**Chlamydia Infection Promotes Host DNA Damage and Proliferation but Impairs the DNA Damage Response**

Cindrilla Chumduri,1,2 Rajendra Kumar Gurumurthy,1,2 Piotr K. Zadora,1 Yang Mi,1 and Thomas F. Meyer1,*

1Department of Molecular Biology, Max Planck Institute for Infection Biology, 10117 Berlin, Germany
2These authors contributed equally to this work
*Correspondence: meyer@mpiib-berlin.mpg.de

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**SUMMARY**

The obligate intracellular bacterial pathogen *Chlamydia trachomatis* (Ct) has been associated with cervical and ovarian cancer development. However, establishment of causality and the underlying mechanisms remain outstanding. Our analysis of Ct-induced alterations to global host histone modifications revealed distinct patterns of histone marks during acute and persistent infections. In particular, pH2AX (Ser139) and H3K9me3, hallmarks of DNA double-strand breaks (DSBs) and senescence-associated heterochromatin foci (SAHF), respectively, showed sustained upregulation during Ct infection. Ct-induced reactive oxygen species were found to contribute to persistent DSBs, which in turn elicited SAHF formation in an ERK-dependent manner. Furthermore, Ct interfered with DNA damage responses (DDR) by inhibiting recruitment of the DDR proteins pATM and 53BP1 to damaged sites. Despite impaired DDR, Ct-infected cells continued to proliferate, supported by enhanced oncogenic signals involving ERK, CyclinE, and SAHF. Thus, by perturbing host chromatin, DSB repair, and cell-cycle regulation, Ct generates an environment favorable for malignant transformation.

**INTRODUCTION**

*Chlamydiae* are gram-negative, obligate intracellular bacterial pathogens responsible for a broad spectrum of diseases (Brunham and Rey-Ladino, 2005). *Chlamydia trachomatis* (Ct) is the leading cause of bacterial sexually transmitted disease. Seventy to seventy-five percent of women infected remain asymptomatic, and these bacteria can persist for months, or even years (WHO, 2001). Chronic bacterial infections have been implicated in the occurrence of cancer (Vogelmann and Amieva, 2007), the broadly accepted example being the gastric bacterium *H. pylori* (Arnheim Dahlström et al., 2011; Koskela et al., 2000) and ovarian (Shanmughapriya et al., 2012) carcinoma. However, the molecular mechanisms by which Ct might contribute to carcinogenesis have not been elucidated.

Ct exhibits a biphasic life cycle: metabolically inactive elementary bodies (EBs) infect host cells and differentiate into metabolically active, replicating reticulate bodies (RBs) within a membrane-bound vacuole called inclusion. At the end of the infectious cycle, RBs redifferentiate into EBs, which are released to initiate new infections (Brunham and Rey-Ladino, 2005). *Chlamydia* can establish asymptomatic, persistent infections by several mechanisms, including antibiotic resistance, immune evasion, and apoptosis suppression (Mpiga and Ravaoarinoro, 2006a). Ct triggers activation of oncogenic Ras-Raf-MEK-ERK pathway components (Gurumurthy et al., 2010; Vignola et al., 2010) and production of reactive oxygen species (ROS) to support its growth (Abdul-Sater et al., 2010).

Chromatin alterations, such as histone modifications, may induce somatically heritable changes of gene activity and thus have oncogenic potential (Füllgrabe et al., 2011). Histone post-translational modifications (PTMs) are typically induced by signal transduction pathways activated in response to cellular stimuli. One prominent pathway implicated in histone PTMs is the mitogen-activated protein kinase (MAPK) cascade, which leads to histone H3 serine 10 (H3S10) phosphorylation in a promoter-specific manner, targeting only a subset of genes (Clayton and Mahadevan, 2003). Bacterial pathogens such as Mycobacteria, *Shigella*, *Listeria*, and *Helicobacter* are known to modify the chromatin architecture of host cells, thus manipulating host transcriptomes, e.g., to suppress immune responses (Hamon and Cossart, 2008). Furthermore, C. *pneumoniae* induces modifications of histones H3 and H4, which play an important role in cytokine production (Schmeck et al., 2008). More recently, the chlamydial nuclear effector protein (Nue) was shown to have histone methyltransferase activity that targets histones H2B, H3, and H4 (Pennini et al., 2010). Collectively, these data establish that bacterial pathogens induce multiple types of histone PTMs, although the mechanisms and extent of this phenomenon requires elucidation.

Recently, the role of chromatin and histone modifications in promoting DNA damage responses (DDRs) and genome stability have gained prominence (Miller and Jackson, 2012). Upon detection of DNA double-strand breaks (DSBs), cells activate DDR pathways that detect DNA lesions and signal their presence by mediating responses such as cell-cycle arrest, DNA repair, and, under some circumstances, apoptosis. Phosphorylation of H2AX Ser139 (γH2AX) is a prominent chromatin modification...
in response to DSBs that acts as a signal for recruitment of repair proteins including pATM and 53BP1 to DNA break sites. Deficiencies in DNA damage signaling and repair pathways lead to genetic instability, which in turn might enhance oncogenesis (O’Driscol and Jeggo, 2006).

