Human Papillomavirus 16 E7 Oncoprotein Attenuates DNA Damage Checkpoint Control by Increasing the Proteolytic Turnover of Claspin

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Abstract

The human papillomavirus (HPV) 16 E7 oncoprotein has been reported previously to stimulate DNA damage and to activate host cell DNA damage checkpoints. How HPV-16 E7 maintains proliferation despite activated DNA damage checkpoints is incompletely understood. Here, we provide evidence that cells expressing the HPV-16 E7 oncoprotein can enter mitosis in the presence of DNA damage. We show that this activity of HPV-16 E7 involves attenuation of DNA damage checkpoint control by accelerating the proteolytic turnover of claspin. Claspin mediates the activation of CHK1 by ATR in response to replication stress, and its degradation plays a critical role in DNA damage checkpoint recovery. Expression of a nondegradable mutant of claspin was shown to inhibit mitotic entry in HPV-16 E7–expressing cells. Multiple components of the SCFβ-TRCP–based claspin degradation machinery were found deregulated in the presence of HPV-16 E7, including cullin 1, β-TRCP, Aurora A, and Polo-like kinase-1 (PLK1). In contrast, no difference in the expression level of the claspin deubiquitinating enzyme USP7 was detected. Levels of Aurora A and PLK1 as well as phosphorylated PLK1 at threonine 210, a prerequisite for DNA damage checkpoint recovery, remained detectable following replication stress in HPV-16 E7–expressing cells but not in control cells. In summary, our results suggest that the HPV-16 E7 oncoprotein attenuates DNA damage checkpoint responses and promotes mitotic entry by accelerating claspin degradation through a mechanism that involves deregulation of components of the SCFβ-TRCP–based claspin degradation machinery. [Cancer Res 2009;69(17):7022–9]

Introduction

Infection with high-risk human papillomaviruses (HPV), such as HPV-16, is intimately associated with squamous cell carcinomas of the anogenital tract as well as a subset of oropharyngeal cancers (1). HPV-16 encodes two oncoproteins, E6 and E7, which play central roles in the viral life cycle and are commonly found overexpressed in high-risk HPV-associated tumors (2). The HPV-16 E7 oncoprotein is a multifunctional protein that binds and degrades the retinoblastoma tumor suppressor protein (pRB) as well as the related proteins p107 and p130 (3). The HPV-16 E7 oncoprotein further disrupts G1-S cell cycle checkpoint control by inhibiting the cyclin-dependent kinase inhibitors p21Cip1 and p27Kip1 as well as various other activities (4–7). The cooperating HPV-16 E6 oncoprotein induces the degradation of the p53 tumor suppressor protein, stimulates hTERT expression, and has several other functions that promote proliferation (8, 9). Together, the high-risk HPV oncoproteins relax G1-S checkpoint control to induce unscheduled entry into S phase and promote a S-phase–like milieu conducive for viral genome replication in differentiated human keratinocytes (7, 10).

Several studies have suggested that deregulated S-phase entry is associated with DNA replication stress (11–13). In line with this notion, the HPV-16 E7 oncoprotein has been shown to activate the Fanconi anemia pathway (14), a DNA damage response pathway that responds primarily to replication stress and stalled DNA replication forks (15). Replication stress can ultimately lead to DNA breakage and several lines of evidence suggest that expression of the high-risk HPV-16 E7 oncoprotein triggers host cell DNA damage. HPV-16 E7 has been reported to induce structural chromosomal changes (16, 17) as well as promote the integration of foreign DNA, an event that requires the formation of DNA double-stranded breaks (18). HPV-16 E7 expression has also been shown to stimulate an increase of nuclear foci that contain γ-H2AX, a marker of double-stranded DNA breaks (17).

