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# Human Papillomavirus E7 Induces Rereplication in Response to DNA Damage

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**Human papillomavirus (HPV) infection is necessary but not sufficient for cervical carcinogenesis. Genomic instability caused by HPV allows cells to acquire additional mutations required for malignant transformation. Genomic instability in the form of polyploidy has been demonstrated to play an important role in cervical carcinogenesis. We have recently found that HPV-16 E7 oncogene induces polyploidy in response to DNA damage; however, the mechanism is not known. Here we present evidence demonstrating that HPV-16 E7-expressing cells have an intact G<sub>2</sub> checkpoint. Upon DNA damage, HPV-16 E7-expressing cells arrest at the G<sub>2</sub> checkpoint and then undergo rereplication, a process of successive rounds of host DNA replication without entering mitosis. Interestingly, the DNA replication initiation factor Cdt1, whose uncontrolled expression induces rereplication in human cancer cells, is upregulated in E7-expressing cells. Moreover, downregulation of Cdt1 impairs the ability of E7 to induce rereplication. These results demonstrate an important role for Cdt1 in HPV E7-induced rereplication and shed light on mechanisms by which HPV induces genomic instability.**

Genomic instability is a hallmark of cancer progression (1). Genomic instability in the form of polyploidy, wherein cells have more than two sets of chromosomes, has been implicated as a causal factor in tumorigenesis (2, 3). Tetraploidy in basal keratinocytes has been found in low-grade squamous intraepithelial lesions of the cervix infected with high-risk but not low-risk human papillomavirus (HPV) types (4). Significantly, it was demonstrated that tetraploidy occurred as an early event during cervical carcinogenesis and predisposed cells to aneuploidy that is consistently observed in all cancers (5). Polyploidy can be formed via rereplication, a process in which origins fire more than once within a single S phase, or endoreduplication, in which multiple S phases occur without an intervening mitosis (6). Endoreduplication can occur in G<sub>2</sub>, early mitosis, or S phase. In the literature, the term rereplication has often been used for both rereplication and endoreduplication (6). To reduce confusion, we will use the term rereplication for DNA rereplication that occurs within the same interphase and endoreduplication for DNA rereplication that occurs after cells enter into mitosis. Rereplication can lead not only to polyploidy but also to gene amplification (7), DNA fragmentation (8), DNA breaks (9), and cellular DNA damage response (reference 10 and references therein).

Papillomaviruses are small DNA viruses that replicate in the stratified layers of skin and mucosa. Human papillomaviruses (HPVs) can be classified as either high or low risk depending on their clinical associations. The high-risk HPV types, such as HPV-16 and HPV-18, are commonly associated with lesions that can progress to high-grade cervical intraepithelial neoplasia and cervical carcinoma (for a review, see reference 11). The transforming properties of high-risk HPVs reside primarily in the E6 and E7 oncogenes, and the sustained expression of these genes appears to be essential for the maintenance of the transformed state of HPV-positive cells (reference 12 and references therein). E6 and E7 from high-risk HPV types induce genomic instability that occurs early in preneoplastic lesions, when the viral genome still persists in an episomal state (13, 14). The ability of the high-risk HPV E7 protein to bind and promote the degradation of pRb has been suggested as a mechanism by which HPV oncogenes promote tu-

mor formation, although E7 also has functions independent of inactivating pRb (reviewed in reference 13).

Cell cycle progression is regulated at several checkpoints whose defects contribute to genomic instability (15). The checkpoints in eukaryotic cells include the G<sub>1</sub> checkpoint, the G<sub>2</sub> checkpoint, the spindle assembly checkpoint, and the postmitotic checkpoint (16). The cell cycle is driven mainly by cyclins and cyclin-dependent kinases (Cdks) (17) and is partly controlled by p53 and pRb (17, 18). Although it is well documented that HPV E7 abrogates the G<sub>1</sub> checkpoint (13), its effect on the G<sub>2</sub> checkpoint is not as clear. In primary human keratinocytes (PHKs) expressing HPV-16 E7, an intact G<sub>2</sub> checkpoint was implicated after treatment with doxorubicin (Adriamycin) (19). However, interpretation of this observation is complicated by the fact that doxorubicin inhibits topoisomerase II that triggers a decatenation checkpoint (20) in addition to inducing DNA breaks (21). In contrast, mouse NIH 3T3 cells expressing HPV E7 were thought to be unable to maintain a G<sub>2</sub> arrest after doxorubicin treatment (22). In response to <sup>60</sup>Co, the G<sub>2</sub> checkpoint is maintained in human fibroblasts expressing HPV-16 E7 (23). On the other hand, following hydroxyurea treatment, more HPV-16 E7-expressing PHKs than control cells were found in mitosis (24).

We have recently demonstrated that in response to microtubule disruption, E6 and E7 induced polyploidy through endoreduplication, a process including cell arrest at the spindle checkpoint, mitotic slippage, and abrogation of the postmitotic checkpoint (25, 26). Induction of polyploidy by HPV E7 in mouse NIH 3T3 cells treated with doxorubicin was proposed by Polager

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and Ginsberg to be a result of endoreduplication, though evidence was not provided (22). The mechanism by which E6 or E7 induces polyploidy in response to DNA damage therefore remains unknown.

In the present study, we examined the effect of HPV-16 E7 on the G<sub>2</sub> checkpoint in PHKs and explored the mechanism by which E7 induces polyploidy in response to DNA damage. We show that HPV E7-expressing cells have an intact G<sub>2</sub> checkpoint. In response to DNA damage, HPV E7 induces rereplication. We also demonstrate a role for the DNA replication initiation factor Cdt1 in E7-induced rereplication.

## MATERIALS AND METHODS

**Cell culture.** PHKs and cervical epithelial cells (CEs) were obtained from the University of Massachusetts Hospital. Cervical epithelial cells containing the HPV-16 genome (CE-HPV) (27) were provided by Aloysius Klingelutz, University of Iowa. These cells were cultured on mitomycin C-treated J2-3T3 feeder cells in E medium composed of three parts Dulbecco's modified Eagle medium (DMEM) and one part Ham's F12 medium plus 5% fetal bovine serum (FBS), with all supplements as previously described (26). The human telomerase reverse transcriptase-expressing human retinal pigment epithelium cell line (RPE1) was maintained in a 1:1 dilution of DMEM-Ham's F-12 medium plus 10% FBS.

PHKs and RPE1 cells expressing HPV-16 E7 were established using the pBabe retroviral system. Early-passage PHKs (within 3 passages) were used for infection. Retroviruses encoding HPV-16 E7 or vector were produced by transfection of PA317 or LinXA packaging cells. After transfection, retrovirus was collected in E medium or RPE1 medium. The PHKs or RPE1 cells were infected with retroviruses in 8 µg/ml hexadimethrine bromide (Polybrene) for 6 h. Cells were allowed to recover for 24 h and then selected with 1.25 µg/ml puromycin (PHKs) or 12.5 µg/ml puromycin (RPE1) for 3 to 6 days. Populations of infected cells were pooled and expanded. To ensure that a high percentage of cells will express E7 or contain the retroviral vector, PHKs and RPE1-derived cell lines were maintained in puromycin (0.5 µg/ml and 6.5 µg/ml, respectively) and used within 10 passages. Parental PHKs do not proliferate well after long-term culture with such puromycin concentrations.

