydia* cells (Fig. S3). Thus, in addition to the known effect on cytokinesis, which leads to centrosome amplification and multinucleation, *Chlamydia* appears to disrupt progression through interphase. This *Chlamydia*-induced cell cycle arrest is likely in G1 and may be the consequence of either centrosome amplification or multinucleation. Centrosome amplification and multinucleation have been reported to lead to a G1 arrest through p53-dependent or p53-independent mechanisms, respectively (Hart et al. 2021; Mikule et al. 2007). However, as *Chlamydia* infection is known to induce p53 degradation, the presence of multiple nuclei in an infected cells is more likely to induce this G1 arrest in a p53-independent manner (González et al. 2014:2; Hart et al. 2021; Siegl et al. 2014). In HPV+*Chlamydia* cells, the presence of E6 and E7 may release the G1 arrest, possibly by degrading retinoblastoma protein (pRB) (Boyer, Wazer, and Band 1996; Giacinti and

Giordano 2006), resulting in cell cycle progression and the formation of aberrant mitotic spindles.

We hypothesize that these combined effects on the centrosome occur through co-infection, with HPV infection preceding the *Chlamydia* infection. Both sexually transmitted agents are highly prevalent, making co-infection likely. These pathogens each infect the stratified epithelia of the cervix, but they do so at different locations, with HPV infecting basal cells (Spurgeon and Lambert 2017), while *Chlamydia* infects the superficial cell layer (Murall et al. 2019). It is known, however, that HPV-infected basal cells divide, differentiate and migrate to the epithelial surface (Pinidis et al. 2016), thus providing a population of HPV-infected cells that can be infected acutely by *Chlamydia*. Our HPV/*Chlamydia* cell culture model mimics this sequence of events by taking cells expressing HPV E6 and E7 and then infecting them with *C. trachomatis*.

Our data is consistent with a 'hit-and-run' model, in which *C. trachomatis* infects a cervical cell that has an on-going HPV infection and contributes to HPV-induced carcinogenesis by augmenting centrosomal defects in these cells. *Chlamydia* typically causes a lytic infection, but we propose that some co-infected cells survive the *Chlamydia* infection and have enhanced centrosomal defects that promote their progression into cancer cells. This model is supported by data showing that cervical cancer cells do not have evidence of active *Chlamydia* infection (Wallin et al. 2002). In addition, cervical cancer has been associated with serological evidence of past, rather than current, *Chlamydia* infection (Wallin et al. 2002). There is also evidence that cells can be cleared of a *C. trachomatis* infection with antibiotics, while retaining amplified centrosome number (Grieshaber et al. 2006) or can survive through a non-lytic process called extrusion (Hybiske and Stephens 2007).

Our findings provide biologic plausibility for *C. trachomatis* as a co-factor for HPV in carcinogenesis and have implications for the management of HPV and *C. trachomatis* infections. Based on this study, HPV-infected women who have had a prior *C. trachomatis* infection may be at a higher risk for the development of cervical cancer. Our findings suggest that current screening for cervical cancer, which is based on Pap smear identification of premalignant cells and HPV test may not be adequate (Fontham et al. 2020). Enhanced screening for past *C. trachomatis* infection could be performed with an antibody blood test, but not with the standard *C. trachomatis* test, which is a nucleic acid amplification test (NAAT) that only detects an active or resolving infection (Meyer 2016). If *Chlamydia* does contribute to cervical cancer, there will also be a greater need to identify and treat active infections and to develop a vaccine.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Take Away:**

- *Chlamydia* and HPV induce centrosome amplification in an additive manner
- *Chlamydia*-induced centrosome amplification is linked to host cell multinucleation
- *Chlamydia*-induced centrosome amplification requires cell cycle progression
- *Chlamydia* and HPV cause centrosome amplification through different mechanisms
- This study supports *Chlamydia* as a co-factor for HPV in carcinogenesis