Despite signs of DNA damage and checkpoint activation, HPV-16 E7–expressing cells remain proliferative. Although several reports suggest that the ability of HPV-16 E7–expressing cells to maintain proliferation following DNA damage is associated with manipulation of cell cycle checkpoints (19, 20), it is not known in detail whether the HPV-16 E7 oncoprotein can also interfere more directly with the host cell DNA damage checkpoint response to overcome antiproliferative stimuli. Here, we show that HPV-16 E7–expressing cells can enter mitosis in the presence of DNA damage. We provide evidence that this is not simply a reflection of increased overall DNA damage but that the HPV-16 E7 oncoprotein triggers host cell DNA damage. HPV-16 E7 oncoprotein attenuates the DNA damage checkpoint response to overcome antiproliferative stimuli.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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HPV-16 E7. We show that Aurora A and PLK1 protein levels, as well as Aurora A–mediated phosphorylation of PLK1, a prerequisite for DNA damage checkpoint recovery, are maintained in the presence of replication stress in HPV-16 E7–expressing cells but not in control cells. Because claspin itself as well as components of its degradation machinery are E2F responsive, our results suggest a model in which HPV-16 E7–mediated disruption of the pRB/E2F signaling axis up-regulates claspin to promote efficient DNA replication in S phase but also leads to accelerated degradation of claspin as cells approach the G2-M transition of the cell division cycle. Our results highlight the astonishing ability of the HPV-16 E7 oncoprotein to promote DNA replication and cell division despite a DNA damage response-activated, antiproliferative host cell environment.

Materials and Methods

Cell culture, treatments, and transfections. Primary human foreskin keratinocytes (HFK) were harvested from foreskins and maintained in serum-free keratinocyte growth medium (Epilife; Cascade Biologies/Invitrogen) supplemented with human keratinocyte growth supplement (Invitrogen), 50 units/ml penicillin (Cambrex), 50 µg/ml streptomycin (Cambrex), and fungizone (Invitrogen). Human foreskin fibroblasts (BJ; American Type Culture Collection) and C33A cells (American Type Culture Collection) were maintained in DMEM (Cambrex) supplemented with 10% fetal bovine serum (Mediatech) and antibiotics as indicated above. Ca Ski cells (American Type Culture Collection) were maintained in RPMI (Biowhitaker/Lonza) supplemented with 1% t-glutamine (Invitrogen) and antibiotics as indicated above. To assess protein stability, cells were treated with 30 µg/ml cycloheximide (Calbiochem) or distilled H2O for the indicated times. The proteasome was inhibited by treating cells with 1 µmol/L Z-Leu-Leu-Leu-vinyl sulfone (BioMol International/Enzo Life Sciences) or DMSO for 24 h. Stalled replication forks were induced by treating cells with 1 µmol/L hydroxyurea (Calbiochem) or distilled H2O for 24 h.

Primary HFKs were transduced with a LXSN-based high-risk HPV-16 E7 construct or LXSN empty vector followed by selection in G418-supplemented medium. Expression was confirmed through immunoblot analysis. Transient transfections of primary HFKs stably expressing HPV-16 E7 was done using nucleofection (Lonza) with 2 µg plasmids encoding a HA-tagged mutant claspin S30/34A (kindly provided by Michele Pagano, New York University School of Medicine) or empty vector control (neo-HA) in combination with 0.5 µg dsRED plasmid DNA (Clontech) as transfection marker. Tissue culture medium was replaced 24 h after transfection and cells were fixed and mitotic index was assessed 48 h after transfection. BJ fibroblasts were stably transfected using nucleofection (Lonza) with 2 µg HA-tagged HPV-16 E7, mutant HPV-16 E7 ∆21-24, or empty vector control (neo-HA) plasmids kindly provided by Karl Münger (Channing Laboratory, Brigham & Women’s Hospital). Cells stably transfected with HPV-16 E7-HA or empty vector control were selected using 0.5 µg/mL G418 for ~7 days. Cells stably transfected with HPV-16 E7 ∆21-24-HA were selected using 0.125 µg/mL G418 for 2 days. Plasmid expression was verified by determining pRB protein destabilization (data not shown).