Here, we provide a comprehensive analysis of global changes to host chromatin induced upon infection with a bacterial pathogen. We show that Chlamydia infection alters histone PTMs, leading to four distinct patterns of histone marks, which vary between acute and persistent infections. Among others, γH2AX and H3K9me3, hallmarks of DSBs and senescence associated heterochromatin foci (SAHF), respectively, showed sustained upregulation during Ctr infection. Reactive oxygen species (ROS) induced by Ctr were found to contribute to persistent DSBs. SAHF formation was selectively induced in an ERK-dependent manner in response to Ctr-induced DSBs, in contrast to DNA damage induced by etoposide. Strikingly, Ctr infection suppressed DNA damage repair activities despite the presence of extensive DSBs in host cells. Infected cells containing DSBs continued to proliferate, facilitated by SAHF formation. Thus, we report that Ctr infection causes DSB generation, which could predispose host cells to genomic instability and transformation via the unusual combination of impaired repair and prosurvival signaling.

RESULTS

Ctr Infection Induces Alterations to Host Histone Posttranslational Modifications

Intracellular pathogens create conducive environments to promote their own survival by manipulating various cellular processes including epigenetic alterations (Hamon and Cossart, 2008). We hypothesized that epigenetic modifications induced by infection is a primary mechanism that could contribute to heritable changes in host cells. Therefore, we investigated the ability of Ctr to alter the host histone PTMs. We assessed global changes to host cell histone PTMs in End1 E6/E7 cells persistently infected with Ctr for 6 days using PTM-specific antibodies. Our analysis focused on methylation, acetylation, and phosphorylation marks of the core histones H2A, H2B, H3, and H4. Persistent Ctr infection was established by treatment of infected cells with doxycycline from 24 h postinfection (hpi) up to 6 days postinfection (dpi) (Figure S1A available online) (Mpig and Ravaoarinoro, 2006b). Persistent infection was monitored by immunofluorescence with an antibody specific to Ctr major outer-membrane protein (MOMP) (Figures S1B and S1C) and immunoblot analysis with a Ctr heat shock protein 60 (Hsp60)-specific antibody (Figure S1D).

Our data reveal that Ctr infection induces extensive alterations to histone PTMs, the pattern of which varies between acute (up to 2 dpi) and persistent infection (from 2 dpi in the presence of doxycycline). All histone modifications analyzed by immunoblotting were manually assigned to four distinct groups, based upon the fold change of histone marks compared to uninfected cells and are depicted in a heatmap (Figures 1A and 1D and Table S1). Group I includes histone PTMs that were consistently elevated during acute and persistent infection. Group II includes modifications that were consistently downregulated in both phases of infection. Group III includes modifications that were upregulated in the acute and downregulated in the persistent phase. Group IV includes modifications that were downregulated in the acute and upregulated in the persistent phase. While total H3 remained unaltered at all times, Ctr infection induced global histone hypoacetylation and hypermethylation, indicative of changes to higher order chromatin structure.

We then investigated the DNA damage marker γH2AX and the SAHF marker H3K9me3, which showed sustained upregulation upon infection. These markers are connected with cellular senescence and cancer (Cann and Dellaire, 2011; Di Micco et al., 2011). To assess whether the observed modifications were cell-type specific, we analyzed γH2AX and H3K9me3 levels in primary human fallopian-tube-derived epithelial cells and IMR90 fibroblasts, the latter being a commonly used system for DDR studies. Ctr infection for 48 hr increased γH2AX and H3K9me3 levels in both cell types as determined by immunofluorescence and immunoblot analysis, respectively (Figures 1B and 1C). To investigate the chlamydial strain dependency of the histone modifications, we subjected End1 E6/E7 cells infected with Ctr L2 and Ctr D for 48 hr and C. pneumonia (Cpn) for 96 hr to immunoblot analysis. γH2AX and H3K9me3 protein levels were increased in Ctr-L2- and Ctr-D- but not in Cpn-infected cells, indicating species-specific differences in the regulation of these histone PTMs (Figure 1D).