For UV treatment, the RPE1-Babe or E7 cells were rinsed twice with phosphate-buffered saline (PBS) and resuspended in PBS during UV exposures. Dishes were uncovered and exposed to UV irradiation using the germicidal UV lamp in a tissue culture hood for 1 min and then released to regular medium and incubated for 48 h.

**Immunoblotting.** Protein extraction was prepared in lysis buffer (10 mM Tris [pH 7.4], 1% SDS, 1.0 mM sodium orthovanadate). The protein concentration was measured by the bicinchoninic acid (BCA) protein assay reagent (Pierce) and confirmed by Coomassie blue staining of membranes after blotting. Equal amounts of protein from each cell lysate were separated in an SDS polyacrylamide gel (PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blotted with antibodies against Cdt1 (a gift from Anita Dutta, Millipore, number 071383, and Santa Cruz Biotechnology, H-300, sc-28262), Cdc25c (Cell Signaling; number 4688s), p-Cdc25c (Cell Signaling; number 4901s), β-tubulin (Sigma; T-4026), and β-actin (Sigma; A-2066). For E7 detection, whole-cell extracts (150 µg) in ML buffer (300 mM NaCl, 0.5% Nonidet P-40 [NP-40], 20 mM Tris-HCl [pH 8.0], 1 mM EDTA) were separated by SDS-PAGE, transferred onto a PVDF membrane, and subsequently probed with a mixture of 8C9 (Zymed/Invitrogen; number 28-0006, at a 1:150 dilution) and ED17 (Santa Cruz Biotechnology; sc-6981, at a 1:200 dilution). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit were used as secondary antibodies. β-actin or β-tubulin was used to indicate the loading amount of total proteins. ImageJ (NIH) was used to quantify gel images.

**Immunofluorescence.** For phospho-histone H2AX staining, 1.5 × 10<sup>5</sup> cells were seeded onto a 60-mm dish and grown on coverslips. The

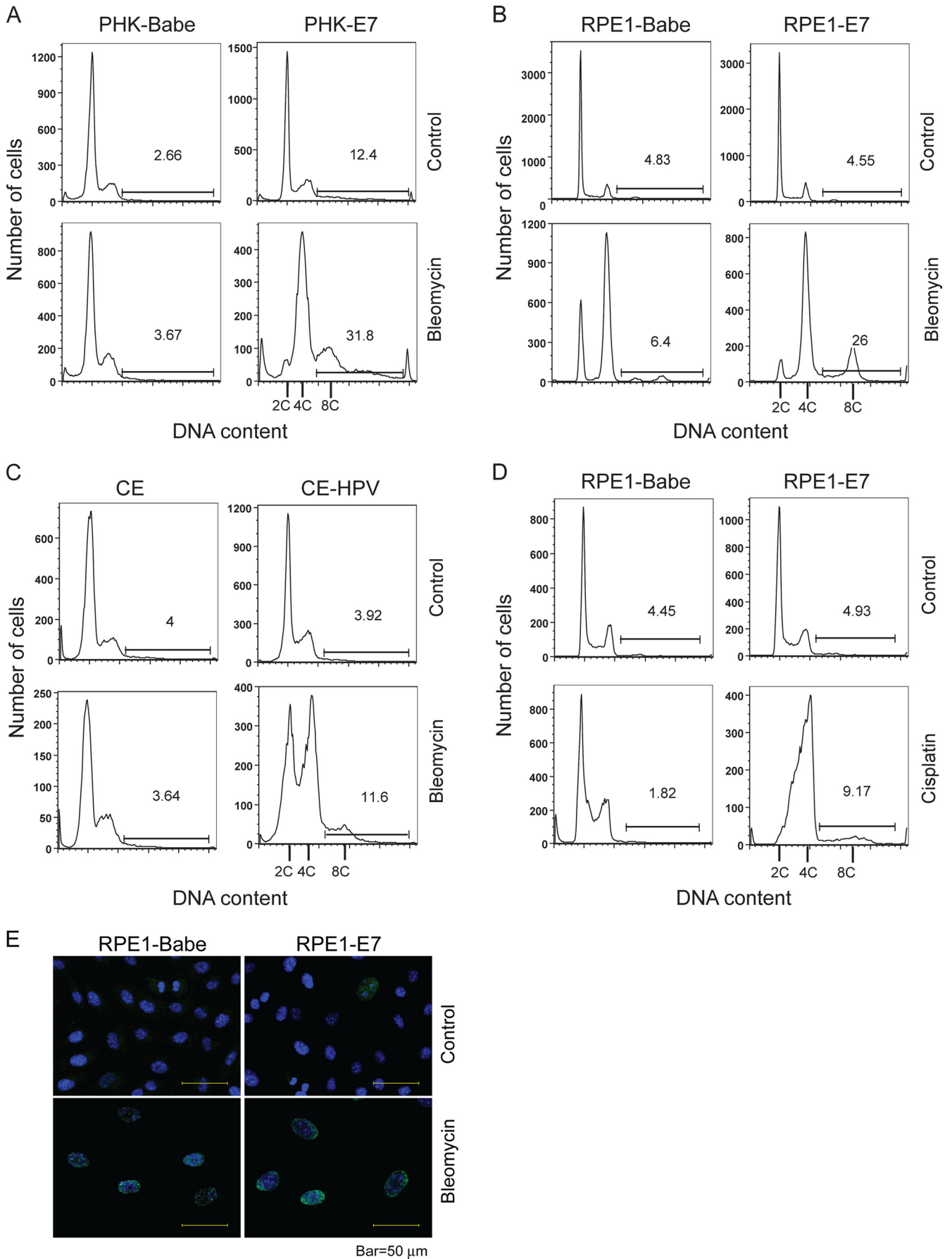
following day, cells were treated with bleomycin (5 µg/ml). During 48 h of treatment, bleomycin was replenished in 24 h. Cells were fixed with 95% ethanol/5% acetic acid for 5 min at room temperature and blocked with 3% bovine serum albumin (BSA)/Tris-buffered saline (TBS) buffer for 30 min at room temperature. Cells were then incubated with antibody against phospho-histone H2AX (Millipore; 05-636) for 1 h at a 1:100 dilution, followed by incubation with a fluorescein isothiocyanate (FITC)-labeled anti-mouse secondary antibody. Cells were washed in PBS, counterstained with 4,5-diamidino-2-phenylindole dihydrochloride (DAPI; Vector Laboratories), and analyzed with the Olympus BX51 epifluorescence microscope equipped with a multiband filter set. Two-color images were overlaid using the Nikon NIS-Elements BR 3.10 imaging software.