Immunologic methods. Whole-cell lysates were prepared and immunoblot analysis was done as described previously (14). Quantification of band intensities was done using NIH ImageJ software.

Immunofluorescence analysis of cells grown on coverslips was done as described previously (17). Briefly, cells were fixed in 4% paraformaldehyde for 10 min at room temperature, washed in PBS, and permeabilized using 1% Triton X-100 for 15 min at room temperature. Following a PBS wash, cells were blocked with 10% normal donkey serum (Jackson Immunoresearch) in distilled water for 15 min at room temperature. Cells were then incubated in primary antibodies overnight at 4°C and again for at least another 2 h at 37°C in a humidified chamber. Following primary antibody incubation, cells were washed in PBS, incubated with FITC-conjugated rhodamine red–conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson Immunoresearch) for at least 2 h at 37°C, washed in PBS, and counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Cells were analyzed using an Olympus AX70 epifluorescence microscope equipped with a SpotRT digital camera.

Paraffin-embedded tissue samples were retrieved from the archives of the Department of Pathology of the University of Pittsburgh School of Medicine (institutional review board approval #0505181) and HPV typed using the Rembrandt/PanPath in situ hybridization kit (Invitrogen). Immunofluorescence microscopic analysis of paraffin-embedded tissue samples was done as described previously (14), with the exception that slides were not subjected to pepsin digest. Briefly, slides were deparaffinized by baking and xylene treatment followed by dehydration in 100% ethanol. After rehydration in a graded ethanol series (90%, 70%, and 50%), slides were washed twice in distilled H2O and microwaved at ~0.01 mol/L citrate buffer (pH 6.0) for 30 min. Slides were allowed to cool and washed once in distilled H2O and twice in PBS. Slides were then blocked in 10% normal donkey serum for 30 min at room temperature and primary and secondary antibodies were applied as described above, with the exception that primary antibody solution was applied for two to three nights at 4°C followed by several hours at 37°C. Slides were incubated in secondary antibody overnight at 4°C followed by 2 to 3 h at 37°C followed by counterstain using DAPI.

Primary antibodies used for immunoblotting and immunofluorescence were 53BP1 (Novus Biologicals), actin (Sigma), phosphorylated ATM at serine 1981 (Gene Tex), Aurora A (Cell Signaling Technology), claspin (antibody kindly provided by Raimundo Freire, Unidad de Investigacion, Hospital Universitario de Canarias), cullin 1 (Santa Cruz Biotechnology), cyclin A (Novocastra Laboratories), phosphorylated H2AX at serine 139 (Upstate), HPV-16 E7 (Santa Cruz Biotechnology), phosphorylated PLK1 at threonine 210 (BD Pharmingen), PLK1 (Santa Cruz Biotechnology), and β-TrCP (Zymed/Invitrogen).

Statistical analysis. Student’s two-tailed t test for independent samples was used to assess statistical significance.

Results

HPV-16 E7–expressing cells enter mitosis despite DNA damage. To determine the frequency of DNA damage in mitosis, an immunofluorescence microscopic analysis for the DNA damage marker γ-H2AX was done in tissue samples obtained from high-risk HPV-positive (types 16/18 and/or 31/33) anal neoplasms or nonmalignant tissue. A total of six carcinoma in situ samples, seven squamous cell carcinomas, and six nonmalignant controls (hemorrhoids) were analyzed for the presence of γ-H2AX foci in mitotic cells. Microscopic analysis revealed that high-risk HPV-positive tissue samples displayed a statistically significant increase in the percentage of γ-H2AX–positive mitotic cells (Fig. 1A, left) from 29.5% in nonmalignant tissue to 52.6% in carcinoma in situ and 62.4% in squamous cell carcinoma tissue (P ≤ 0.01 and P ≤ 0.0001, respectively; Fig. 1A, right). Because DNA damage checkpoints are crucial to prevent mitotic entry in the presence of unrepaired DNA damage (21), these results suggest that high-risk HPV oncoproteins can attenuate DNA damage checkpoint control that would normally lead to cell cycle arrest in G2-M.