Ctr-Induced ROS Contribute to DNA Double-Strand Breaks

To expand on these findings, we dissected the underlying mechanisms that result in infection-induced perturbations of γH2AX and H3K9me3 levels and their functional consequences. γH2AX levels increased predominantly by 48 hpi and remained elevated during persistent Ctr infection (Figure 2A). Similar to doxycycline treatment, inhibition of Ctr growth by blocking of protein synthesis at 25 hpi with chloramphenicol did not repress γH2AX formation (Figure S2A). Since γH2AX is a hallmark of DNA damage, DSB induction during persistent Ctr infection was assessed by neutral comet assay. Infected cells showed increased levels of DSBs that were sustained during persistent Ctr infection, while levels in uninfected cells did not change (Figures 2B and 2B). To exclude the possibility that fragmented DNA is of Ctr origin, we fractionated cells into nuclear and cytoplasmic fractions. Half of the nuclear fraction was subjected to neutral comet assay (Figure S2C), and the remaining nuclear and the cytoplasmic fractions were analyzed for Ctr Hsp60 by immunoblotting (Figure S2D). Nuclear fractions from infected cells demonstrated enhanced DSBs despite the absence of detectable Ctr Hsp60, confirming that the DNA detected in the comet tails was not of Ctr origin. Chlamydiae are known to inhibit host cell apoptosis (Sharma and Rudel, 2009). However, to ensure that Ctr-induced DSBs were not due to apoptosis-associated DNA fragmentation, we measured processing of caspase-3 to its active p17 and p13 subunits. Caspase-3 was not cleaved in Ctr-infected cells, whereas cells treated for 24 hr with etoposide, a known proapoptotic stimulus, showed clear processing of caspase-3 (Figure 2C). Thus, production of DSBs upon Ctr infection occurs in the absence of apoptosis induction.

A recent study showed that Ctr induces ROS production, which is essential for optimal growth of this pathogen (Abdul-Satter et al., 2010). Here, we demonstrate that Ctr infection induces
increased ROS levels at 36 hpi using carboxy-H2DCFDA staining followed by microscopic analysis (Figure S2E). For evaluation of whether ROS mediated the Ctr-induced DSBs, ROS production was inhibited through the use of either diphenyleneiodonium (DPI), a small-molecule inhibitor of NOX, or small interfering RNA (siRNA) against DUOX-1 and DUOX-2 (Figure S2F), and cells were subjected to γH2AX analysis. Inhibition of ROS production reduced γH2AX levels in infected cells as observed by immunofluorescence (Figures 2D and S2G) and immunoblots of total cell extracts (Figures 2E and S2H). Inhibition of ROS production with DPI for the remaining 24 or 9 hr of infection in cells preinfected with Ctr also reduced DSB levels as assessed by neutral comet assay (Figure 2F). Moreover, infected cells showed increased levels of 8-oxo-dG, indicating that ROS...
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mediated DSBs (Figure 2G). Furthermore, consistent with published data, inhibition of ROS production decreased Ctr infection significantly and slightly reduced primary inclusion size but had no effect on the number of inclusions in primary infection (Figures 2H and S2I–S2J). Thus, Ctr induces ROS production to support its own growth and, as a result, induces DSB and γH2AX formation.

**DSB Repair Activities Are Suppressed in Ctr-Infected Cells**

The key transducer and mediator of DSB signals are nuclear protein kinase ataxia telangiectasia mutated (ATM) and tumor protein 53 binding protein 1 (53BP1), respectively. To investigate whether Ctr-induced DSBs activate ATM signaling, we infected cells for 36 hr with or without etoposide (2 hr) and subjected them to immunofluorescence analysis of phospho-ATM Ser1981 (pATM), γH2AX, and DNA. Ctr-infected cells lacked pATM foci at DSB sites (marked by γH2AX; Figure 3A), in contrast to spontaneous breaks in uninfected or etoposide-treated cells, which colocalized with pATM foci, as demonstrated by line traces (Figure S3A). Moreover, compared to Ctr-infected or etoposide-only controls, etoposide-induced ATM phosphorylation was suppressed in infected cells despite enhanced DSBs (Figures 3A, 3B, 3E, and S3B–S3D). We next investigated the regulation of 53BP1 and pMRE11, which plays a role of other phosphatidylinositol-3-kinase-related kinases in γH2AX formation was assessed. RNA interference analysis revealed that DNA-PKc but not ATM or ATR is involved in γH2AX formation in Ctr infections (Figures 3F and S3F). To assess the implication of impaired DDR signaling on joining double-strand DNA ends by the NHEJ pathway, we transfected Ctr-infected cells with restriction-enzyme-digested, linearized plasmids containing either blunt-blunt or incompatible 3′-3′ overhang ends. DNA sequencing analysis of recircularized plasmids recovered 48 hpi revealed that the junctions in plasmids from Ctr-infected cells have relatively more nucleotide loss and misincorporation of nucleotides than uninfected cells, irrespective of the nature of the DNA ends (Figures 3H–3J).

Besides, infected cells failed to display radiosensitivity (Figures 3G, 3S, and S3H), which is in line with Ctr’s ability to inhibit apoptosis (Sharma and Rudel, 2009). Thus, Ctr infection results in considerable levels of DSBs, as shown by the accumulation of γH2AX, but inhibits subsequent downstream repair and cell-cycle checkpoint activation that typically follow DSB induction.