**Flow cytometry.** For cell cycle and polyploidy analysis, asynchronous cultures of PHKs and RPE1 cells expressing HPV E7 or vector alone were treated with PBS or bleomycin (Alexis Biochemicals; 5 µg/ml in PBS). For CE-HPV or the control, the cells were treated with 200 ng/ml bleomycin. For cisplatin treatment, cells were incubated for 6 h with 50 µM cisplatin (Sigma), washed with PBS, and incubated with cisplatin-free medium for 42 h. Forty-eight hours later, after the feeder cells were removed, cells were collected, fixed in 70% ethanol overnight, resuspended in PBS, stained in propidium iodide (PI) (50 µg/ml in PBS; Sigma) staining solution supplemented with 70 µg/ml RNase A (Sigma), and analyzed by flow cytometry. Cell cycle analysis was done using FlowJo software (Tree Star, Inc., Ashland, OR). For a nocodazole trapping assay, cells were first treated with 10 µg/ml bleomycin for 12 h and then incubated with bleomycin (10 µg/ml) plus nocodazole (50 ng/ml) for an additional 24 h. For the mitotic marker phospho-histone H3 (P-HH3) staining, cells were fixed in 70% ethanol, permeabilized in 0.25% Triton X-100, stained with 0.5 µg of the rat anti-phospho-histone H3 (anti-PHH3) immunoglobulin G 2a (IgG2a) (Sigma), followed by staining with fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG2a (BD Biosciences), and counterstained with PBS-PI-RNase A.

For the bromodeoxyuridine (BrdU) labeling experiment, asynchronous cultures of cells were treated with PBS or bleomycin. BrdU (final, 20 µM) was added to the medium 2 h before collection of cells. Cells were then harvested and fixed in 70% ethanol overnight. The cells were permeabilized with 2 N HCl-0.5% Triton X-100, neutralized with 0.1 M sodium tetraborate, stained with monoclonal anti-BrdU (BD Biosciences), followed by anti-mouse IgG F(ab)2-FITC (Sigma) staining, and counterstained with PBS-PI-RNase A.

**RNA interference (RNAi).** Small interfering RNA (siRNA) oligonucleotides were purchased from Dharmacon, Inc. The siRNA duplexes were the nonsilencing control siRNA sense strand (5'-UUCUCCGAACGUGUCACGU-3'), Cdt1-siRNA001 (5'-AACGUGGAUGAAGUACCCGAC-3') (sense strand, described in reference 28), and Cdt1-siRNA002 (5'-CCUACGUAAGCUGGACAA-3') (sense strand, described in reference 29). For polyploidy analysis, 1.2 × 10<sup>5</sup> cells were seeded onto a 60-mm dish the day before transfection. Cells were transfected with a final concentration of 20 nM siRNA per target gene using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions (Invitrogen). The next day after transfection, cells were treated with 4 µg/ml bleomycin, incubated for an additional 36 to 48 h, and then harvested 72 h posttransfection. For protein knockdown analysis, cells were seeded onto a 60-mm dish the day before siRNA transfection. Seventy-two hours after transfection, cells were harvested and specific protein levels were analyzed by immunoblot. For the BrdU labeling experiment, RPE1-E7 cells were transfected with 20 nM siRNA. Thirty-six hours later, the cells were treated with bleomycin (4 µg/ml) for an additional 36 h. BrdU (final, 20 µM) was added to the medium at 2 h before collection of cells. Cells were then stained and analyzed as described above.

**Statistical analysis.** All data are shown as means and standard deviations (SDs). The Student *t* test was used to compare the differences between means. Significance was set at a *P* value of <0.05.



## RESULTS

**PHKs expressing HPV-16 E7 have an intact G<sub>2</sub> checkpoint in response to DNA damage.** We have shown previously that upon DNA damage, PHKs expressing HPV-16 E7 or both E6 and E7 bypassed the G<sub>1</sub> checkpoint and accumulated at the G<sub>2</sub>/M phase with an increase in cells with more than 4C DNA content (26). Upon treatment with bleomycin, a radiomimetic (30), newly prepared PHKs from a different individual reproducibly demonstrated E7-induced polyploidy (Fig. 1A). Similar results were obtained in RPE1 cells expressing HPV-16 E7 (Fig. 1B). Expression of E7 in both cell types was demonstrated by a Western blot (data not shown). Significantly, cervical epithelial cells containing the HPV-16 genome also became polyploid after bleomycin treatment (Fig. 1C). Cisplatin, another DNA damage agent that has been widely used to treat cervical cancer, also induced polyploidy of HPV E7-expressing RPE1 cells (Fig. 1D). The treatment with the DNA damage agent is biologically relevant, as both E6 and E7 oncogenes from high-risk HPV types induce DNA damage and genomic instability (31–33). DNA damage may also result from spontaneous decay, replication errors, and cellular metabolism (34). Consistent with this notion, spontaneous polyploidization in PHKs expressing E7 was observed (Fig. 1A). Although no significant spontaneous polyploidy was seen in early-passage RPE1 cells expressing E7 (Fig. 1B), over an extended period of culture, a small but statistically significant increase in the percentage of polyploidy in these cells has been found (25). Bleomycin treatment enhanced the extent of DNA damage (Fig. 1E).