The HPV-16 E7 oncoprotein stimulates DNA damage in a cell cycle–dependent manner (Supplementary Fig. S1) and activates DNA damage checkpoints associated with DNA replication stress (14). Because HPV-16 E7–expressing cells continue to proliferate and enter mitosis, we asked whether HPV-16 E7 plays a role in overcoming DNA damage checkpoint control. We performed an immunofluorescence microscopic analysis for γ-H2AX in primary HFKs stably transduced with HPV-16 E7 or empty vector control (LXSN). We first analyzed interphase cells and found a statistically

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significant 1.8-fold increase of the percentage of cells with \( \gamma \)-H2AX foci in HPV-16 E7–expressing cells in comparison with empty vector controls (53.1% and 28.3%, respectively; \( P \leq 0.001 \)) similar to what has been reported previously (17). We then specifically analyzed mitotic cells and found that a significant proportion of HPV-16 E7–expressing cells display \( \gamma \)-H2AX foci in prophase/metaphase as well as later stages of mitosis (anaphase/telophase; Fig. 2B, left). A statistically significant 1.6-fold increase in the percentage of

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**Figure 1.** HPV-16 E7 oncoprotein promotes mitotic entry despite the presence of DNA damage. A, immunofluorescence microscopic analysis of \( \gamma \)-H2AX foci in biopsy samples obtained from high-risk HPV-positive anal carcinomas in situ (CIS), squamous cell carcinomas (SCC), or nonmalignant tissue samples (hemorrhoids). Nuclei stained with DAPI. Bar, 10 \( \mu \)m (left). Quantification of the proportion of mitotic cells with \( \gamma \)-H2AX foci in carcinomas in situ, squamous cell carcinomas, or nonmalignant tissue samples. Red bar, mean percentage of \( \gamma \)-H2AX–positive mitotic cells for each category. At least 6 cases and a total of at least 350 mitotic cells were analyzed for each category (right). B, immunofluorescence microscopic analysis of \( \gamma \)-H2AX foci in primary HFKs stably expressing the HPV-16 E7 oncoprotein or empty vector control (LXSN). Nuclei stained with DAPI. Arrows, \( \gamma \)-H2AX foci. Bar, 10 \( \mu \)m (left). Quantification of fold changes of the percentage of total mitotic cells (top), prophase/metaphase cells (middle), or anaphase/telophase cells (bottom) that contain \( \gamma \)-H2AX foci. Five counts of at least 50 cells were analyzed from a representative experiment (right). C, quantification of the percentage of mitotic cells detected in primary HFKs stably expressing HPV-16 E7 or empty vector control (LXSN) following treatment with either 1 mmol/L hydroxyurea (HU) or distilled \( \text{H}_2\text{O} \) for 24 h. Mean ± SE of two independent experiments with triplicate quantification of a minimum of 50 cells. Asterisk, statistically significant differences in comparison with controls.
total γ-H2AX–positive mitotic cells was observed in HPV-16 E7–expressing cells (72.3%) compared with controls (44%; \( P < 0.005 \); Fig. 1B, top right). Furthermore, a significant 1.5-fold increase of γ-H2AX–positive prophase/metaphase was observed in HPV-16 E7–expressing cells (75.2%) compared with controls (49.5%; \( P < 0.005 \); Fig. 1B, middle right) and a significant 2.0-fold increase of γ-H2AX–positive anaphase/telophase in HPV-16 E7–expressing cells (68%) compared with controls (33.7%; \( P < 0.005 \); Fig. 1B, bottom right). Together, these results underscore that the HPV-16 E7 oncoprotein not only stimulates DNA damage but also attenuates DNA damage checkpoint responses that would normally prevent mitotic entry following DNA replication stress.