**Ctr-Induced DSBs Selectively Promote the Formation of SAHF and γH2AX in an ERK-Dependent Manner**

The recruitment of specific DSB repair factors and the regulation of repair output are likely to be influenced by chromatin modifications (Shi and Oberdoerffer, 2012). To investigate the role of Ctr-induced histone PTMs γH2AX and H3K9me3 in the context of DSBs, we stained Ctr-infected cells for the SAHF components H3K9me3 and heterochromatin protein 1 beta (HP1β) and γH2AX. Infected cells showed enhanced signal intensities for H3K9me3, HP1β, and γH2AX (Figure 4A). Analysis of colocalization coefficients as indicated in the bottom right of the images and line traces drawn through cells revealed clear colocalization of H3K9me3 foci with DNA dense regions (Figure S4A). Interestingly, although H3K9me3 foci were observed proximally to γH2AX foci, they did not colocalize (Figure S4B). Increased HP1β staining in Ctr-infected cells was distributed between H3K9me3 and γH2AX but predominantly colocalized with γH2AX foci (Figure 4A). H3K9me3 protein levels remained upregulated during persistent infection (up to 6 dpi) as determined by immunoblotting of whole-cell lysates (Figures 1A and 4B). To investigate the interplay between DSBs and SAHF during Ctr infection, we determined the intensities of γH2AX (indicative of DSBs) and H3K9me3 (indicative of SAHF) at single-cell level after treatment of infected cells with DPI. Interestingly, ROS inhibition reduced both γH2AX and H3K9me3 intensities (Figures 4C–4E). This suggests that SAHF are formed in response to ROS in Ctr-infected cells.

**Figure 2. ROS-Mediated Induction of DSBs upon Ctr Infection Does Not Lead to Apoptotic Cell Death**

End1 E6/E7 cells were transfected with Ctr DNA. (A) Cells were harvested at indicated time points and total cell lysates subjected to immunoblot analysis for γH2AX, chlamydial Hsp60, and β-actin. (B) Neutral comet assays were performed at the indicated time points. Quantity of DSBs is represented as percentage of DNA in tail. Cells treated with etoposide (50 μM) for the indicated time points were used as a positive control. (C) End1 E6/E7 cells uninfected or infected with Ctr (48 hr) were analyzed by immunoblotting for procaspase-3, chlamydial Hsp60, and β-actin. Molecular masses of active caspase-3 proteins are indicated. Cells treated with etoposide for the indicated time points were used as a positive control. (D) Uninfected and Ctr-infected cells with or without DPI (10 nM) added 9 hr before fixation were subjected to immunofluorescence analysis 36 hpi for γH2AX and DNA (Draq5). (E and F) Uninfected and Ctr-infected cells with or without DPI treatment were harvested 48 hpi and analyzed by immunoblotting for γH2AX, chlamydial Hsp60, and β-actin (DPI was added 9 hr before harvesting) or (F) subjected to neutral comet assay 48 hpi. Quantity of DSBs is represented as percentage of DNA in the tails. DPI was added to cells 9 or 24 hr before comet assays. (G) Uninfected and Ctr-infected cells were subjected to immunofluorescence analysis 36 hpi for γH2AX, 8-oxo-dG, and DNA (Draq5). Cells treated with tert-Butyl hydroperoxide (TBHP) were used as a positive control. (H) Ctr-infected cells with or without DPI were lysed at 48 hpi, and lysate was used to infect freshly seeded cells, which were fixed 24 hpi for quantification of infectivity. DPI was added 9 or 24 hr before lysis. Blots in (A), (C), and (E), images in (D), and data in (F) are representative of three independent experiments. Data in (B) and (F) are represented as the mean ± SEM. Data in (H) show the mean ± SD of three experiments normalized to mock-treated, infected cells. A Student’s t test was used to determine p value; **p < 0.0001 and ***p < 0.001. n is the minimum number of cells analyzed per condition. See also Figure S2.
Ct-infected cells activate ERK signaling (Gurumurthy et al., 2010), and ERK has an emerging role in the DNA damage response (Wu et al., 2006). Therefore, we investigated the role of ERK signaling in Ct-infected DSBs, enhanced γH2AX, and H3K9me3 modifications. Ct-induced ERK phosphorylation was reduced in infected cells upon inhibition of ROS formation by DPI treatment (Figure 4E). Furthermore, use of U0126, a specific inhibitor of MEK, or siRNA-mediated depletion of MEK, which is an upstream activator of ERK, γH2AX and H3K9me3 upregulation was shown to be mediated by ERK activation in Ct-infected cells (Figures 4F, S4D, S4F, and S4G). Additionally, overexpression of dominant negative MEK led to reduction in H3K9me3 levels (Figure S4H). By contrast, etoposide did not activate ERK or upregulate H3K9me3, indicating that SAHF are preferentially formed in infected cells by an ERK-dependent mechanism. Ct-infected cells treated with U0126 were also subjected to neutral comet assay. There were no significant differences in the percentage of DSBs in DNA tails between U0126-treated and untreated Ct-infected cells (Figure 4G). Consistent with previous observations (Gurumurthy et al., 2010), the activation of ERK by Ct supports its developmental cycle, demonstrated by a significant reduction in the percentage of infectious progeny when ERK activation was inhibited (Figures 4H and S4E). ERK inhibition did not alter primary infection, but led to a slight reduction in inclusion size (Figures S4C and S4E). These findings suggest that Ct-induced ROS lead to DSBs and ERK activation, which upregulates γH2AX and H3K9me3.