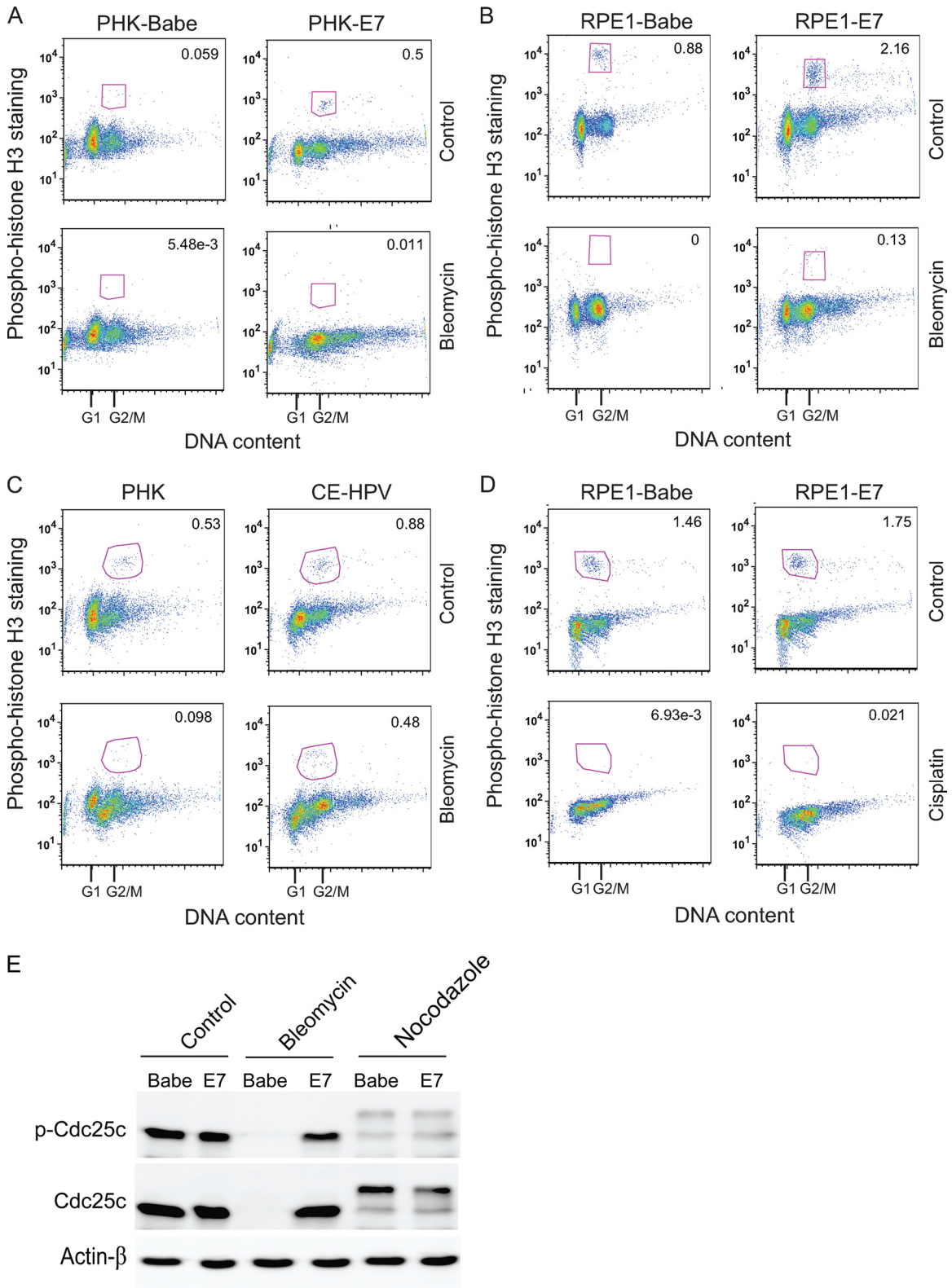
Next, we extended our studies to determine the integrity of the G<sub>2</sub> checkpoint in early-passage cells expressing E7. Cells in the G<sub>2</sub> phase and in mitosis normally have similar DNA contents of 4C. To distinguish between these two cell populations, we used the mitotic specific marker P-HH3 (35) to determine which cells were in mitosis. Using P-HH3 as a marker may underestimate the number of mitotic cells due to sensitivity and tendency of mitotic cells to detach from the culture dish. The trypsinization and removal steps for feeder cells before collection of PHKs would further reduce the number of mitotic cells. For this concern, we emphasize the relative numbers of mitotic cells with and without DNA damage. After bleomycin treatment, PHKs expressing E7, like vector control cells, showed a reduced number of cells expressing P-HH3, demonstrating that these cells with 4C DNA content are mainly at G<sub>2</sub> and not in mitosis (Fig. 2A). This indicates that the G<sub>2</sub> checkpoint in these cells is intact. Similar results were obtained in E7-expressing RPE1 cells (Fig. 2B), cervical epithelial cells containing the HPV-16 genome (Fig. 2C), and RPE1-E7 cells treated with cisplatin (Fig. 2D). We also used DAPI to stain cells in mitosis. We counted a total of approximately two thousand regularly cultured RPE1 cells and identified 1.7% and 1.9% mitotic cells (as indicated by cells with condensed chromosomes and a lack of a

nuclear membrane) in vector- and E7-expressing cells, respectively. Upon bleomycin treatment, however, no apparent DAPI-positive cells can be identified from more than one thousand vector- or E7-expressing RPE1 cells (data not shown). These results are consistent with what was observed when P-HH3 was used as the mitotic marker.

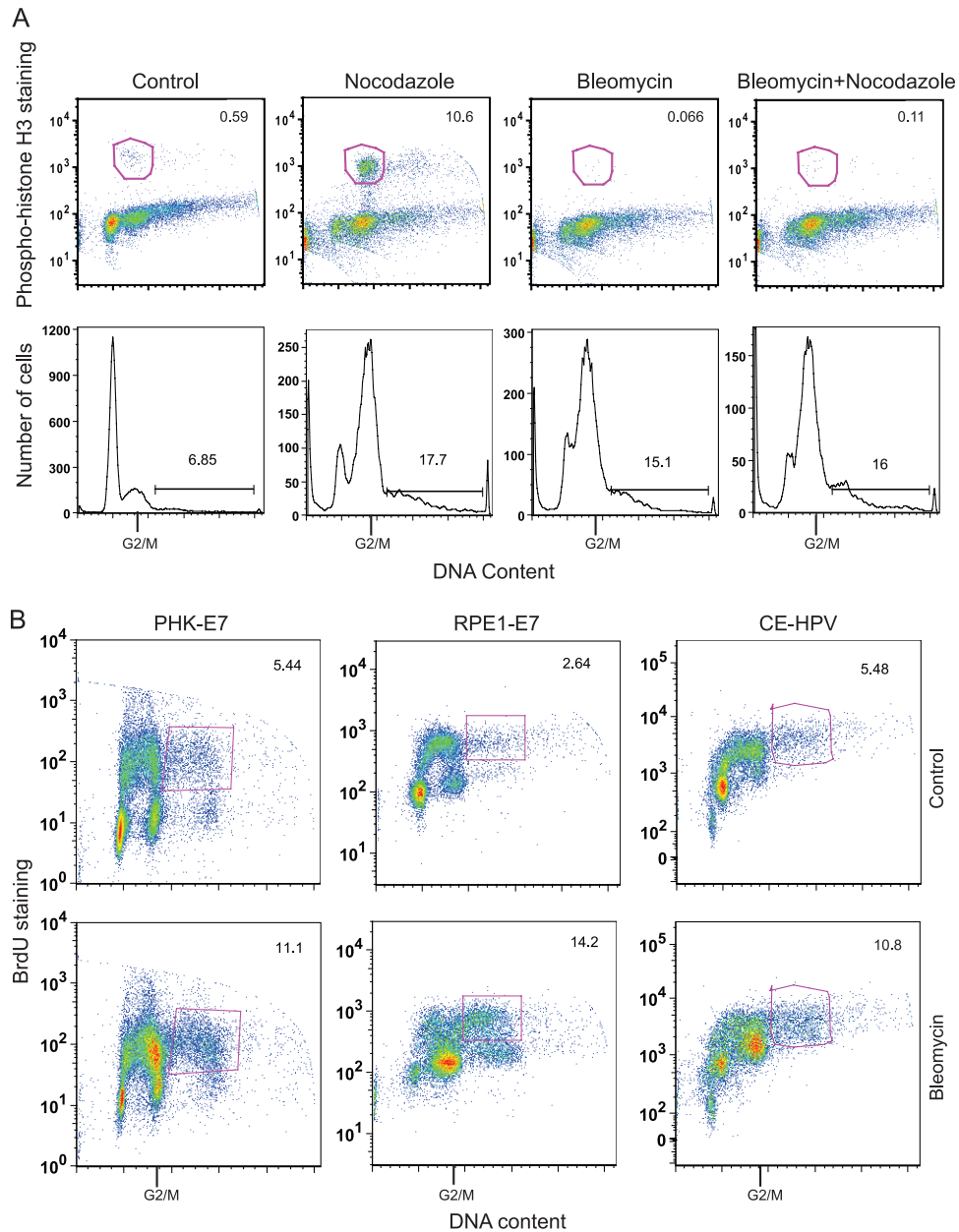
To provide more evidence that the bleomycin-treated cells are in G<sub>2</sub> instead of mitosis, we examined the protein phosphatase Cdc25c, a protein responsible for dephosphorylation and activation of Cdk1 (36–38). During interphase, Cdc25c is phosphorylated on Ser216 [phospho-Cdc25c (Ser216)] (39). During the transition from interphase to mitosis, Cdc25c becomes hyperphosphorylated (40). As shown in Fig. 2E, upon bleomycin treatment, while undetectable in the vector control cells, Cdc25c in E7-expressing RPE1 cells is phosphorylated on Ser216 in a form similar to those in asynchronous cells but different from those in mitosis (hyperphosphorylated, as demonstrated by the appearance of an upper band in nocodazole-treated cells using antibody against total Cdc25c). The weak band detected by the antibody specific to phospho-Cdc25c (Ser216) after nocodazole treatment suggests that a small percentage of cells are still in interphase, while the hyperphosphorylated band suggests that some phospho-Cdc25c (Ser216) may persist during mitosis and can be further phosphorylated at additional amino acids. These results indicate that RPE1-E7 cells remain largely in interphase instead of mitosis after bleomycin treatment. In conjunction with results from flow cytometry analysis (Fig. 1B) that indicate that the majority of RPE1-E7 cells contains 4C DNA content, the evidence suggests that these cells are in the G<sub>2</sub> stage of the cell cycle. Based on these results, we conclude that in HPV E7-expressing cells, while the G<sub>1</sub> checkpoint is defective, the G<sub>2</sub> checkpoint is largely intact in response to DNA damage.