HPV-16 E7 attenuates DNA damage checkpoint control and promotes mitotic entry in the presence of replication stress. We next sought out to determine that the increase of mitotic cells with γ-H2AX foci in HPV-16 E7 cell populations is not simply a result of an overall increase of cells with DNA breakage. HPV-16 E7–expressing or control primary HFKs were treated for 24 h with hydroxyurea, a ribonucleotide reductase inhibitor that causes replication forks to stall by depleting the intracellular deoxynucleotide pool followed by quantification of the percentage of cells in mitosis. Hydroxyurea was used because it mimics certain aspects of the DNA damage response observed in HPV-16 E7–expressing cells, such as activation of the Fanconi anemia pathway (14). No significant difference in the mitotic index of HPV-16 E7–expressing cells (1.8%) compared with control cells (1.5%; \( P > 0.05 \)) was observed following control treatment (Fig. 1C). In contrast, a statistically significant 4.5-fold increase of HPV-16 E7–expressing cells were found in mitosis (0.9%) following hydroxyurea treatment compared with control cells (0.2%; \( P < 0.001 \); Fig. 1C). These results underscore that the HPV-16 E7 oncoprotein not only stimulates DNA damage but also attenuates DNA damage checkpoint responses that would normally prevent mitotic entry following DNA replication stress.
HPV-positive cells show altered claspin protein stability. Claspin facilitates replication stress-associated ATR phosphorylation of CHK1 (22, 23) and its degradation promotes recovery from DNA damage checkpoint arrest (24–26). Therefore, we next asked whether claspin expression is altered in the presence of high-risk HPV oncoproteins. HPV-16–positive cervical cancer cells (CaSki) or HPV-negative cervical cancer cells (C33A) were treated with cycloheximide for up to 8 h and claspin protein levels were assessed by immunoblot analysis (Fig. 2A, top). CaSki cells overexpress HPV-16 E6 and E7 and hence resemble high-risk HPV-associated lesions. As expected from previous studies in cancer cell lines (27), both C33A and CaSki cells contain elevated baseline claspin levels. However, calculation of claspin protein half-life revealed a half-life of 4 to 6 h in C33A cells, whereas the claspin half-life was significantly reduced and close to only 2 h in HPV-16 E7–expressing HFKs (Supplementary Fig. S2). These results suggest that claspin protein stability is decreased in the presence of high-risk HPV oncoproteins.

HPV-16 E7 oncoprotein accelerates the proteolytic turnover of claspin. To determine the individual contributions of HPV-16 oncoproteins, we analyzed claspin protein stability in BJ fibroblasts stably expressing empty vector, HPV-16 E7 alone, or HPV-16 E7 in combination with HPV-16 E6. Coexpression of HPV-16 E7 with HPV-16 E6 was not associated with any significant changes in claspin protein stability compared with cells expressing HPV-16 E7 alone (Supplementary Fig. S2). These results suggest that the reduced claspin stability in HPV-16–positive cells is primarily due to expression of the HPV-16 E7 oncoprotein. To further corroborate these results, we analyzed claspin protein stability in primary HFKs stably expressing HPV-16 E7 or control cells (Fig. 2B). Immunoblot analysis showed increased baseline levels of claspin in HPV-16 E7–expressing cells in comparison with control cells. The differences in baseline levels of claspin between BJ fibroblasts and primary HFKs are very likely due to the different cellular background. Importantly, however, half-life calculation revealed a claspin half-life of 1 to 2 h (closer to 1 h than 2 h) in HPV-16 E7–expressing HFKs, whereas no significant drop of claspin protein levels was detected in empty vector controls within the time interval analyzed (Fig. 2B). These results are in agreement with the shortened claspin half-life in HPV-16 E7–expressing BJ fibroblasts (Supplementary Fig. S2). We also analyzed mutant HPV-16 E7 Δ21-24 for both claspin protein stability and mitotic entry and no significant differences were detected in comparison with empty vector controls (Supplementary Fig. S2). These results indicate that, despite the overall increase of claspin protein levels in HPV-16 E7–expressing cells, the proteolytic turnover of claspin is accelerated in the presence of the HPV-16 E7 oncoprotein. Our mutational analysis furthermore suggests that disruption of the pRb/E2F signaling axis by HPV-16 E7 may contribute to accelerated claspin degradation.