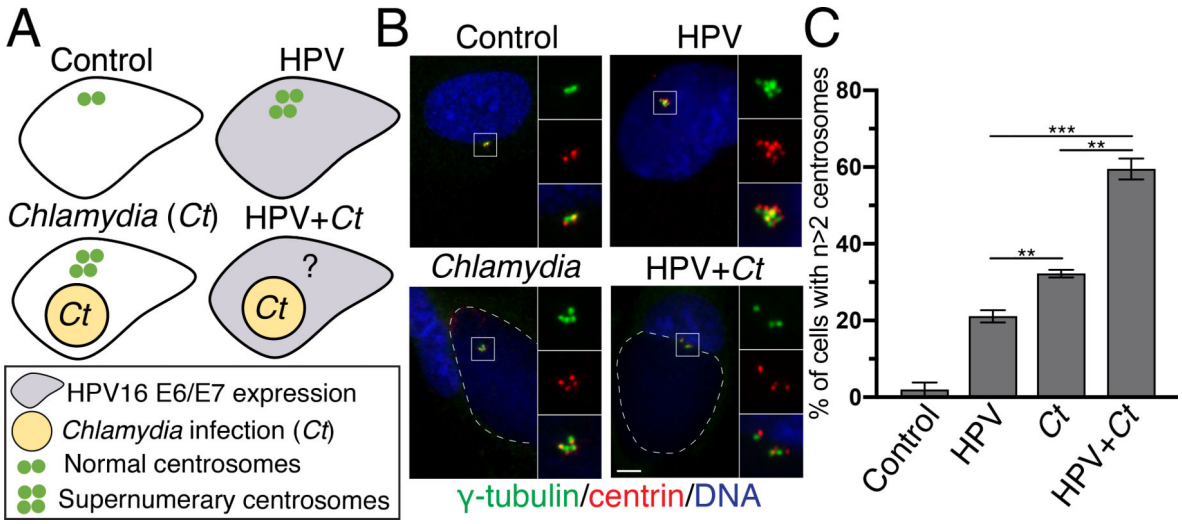


**Figure 1. *Chlamydia* and HPV have additive effects on the host cell centrosome**

(A) Schematic representation of the experimental design to study the separate and combined effect of *Chlamydia* and HPV on the centrosome. Control cells are RPE-1 cells transfected with an empty plasmid. "HPV cells" are RPE-1 cells that stably express HPV16 oncoproteins E6 and E7 (gray cell). "*Chlamydia* cells" are RPE-1 cells infected with *Chlamydia trachomatis* (yellow circle), while "HPV+*Chlamydia*" cells are RPE-1 cells that stably express HPV16 oncoproteins and that are infected with *C. trachomatis*. Individual centrosomes are presented as green dots.

(B) Control RPE-1 cells or HPV cells, grown on coverslips, were either mock-infected or infected with *C. trachomatis* L2 at an MOI of 3. Samples were fixed at 36 hours post infection (hpi). Centrosomes were visualized with antibodies to γ-tubulin (green) and centrin (red), host and chlamydial DNA was detected with DAPI (blue). Chlamydial inclusions are outlined with white dashed lines. Scale bar: 5 μm.

(C) The percentage of host cells with supernumerary centrosomes (n>2 centrosomes) from the four different conditions is shown. 100 cells were analyzed for each condition. For the samples with a *Chlamydia* infection, only infected cells were examined and quantified. Data are represented as mean ± SD (n=3); $^{**}P \leq 0.01$ and $^{***}P < 0.001$.