Perturbation of SAHF Augments Ct-Induced DNA Damage

It has been suggested that heterochromatin restrains DDR signaling (Cann and Delaire, 2011; Murga et al., 2007). We reasoned that global heterochromatin formation induced by Ct infection might modulate DDR signaling. Thus, cells were depleted of Suv39h histone methyltransferase, the key enzyme involved in H3K9 methylation, either by treatment of infected cells with a small-molecule inhibitor (SUVi) or use of siRNA against SUV39H1 and SUV39H2 to perturb heterochromatin formation (Di Micco et al., 2011), and H3K9me3 and γH2AX formation was analyzed. Quantification of integrated intensities from single cells or western blotting with whole-cell lysate showed that SUVi or double knockdown of SUV39H1 and SUV39H2 reduced H3K9me3 intensities in infected cells (Figures 5A, 5F, 5S, and 5D). Inhibition of H3K9me3 formation enhanced γH2AX levels in infected cells (Figures 5B, 5F, 5S, and 5D) but did not increase pATM levels in infected cells even when additionally treated with etoposide (Figures 5C, 5F, and 5D).

To assess whether SUVi-mediated perturbation of heterochromatin altered the distribution of γH2AX foci, we analyzed H3K9me3, HP1β, and γH2AX by immunofluorescence. Line trace and colocalization analysis revealed that in Ct-infected cells HP1β colocalized with γH2AX to a much greater extent than with H3K9me3. In the presence of SUVi, however, colocalization of H3K9me3 and γH2AX was enhanced (Figure 5A), indicating spreading of γH2AX due to overall loss of heterochromatin structures. Furthermore, there was a significant increase in DSB formation upon perturbation of heterochromatin in Ct-infected cells, as shown by neutral comet assay (Figure 5D). Thus, Ct appears to induce heterochromatin formation to prevent excessive DNA damage by limiting DSBs.

As the functional consequences of increased H3K9me3 levels for Ct are unknown, we examined the effects of perturbed heterochromatin on Ct growth and proliferation. Perturbation of heterochromatin significantly decreased Ct infectivity (Figures 5E and S5E) with no effect on primary infection but with slight reduction in inclusion size (Figures S5E–S5F). This suggests that heterochromatin perturbation leads to aberrant Ct development.

Ct Induces Persistent Proliferative Signals and SAHF to Overwhelm Impaired DDR

Inactivation of DNA repair activities, suppressed checkpoint activation, and inhibition of apoptotic cell death in the context of profound DSB induction in Ct-infected cells was unanticipated. We hypothesized that activated ERK signaling and SAHF might enforce cell proliferation during Ct infection, despite deregulated DSB responses, which typically induce cellular senescence/apoptosis. Thus, to investigate proliferative capacities, we determined cell numbers and bromodeoxyuridine (BrdU) uptake over 6 days in persistently infected End1 E6/E7 cells treated with U0126 or SUVi. Uninfected and etoposide-treated cells were used as positive and negative controls.
Figure 4. Ctr-Induced DSBs Selectively Activate SAHF Formation and γH2AX in an ERK-Dependent Manner

(A) Uninfected and Ctr-infected End1 E6/E7 cells were subjected to immunofluorescence analysis 36 hpi. Representative confocal images for H3K9me3, HP1β, and γH2AX are shown.

(B) End1 E6/E7 cells persistently infected with Ctr were harvested at indicated time points and subjected to immunoblot analysis for H3K9me3, chlamydial Hsp60, and β-actin.

(C and D) Uninfected and Ctr-infected End1 E6/E7 cells were subjected to immunofluorescence analysis with γH2AX and H3K9me3 antibodies 36 hpi with or without DPI for the final 9 hr. Mean pixel intensities of (C) γH2AX and (D) H3K9me3 per cell were quantified from confocal images with ImageJ. Data shown are the means ± SEM.