To further corroborate the role of HPV-16 E7 in claspin degradation, we next tested whether inhibition of the proteasome leads to stabilization of claspin protein levels. Treatment of primary HFKs stably expressing HPV-16 E7 or control cells with the proteasome inhibitor Z-Leu-Leu-Leu-vinyl sulfonyl for 24 h led to a significant increase of claspin protein levels in HPV-16 E7–expressing cells compared with control cells (Fig. 2C). These results underscore that the HPV-16 E7 oncoprotein destabilizes claspin protein through a mechanism that involves the ubiquitin-proteasome pathway.

Claspin degradation is required for mitotic entry in HPV-16 E7–expressing cells. In order for cells to enter mitosis following DNA damage checkpoint responses, claspin must be degraded, thereby relieving further ATR-mediated CHK1-associated cell cycle arrest (24–26). To directly test whether claspin degradation facilitates entry into mitosis in HPV-16 E7–expressing cells, we transiently transfected empty vector–transduced HFKs or HPV-16 E7–expressing HFKs with a nondegradable mutant of claspin, in which two conserved serine residues at amino acid positions 30 and 34 within the phospho-degron motif of claspin have been mutated to alanines (S30A/S34A; kindly provided by Michele Pagano) or vector control and monitored the mitotic index 48 h following transfection (Fig. 3). No effect of degradation-impaired claspin on mitotic entry was detected in control HFKs, which is in line with the notion that nondegradable claspin does not significantly affect entry into mitosis in the absence of DNA damage as suggested previously (24). However, a significant 1.9-fold increase in the percentage of mitotic cells was observed in HPV-16 E7–expressing primary HFKs transiently transfected with the phospho-degron mutant claspin S30A/S34A (1.2%) compared with cells transfected with empty vector controls (2.2%; P ≤ 0.0001). These findings underscore not only that HPV-16 E7 induces DNA damage but also that acceleration of claspin degradation by the HPV-16 E7 oncoprotein is necessary to facilitate entry into mitosis. Furthermore, they suggest that accumulation of claspin in HPV-16 E7–expressing cells can reinforce the G2–M checkpoint.

HPV-16 E7–induced claspin degradation involves deregulation of multiple components of the SCFe−TrCP–based claspin degradation machinery. DNA damage checkpoint recovery is facilitated, in part, by the activities of the mitotic kinases Aurora A and PLK1, which promote SCFe−TrCP–mediated claspin proteolysis (25, 26, 28, 29). To test whether the HPV-16 E7 oncoprotein affects claspin protein stability by deregulating the SCFe−TrCP–based claspin degradation machinery, we examined the expression levels of proteins involved in claspin ubiquitination in primary HFKs