**HPV E7-expressing cells undergo rereplication upon DNA damage.** Since E7-expressing cells have an intact G<sub>2</sub> checkpoint and polyploidy occurred without the cells entering mitosis, we conclude that endoreduplication is not the major mechanism for E7-expressing cells to become polyploid. However, it remains possible that a small percentage of cells expressing E7 can enter into mitosis after DNA damage and lead to endoreduplication. To test this possibility, we added nocodazole to trap cells that may have bypassed the G<sub>2</sub> checkpoint in mitosis. Our previous studies have demonstrated that HPV E6 and E7 do not have much effect on the spindle checkpoint and that cells expressing E7 arrest in mitosis for an extended period of time (25). As shown in Fig. 3A, after addition of nocodazole following DNA damage, the percentage of P-HH3-positive cells is still very small (0.11%), indicating that few cells entered mitosis after bleomycin treatment. Furthermore, the percentage of polyploidy formed in PHKs expressing E7 after bleomycin treatment in the presence of nocodazole did not

**FIG 1** HPV-16 E7 induces polyploidy upon DNA damage. Asynchronous cultures of cells were treated with PBS, bleomycin, or cisplatin. Forty-eight hours later, cells were collected, fixed, stained with PI, and analyzed by flow cytometry. (A) PHKs expressing HPV-16 E7 (PHK-E7) or containing the vector (PHK-Babe). Cells were treated with 5 μg/ml bleomycin for 48 h. Data from a representative experiment of 2 individual experiments are shown. (B) RPE1 cells expressing HPV-16 E7 (RPE1-E7) or containing the vector (RPE1-Babe) were treated as described above. Data from a representative experiment of 5 individual experiments are shown. (C) Cervical epithelial cells containing the HPV-16 genome (CE-HPV) or control cells (CE) were treated with 200 ng/ml bleomycin for 48 h. Data from a representative experiment of 4 individual experiments are shown. (D) RPE1-E7 and RPE1-Babe cells were treated with PBS or cisplatin (50 μM) for 6 h, followed by incubation with regular medium for an additional 42 h. Data from a representative experiment of 4 individual experiments are shown. The percentages of cells with 2C, 4C, and more than 4C DNA content are indicated. (E) RPE1-E7 and RPE1-Babe cells were treated with PBS or bleomycin (5 μg/ml) for 48 h. Indirect immunofluorescence microscopy was performed to detect phospho-histone H2AX (green). DAPI (blue) was used to counterstain the nucleus. Images were captured at ×40 magnification.



**FIG 2** HPV-16 E7-expressing cells have an intact G<sub>2</sub> checkpoint upon DNA damage. Asynchronous cultures of cells were treated with bleomycin, cisplatin, or PBS. Cells were collected, fixed, stained with rat anti-PHH3 IgG2a and FITC-conjugated anti-Rat IgG2a, counterstained with PI, and analyzed by flow cytometry. (A) PHKs expressing E7 or vector control treated with bleomycin (5 μg/ml) for 48 h. (B) RPE1-E7 and RPE1-Babe cells treated with bleomycin (5 μg/ml) for 48 h. (C) Cervical epithelial cells containing the HPV-16 genome or control (PHK) cells treated with bleomycin (200 ng/ml) for 48 h. (D) RPE1-E7 and RPE1-Babe cells treated with cisplatin (50 μM) for 6 h and incubated in regular medium for an additional 42 h. Data from a representative of at least two independent experiments are shown. The percentages of mitotic cells with 4C DNA content are indicated. (E) p-CDC25c and Cdc25c levels in RPE1 cells expressing E7 or vector were examined by Western blotting. Asynchronous RPE1 cells expressing E7 or vector were treated with bleomycin (5 μg/ml) for 48 h and harvested for Western blotting or treated with nocodazole (50 ng/ml) for 20 h. Mitotic cells were shaken off the dish and used for a Western blot analysis.