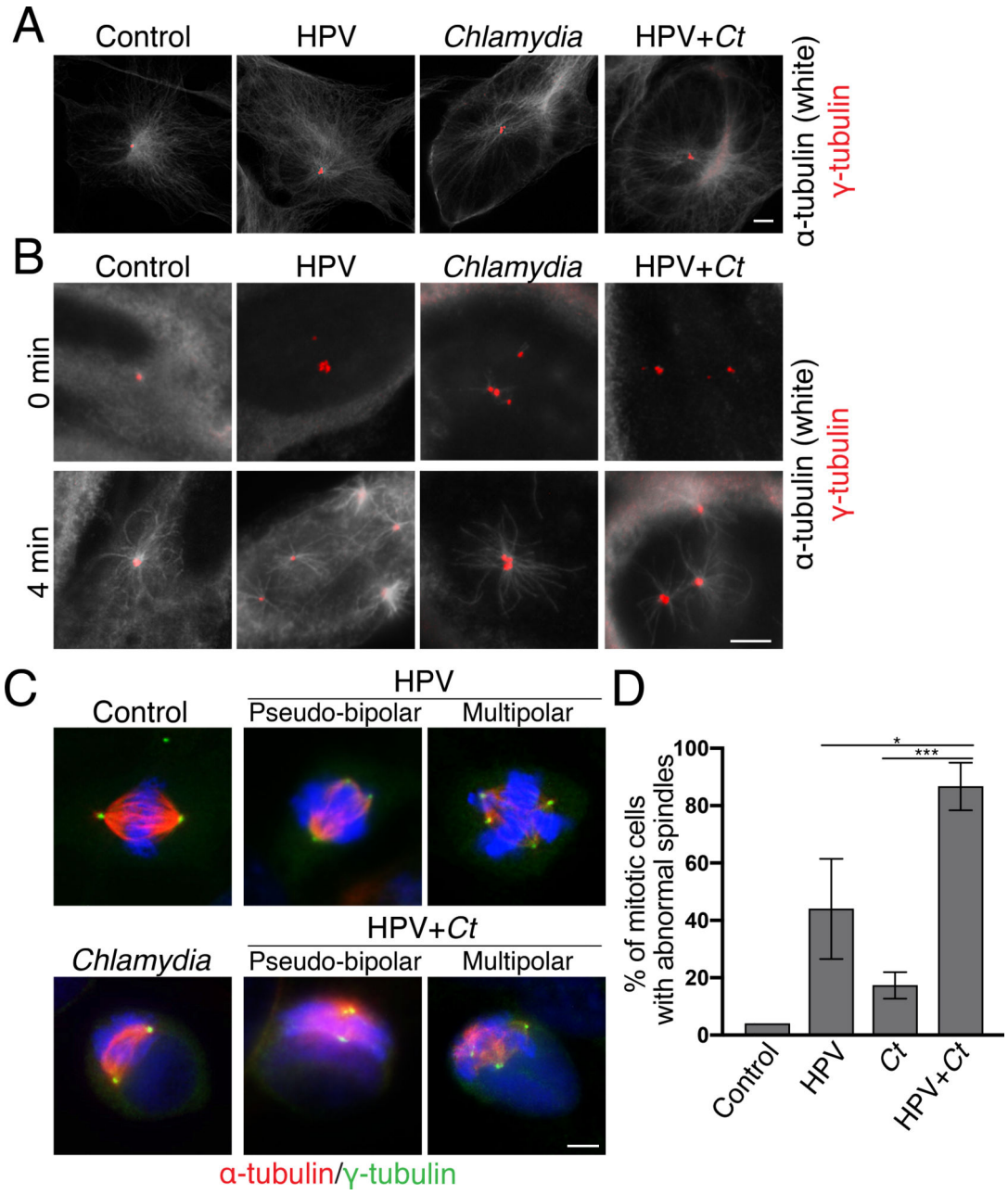


**Figure 2. Centrosome amplification does not disrupt centrosome function**

(A) Immunofluorescence images of mock or *Chlamydia*-infected RPE-1 or HPV cells fixed at 36hpi in interphase. Microtubules were visualized with α-tubulin antibodies (white) and centrosomes were detected with γ-tubulin antibodies (red). Scale bar: 5μm.