Figure 3. A nondegradable mutant of claspin inhibits mitotic entry in HPV-16 E7–expressing cells. The mitotic index was assessed in HPV-16 E7–expressing or empty vector control (LXSN) primary human keratinocytes 48 h after transient transfection with either a phospho-degron mutant of claspin (claspin S30A/S34A) or empty vector (control) using diRED as a transfection marker. Mean ± SE for LXSN cells were generated from a representative experiment with at least 100 cells counted in triplicate. Mean ± SE for HPV-16 E7–expressing cells were generated from three independent experiments with at least 100 cells counted in triplicate. Asterisk, statistically significant differences in comparison with cells transiently transfected with empty vector (control).
stably expressing HPV-16 E7 or control. Multiple proteins implicated in SCF$^{β-TrCP}$-mediated claspin degradation were up-regulated in HPV-16 E7–expressing primary HFKs, including CUL1, β-TrCP, Aurora A, and PLK1 (Fig. 4A). Claspin protein stability, however, is also regulated by the deubiquitinating enzymes USP7 and USP28 (28, 30, 31). No significant difference in the expression level of USP7, which primarily counteracts SCF$^{β-TrCP}$-based claspin ubiquitination in G$_2$-M (28), was detected in HPV-16 E7–expressing cells compared with control cells (Fig. 4B). A slight increase of USP28, which opposes APC/C$^{Cdh1}$-based claspin degradation in G$_1$ (28, 30), was observed in HPV-16 E7–expressing cells compared with controls. Together, these findings suggest that HPV-16 E7–associated acceleration of claspin degradation involves the deregulation of components of the SCF$^{β-TrCP}$-based claspin degradation machinery but not the deregulation of the deubiquitinating enzyme that thwarts SCF$^{β-TrCP}$-mediated claspin degradation.

The activation of PLK1 before DNA damage checkpoint recovery is mediated by the Aurora A kinase (29). We therefore analyzed Aurora A and PLK1 expression as well as PLK1 activation in primary HFKs stably expressing HPV-16 E7 or control cells following DNA replication stress. We found that HPV-16 E7–expressing cells maintain high expression levels of Aurora A in response to hydroxyurea-induced replication stress compared with control cells (Fig. 4C). In addition, HPV-16 E7–expressing cells also showed higher PLK1 protein levels and PLK1 phosphorylation at threonine 210. Phosphorylation at this residue has been shown to be a prerequisite for PLK1 to promote mitotic entry after DNA damage checkpoint arrest (29). Together, these results show that multiple layers of control involved in recovery from the DNA damage checkpoint are disrupted in HPV-16 E7–expressing cells.

**Discussion**

In the present report, we show that the HPV-16 E7 oncoprotein can promote mitotic entry in the presence of DNA damage. We provide evidence that attenuation of DNA damage checkpoint control is mediated by HPV-16 E7–associated accelerated proteolysis of claspin, a critical mediator of the ATR/CHK1 signaling axis (22, 23). Several previous reports have shown that claspin degradation by the SCF$^{β-TrCP}$–based ubiquitin ligase in G$_2$-M is linked to recovery from DNA damage checkpoint activation (24–26). We found that several proteins involved in this process are up-regulated in HPV-16 E7–expressing cells, thus creating a cellular environment that promotes aberrant mitotic entry.

Although our results clearly show that the HPV-16 E7 oncoprotein attenuates DNA damage checkpoint control (Fig. 1C), it is noteworthy that we discovered γ-H2AX–positive mitotic cells in nonmalignant tissue samples as well as in control keratinocytes. It is

![Figure 4](https://example.com/figure4.png)
possible that genotoxic stress from unfavorable growth conditions contributes to these findings. Remarkably, several studies suggest that cells with DNA damage avoid a prolonged cell cycle arrest and enter mitosis through checkpoint adaptation (32–36). It is hence possible that HPV-16 E7 manipulates a process that, to a certain degree, represents a physiologic response to DNA damage.

Our finding that HPV-16 E7–expressing cells show an accelerated proteolytic turnover of claspin provides an explanation for aberrant mitotic entry in the presence of DNA damage. Although we assume that claspin degradation occurs at the G2-M transition, the technical difficulties to efficiently synchronize HPV-16 E7–expressing keratinocyte populations prevented us from directly testing this hypothesis.