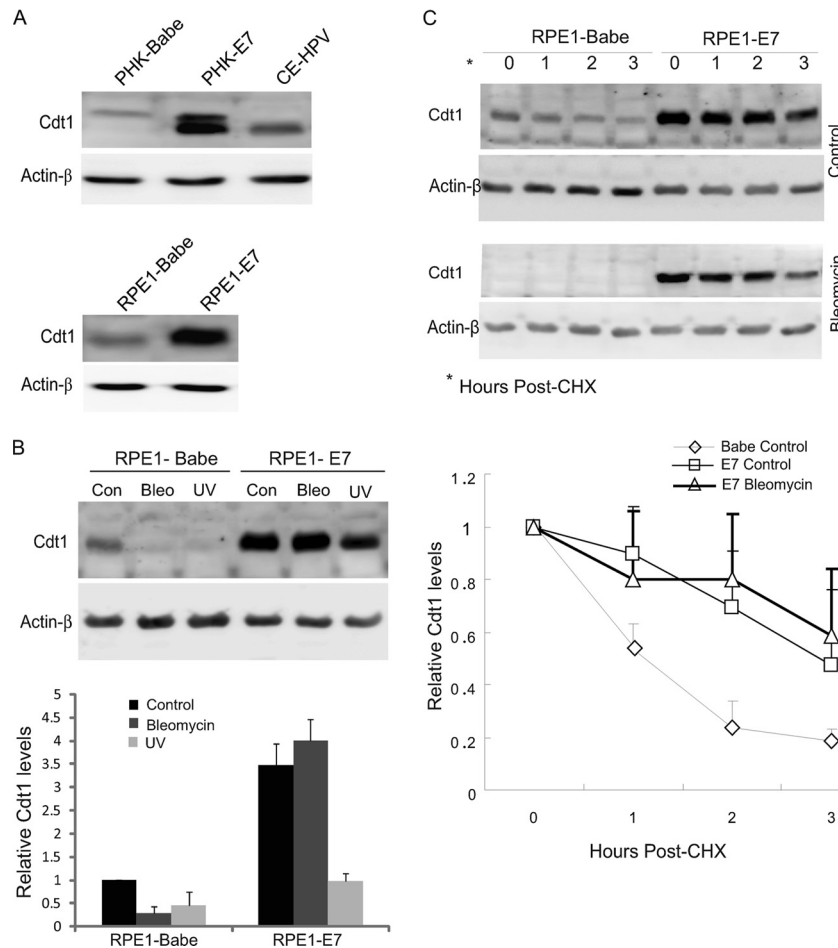
**FIG 3** HPV-16 E7 induces DNA rereplication upon DNA damage. (A) Asynchronous cultures of PHK-E7 cells were treated with bleomycin (10  $\mu\text{g}/\text{ml}$ ) for 12 h. Nocodazole (50 ng/ml) was added, and cells were cultured for an additional 24 h. Cells were collected, fixed, stained with anti-P-HH3 antibody, counterstained with PI, and analyzed by flow cytometry. Percentages of mitotic cells with 4C ( $G_2/M$ ) DNA content are indicated. (B) Asynchronous cultures of PHKs, RPE1 cells expressing E7, and cervical epithelial cells containing the HPV-16 genome were treated with bleomycin (10  $\mu\text{g}/\text{ml}$ , 5  $\mu\text{g}/\text{ml}$ , and 200 ng/ml, respectively) or PBS for 16 (PHKs) or 48 (other cells) h. Cells were labeled with BrdU for 2 h before collection. Then the cells were stained with anti-BrdU antibody, counterstained with PI, and analyzed by flow cytometry. The percentages of BrdU-positive cells with DNA content  $>4C$  are indicated. Data from a representative of at least 2 experiments are shown.

change significantly compared with that in PHKs treated with bleomycin alone, suggesting that endoreduplication does not contribute much to polyploidy formation under this experimental condition. These results suggest that rereplication is the major mechanism by which E7 induces polyploidy formation in response to DNA damage.

To confirm that polyploidy formation in E7-expressing cells is a result of DNA replication, we measured BrdU incorporation in E7-expressing cells. As shown in Fig. 3B, after 16 h of bleomycin

treatment, increasing numbers of E7-expressing PHKs with  $>4C$  DNA content stained with BrdU and became polyploid, indicating that rereplication had occurred. Notably, a significant number ( $\sim 5\%$ ) of E7-expressing PHKs underwent rereplication without bleomycin treatment, a finding which may be a result of DNA damage induced by E7. The number of cells with  $>4C$  DNA content continued to increase up to 48 h after DNA damage while the number of P-HH3-positive cells did not change significantly (data not shown). Similar results were observed in RPE1 cells expressing





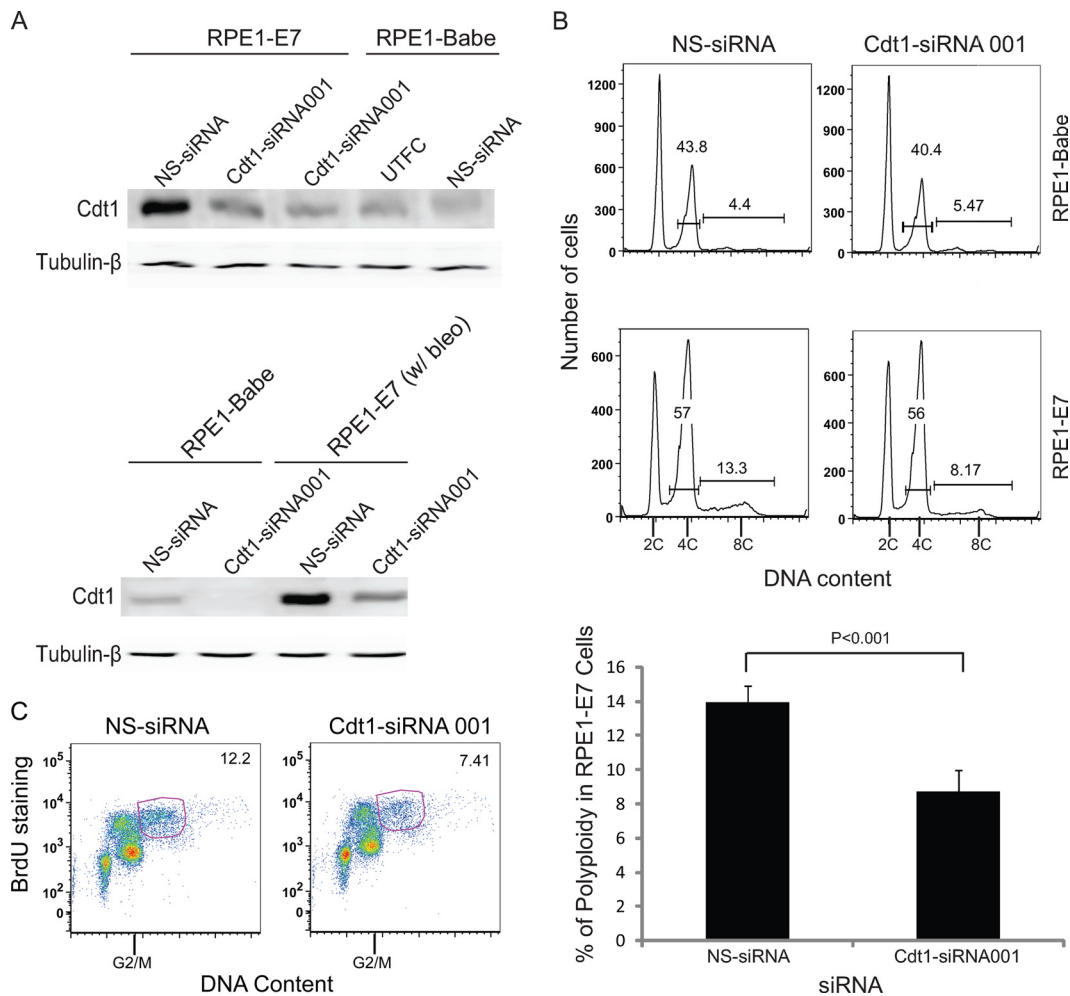
**FIG 4** Upregulation of Cdt1 in E7-expressing cells. (A) Cdt1 levels in PHKs (upper panel) and RPE1 cells (lower panel) expressing E7 or vector were examined by Western blotting.  $\beta$ -actin was used as a loading control. CE-HPV cells (upper panel) were also examined for Cdt1 expression. (B) Cdt1 levels in RPE1 cells after bleomycin (5  $\mu$ g/ml for 48 h) and UV (1 min, followed by 48 h of UV-free incubation) treatment were examined by Western blotting (upper panel).  $\beta$ -actin was used as a loading control. Steady-state levels of Cdt1 were quantified and normalized to  $\beta$ -actin (lower panel). The ratio between Cdt1 and  $\beta$ -actin in untreated control RPE1-Babe cells was set as 1. Con, control; Bleo, bleomycin. (C) RPE1 E7-expressing cells were treated with bleomycin (5  $\mu$ g/ml) or PBS. Forty-eight hours later, cells were incubated with MG132 for 4 h, treated with 25  $\mu$ g/ml cycloheximide (CHX) in the absence of MG132, and harvested at the indicated times. The stability of Cdt1 was monitored by immunoblot analyses (upper panel). Relative quantification of Cdt1 protein levels was assessed by densitometric analysis (lower panel). The average of 3 independent experiments is given.