(E) Uninfected and Ctr-infected cells with or without DPI treatment were harvested 48 hpi and analyzed by immunoblotting for H3K9me3, pERK1/2, ERK2, chlamydial Hsp60, and β-actin. DPI was added to the cells 9 hr before harvesting.

(legend continued on next page)
respectively, for cell proliferation. Over 6 days, numbers of cells increased at similar rates and to similar levels in infected and uninfected cell populations (Figure 6A). By contrast, U0126 and SUVi treatment decreased cell proliferation, with Ctr-infected cells being relatively more susceptible than uninfected cells. Etoposide treatment induced cell death as early as 24 hr, as demonstrated by decreased cell numbers. Similar observations were made in IMR90 cells, excluding a role of E6/E7, as these cells continue to proliferate despite senescence-associated β-galactosidase activity, indicating that Ctr infection bypasses the senescence barrier (Figures S6A and S6D). No significant difference in BrdU incorporation was observed between uninfected and Ctr-infected End1 E6/E7 cells, indicating similar replication efficiencies (Figure 6B). Furthermore, continuous proliferation of Ctr-infected cells is demonstrated by live-cell imaging of H2B-GFP-expressing HeLa cells (Movie S1). Together, our data demonstrate the striking phenomenon that Ctr-infected cells retain their proliferative capacity despite increased levels of DSBs.

Cell-cycle deregulation and unrestricted cell proliferation, often driven by upregulated ERK signaling, is a common feature of many cancers (Deshpande et al., 2005; Roberts and Der, 2007). Analysis of ERK activation kinetics revealed prolonged upregulation of pERK levels up to 5 dpi (Figure 6C). We examined the expression of cell-cycle regulatory proteins that might support the proliferation of Ctr-infected cells. Interestingly, infected cells exhibited consistently higher levels of the proto-oncogenic CyclinE protein compared to uninfected cells for the duration of the infection. CyclinB1 was also upregulated during acute infection but was reduced almost to basal levels during later infection time points (Figures S6B and S6C). By contrast, CyclinD levels decreased below basal levels during acute infection but increased by 5 dpi. CyclinA levels remained essentially unchanged at all time points except for a slight upregulation at 6 dpi. We also investigated the possible interplay between ERK signaling and regulation of cyclins during Ctr infection. Interestingly, ERK inhibition did not influence Ctr-induced CyclinE upregulation. However, CyclinB, CyclinD, and CyclinA protein levels were reduced upon ERK inhibition compared to untreated infected cells (Figure 6D). Taken together, the data clearly suggest that Ctr-infected cells continue to proliferate despite extensive DSBs, facilitated by SAHF formation and sustained upregulation of ERK and oncogenic CyclinE.

**DISCUSSION**

Here, we present a comprehensive analysis of histone PTMs induced upon Ctr infection of endocervical epithelial cells. We show that Ctr infection profoundly alters global histone PTMs of host cells. In particular, infected cells displayed global hypoacetylation and hypermethylation of lysine residues on core histones, suggesting an overall decrease in chromatin accessibility and increased higher-order chromatin structure. Host chromatin perturbations occurred in the context of infection-induced ROS-mediated DNA damage and inhibited DDR. Moreover, infected cells failed to activate cell-cycle checkpoints and continued to proliferate. Thus, these data provide evidence of a cellular mechanism that supports the epidemiological observations associating Ctr infection with cancers of the female reproductive system.

Histone modifications are increasingly implicated in DDR and regulation of genome stability, in addition to their accepted roles in transcriptional regulation (Miller and Jackson, 2012). Currently, there are a limited number of examples that describe the ability of bacterial pathogens to perturb the host epigenome (Lebretton et al., 2011). Notably, the prototypical carcinogenic bacterium *Helicobacter pylori* has been shown to alter histone H3 phosphorylation in a type-IV-secretion-system-dependent mechanism (Fehri et al., 2009). More recently, the chlamydial effector protein Nue was shown to have histone methyltransferase activity, which could directly modify mammalian histones (Pennini et al., 2010). We show here that Ctr infection can lead to extensive alterations of global host histone PTMs. Specifically, Ctr infection consistently increased levels of phosphorylated H2AX. γH2AX is a prominent chromatin modification that is up-regulated in response to DSB induction and is important as a signal for the recruitment of repair proteins to DSB sites. Another histone modification that is a constituent of the DNA damage histone code is phosphorylation of H2B at Ser14 (Fernandez-Capetillo et al., 2004), which was also found to be modulated in Ctr-infected cells. Phosphorylation of H4 at Ser1 (pH4Ser1) has been implicated in restoration of chromatin structure by preventing reacetylation and, thereby, shutting down DNA damage signaling after DNA repair (Utley et al., 2005). Ctr infection induced elevated levels of the phosphorylated form of H4Ser1 during acute infection, which might reflect its role in DNA repair activities. We also observed decreased levels of H3K9Ac and H3K56Ac in infected cells, which has previously been shown to be reduced in response to DNA damage in human cells (Tjeertes et al., 2009).