Because claspin is involved in the ATR/CHK1 signaling cascade (22, 23), it is important to mention that phosphorylation of CHK1 was observed in HPV-16 E7–expressing cells (data not shown). These results suggest that claspin remains functional within the ATR pathway in HPV-16 E7–expressing cells but that it is more efficiently degraded during the G2 phase of the cell cycle to attenuate DNA damage checkpoint responses and promote mitotic entry. This notion is supported by our finding that a nondegradable mutant of claspin inhibits mitotic entry in HPV-16 E7–expressing cells, thus underscoring that claspin accumulation reinforces G2-M checkpoint control in HPV-16 E7–expressing cells.

We found that several proteins involved in SCF<sup>β-TRCP</sup>–mediated claspin degradation are up-regulated in HPV-16 E7–expressing cells, including CUL1, β-TRCP, PLK1, and Aurora A. Claspin protein stability is also regulated by the deubiquitinating enzymes USP7 and USP28, which counteract SCF<sup>β-TRCP</sup>–mediated and APC/C<sup>Cdh1</sup>–mediated claspin degradation, respectively (28, 30, 31). Despite the slight up-regulation of USP28 levels, no such increase in USP7 protein expression, which opposes SCF<sup>β-TRCP</sup>–based claspin degradation in G2 (28), was detected in HPV-16 E7 keratinocyte populations compared with controls.

The degradation of claspin during DNA damage checkpoint recovery involves its phosphorylation by PLK1, which creates a recognition motif for the SCF<sup>β-TRCP</sup> ubiquitin ligase (24–26). PLK1 is activated before DNA damage checkpoint recovery by Aurora A–mediated phosphorylation of PLK1 at threonine 210 (29). Here, we show that HPV-16 E7–expressing cells maintain high levels of Aurora A and PLK1 when compared with control cells in the presence of hydroxyurea-induced replication stress. Furthermore, we found that the level of phosphorylated PLK1 at threonine 210 also remains detectable in HPV-16 E7–expressing cells treated with hydroxyurea. The finding that hydroxyurea-treated control cells show significantly decreased Aurora A and PLK1 protein levels could be explained by the fact that these cells are more efficiently phosphorylated by hydroxyurea treatment in early S phase, when PLK1 and Aurora A levels are still low (37–39). In contrast, the higher levels of Aurora A and PLK1 found in HPV-16 E7–expressing cells treated with hydroxyurea may suggest that cells do not arrest properly following hydroxyurea and/or that the regulation of these two kinases is disrupted by HPV-16 E7.

Together, our findings indicate that the HPV-16 E7 oncoprotein increases claspin degradation by disrupting the balance between positive and negative regulators involved in SCF<sup>β-TRCP</sup>–mediated claspin protein stability during DNA damage checkpoint recovery. However, we cannot rule out the possibility that the HPV-16 E7 oncoprotein may accelerate the proteolytic turnover of claspin in a more direct manner.

Claspin, PLK1, and Aurora A have been found to harbor E2F-responsive promoter elements (40–42). Therefore, it is likely that degradation of pRB by HPV-16 E7 and the associated increase in E2F-mediated gene transcription may contribute to claspin expression levels in both a positive and a negative manner. Deregulation of E2F-mediated gene transcription is likely to stimulate high levels of claspin in S phase, which may help to promote efficient DNA replication. This is consistent with the increased baseline levels of claspin protein expression observed in HPV-16 E7–expressing HFKs (Fig. 2B). However, enhanced E2F-mediated gene transcription would also up-regulate components of the SCF<sup>β-TRCP</sup>–based claspin degradation pathway leading to accelerated and/or premature degradation of claspin and hence attenuated DNA damage checkpoint control and aberrant entry into mitosis (ref. 25; Fig. 4D). Our finding that pRB degradation–deficient mutant HPV-16 E7 Δ21-24 does not significantly alter claspin stability supports this hypothesis. The fact that inhibition of claspin degradation prevents mitotic entry in HPV-16 E7–expressing cells suggests that Aurora A or PLK1 inhibitors may have therapeutic potential in high-risk HPV-associated neoplasms and potentially in cancers in which deregulated claspin degradation is observed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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