HPV-16 E7 and cervical epithelial cells containing the HPV-16 genome (Fig. 3B). Together with results shown in Fig. 3A, our results demonstrate that rereplication is the major mechanism by which E7 induces polyploidy formation in response to DNA damage.

**The DNA replication initiating factor Cdt1 is upregulated in E7-expressing cells.** As an initial step toward understanding the mechanism by which E7 induces rereplication, we examined the expression of Cdt1, which is the only known mammalian gene that efficiently triggers rereplication when overexpressed in cancer cells (9, 41, 42). As shown in Fig. 4A, the steady-state level of Cdt1 was significantly increased (by more than 4-fold) in E7-expressing PHKs compared to that in the vector control PHKs. Notably, an extra band that is more evident in PHK-E7 cells was observed by using the antibody (Ab) from Santa Cruz (sc-28262, H-300) (Fig. 4A, upper panel). However, this band is absent in cervical epithelial cells containing the HPV-16 genome and RPE1 cells (Fig. 4A, lower panel) and it may represent a cell-type-specific posttranslational modification of Cdt1 in PHKs. Upregulation of Cdt1 pro-

tein also occurs in RPE1-E7 cells (Fig. 4A, lower panel). Also, while bleomycin treatment led to a reduction of Cdt1 in the vector control cells, no significant change in the steady-state level of Cdt1 was seen in RPE1-E7 cells (Fig. 4B). Consistent with what was observed previously (43), Cdt1 levels dropped after UV treatment in RPE1-Babe cells (Fig. 4B). Interestingly, unlike what was observed with bleomycin treatment, UV treatment reduced the steady-state level of Cdt1 in E7-expressing RPE1 cells, although the reduction is not as much as that in the vector control cells (Fig. 4B).

We also examined Cdt1 protein stability in E7-expressing RPE1 cells. Consistent with the steady-state level of Cdt1, the half-life of Cdt1 in E7-expressing cells is longer than that in the vector-containing cells (~3 h versus ~1 h) (Fig. 4C). The half-life of Cdt1 in the vector-containing RPE1 cells upon DNA damage was not able to be measured, as the steady-state level of Cdt1 in these cells was very low upon bleomycin treatment, even in the presence of MG132, the proteasome inhibitor (Fig. 4C). In contrast, upon bleomycin treatment, the half-life of Cdt1 in E7-expressing cells did not change significantly (Fig. 4C). These results dem-



**FIG 5** Downregulation of Cdt1 impairs the ability of E7 to induce rereplication. (A) RPE1-E7 and RPE1-Babe cells were transfected with Cdt1-siRNAs. Some dishes of RPE1-E7 cells were treated with bleomycin (4  $\mu$ g/ml) for the final 36 h. Seventy-two hours posttransfection, cells were harvested and lysed and Cdt1 protein levels were analyzed by immunoblot.  $\beta$ -tubulin or  $\beta$ -actin was used as a loading control. Bleo, bleomycin. (B) RPE1-E7 and RPE1-Babe cells were transfected with Cdt1-siRNAs. The transfected cells were then treated with bleomycin (4  $\mu$ g/ml). Seventy-two hours posttransfection, cells were harvested, stained with PI, and analyzed by flow cytometry. A representative experiment of 4 individual experiments is shown in the upper panel. Data of 4 experiments from RPE1-E7 cells are summarized in the lower panel. Error bars reflect the standard deviations of the mean. (C) RPE1-E7 cells were transfected with Cdt1-siRNA001. Thirty-six hours later, cells were treated with bleomycin (4  $\mu$ g/ml) for an additional 36 h. Cells were labeled with BrdU for 2 h before collection. Cells were then stained with anti-BrdU antibody, counterstained with PI, and analyzed by flow cytometry. The percentages of BrdU-positive cells with DNA content  $>4C$  are indicated. Data from a representative of 3 experiments are shown. UTFC, untransfected control; NS-siRNA, nonsilencing siRNA.

onstrate that Cdt1 expression in E7-expressing cells is regulated at the posttranslational level. Since Cdt1 is an E2F-regulated gene that is negatively regulated by pRb (44), it is expected that Cdt1 transcription should also be upregulated in E7-expressing cells.

**Cdt1 plays an important role in E7-induced rereplication.** To confirm the role of Cdt1 in E7-induced rereplication, we used siRNAs targeting Cdt1. These siRNAs were previously demonstrated to downregulate Cdt1 expression after transfection into cultured cells (28, 29). Transfection of siRNAs targeting Cdt1 efficiently reduced the steady-state levels of Cdt1 in RPE1-E7 and RPE1-Babe cells (Fig. 5A). For RPE1-E7 cells, the Cdt1 levels were also reduced by siRNA after bleomycin treatment (Fig. 5A, lower panel). In one case (Cdt1-siRNA001), the level of Cdt1 was still higher than that in the vector control cells; in the other case (Cdt1-siRNA002), the level of Cdt1 was reduced to that in the vector

control cells (Fig. 5A, upper panel). Therefore, knocking down of Cdt1 by these siRNAs should not disrupt normal DNA replication initiation. We then used these siRNAs to assess the role of Cdt1 in E7-induced rereplication. Notably, the percentage of polyploidy in siRNA-transfected RPE1 cells expressing E7 was lower than those shown in Fig. 1A (13% versus 26%), probably due to the toxicity of the transfection reagent. Nonetheless, knocking down Cdt1 by Cdt1-siRNA001 led to a statistically significant reduction in polyploidy formation in RPE1 cells expressing E7 after bleomycin treatment (Fig. 5B). Similar results were obtained using Cdt1-siRNA 002 (data not shown). Moreover, knocking down of Cdt1 by Cdt1-siRNA001 led to a significant reduction of BrdU incorporation for RPE1-E7 cells with  $>4C$  DNA content (Fig. 5C). These results demonstrate that upregulation of Cdt1 is important for E7 to induce rereplication and contributes to the polyploid formation in E7-expressing cells.