We investigated in more depth the origin and functional significance of infection-induced perturbations to two histone PTMs, γH2AX, the DSB marker, and H3K9me3, a marker for SAHF. This study describes DSB induction during Ctr infection, which was dependent on Ctr-induced ROS production. It has been reported that Ctr induces NOX- and NLRX1-mediated ROS production (Abdul-Sater et al., 2010). Ctr induces activation of the Ras GTPase superfamily members H-RAS, N-RAS, and K-RAS (Vignola et al., 2010) upon infection, and RAS GTPases are known to induce and regulate ROS production in cancers and contribute to cancer cell proliferation (Ferro et al., 2012).
Increased levels of intracellular ROS lead to activation of ERK, JNK, or p38 MAPK (Martindale and Holbrook, 2002). We demonstrated here that ERK activation is partially dependent on ROS and is essential for γH2AX upregulation in Ctr-infected cells. Chlamydia uses ROS for its own benefit by activating caspase-1, which contributes to chlamydial growth (Abdul-Sater et al., 2010).

Ctr-induced DSBs were observed throughout the course of infection. To date, few studies have described the induction of DSBs in host genomes by bacterial pathogens, e.g., Helicobacter pylori, which induces DSBs by both ROS-dependent and -independent mechanisms (Katsurahara et al., 2009; Toller et al., 2011). Similarly, colibactin from pathogenic gastrointestinal E. coli has been implicated in DNA damage induction and checkpoint activation (Nougayrède et al., 2006). Damaged DNA is repaired through the coordinated activation of cell-cycle checkpoints and DNA repair machineries, which involve protein sensors, transducers, and effectors (Ciccia and Elledge, 2010). Surprisingly, we observed that Ctr-induced DSBs fail to recruit the crucial DNA damage sensor pMRE11, transducer pATM, and mediator 53BP1 to the site of breaks. Moreover, this was followed by failed

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**Figure 5. Perturbation of SAHF Augments Ctr-Induced DNA Damage and γH2AX Foci, but Not pATM Responses**

(A and B) Uninfected and Ctr-infected End1 E6/E7 cells treated with or without SUVi were subjected to immunofluorescence analysis. Images were acquired with an automated microscope (Olympus Soft Imaging Solutions). Integrated intensities of (A) H3K9me3 and (B) γH2AX per cell were quantified with ScanR analysis software (Olympus). Black bars indicate the means ± SEM.

(C) End1 E6/E7 cells uninfected or Ctr-infected (36 hr) with or without SUVi (5 nM) or treated with etoposide (2 hr) were subjected to immunofluorescence analysis. Representative confocal images of three independent experiments for γH2AX, pATM, and DNA (Drag5) are shown with overlays.

(D) The quantity of DSBs was determined in uninfected and Ctr-infected cells with or without SUVi at 48 hpi via neutral comet assays. Data are represented as percentage of DNA in tail. Cells treated with etoposide (2 hr) were used as a positive control. n is the minimum number of cells analyzed per condition. Data are represented as the mean ± SEM.

(E) Ctr-infected cells treated with or without SUVi were lysed 48 hpi, and these samples were used to infect freshy seeded cells. Cells were fixed 24 hpi and infectivity quantified. Data shown are the mean ± SD of three experiments normalized to mock-treated infected cells.

(F) Uninfected and Ctr-infected END1 E6/E7 cells transfected with siRNAs targeting luciferase or SUV39h1 and SUV39h2 with or without etoposide treatment were harvested 48 hpi and analyzed by immunoblotting for γH2AX, H3K9me3, pATM, ATM, chlamydial Hsp60, and β-actin. Representative blots of three independent experiments are shown.

A Student’s t test was used to determine p values; ****p < 0.0001, ***p < 0.001, and **p < 0.01. See also Figure S5.
pChk1- and pChk2-mediated DNA damage checkpoint activation in infected cells. Unlike Ctr-induced DSBs, but consistent with earlier observations (Tanaka et al., 2007), etoposide-induced DSBs elicit recruitment of pATM and 53BP1 to DSBs and activation of Chk1 and Chk2. Moreover, Ctr infection also inhibited etoposide-induced recruitment of pATM 53BP1 and pMRE11. Further, infected cells exhibit defective NHEJ process, indicating impaired DSB repair. These data clearly suggest that suppression of DSB repair activities and lack of checkpoint activation are characteristic features of Ctr-infected cells. Comparably, in oncogene-expressing cells, DDR inactivation abolishes oncogene-induced senescence and promotes cellular transformation (Di Micco et al., 2011).