(B) Microtubule regrowth assays are shown for cells of each of the four experimental conditions. Cells were incubated on ice to depolymerize microtubules (0 min) and then shifted to 37°C for 4 minutes to allow microtubule nucleation and polymerization (4 min). Cells were fixed at the indicated time points and analyzed by immunofluorescence microscopy, detecting microtubules and centrosomes as described in (A). Scale bar: 5μm.

(C) Immunofluorescence images of mitotic cells in the samples in (A). Mitotic spindles were identified with antibodies to α-tubulin antibodies (red) and centrosomes were visualized with γ-tubulin antibodies (green). DNA was visualized with DAPI (blue). Scale bar: 5μm. (D) The percentage of mitotic cells with abnormal spindles in each of the four conditions is shown. Both pseudo-bipolar (centrosome clustering) and multipolar spindles were considered abnormal. 50 mitotic cells were analyzed for each condition. For the samples with a *Chlamydia* infection, only infected cells were examined and quantified. Data are represented as mean ± SD (n=3); $*P \leq 0.05$ and $***P < 0.001$.

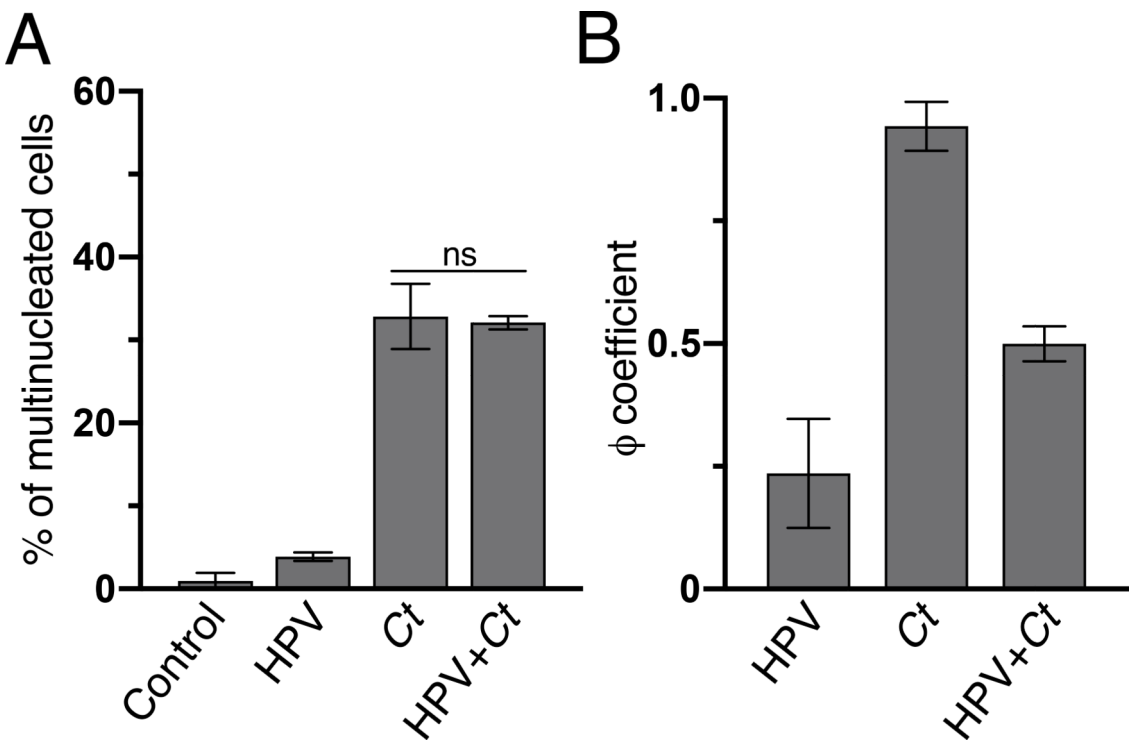


**Figure 3. Multinucleation is strongly correlated with centrosome amplification in *Chlamydia* cells but not in HPV cells**

(A) The percentage of multinucleated host cells described in Figure 1C is shown. 100 cells were examined for each condition. For *Chlamydia* cells, only infected cells were counted. Data are shown as mean ± SD (n=3); ns: not statistically significant.