## DISCUSSION

We have recently shown that in response to microtubule disruption, HPV E7 abrogates the postmitotic checkpoint to induce endoreduplication (25). In this study, we demonstrated that upon DNA damage, cells expressing HPV oncogene E7 did not arrest at the G<sub>1</sub> checkpoint, likely due to G<sub>1</sub> checkpoint abrogation as previously described (45–47). However, E7 does not alleviate the G<sub>2</sub> checkpoint. While being arrested at G<sub>2</sub>, cells expressing E7 induce rereplication. HPV E7 therefore induces polyploidy via different mechanisms in response to different insults. We also show that the DNA replication initiation factor Cdt1 is upregulated in E7-expressing cells and is important for E7-induced rereplication.

Compared with the G<sub>1</sub> checkpoint, which is defective in HPV E7-expressing cells (45–47), less attention has been paid to the effect of HPV on the G<sub>2</sub> checkpoint. While some studies suggest that HPV E7 does not abrogate the G<sub>2</sub> checkpoint in early-passage cells, several groups of investigators had different opinions. Specifically, mouse NIH 3T3 cells expressing HPV E7 were believed to be defective for the G<sub>2</sub> checkpoint in response to doxorubicin treatment (22). However, the ability of E7-expressing cells to enter into mitosis was not examined in this study. Notably, upon hydroxyurea treatment, more HPV-16 E7-expressing PHKs than control cells were found in mitosis (24). It is likely that the G<sub>2</sub> checkpoint in E7-expressing PHKs may respond differently to different types of DNA-damaging agents. However, it is evident that the percentage of E7-expressing cells entering mitosis in this case is relatively small (0.9%) while the percentage of cells in G<sub>2</sub> upon hydroxyurea treatment is expected to increase significantly. Collectively, these data suggest that although a small number of E7-expressing cells can enter into mitosis in the presence of DNA damage, the G<sub>2</sub> checkpoint in these cells is largely intact. The controversy between the previous studies and our current observation regarding the function of the G<sub>2</sub> checkpoint can therefore be explained by differences in data interpretation as well as experimental conditions.

During the HPV life cycle, several mechanisms that arrest cells at the G<sub>2</sub> checkpoint have been proposed. For example, the HPV-16 E4 protein prevents the nuclear entry of the mitotic cyclin/Cdk (48). In suprabasal cells, E7 expression induced cytoplasmic accumulation of cyclin B1 and Cdk1 with inactivating phosphorylation and G<sub>2</sub> arrest following S phase reentry (31). Arresting cells at G<sub>2</sub> may facilitate viral replication, as several viruses were found to replicate their genomes in the G<sub>2</sub> phase (49). Interestingly, it was suggested that Cdt1 contributes to HIV Vpr-induced G<sub>2</sub> arrest (50). On the other hand, activation of some of the host DNA replication machinery might be needed for viral DNA replication. In the case of HPV, whose productive life cycle is linked to epithelial differentiation, E7 establishes conditions permissive for viral DNA amplification (reference 31 and references therein). Having an intact G<sub>2</sub> checkpoint and activation of the host DNA replication machinery, such as Cdt1, is therefore consistent with what is expected for a role of E7 in the viral life cycle. However, the E7 from the high-risk HPV types may also upregulate Cdt1 in the basal layer of stratified epithelia and induces the host genome instability that contributes to carcinogenesis. Future studies should examine the role of Cdt1 in HPV genome amplification and the ability of E7 from the low-risk HPV types to upregulate Cdt1.

Since Cdt1 is the only known mammalian gene that efficiently

triggers rereplication when overexpressed (14, 41, 42), it is not surprising that Cdt1 is upregulated in E7-expressing cells and plays an important role in E7-induced rereplication. Notably, Cdt1 overexpression-induced rereplication was observed only in cancer cells previously; here, we show that E7 induces rereplication that is mediated by Cdt1 in primary and immortalized cells as well. Unexpectedly, the Cdt1 protein remains relatively stable in E7-expressing cells upon bleomycin treatment (Fig. 4C). Three E3 ubiquitin ligases, SCF-Skp2, APC/C, and DDB1-Cul4, have been identified to target Cdt1 for proteolysis (43, 51–54). UV-dependent Cdt1 destruction is mediated by DDB1-Cul4 ubiquitin ligase, which appears to be functioning in E7-expressing cells (Fig. 4B). In PHKs expressing E6 and E7, the APC/C ubiquitin ligase complex was shown to be active (55). It is possible that the SCF-Skp2 ligase is somehow compromised in cells expressing E7. Consistent with this notion, in cervical cancers, the Skp2 ligase does not appear to be functioning (56). Interestingly, E7 was shown to bind Skp2 and was ubiquitinated by the SCF-Skp2 ligase (57). It remains to be determined whether the SCF-Skp2 ligase or some other as-yet-unidentified E3 ubiquitin ligases are indeed altered in E7-expressing cells. While we have demonstrated that Cdt1 expression in E7-expressing cells is regulated at posttranslational levels, this does not rule out the possibility that E7 also regulates Cdt1 expression at the transcriptional level. It is known that Cdt1 is an E2F-regulated gene that is negatively regulated by pRb (44). pRb degradation may therefore contribute to E7-induced Cdt1 upregulation and rereplication.

Although our data demonstrate that Cdt1 plays an important role in E7-induced rereplication, how DNA damage enhances rereplication in E7-expressing cells is still not clear, since the level of Cdt1 is not further increased in these cells upon DNA damage. A straightforward explanation for DNA damage-enhanced rereplication in E7-expressing cells is that the G<sub>2</sub> arrest allows extra time for licensing of DNA replication to occur. However, merely extra time may not be sufficient for the initiation of rereplication, as DNA replication licensing factors are subjected to multiple regulations in the G<sub>2</sub> phase (58). DNA damage in E7-expressing cells may inhibit cyclin A-Cdk1 activity and lead to an increased Cdt1 activity for licensing and therefore induces rereplication. It was observed that Cdt1 phosphorylation by cyclin A-dependent kinases reduces its affinity to DNA and that Cdk1 inactivation results in Cdt1 dephosphorylation and rebinding to chromatin/nuclear matrix (59). Consistent with this observation, Cdk1 inactivation in G<sub>2</sub> phase promotes relicensing (60, 61). Alternatively, the level of binding affinity to Cdt1 by its inhibitory protein geminin might be reduced upon DNA damage in E7-expressing cells. Further studies are needed to test these possibilities.

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