It has been reported that heterochromatin formation limits DDR signaling (Di Micco et al., 2011) and that SAHF are formed preferentially upon oncogene activation. We now show that in response to DSBs, Ctr infection induces SAHF formation, unlike etoposide treatment. Establishment of SAHF in infected cells was mediated by ERK signaling, and SAHF were required for the proliferative phenotype of infected cells. In agreement with previous evidence that heterochromatin might be more resistant to DNA damage (Falk et al., 2010; Murga et al., 2007), heterochromatin formation in Ctr-infected cells restrained the amount of DSB induction and impaired local signal amplification of the damage sensor $\gamma$H2AX. SAHF induction was shown to be crucial for preserving viability in oncogene-expressing cells (Di Micco et al., 2011). However, during Ctr infection, inhibition of SAHF formation did not facilitate pATM recruitment to DSBs, suggesting that Ctr-induced SAHF are not responsible for deregulated ATM signaling.

Consistent with inhibited DNA damage checkpoint activation and apoptosis, Ctr-infected cells with apparently high levels of DSBs continue to proliferate at levels similar to uninfected control cells. It is noteworthy that failed checkpoint activation and...
insufficient time to repair DSBs could induce error-prone chromosomal end joining and genomic instability in infected cells. In support of this, chlamydial infection causes cytokinesis defects, leading to multinucleated host cells (Brown et al., 2012). Furthermore, Chlamydia infection has been shown to increase supernumerary centrosomes, abnormal spindle poles, and chromosomal segregation defects (Grieshaber et al., 2006). Inappropriate activation of ERK and elevated global heterochromatin is a hallmark of many cancers (Di Micco et al., 2011; Roberts and Der, 2007). Interestingly, interference with Ctr-induced ERK activation or global heterochromatin formation inhibited proliferation of host cells. In addition, upregulation of CyclinE bypasses oncogene-induced senescence and contributes to cancer (Vredeveld et al., 2010). Continued upregulation of CyclinE, as observed during persistent Ctr infection, might therefore contribute to aberrant cell proliferation together with other proliferative signals such as ERK, in the presence of DSBs.

Our data demonstrate that Ctr modulates host cell function in ways that convey benefits to the pathogen but have severe consequences for the fate of host cells. These may be of particular relevance during chronic infection. Ctr induces the formation of DSBs, which leads to the induction of γH2AX. Remarkably however, the induction of γH2AX is not followed by a DDR that would either stimulate the induction of appropriate repair processes or lead to cellular senescence or apoptosis. Instead, infection promotes cellular viability by limiting the extent of DNA damage signaling. Together, the orchestrated deregulation of host cell signaling and perturbations to host cell chromatin lead to the enforced survival of damaged host cells, which is likely to predispose them to transformation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

End1 E6/E7 (End1) (American Type Culture Collection [ATCC] CRL-2615) and human primary fibroblast IMR90 (ATCC CCL-186) cells were cultured in HEPES-buffered growth medium (Dulbecco’s modified Eagle’s medium [DMEM; GibCo] supplemented with 10% fetal calf serum [FCS; Biochrome], 2 mM glutamine, and 1 mM sodium pyruvate), at 37°C in a humidified incubator containing 5% CO₂. Human fallopian tube cells were cultured in advanced DMEM/F12 (GIBCO) supplemented with 10% FCS (Biochrome), 2 mM glutamine, and 1 mM sodium pyruvate and 100 μg/ml epidermal growth factor (Invitrogen).

**Chlamydia Infections**

Ctr L2 (ATCC VR-902B), Ctr D (ATCC VR-885), and Cpr (ATCC VR-1310) stocks were prepared as described earlier (Gurumurthy et al., 2010). Ctr D and Cpr were used only in the experiments shown in Figure 1D. Chlamydia infection experiments were performed at a multiplicity of infection (MOI) of 5 in infection medium (DMEM supplemented with 5% FCS, 2 mM glutamine, and 1 mM sodium pyruvate). The medium was refreshed 2 hpi, and cells were grown at 35°C in 5% CO₂. For persistent Ctr infection, cells were infected (MOI 5) for 24 hr. Twenty-four hours postinfection, 250 ng/ml doxycycline was added to both uninfected and infected cells, and cells were allowed to grow for 6 dpi.

**Antibodies and Chemicals**

Details of the antibodies for screening histone PTMs are given in the Table S1 and details of other antibodies used are provided in the Supplemental Experimental Procedures.

**Statistical Analysis**

Statistical analyses were performed with GraphPad Prism 5 software. Student’s t tests were used to determine p values. Significance levels were set at ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05; p > 0.05, not significant (ns).

For information regarding infectivity assays, SDS-PAGE and western blotting, indirect immunofluorescence microscopy, BrdU labeling, antibodies, neutral comet assays, in vivo DNA-Ligation Assay, SA-beta-gal staining, IR sensitivity assay, siRNA transfection and knockdown analysis, and ROS measurements, see the Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, one table, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.05.010.

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