(B) ϕ coefficients between multinucleation and centrosome amplification were calculated for the HPV, *Chlamydia*, and HPV+*Chlamydia* cells of Figure 1C. ϕ coefficients range from −1 to 1, with −1 or +1 indicating perfect negative or positive relationships, respectively, while 0 shows no relationship between the two phenotypes. The control sample was not included due to low level of centrosome amplification in these cells. Data are shown as mean ± SD (n=3).

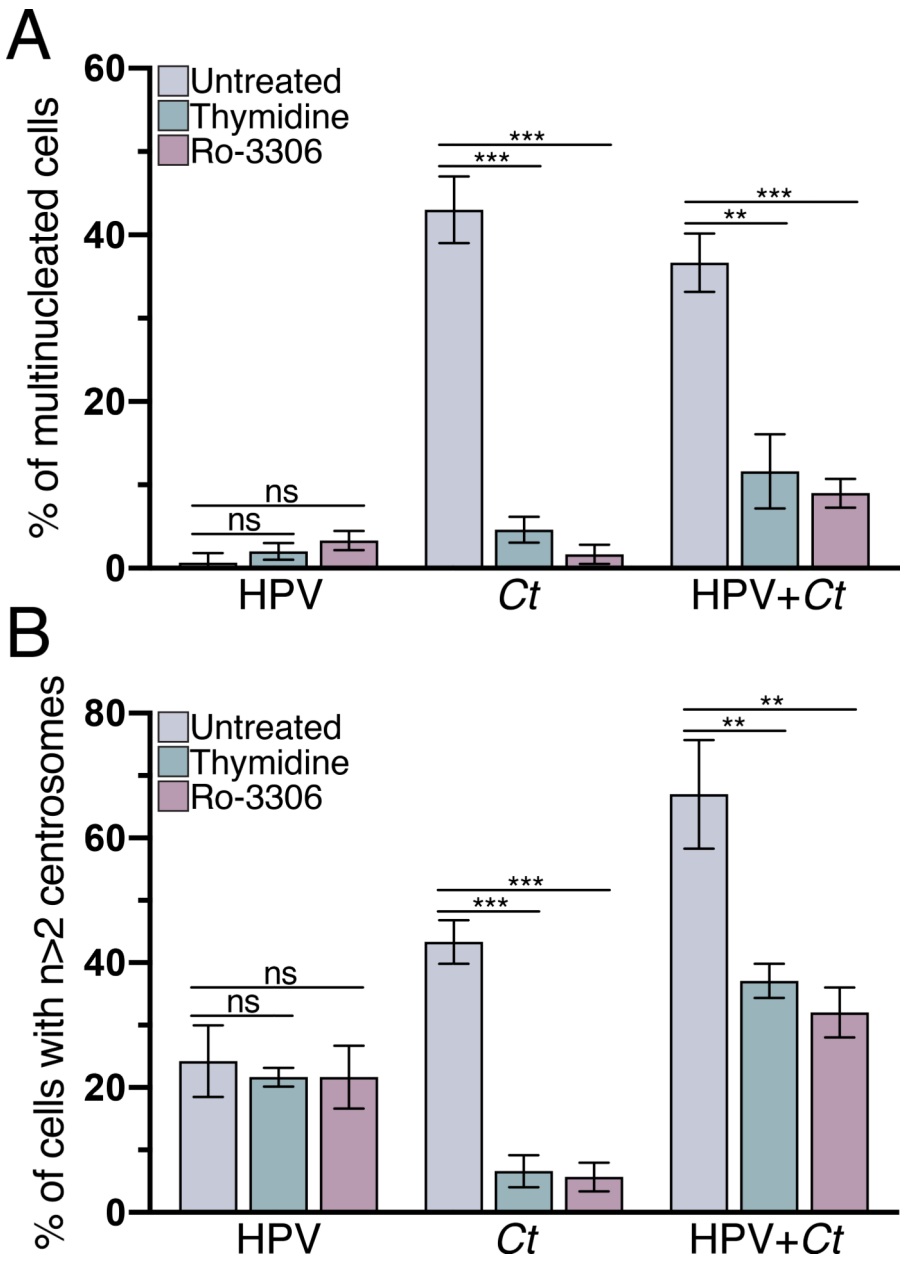


**Figure 4. Multinucleation and centrosome amplification in *Chlamydia*-infected cells require mitotic progression**

The cells of our four experimental conditions were arrested in S-phase with thymidine, or in G2 by the addition of RO-3306. Untreated samples were incubated in equivalent volume of DMSO. The percentage of cells with (A) multiple nuclei and (B) supernumerary centrosomes is shown. 100 cells were analyzed for each condition at 36hpi. Data are represented as mean ± SD (n=3); ** $P \leq 0.01$, *** $P < 0.001$, ns: not statistically significant.

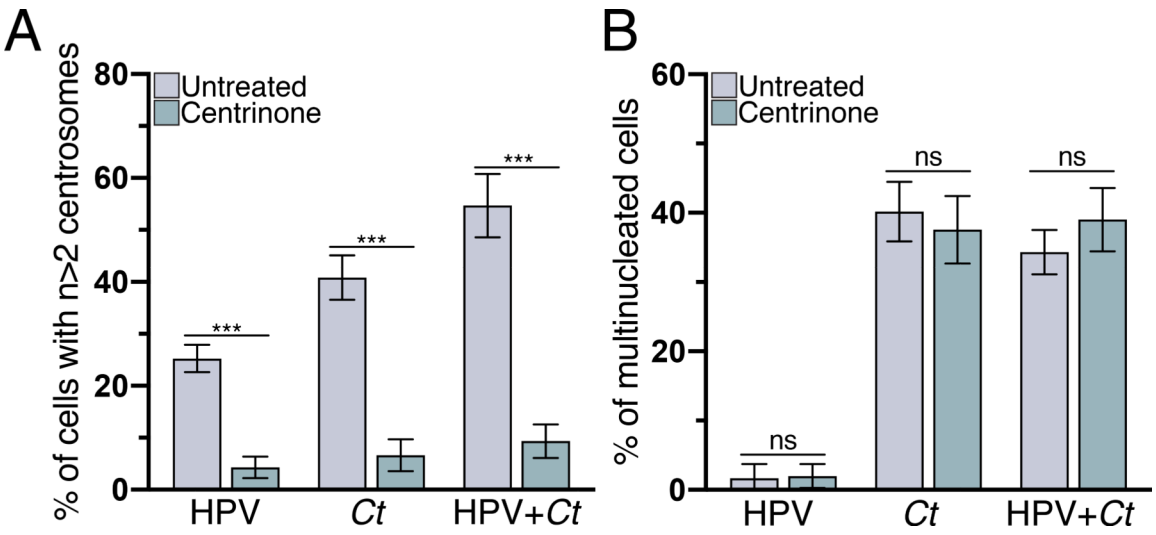


**Figure 5. Multinucleation in *Chlamydia*-infected cells does not depend on centrosome amplification**

The percentage of HPV, *Chlamydia*, and HPV+*Chlamydia* cells with (A) supernumerary centrosomes or (B) multiple nuclei after treatment with the Plk4 inhibitor centrinone is shown. Untreated samples were incubated in equivalent volume of DMSO. 100 cells were analyzed for each condition at 36hpi. Data are represented as mean ± SD (n=3); *** $P<0.001$.